BBA 45790

BIOGENESIS OF CHLOROPLAST MEMBRANES

III. LIGHT-DEPENDENT INDUCTION OF PROTON PUMP ACTIVITY IN WHOLE CELLS AND ITS CORRELATION TO CYTOCHROME *f* PHOTOOXIDATION DURING GREENING OF A *CHLAMYDOMONAS REINHARDTI* MUTANT (y-1)*

S. SCHULDINER AND I. OHAD

Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem (Israel)

(Received December 4th, 1968)

SUMMARY

A proton pump activity in light-grown whole cells which is sensitive to low concentrations of electron transfer inhibitors and uncouplers is described. Carbonyl cyanide *m*-chlorophenylhydrazone inhibits the formation of the pH gradient at concentrations which slightly enhance the oxygen evolution in the light. The activity is proportional to chlorophyll concentration, the maximal rate being 1–2 μmoles H^+ per mg chlorophyll per min. The activity is absent in dark-grown y-1 cells, having only trace amounts of chlorophyll, but present in dark-grown wild-type cells, having a normal chloroplast. During light-induced formation of membranes in the dark-grown y-1 cells, the proton pump activity is completely restored after 2 h of illumination, when only small amounts of chlorophyll have been synthesized. The reactivation of the proton pump during the initial phase of the greening parallels the photoactivation of cytochrome *f*. Cytochrome *f*, present in dark-grown y-1 cells, becomes photooxidizable only at about 2 h of greening, before additional cytochrome is synthesized. The activity of Photosystem II (oxygen evolution and ferricyanide reduction) and Photosystem I (cyclic photophosphorylation) could be detected before photoactivation of cytochrome *f* and proton pump activity. It is concluded that the cytochrome *f* present in the dark-grown cells is not coupled with the two photosystems. The coupling or activation occurs within the first 2 h of the greening and is accompanied by the appearance of proton pump activity.

INTRODUCTION

The light-induced formation of the photosynthetic membranes and electron transfer system activity in a Chlamydomonas mutant have previously been investigated. The greening process showed essentially two distinct phases: an initial or lag

Abbreviations: DCMU, 3-(3,5-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PMS, *N*-methylphenazonium methosulfate.

* For previous numbers of this series see refs. 1 and 2.

phase lasting 1.5–3 h, during which chlorophyll synthesis proceeds at a slowly increasing rate, and a second phase during which the rate of synthesis becomes maximal and constant for the rest of the process (5–6 h).

The electron transfer system activity of both Photosystems I and II parallel the chlorophyll synthesis during the second phase, indicating that membrane assembly might occur through a simultaneous or "one-step" process[1].

The ultrastructure of the developing photosynthetic membranes changed continuously, showing also at least two distinct phases: (a) flattening and elongation of irregular vesicles initially present to form thylakoids, and (b) increase in length and number of the thylakoids, followed by their pairing and fusion to form a grana system. Whereas the second phase of structural changes was correlated with the appearance of electron transfer system activity of both photosystems, no such correlation was clearly observed for the initial phase of transition from vesicular to thylakoidal elements.

In the present work, an additional parameter of activity in photosynthetic membranes was measured, with special attention to its function during the initial phase of the greening process. It was found that light-grown y-1 and the wild-type cells exhibit a light-dependent proton uptake activity which is absent in dark-grown cells. During the initial phase of the greening, the proton pump is reactivated, the process being correlated with the photoactivation of cytochrome *f*.

MATERIALS AND METHODS

Chlamydomonas reinhardti wild-type and y-1 mutant cells were used. Cell growth, light-induced greening, and chlorophyll content were as previously described[1,2].

Proton uptake

Cells were harvested in the cold by centrifugation at 2000 × *g* for 5 min, washed and resuspended in deionised water at a final concentration of 10^8 cells per ml. The cell suspension was kept at room temperature during the experiments for periods of up to 2 h. No loss of activity was observed during this time. Light-induced pH changes were measured in an 8-ml reaction vigorously stirred vessel surrounded by a water jacket which was kept at 25°. A Radiometer pH meter type 28 equipped with a Radiometer glass electrode type G2027 KW was used. Differential expanded scale recording was achieved with a potentiometric recorder and a "backing off" circuit, obtained from a Radiometer pH calibrator type PHN2H No. 77298. The sensitivity was such that full deflection of the scale for 0.5 pH unit was obtained. The light source was a Norris slide projector equipped with 300-W tungsten lamp placed at 10 cm. The pH changes were expressed as μequiv of H^+ taken or released per 10^7 cells or per mg chlorophyll as a function of time. The cell suspension had a certain buffer capacity. In order to obtain linearity between pH changes and cell concentration it was necessary to titrate the buffer content of the cell suspensions. This was done by calibrating the pH scale with NaOH against cell concentration. Unless otherwise specified, the initial pH was brought to 6.2.

Conductivity measurements were done simultaneously with pH measurements by means of a Radiometer type CDM2d conductivity meter with a CDD114 type electrode whose constant was 0.64 cm.

For measurement of pH changes in the absence of CO_2, a closed reaction vessel was used, containing a central well with 40% KOH, and provisions for addition of base, acid and cell suspensions from closed sidearms. The pH was first lowered to pH 3 by HCl addition to chase the dissolved CO_2 and raised to pH 6.2 by the addition of NaOH. This was repeated twice before adding the cells and starting the measurements.

Photoreduction of ferricyanide and photooxidation of cytochrome f

Cells were washed and resuspended in 0.02 M phosphate buffer (pH 7.0) at a final concentration of 10^8 cells per ml. The cells were disrupted with the help of a French press operated in the cold at 5000 lb/inch^2. For photoreduction of ferricyanide the disrupted cells were centrifuged at 1000 $\times$ *g* for 3 min, the pellet discarded and the supernatant used for measurements. The reaction was carried out under conditions of linearity with respect to chlorophyll concentration and incubation time.

The effect of different buffers during homogenate preparation and on the assay system were tested in order to define the conditions for maximum activity. The best results were obtained when phosphate buffer was used for homogenate preparation and glycylglycine buffer was used in the assay system. The pH optimum of the reaction was found to be 7.0. Formation of ferrocyanide was measured with sulfonated 4,7-diphenyl-1,10-phenanthroline obtained from F. Smith Co. (Ohio), using the method described by AVRON AND SHAVIT[3]. Cytochrome *f* photooxidation was measured spectrophotometrically directly in homogenates prepared as above, using an Aminco–Chance double-beam spectrophotometer. The homogenate was exposed first to light of 640 mμ and then to light of 715 mμ wavelength, and the difference ΔA (553$-$540 mμ) recorded.

Total content of cytochrome was measured by the difference in absorbance as above between the reduced (ascorbate) and oxidized (ferricyanide) state.

Cyclic photophosphorylation

Cells were washed and resuspended at a final concentration of 10^8 cells per ml in 0.5 M mannitol containing 0.03 M phosphate buffer (pH 8.0). The cells were disrupted in the French press in the cold at 3000 lb/inch^2. The disrupted cells were homogenized with the aid of a glass–Teflon Potter–Elvehjem homogenizer (0.25 mm clearance) for 3 min in the cold. The homogenate was centrifuged for 15 min at 6000 $\times$ *g*. The supernatant was centrifuged again at 200000 $\times$ *g* for 30 min in a Spinco preparative L2 model centrifuge at 5°. The resulting pellet was resuspended in 0.5 M mannitol containing 0.03 M glycylglycine buffer (pH 8.0) and kept in ice until use. Photophosphorylation was carried out using a modification of the reaction mixture described by GORMAN AND LEVINE[4].

The reaction was carried out at 25° in a photosynthetic Warburg apparatus under N_2 and illuminated with 300-W tungsten lamps placed at a distance of 10 cm from the incubation vessel. The reaction was terminated after 6 min by addition of 0.2 ml of 0.5 M perchloric acid. Esterified ^{32}P was measured as described by AVRON[5], using a Packard-Tricarb scintillation counter.

Oxygen measurements

Oxygen evolution was measured in suspensions of whole cells in growth medium at 25° using a Gilson Oxygraph (Gilson Medical Electronics, Middleton, Wisc.)

equipped with a platinum vibrating electrode polarized at 0.6 V. The light source was the same as used for pH change measurements. The changes in oxygen concentration (light *minus* dark) were proportional to cell (chlorophyll) concentration at 0.02–2.0 μg chlorophyll per ml and to illumination time. The sensitivity set was such that $2 \cdot 10^{-8}$ moles O_2 were recorded as a 3.5-cm deflection on the chart.

3-(3,5-Dichlorophenyl)-1,1-dimethylurea (DCMU) was purchased from Abic Co. (Tel Aviv), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was kindly supplied by Dr. B. Z. Ginzburg from the Department of Plant Physiology, The Hebrew University of Jerusalem. ^{32}P was supplied by the Committee for Atomic Energy, Nahal Sorek, Israel. ADP, AMP and *N*-methylphenazonium methosulfate (PMS) were purchased from Calbiochem (Los Angeles, Calif.), hexokinase from Boehringer (Mannheim) and Nonidet P-40, a nonionic detergent, was purschased from British Drug Houses, England. All other reagents were of analytical grade.

RESULTS

1. Characterization of proton pump activity in whole cells

Light-grown y-1 and wild-type cells, as well as dark-grown wild-type cells, showed a reversible, light-dependent proton uptake (Fig. 1). Upon first illumination a lag of about 10–40 sec was observed, followed by proton uptake at a rate of about 0.2–0.4 μmole H^+ per mg chlorophyll per min. On subsequent illumination cycles, the lag period was shorter and the proton uptake rate increased. It was found that repeated light–dark cycles or continuous illumination for 3–15 min (depending on cell batch) was sufficient to abolish the lag period almost completely and give a maximal and constant proton uptake rate, varying for different cell batches from 1 to 2 μmoles H^+ per mg chlorophyll per min. The results of this experiment are shown in Fig. 2. All pH change measurements were therefore carried out after an appropriate "conditioning" period. The conditioning phenomenon was not simply the result of exposing the cells to changes in pH due to illumination, since alternating the pH of

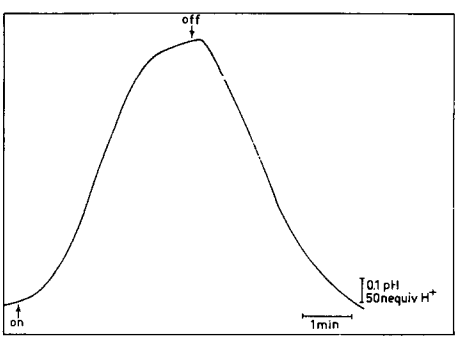


Fig. 1. Recorder tracing of pH changes induced by light. Light-grown y-1 cells were used. The system contained $2.8 \cdot 10^7$ cells (70 μg chlorophyll). ΔpH/μmole H^+ was 2.4. Similar results were obtained when dark-grown wild-type cells were used.

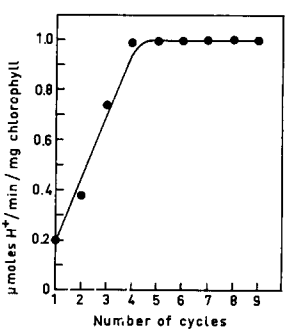


Fig. 2. Increase in proton uptake rate as a function of number of light–dark cycles. Light-grown y-1 cells were used. The system contained $1.8 \cdot 10^7$ cells (60 μg chlorophyll). ΔpH/μmole H^+ was 2.8. Similar results were obtained when dark-grown wild-type cells were used.

the cell suspension by proper addition of HCl or NaOH in the dark did not induce any increase in proton uptake activity. The reaction rates were dependent on the initial pH of the system, as shown in Fig. 3.

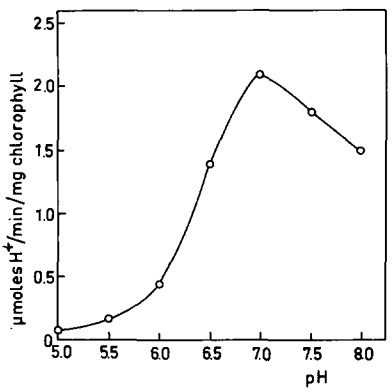


Fig. 3. Influence of initial pH on the initial rate of proton uptake. Light-grown y-1 cells were used. The system contained $3.8 \cdot 10^7$ cells (107 μg chlorophyll). The ratios ΔpH/μmole H^+ for the different initial pH were as follows: 4.5 at pH 5.0; 2.0 at pH 5.5; 0.6 at pH 6.0; 0.35 at pH 6.5; 0.3 at pH 7.0; 0.23 at pH 7.5; and 0.16 at pH 8.0.

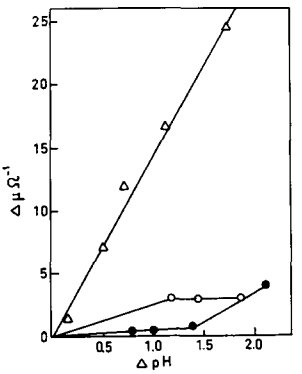


Fig. 4. Changes in conductivity as a function of pH rise due to illumination or addition of NaOH. Light-grown cells were used. The system contained $3 \cdot 10^7$ cells (75 μg chlorophyll). Conductivity changes induced in the light phase (○—○), dark phase (●—●) and during rise of pH to a similar extent as in the light phase by addition of NaOH (△—△).

The proton uptake was not affected by the presence or removal of CO_2 in the reaction vessel (Table I). To minimize the buffer capacity of dissolved CO_2, all measurements were performed at pH 6.2*. It was also found that for a given change in pH induced by light, the change in conductivity was much less than expected if the same pH change was to be obtained in the system by addition of NaOH (Fig. 4).

TABLE I

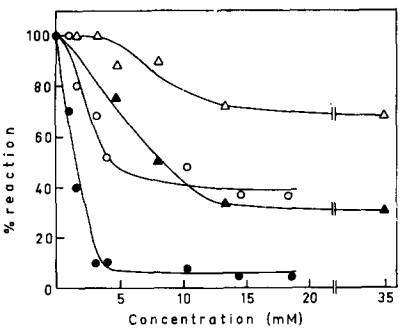
RATE AND EXTENT OF pH CHANGES INDUCED BY LIGHT IN CELL SUSPENSIONS IN THE PRESENCE OR ABSENCE OF CO_2

The system contained $2 \cdot 10^7$ cells (30 μg chlorophyll). For other experimental conditions, see MATERIALS AND METHODS.

System	*Rate (ΔpH/min)*	*Extent (ΔpH)*
With CO_2	0.12	0.65
Without CO_2	0.14	0.67

The influence of KCl, $CaCl_2$ and sucrose on the proton uptake rate and extent is shown in Figs. 5 and 6. It was found that on a molar basis, proton uptake was more sensitive to $CaCl_2$, which at 6 mM inhibited the rate 50% and the extent 95%, whereas KCl, at a similar concentration, inhibited the rate only 10% and the extent

* The properties of the system were not basically different when measurements were carried out in the range of pH 6.8–7.2.



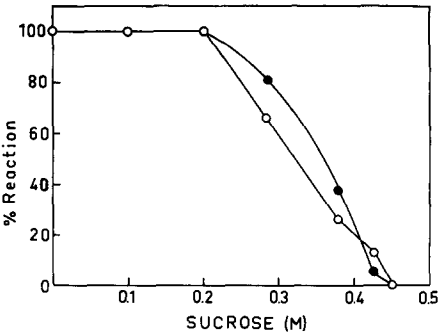


Fig. 5. Influence of KCl and $CaCl_2$ on the rate and extent of proton uptake. Light-grown cells were used. The system contained $2 \cdot 10^7$ cells (55 μg chlorophyll). Data are from two different experiments. The maximal rate (100 %) for KCl was 1.9 μmoles H^+ per mg chlorophyll per min (△—△) and the extent, 7.8 μmoles H^+ per mg chlorophyll (▲—▲). For $CaCl_2$, the maximal rate (100 %) was 2.1 μmoles H^+ per mg chlorophyll per min (○—○) and the extent, 8.8 μmoles H^+ per mg chlorophyll (●—●). ΔpH/μmole H^+ was 2.3.

Fig. 6. Influence of sucrose concentration on proton-pump activity. Light-grown cells were used. The system contained $3 \cdot 10^7$ cells (64 μg chlorophyll). The maximal (100%) rate (○) and extent (●) were 2.1 μmoles H^+ per min per mg chlorophyll and 9.2 μmoles H^+ per mg chlorophyll, respectively. ΔpH/μmole H^+ was 2.4.

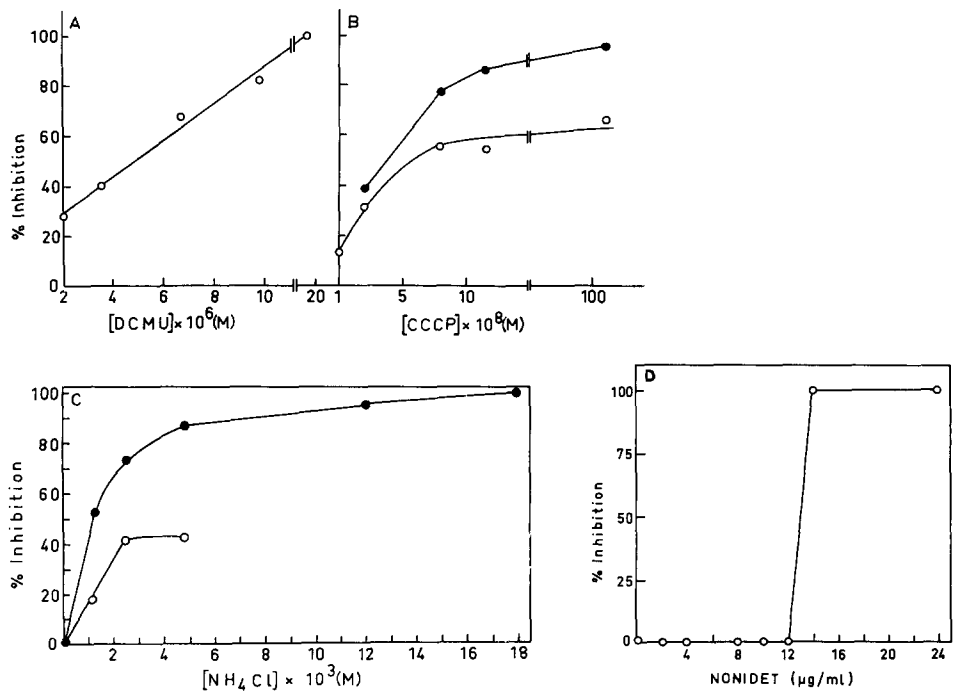


Fig. 7. Inhibition of the proton uptake by different inhibitors. Light-grown cells were used. The system contained $3 \cdot 10^7$ cells (65 μg/chlorophyll). ΔpH/μmole H^+ was 2.4. A. Effect of DCMU (rate). B. Effect of CCCP on the rate (○—○) and extent (●—●). C. Effect of NH_4^+ on the rate (○—○) and extent (●—●). D. Effect of Nonidet P-40 (rate). The maximal rates (μmoles H^+ per min per mg chlorophyll) were 2.2 (DCMU); 1.8 (CCCP); 2.5 (NH_4^+) and 2.4 (Nonidet P-40). The maximal extents (μmoles H^+ per mg chlorophyll) were 9.7 (DCMU); 10.8 (CCCP); 7.5 (NH_4^+) and 8.4 (Nonidet P-40). CCCP at a concentration of 10^{-6} M slightly increased the rate of oxygen evolution.

35 %. If the effect of ion concentration is calculated on the basis of ionic strength, the results show that the reaction extent inhibition is similar for both Ca^{2+} and K^+, whereas the reaction rate is more sensitive to Ca^{2+}.

Increasing concentration of sucrose inhibited both the rate and extent of the reaction (Fig. 6). The reaction was blocked by photosynthetic electron transfer inhibitors like DCMU, photophosphorylation uncouplers like CCCP and NH_3, and nonionic detergents like Nonidet P40, at concentrations similar to those used by other workers with cell-free systems (Fig. 7). The inhibition by DCMU, CCCP and ammonia were fully reversible by removal of the inhibitors through washing, whereas that of the detergent was only partially reversible (about 30 %). CCCP at a concentration which completely inhibited proton uptake (10^{-6} M) did cause a slight increase in the oxygen evolution rate. The rate of proton release in the dark period following illumination was not significantly increased by addition of CCCP at 10^{-8} M.

2. Proton pump and electron transfer system activity during the greening process

The chlorophyll content of dark-grown y-1 cells is a function of the number of divisions in the dark[2]. In this work, cells having initial chlorophyll levels of ≤ 2 μg/10^7 cells (as compared with 20–30 μg in light-grown cells) were used. The chlorophyll present in these cells is capable of promoting the reduction of ferricyanide and oxygen evolution (Fig. 8), but not the photooxidation of cytochrome *f* (Fig. 9), nor the uptake of protons (Fig. 10).

The oxygen evolution per unit chlorophyll remains constant during a 20-fold increase in chlorophyll, and the ferricyanide reduction increased 2-fold during a 15-fold increase in chlorophyll (Fig. 8).

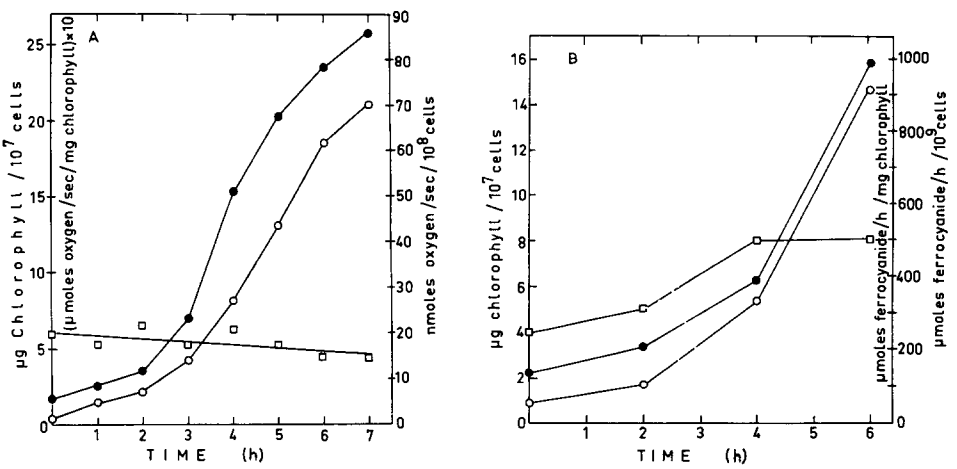


Fig. 8. Oxygen evolution and ferricyanide photoreduction as a function of chlorophyll synthesis during the greening of y-1 cells. Dark-grown cells were washed and suspended in growth medium at a final concentration of $5 \cdot 10^6$ cells per ml. Cells were incubated in light (700 ftcandles) at 25°. Samples were taken at different times and oxygen evolution (whole cells), photoreduction of ferricyanide (cell homogenates) and chlorophyll content measured as described in MATERIALS AND METHODS. The assay system for ferricyanide photoreduction contained in a final volume of 3 ml 40 μmoles glycylglycine buffer (pH 7.0); 80 μmoles KCl; 10 μmoles $MgCl_2$; 2 μmoles $Fe(CN)_6^{3-}$ and 5–50 μg chlorophyll. A. ●—●, oxygen evolution; ○—○, chlorophyll content; □—□, ratio μmoles O_2 per mg chlorophyll per sec. B. ○—○, ferricyanide reduction; ●—●, chlorophyll content; □—□, ratio μmoles ferrocyanide per mg chlorophyll per h.

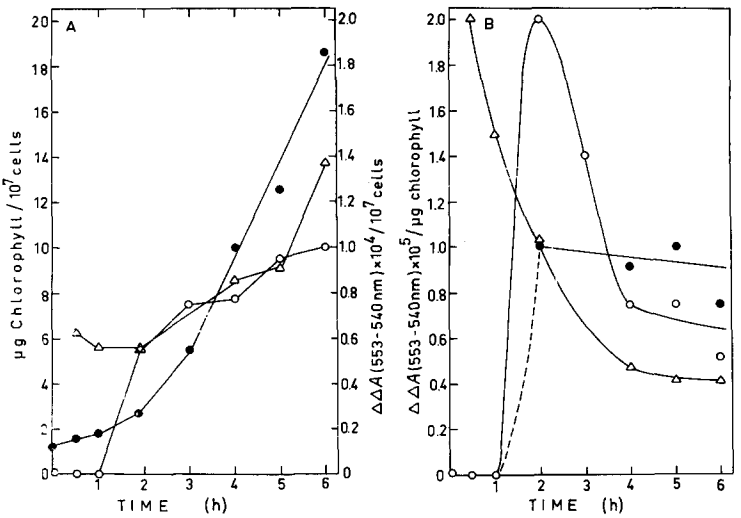


Fig. 9. Changes in content and photooxidation of cytochrome *f* during the greening of y-1 cells. Same experimental conditions as in Fig. 8. A. Increase in chlorophyll (●—●), cytochrome *f* content (△—△), and its photooxidation (○—○). B. Ratio of cytochrome to chlorophyll (△—△), ratio of photoactive cytochrome *f* to total cytochrome content (●—●), ratio of photoactive cytochrome to chlorophyll content (○—○). The content and photooxidation of cytochrome *f* were determined as described in MATERIALS AND METHODS.

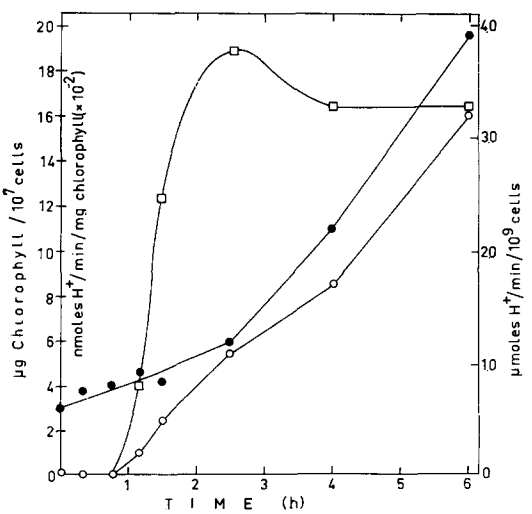


Fig. 10. Proton uptake and chlorophyll synthesis during the greening of y-1 cells. Dark-grown cells were suspended in growth medium at a final concentration of $3.4 \cdot 10^8$ cells per ml and incubated in light (700 ftcandles) at 25°. Samples were taken at different times, the cells washed in deionised water and chlorophyll content and proton-uptake activity measured as described in MATERIALS AND METHODS. $\Delta pH/\mu$mole H^+ was between 1 and 5 for the different samples. Appropriate cell concentrations were used to ensure a significant response for each time point. The system contained $8 \cdot 10^7$ cells at zero time and only 10^7 cells at 6 h light. ●—●, chlorophyll; ○—○, H^+ uptake rate; □—□, ratio proton uptake to chlorophyll.

Cytochrome *f*, although present in dark-grown cells at levels about 40 % that found in light-grown cells[2], cannot be photooxidized in these cells during the initial phase of the greening. The first response to light could be detected after 2 h of illumination, cytochrome *f* becoming completely photooxidizable at 2 h of greening, when chlorophyll increased only 2-fold (Fig. 9).

During the rest of the process more cytochrome *f* was synthesized, the whole content being completely photoactive. The ratio of photoactive cytochrome to chlorophyll becomes constant during the second phase of the greening process, when the chlorophyll synthesis rate is constant (Fig. 9).

A similar situation was found for the proton pump. Its activity could only be detected after 1 h of greening. The specific activity (H^+ uptake per min per mg chlorophyll) increased rapidly, reaching a peak at 2.5 h when chlorophyll increased less than 15 %. The specific activity slightly decreased afterwards and remained constant for the rest of the process (Fig. 10).

TABLE II

CYCLIC PHOTOPHOSPHORYLATION RATES BY CHLOROPLAST FRAGMENTS FROM LIGHT- AND DARK-GROWN y-1 CELLS

Chloroplast fragments were incubated in a final volume of 2 ml containing: 40 μmoles glycylglycine buffer (pH 8.0); 10 μmoles sodium phosphate (10^5 counts/min per μmole); 5 μmoles of each, ADP and AMP; 10 μmoles glucose; 40 μmoles KCl; 4 μmoles $MgCl_2$; 6.7 nmoles PMS; 0.6 μmole ascorbate; 10 μg of hexokinase and a suspension of chloroplast fragments containing between 25 and 150 μg chlorophyll for light-grown cells and 3–15 μg chlorophyll for dark-grown cells. The content of chlorophyll of the dark-grown cells used was $\leq$ 2 μg/10^7 cells in all experiments. Data are presented as the average of 4 different experiments.

System	*Specific activity (μmoles ATP formed/mg chlorophyll per h)*
Light-grown cells	90 ± 20
Dark-grown cells	100 ± 70

Light-dependent cyclic phosphorylation activity was present in both light- and dark-grown cells (Table II). The reaction was dependent on chlorophyll concentration and incubation time in chloroplast fragments prepared from light-grown cells when high concentration of chlorophyll could be used.

In chloroplast fragments prepared from dark-grown cells having a low chlorophyll content, the reaction did not show a linear dependency on illumination time and chlorophyll concentration. However, in four repeated experiments there was always a net light-dependent incorporation of phosphate into ATP. As shown in Table II, calculation of rates under these conditions is difficult and the data should be regarded as only an approximation.

DISCUSSION

Proton pump activity in whole cells

The properties of the photosynthetic proton pump of isolated chloroplasts and chromatophores have been described in detail by several workers[6–12]. Photosynthetic

ion pump activity in whole cells has also been reported[12,14–16]. The light-induced pH changes in suspensions of whole cells described in this work appear to be the expression of the proton pump operating at the chloroplast level. This conclusion is based on the following properties of the system: (1) reversible, light-dependent proton uptake independent of the presence of CO_2 in the medium, (2) sensitivity to inhibitors of photosynthesis at concentrations similar to those effective in cell-free systems, and (3) absence of activity in dark-grown nonphotosynthetic mutant cells. Thus DCMU inhibits completely both the oxygen evolution and the proton uptake in whole cells at $5 \cdot 10^{-5}$ M. CCCP, which was shown to act as an uncoupler and seems to increase the rate of decay of the high-energy intermediate synthesized during the light reaction[17], causes a complete inhibition of the proton uptake and slightly increases the rate of oxygen evolution, as in isolated chloroplasts[18]*. The extent of light-dependent proton uptake per mg chlorophyll by whole cells is about 4–5 times higher than that observed in cell-free systems, while the rates are similar to those obtained in isolated chloroplasts[6–12]. This is not surprising when one considers the intactness of the chloroplast in its natural environment, the cell. The chloroplast in Chlamydomonas represents a major compartment occupying about 45–55 % of the cell volume[1,19].

The width of the plasma layer separating the chloroplast from the cell membrane, as seen in electron micrographs[1,2], is less than 0.4 μ. If the plasma membrane were rendered permeable to protons, it would be expected that massive proton movement to or from the chloroplast would also be rapidly expressed in the medium surrounding the cell. A similar effect was detected in other algae having a similar morphology by BEN-AMOTZ AND GINZBURG[20] working with the unicellular alga Dunaliella**. It is not possible at the moment to assess the physiological implication of this phenomenon. It is conceivable that the proton gradient generated by the chloroplast is rapidly compensated for in the cytoplasm by proton uptake from the surrounding medium, so that proton concentration in the cytoplasm is kept constant. However, the possibility should be considered that the light-induced proton movements, or the accompanying ion movements, also occur in the cytoplasm and might play a role in the control of the cellular metabolism.

The Chlamydomonas cells are bounded by a tough cell wall reinforced by cellulose fibrils[2] which can prevent swelling and lysis when cells are suspended in an hypotonic environment. The normal osmotic pressure of these cells is in the range of about 2–4 atm. Higher osmotic pressure causes shrinkage of the protoplast. Sucrose solutions at concentrations above 0.2 M strongly inhibit the proton uptake extent and rate. This can be explained by a reduction in the cell volume due to shrinkage of the protoplast without immediate damage to the structure of the membranes, since the inhibition is reversible.

The integrity of cellular compartments is an obvious prerequisite for ion transport and accumulation[13,22]. This is demonstrated by the high sensitivity of the system to detergents.

* Apparently CCCP does not increase the decay rate of the pH gradient in the dark in whole cells. However, preliminary experiments indicate that such an effect is present in Chlamydomonas chloroplast fragments.

** We would like to mention that Mr. A. Ben Amotz and Dr. B. Z. Ginzburg, from the Department of Botany of this University, first observed a light-dependent pH change in a halophylic alga, Dunaliella and in Chlamydomonas and have investigated the properties of this system in Dunaliella parallel to our work on Chlamydomonas.

Influence of ions on proton uptake

Certain ion species and concentrations are necessary for optimal proton uptake in isolated chloroplasts[7–12]. It should be mentioned that isolated chloroplasts are osmotically fragile, and that the optimum salt concentration might be correlated at least in part with osmotic requirements. The ion species and concentrations necessary for optimal activity of the proton pump cannot simply be studied in whole cells, since the internal concentration of different ions is not known. The results show that K^+ and Ca^{2+} inhibit the extent of proton uptake by whole cells at similar and very low ionic strength, whereas the rate is more sensitive to Ca^{2+}.

Measurements of conductivity changes during illumination might indicate that no extensive net fluxes of ions are superimposed on the proton uptake activity.

The light-induced proton uptake activity, present in light-grown y-1 cells and in the wild-type grown either in the light or in the dark, was absent in dark-grown y-1 cells, in which the photosynthetic membrane system is absent[2]. During the greening of dark-grown y-1 cells by exposure to light, the proton uptake activity was restored after about 1–2 h illumination before a marked increase in chlorophyll could be detected. The absence of activity in dark-grown cells might be due to changes in the internal distribution of ions, substrates or cofactors associated with the heterotrophic growth. Thus the lag phase might represent the time necessary for reequilibration or redistribution of these compounds within the different cellular compartments. That this is not the case is demonstrated by the wild-type cells which when grown heterotrophically in the dark do not show any lag period when proton uptake activity is measured. Thus this lag phase is clearly associated with the state of organization of the chloroplast. However, the phenomenon of "conditioning" before maximal and constant activity is observed in both wild-type and y-1 cells (in which activity is already present) and might be associated with equilibration as suggested above or with a change in permeability of the plasma membrane to ions.

Light-induced formation of proton pump and photooxidation of cytochrome f

Upon activation during greening, the rate of proton uptake (H^+ per mg chlorophyll per min) increased rapidly, reaching a maximal value at a chlorophyll content of about 6 μg per 10^7 cells. During this process, not only the newly synthesized chlorophyll but also the chlorophyll initially present in the dark-grown cells became active. A similar behavior was observed for the photooxidation of cytochrome *f*. The presence of cytochrome *f* in dark-grown y-1 cells and its photooxidation during the greening process have been described in a previous publication[2,1]. In order to increase the sensitivity of the measurement of photooxidation of cytochrome *f*, homogenates were used instead of whole cells and light of 640 mμ and of 715 mμ was used. Although cytochrome *f* was present in dark-grown y-1 cells, it was not photooxidized by light until after about 2 h of greening. From this time on, all the cytochrome present, including the initial content and that synthesized during the greening, amounting to about twice the initial content, was completely photooxidizable. As opposed to proton uptake and cytochrome *f*, the activity of photosystem II measured in whole cells as O_2 evolution and homogenates as ferricyanide photoreduction were practically constant per chlorophyll unit throughout the greening process. Thus a clear correlation exists between the proton pump and activation or coupling of both photosystems. In a previous work, the possibility was considered that photosynthetic

membrane assembly is a spontaneous or one-step process[1,2]. By this it was meant that the components necessary for the activity of both Photosystems II and I are incorporated at once in the developing membranes. The results presented in this work indicate that this might be the case for the Photosystem II complex. However, it seems that the photoactivity of cytochrome *f* goes through two different stages: an initial stage during the first 2 h of the greening when small amounts of chlorophyll are synthesized, and cytochrome *f* oxidation and proton uptake activity is drastically increased; and a second stage of rapid and constant rate of chlorophyll synthesis during which proton uptake activity is constant, and cytochrome *f* becomes completely photoactive. The transition from the first to the second state occurs through an "overshoot" of activity which falls again to a lower and constant level within about 2 h.

Since dark-grown y-1 cells contain rather high levels of cytochrome *f* (this work, also ref. 1), the redox state of the latter during the initial phase of greening deserves special attention. In homogenates as well as in whole cells, cytochrome *f* cannot be photooxidized, and it is found always in the reduced state. This might be due to the presence of a reducing agent having a proper redox potential. The presence of such a component is easily detected in greening cells with a chlorophyll level of about 3 $\mu g/10^7$ cells, which supports cytochrome *f* photooxidation. When light is shut off, cytochrome *f* is rapidly reduced in the dark. Although it does not seem likely, it is still possible that at this stage of the greening cytochrome *f* can accept electrons from Photosystem II also. Its inability to be photooxidized during the initial phase of the greening can be explained in one of two ways: (a) Photosystem I is present but cannot accept or transfer electrons from cytochrome *f*; or (b) Photosystem I is missing. The presence of cyclic photophosphorylation activity with PMS in dark-grown cells before photooxidation of cytochrome *f* could be detected indicates that Photosystem I present indeed. Preliminary observations have also shown that Photosystem I activity tested as electron transfer from ascorbate to diquat is also present at this stage. Thus the y-1 mutant during the initial phase of greening behaves similarly to the Chlamydomonas mutant ac206 lacking cytochrome *f* described by GORMAN AND LEVINE[4], with one difference: the cytochrome is present in the y-1 cells but not "properly connected" to the photosystems. The burst of activity during the initial phase of the greening might represent the repair or coupling of these systems either by incorporation of missing constituents or by structural rearrangements, or both. The ultrastructural changes during the light-induced formation of photosynthetic membranes in the y-1 mutant have been described in detail in a previous work[1]. The first light-induced change to be detected was the flattening and elongation of irregular vesicular elements present in dark-grown y-1 cells. This was completed after about 2 h of illumination. Thus, it seems possible that the coupling of the two photosystems also activates the proton pump and might be correlated with structural modifications. A more detailed analysis of the initial phases of the greening process might throw light on the mechanism of this phenomenon.

The possible implications with respect to the MITCHELL[21] hypothesis of the observation that cyclic photophosphorylation proceeds in the absence of proton pump activity during the initial phase of membrane formation in whole cells cannot be emphasized before data on proton pump activity and phosphorylation in chloroplast fractions from greening cells are available. Experiments devised to provide such data are now being carried out.

ACKNOWLEDGEMENTS

The authors wish to thank Professor M. Avron and Miss G. Ben-Hayyim, from the Weizmann Institute of Science, Rehovoth, for use of their double-beam spectrophotometer and their encouragement, comments and suggestions. Our special thanks are due to Miss G. Ben-Hayyim for measurements of cytochrome *f* oxidation. We also wish to thank Miss Frieda Bachman for performing some of the measurements of oxygen evolution during her stay in our laboratory as a summer student.

REFERENCES

1 I. OHAD, P. SIEKEVITZ AND G. E. PALADE, *J. Cell Biol.*, 35 (1967) 553.
2 I. OHAD, P. SIEKEVITZ AND G. E. PALADE, *J. Cell Biol.*, 35 (1967) 521.
3 M. AVRON AND N. SHAVIT, *Anal. Biochem.*, 6 (1963) 549.
4 D. S. GORMAN AND R. P. LEVINE, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 1669.
5 M. AVRON, *Anal. Biochem.*, 2 (1961) 535.
6 A. T. JAGENDORF AND G. HIND, in *Photosynthetic Mechanisms in Green Plants, Natl. Acad. Sci. – Natl. Res. Council, Publ. 1145* (1963) 599.
7 J. NEUMANN AND A. T. JAGENDORF, *Arch. Biochem. Biophys.*, 107 (1964) 109.
8 S. D. D. KARLISH AND M. AVRON, *Biochim. Biophys. Acta*, 153 (1968) 878.
9 R. A. DILLEY, *Brookhaven Symp. Biol.*, 19 (1967) 258.
10 N. SHAVIT, A. THORE, D. L. KEISTER AND A. SAN PIETRO, *Proc. Natl. Acad. Sci. U.S.*, 59 (1968) 917.
11 G. HIND AND S. IZAWA, *Biochim. Biophys. Acta*, 145 (1967) 377.
12 L. PACKER AND A. R. CROFTS, in D. R. SANADI, *Current Topics in Bioenergetics*, Vol. 2, Academic Press, New York, 1967, p. 23.
13 P. MITCHELL, *Nature*, 191 (1961) 144.
14 D. R. HOAGLAND AND A. R. DAVIS, *J. Gen. Physiol.*, 6 (1923) 47.
15 J. T. CUMMINS, J. A. STRAND AND B. E. VAUGHAN, *Biochim. Biophys. Acta*, 126 (1966) 330.
16 E. A. C. MACROBBIE, *Biochim. Biophys. Acta*, 94 (1965) 64.
17 A. T. JAGENDORF AND J. NEUMANN, *J. Biol. Chem.*, 240 (1965) 3210.
18 M. AVRON AND N. SHAVIT, in *Photosynthetic Mechanisms of Green Plants, Natl. Acad. Sci. – Natl. Res. Council, Publ. 1145* (1963) 611.
19 I. GOLDBERG AND I. OHAD, submitted for publication.
20 A. BEN-AMOTZ AND B. Z. GINZBURG, *Biochim. Biophys. Acta*, 183 (1969) 144.
21 P. MITCHELL, *Federation Proc.*, 26 (1967) 1370.