Photosynthetic Apparatus Formation during the Cell Cycle of Chlorella

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ABSTRACT
Synchronous cell division in cultures of Chlorella vulgaris Beijerinck was induced by intermittent illumination: 9 hours light, 6 hours darkness. The rate of photosynthetic O2 evolution per cell increases 4-fold in a one-step manner at the beginning of the light period, to the same extent as the increase in cell number. Over the division cycle, the following accumulation times during the light period were found: chlorophyll a, between 2 and 8 hours; chlorophyll b, between 5 and 8 hours; reaction centers of photosystems I and II, between 2 and 6 hours; and cytochrome f, between 5 and 5 hours. Cytochrome f accumulation is closely followed by an increase in the delayed fluorescence yield per flash under continuous illumination (caused by the establishment of the pH difference across the thylakoid membrane) maximal by the first hour of the light period.

Studies of cell division in synchronized unicellular green algae have revealed that photosynthetic activity of cells varies within the division cycle, with the greatest activity at the beginning of the cycle and the least activity before the release of autospores (11, 12). It has been shown that changes in photosynthetic capacity of whole cells are correlated to PSI activity measured as photosynthetic activity as a function of 2,6-dichlorophenolindophenol in chloroplasts (7) and in PSII-enriched subchloroplast particles (5). These changes are correlated also with the quantum yield of Chl fluorescence (9), the Emerson enhancement effect (10), and the concentration-dependent inhibition of the Hill reaction by dibromothymoquinone. We have shown that, in a Chlorella culture synchronized with intermittent illumination, the intensity of PSII-generated delayed fluorescence increases stepwise early in the light period (1).

In the present work, an attempt was made to elucidate the correlation between the photosynthetic activities of whole cells and changes in the composition of their photosynthetic apparatus during the cell cycle. Oxygen evolution, light-induced A changes at 520 nm, parameters of delayed fluorescence, and accumulation of Chl a and b, Cyt f, and functional reaction centers of PSI and PSII in synchronous cultures of Chlorella were examined.

MATERIALS AND METHODS
A thermophilic strain of Chlorella vulgaris Beijerinck, S-39/64688, was obtained from the algal collection of the Institute of Biology, Leningrad State University. A stock culture of Chlorella was maintained axenically on an agar slant containing Tamja medium (13). Chlorella cells were preliminarily grown at 37°C for 1 week in liquid Tamja medium under continuous illumination of 10^6 erg cm^{-2} s^{-1}. These cells were transferred asexually into an autoclaved glass culture vessel containing 850 ml of a sterile medium at an initial cell concentration of 10^8 cells ml^{-1}. The vessel was connected with a flow cuvette, which was placed into a cylindrical phosphoroscope for delayed fluorescence measurements (on a time scale >20 ms). Cell suspension was continuously pumped from the vessel into the cuvette by an “air-lift” pump and then returned.

The cells were synchronized under an intermittent illumination 9-h light/6-h dark regime. The light intensity was 10^6 erg cm^{-2} s^{-1} measured at the surface of the culture vessel. The culture was aerated with sterile (filtered and UV-irradiated) air containing 2.5% CO2. Before the beginning of each light period, the culture was diluted with sterile medium to give a cell concentration of 10^8 cells ml^{-1}.

Cell numbers were determined with a Gorjaev’s hemacytometer. Cell diameter was measured with an ocular micrometer. Nuclei were stained with hematoxylin (8). To follow photosynthetic capacity, O2 evolution was measured polarographically with a Clark-type O2 electrode, with the cells suspended in the growth medium. Results were corrected for dark respiration.

Chl a and b contents were calculated from the A of ethanol extracts of algal cells at 665 and 649 nm (17). Ferricyanide-oxidized minus hydroquinone-reduced differential spectra of broken cells were measured with a dual-beam differential spectrophotometer (15). Twenty-five-ml culture samples were centrifuged, and cells were suspended in 1 ml growth medium for measurements. Samples were freeze-thawed twice and solid ferricyanide or hydroquinone was added. With the same spectrophotometer, differential absorption spectra were measured for whole cells differing in age by 1 h. Light-induced A changes of culture suspension were measured on a dual-wavelength spectrophotometer (16), with the samples being placed in a cuvette with a 3-cm light path.

Electron spin resonance (ESR) spectra were recorded at room temperature, with a reflectance X-band spectrometer fitted with 100-kHz modulation. In the sample cuvette, 0.06 ml cell suspension (1.5 × 10^6 cells ml^{-1}) was used.

Delayed fluorescence intensity was measured with a conventional phosphoroscope. The time interval between the illumination and delayed fluorescence registration was 1.25 ms. Excitation was given by continuous light or by a 8- × 10^{-6}s flash from a xenon lamp.

In all measurements of light-induced responses, intensity of continuous actinic illumination from an incandescent lamp (wavelength, >650 nm) was 10^4 erg cm^{-2} s^{-1}.
RESULTS

Parameters of Synchronous Cell Growth and Division. The synchrony of cultures was estimated from cell numbers, cell size distribution, and percentage of multinucleate cells. Under the illumination regime employed, the highest degree of synchrony was seen after three light-dark cycles (Fig. 1). From cycle to cycle, the percentage of cells dividing in the light decreased and the period of autospore release shortened. In the third cycle, cell division was seen only in the dark period for about 2.5 h, i.e. about 20% of the total generation time (Fig. 1).

The cell size distribution pattern of an asynchronous culture reveals cells with diameters from 2 to 6 μm. After the first dark period, cells with diameters >4.5 μm disappeared, and the percentage of small cells increased.

In successive cycles of synchronization, the size distribution at the onset of the light period narrowed (more than 80% of cells having a diameter of 2–2.7 μm; Fig. 1). Over the light period, the cell size distribution shifted toward large sizes and, after 6 h, hardly overlapped with the initial distribution (Fig. 1). This indicates synchronous growth and division of all cells in the culture. Nuclear division in a synchronous culture occurred about 5 h after the onset of the light period, as is evident from Table I.

Photosynthetic O₂ Evolution. Rates of photosynthetic O₂ evolution were measured during synchronous growth under intermittent and continuous illumination of presynchronized cells (Fig. 2). In both cases within the generation time, the O₂ evolution per ml

![Fig. 1](image1)

![Fig. 2](image2)

![Fig. 3](image3)

Table 1. Changes in Percentage of Multinucleate Cells during Developmental Cycle of Synchronous Chlorella Cultures

<table>
<thead>
<tr>
<th>Duration of Light Period</th>
<th>Multinucleate Cells</th>
</tr>
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<tbody>
<tr>
<td>h</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
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<tr>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>62</td>
</tr>
<tr>
<td>11</td>
<td>4</td>
</tr>
</tbody>
</table>

culture increased 4-fold in a single-step manner. With intermittent illumination, this increase occurred immediately after the onset of light, whereas, in continuous illumination, this rise coincided with the release of autospores. These observations indicate that O₂ evolution by each autospore increases rapidly 4-fold, after its release from the mother cell and then remains constant during development into the mother cell.

Chl Content and Absorption Spectra of Cells. During the development of a synchronous culture, we measured absorption difference spectra of whole cells differing in age by 1 h (Fig. 3). Such spectra reveal absorption bands of only those pigments which have accumulated during a given hour. Intensities of the bands reflect the rates of pigment accumulation. The shape and
position (Fig. 3, inset) of the main Chl a absorption band varied during the light period of the cell cycle. This indicates that accumulation of different spectral forms of Chl a during the cell cycle is not simultaneous. In the absorption spectra of the pigments accumulated during the first half of the light period, the shoulder at 650 nm, corresponding to Chl b, is greatly reduced. A prominent shoulder appears after the fifth h of light (Fig. 3). The Chl a absorption band increases during the first half of the light period and then decreases. It seems that the accumulation of Chl b occurs mainly during the second half, whereas that of Chl a occurs throughout most of the light period of the cell cycle.

This conclusion was confirmed by measurements of Chl a and Chl b in ethanol extracts of cells (Fig. 4A). The total Chl content in cells increased to the same extent as the increase in cell number. Chl a was found to accumulate between 2 and 8 h of the light period, with its maximum rate of synthesis 5 h after the onset of the light period (Fig. 4B). Chl b was synthesized at a constant rate between 5 and 8 h of the light period (Fig. 4). As a result of nonuniform Chl a and Chl b accumulation, the Chl a/b ratio undergoes considerable variation during the life cycle of the alga, from 3 at the beginning and end to 6.5 at the fifth h of the light period (Fig. 4A).

Most of all Chl b is localized in the light-harvesting Chl a/b protein complex, in nearly equimolar amounts with Chl a (14). Assuming that the amount of Chl b reflects the amount of light-harvesting Chl-protein complex and that Chl a of this complex is synthesized concurrently with Chl b, one can calculate the content of Chl a associated with the reaction centers of PSI and PSII by subtraction of Chl b from Chl a. The content of Chl and the rate of its accumulation during the cell cycle are presented in Figure 4. Rates of Chl b (Fig. 4, curve 2) and of Chl a minus Chl b (Fig. 4, curve 3) accumulation are high during short, but different, time intervals of the light period.

Accumulation of PSI and PSII Reaction Centers. The content of functional (involved in electron transfer) PSI reaction centers (P700) was determined as the amplitude of the light-induced ESR signal 1 (Δ H = 7-9 g; g-factor, 2.0025). Treatment of cells with 10^{-5} M DCMU, which blocks electron transport between PSII and PSI, does not increase the amplitude of the signal at any stage of the life cycle (data not shown). This indicates that, in native cells, all P700 is oxidized with saturating actinic light. The amplitude of signal 1, and, consequently, the content of functional PSI centers increase between 2 and 6 h of the light period (Fig. 5).

The abundance of functional plus nonfunctional PSI reaction centers was determined from differential (oxidized minus reduced) absorption spectra of P$_{700}$. Figure 6 shows that, in Chlorella cells, some other spectral forms of Chl a, along with PSI centers, are oxidized by ferricyanide. During the cycle, the shape of the spectrum in the 670- to 700-nm region changes, indicating that the accessibility of those Chl forms to oxidation is changed. The amplitude of the 700-nm absorption band increases between 2 and 6 h of the light period (Figs. 5 and 6). A simultaneous increase in content of photooxidizable and chemically oxidizable P$_{700}$ indicates that all PSI reaction centers formed are rapidly integrated into the electron-transport chain.

The accumulation of PSII centers was determined from the intensity of delayed fluorescence. The delayed fluorescence is generated in PSII (4) and, consequently, when excited in whole cells by short light flashes or in DCMU-treated cells under continuous illumination, i.e. under conditions when electron flow and effects of the electrochemical proton gradient were excluded, its intensity must be proportional to the PSI content.

After the illumination with a series of short saturating flashes, the intensity of the delayed fluorescence oscillates with a period of four, due to the transitions between the S-states of the O$_{2}$-evolving system (3). The pattern of oscillation does not change during the cell cycle. Whereas the delayed fluorescence intensity after each flash increases in the middle of the light period. The accumulation of PSII centers was measured from the intensity of the delayed fluorescence after 10 saturating flashes, when the oscillations had been damped. The PSII reaction center content was found to increase between 2 and 6 h of the light period. Similar results were obtained from delayed fluorescence measurements in DCMU-treated cells (Fig. 5). This means that the PSI and PSII centers are formed concurrently with formation of antenna Chl a belonging to PSI and PSII, i.e. both photosystems are formed as complete units.

Cyt f Accumulation. The amount of functional (involved in electron transfer) Cyt f was determined from light-induced A changes at 555 nm with reference wavelength 560 nm in cells with
and without added DCMU. DCMU had no effect on Cyt f oxidation at any stage of the cell cycle (Fig. 7). The amount of functional Cyt f increased 4-fold abruptly between 2.5 and 5 h of the light period (Fig. 7). The total functional plus nonfunctional Cyt f content, estimated from the amplitude of 555-nm absorption band in the difference (oxidized minus reduced) spectra of broken cells at the fifth h was found to be 4-fold greater than at 2.5 h of the light period. This indicates that increase in functional Cyt f content results from synthesis and that all Cyt f formed is rapidly integrated into the electron transport chain.

Light-induced Absorption Changes at 520 nm. The kinetics of light-induced changes at 520 nm are shown in Figure 7. Actinic illumination induces a rapid (\(t_f = 0.2\) s) increase, followed by a further slow (\(t_f = 0.5\) s) increase, after a small lag (\(t_f = 0.2\) s). The two kinetic phases were observed at all stages of the cell cycle their relative amplitudes varying over the entire cycle. The amplitude of the rapid phase increased in the darkened culture and decreased during the first 30 min of the light period. The rapid phase amplitude increased between 2.5 and 5 h illumination (Fig. 7). The amplitude of the slow phase increased between 2.5 and 8 h of the light period and also showed reversible changes upon darkening culture (Fig. 7).

The rapid components of the absorption changes at 520 nm arise from a shift in the carotenoid absorption band due to the development of a light-induced electric field across the photosynthetic membrane (19). Apparently, by the third h of the light period, the intramembranous electric field increases in the chloroplast thylakoids of Chlorella.

Delayed Fluorescence. Measurements of delayed fluorescence with a cultivator's sensor show that delayed fluorescence intensity increased in a step-wise manner between 2.0 and 5 h of the light period (Fig. 2). In addition, the intensity of the delayed fluorescence appeared to undergo changes which are independent of the synchrony of the culture after transfer from light to darkness and vice versa. These changes were observed for about 1 h and were absent during the synchronous division cycle under continuous illumination (Fig. 2). These relatively fast reversible changes in delayed fluorescence intensity and changes in the rapid and slow phases of the light-induced absorption increase at 520 nm (Fig. 7) after light-dark transfer of the culture are probably due to light-dark adaptation of photosynthetic processes in the cells.

Light-induced Proton Concentration Gradient. Changes in the light-induced difference in pH across the thylakoid membrane of whole cells were measured by the increased delayed fluorescence excited by a short light flash in the presence of a continuous light background. Enhancement of the delayed fluorescence under continuous illumination is caused by the establishment of a proton gradient, which increases the delayed light emission (20). Consequently, the ratio of the delayed fluorescence intensities in the presence and absence of continuous illumination (an enhancement coefficient) should reflect the magnitude of the pH difference. The enhancement coefficient increases at the onset of the light period, reaching a maximum 1 h later, whereupon it begins to decrease (Fig. 8).

DISCUSSION

As shown by many authors, the photosynthetic activity of synchronous cultures of microalgae, based on dry weight or cell volume, is maximal in the first half of the developmental cycle (11, 12). In our experiments with Chlorella, the photosynthetic activity, calculated per Chl or reaction center, changes in the same way. When photosynthetic activity is calculated per volume of cell suspension, the photosynthetic capacity increases in a stepwise manner soon after the release of autospores and remains constant for the remainder of the cell cycle. Such a pattern of change in photosynthetic activity resembles change in activity of stable enzymes, which are synthesized once in the cell cycle (6). Obviously, the increase in photosynthetic activity is due to synthesis and/or integration of some rate-limiting component(s) into the photosynthetic apparatus. The fact that this occurs only under
increase in number of electron transfer chains. Rather, it is the result of a series of successive syntheses of its individual components.

Different accumulation kinetics of the rate-limiting component and the reaction centers of PSI and PSII lead us to infer that several electron transport chains can interact at the level of the rate-limiting component. This conclusion is consistent with the suggestion that oxidation of the plastoquinone pool is a rate-limiting step.

The fact that PSI centers and Cyt f accumulate at different stages also indicates that the stoichiometry between these components changes. On the other hand, accumulation of functional PSI and PSII centers and of their antenna Chl a are coordinated by the cell so as to provide constant stoichiometry between them over the entire cell cycle.

From the data of Chl a and b contents, it can be inferred that the formation of the light-harvesting Chl a/b protein complex does not occur until after the formation of the PSI and PSII is complete.

The abrupt changes in the composition of the photosynthetic apparatus results in changes in its functional activities. In particular, the integration of the rate-limiting component into the photosynthetic membrane is accompanied by an increase in the pH gradient across the membrane. The accumulation of Cyt f is accompanied by an increase in the amplitude of the electrochromic absorption changes at 520 nm and the intensity of the ms delayed fluorescence of Chl. It is possible that the increased positive charge on photooxidized Cyt f enhances the intramembranous electric field, which, in turn, would enhance the delayed fluorescence yield.

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