

BIOGENESIS OF CHLOROPLAST MEMBRANES

II. Plastid Differentiation during Greening of a Dark-Grown Algal Mutant (*Chlamydomonas reinhardtii*)

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ABSTRACT

Dark-grown cells of the γ -1 mutant of *Chlamydomonas reinhardtii* contain a partially differentiated plastid lacking the photosynthetic lamellar system. When exposed to the light, a rapid synthesis of photosynthetic membranes occurs accompanied by synthesis of chlorophyll, lipids, and protein and extensive degradation of the starch reserve. The process is continuously dependent on illumination and is completed within 6–8 hr in the absence of cell division. Photosynthetic activity (O_2 evolution, Hill reaction, NADP photo-reduction, and cytochrome *f* photooxidation) parallels the synthesis of pigment and membrane formation. During the greening process, only slight changes occur in the levels of soluble enzymes associated with the photosynthetic process (RuDP-carboxylase, NADP-linked G-3-P dehydrogenase, alkaline FDPase (pH 8)) as compared with the dark control. Also cytochrome *f* concentration remains almost constant during the greening process. The kinetics of the synthesis of chlorophyll, formation of photosynthetic membranes, and the restoration of photosynthetic activity suggest that the membranes are assembled from their constituents in a single-step process.

INTRODUCTION

In a variety of systems including higher plants (1–4) and algae (5–11), it has been demonstrated that during the light-induced greening of previously dark-grown, etiolated organisms a photosynthetic apparatus is formed and photosynthetic activity appears. This process involves the synthesis of lipids, RNA, and proteins (4, 12–17), including many enzymes involved in photosynthesis (4, 8, 18–21), their assembly into the chloroplast lamellar system, and the eventual formation of grana. The entire process amounts to a differentiation of proplastids into plastids.

In the preceding paper (22), it was shown that the dark-grown mutant cells (chyd cells) of *Chlamydomonas reinhardtii* contain a morphologically

distinct, partially differentiated plastid which can be considered a chloroplast in which the amount of photosynthetic membranes has been extensively reduced by dilution through divisions in the dark. It was further found that the concentrations of some of the enzymes involved in the photosynthetic carbon reduction cycle, as well as some of those participating in the photosynthetic electron transport are within $\pm 50\%$ of that found in light-grown cells (chyl cells). Chyd cells also retain the ability to synthesize the whole complement of lipids characteristic of the chyl cells and, upon illumination, rapidly synthesize photosynthetic pigments, produce a lamellar system (chloroplast discs), and show restored photosynthetic activity.

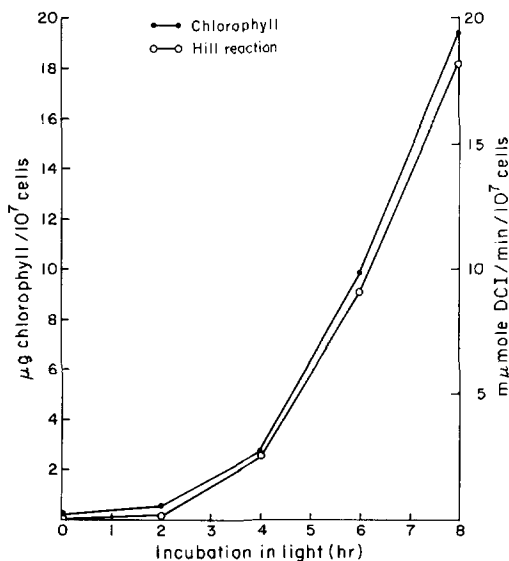


FIGURE 1 Chlorophyll synthesis and Hill reaction during the greening of chyd cells. Dark circles, chlorophyll; open circles, Hill reaction. The cells were grown in the dark in batch culture for 6 days and exposed to light at a final concentration of 6.5×10^7 cells/ml.

A kinetic analysis of the growing process in such cells could yield some information on general mechanisms involved in the biogenesis of photo-

synthetic membranes. For instance, a comparison of the kinetics of appearance of chloroplast membranes (at the morphological level) and of membrane-associated photosynthetic activities (at the biochemical level) could provide an answer to the question whether the assembly of the photosynthetic membrane is a single-step or a multi-step process. In the first alternative, the membrane is expected to become functional upon its assembly; in the second, photosynthetic activity should appear only after the formation of "structural" membranes (discs) when, in a succession of steps, the latter are provided with the whole complement of photosynthetic pigments, carriers, and enzymes. Such a kinetic analysis forms the object of the present work. The results obtained suggest that the first alternative applies.

MATERIALS AND METHODS

Chlamydomonas reinhardtii

These cells were grown either in batch-type cultures or in a semicontinuous culture apparatus as previously described (22). In many experiments, acetate- ^{14}C , carrier-free $^{32}\text{PO}_4^{3-}$, and $^{35}\text{SO}_4^{2-}$ were added, as indicated in the figures, to the incubation medium.

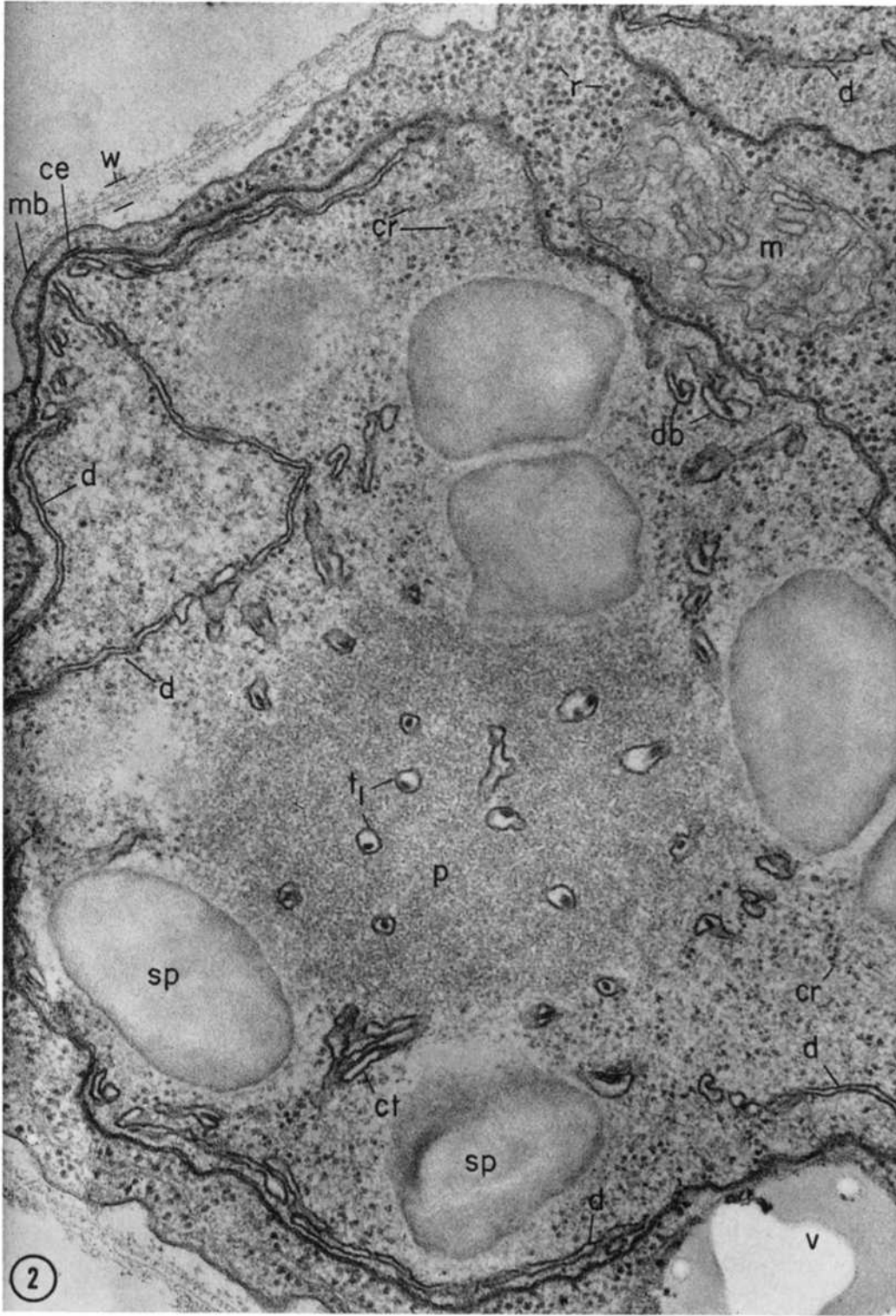
All micrographs show sections of *Chlamydomonas reinhardtii* cells fixed in 2% OsO_4 in 0.1 M phosphate buffer (pH 7.2), embedded in Epon, and sectioned at ≈ 500 Å with diamond knives. The sections were doubly stained with UO_2 acetate and Pb citrate and micrographed in a Siemens Elmiskop I.

General Abbreviations

ce, chloroplast envelope; *cm*, chloroplast matrix; *cr*, chloroplast ribosomes; *d*, disc; *db*, bent or folded over disc; *dc*, dictyosome; *m*, mitochondrion; *mb*, cell membrane; *o*, osmophilic globule; *p*, pyrenoid; *r*, cytoplasmic ribosomes; *sg*, starch granules; *sp*, starch plates; *t*, pyrenoid tubules; *v*, vacuole; *w*, cell wall.

FIGURES 2 and 3 Control cells kept in the dark for the duration of the greening process (9 hr). Batch-type culture grown in the dark for six generations. Chlorophyll concentration: $0.4 \mu\text{g}/10^7$ cells.

FIGURE 2 Section cutting through the base of the chloroplast cup and showing a large pyrenoid (*p*) traversed by tubules (*t*), and surrounded by large, biconvex, or irregular starch plates (*sp*). The plastid contains only a few disc remnants (*d*) most of which run parallel to the envelope (*ce*). A few short, bent over, partially fused discs (*db*) are seen scattered throughout the plastid matrix. Tubules connecting the pyrenoid system with the disc remnants are marked *ct*. The plastid matrix contains a population of ribosome-like particles (*cr*), smaller and less concentrated than cytoplasmic ribosomes (*r*). The hole in the vacuole marked *v* was left behind by dense granular content lost during sectioning (see Figs. 3 and 12 in the companion paper). $\times 58,000$.



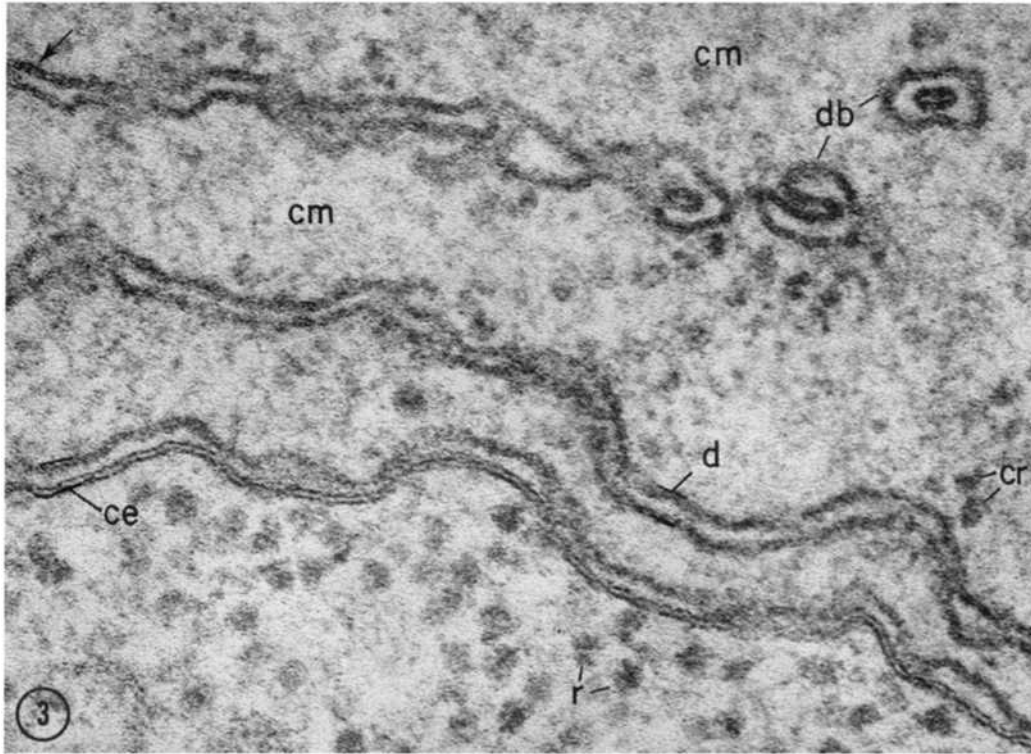


FIGURE 3 Small field at the periphery of the plastid to illustrate the persistence of a normal chloroplast envelope with its two characteristically different membranes, and the presence of a few isolated profiles of disc remnants either elongated (*d*) or short and bent (*db*). The arrow points to a stretch in which the disc membrane appears stratified. Chloroplast ribosomes are marked *cr*, and cytoplasmic ribosomes *r*. $\times 190,000$.

Cell Greening (*Chyd* \rightarrow *Chyl*)

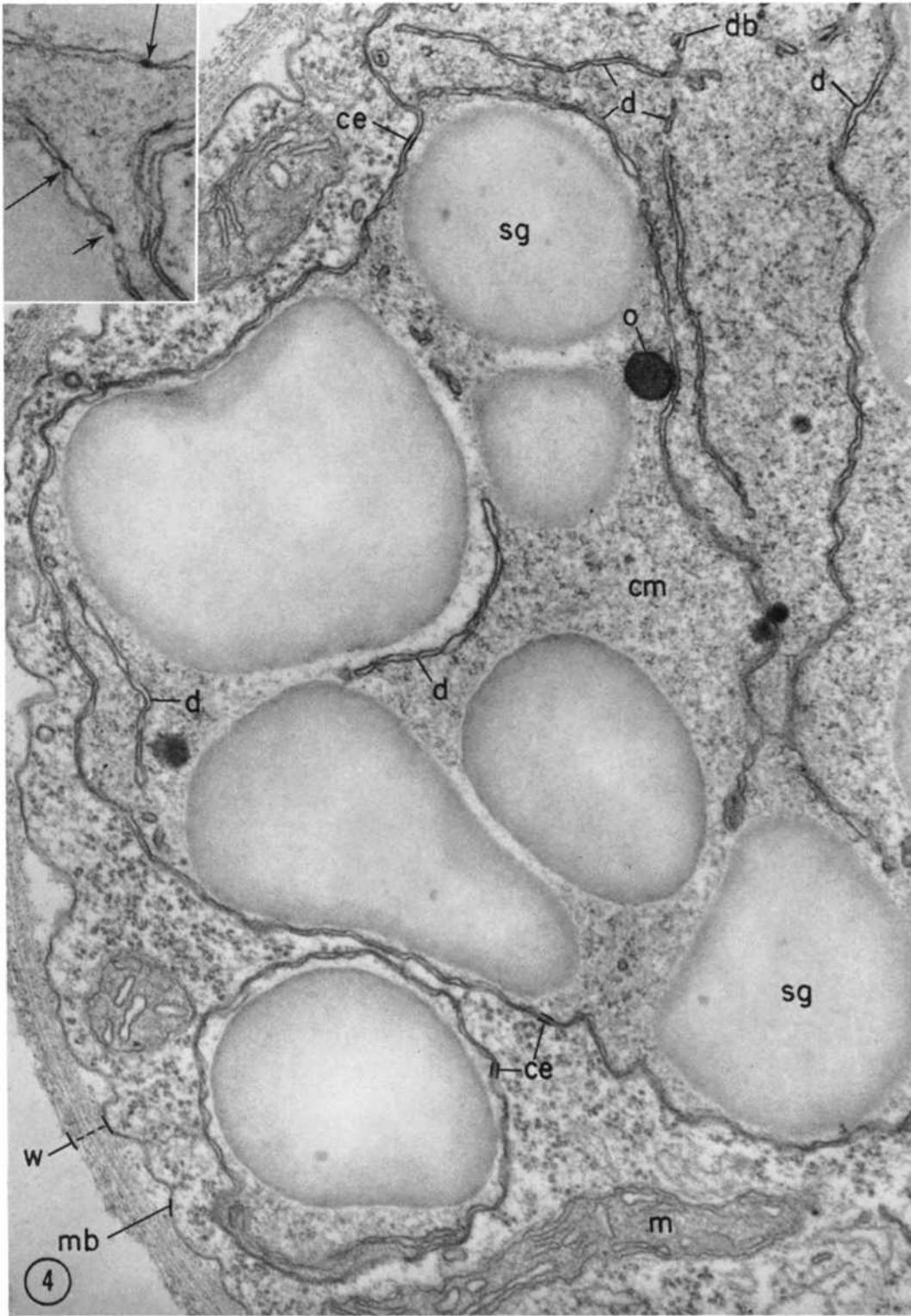
This was performed as described in the companion paper (22). In experiments involving alternating light and dark treatment, *chyd* cells were illuminated for different periods of time and then directly transferred to dark conditions, usually obtained by wrapping the vessels in aluminum foil.

Enzyme Activities Assayed

This was done as previously described (22). In addition, light-induced photooxidation of cytochrome *f* was measured as follows: *chyd* cells were allowed to green under standard experimental conditions at a concentration of $\approx 7.0 \times 10^6$ /cells/ml; aliquots were taken at different times, and their cells were

FIGURES 4-19 Changes in plastid morphology during the greening process of mutant cells grown for six generations in the dark in batch cultures, resuspended in fresh medium at a concentration of 6.5×10^7 cells/ml, and then exposed to light for the times indicated for each figure.

FIGURE 4 Cell after 3 hr of illumination. Chlorophyll concentration: $3.5 \mu\text{g}/10^7$ cells. Long profiles of meandering discs (*d*) are seen not only along the plastid envelope (*ce*) but also around starch grains (*sg*). Osmiophilic globules are marked *o*. In the inset, the long arrows point to regions in which these profiles are reduced to a single dense band which may represent the fusion of two short discs or the obliteration of a disc fenestra. A dense mass (short arrow) appears at the tip of a disc profile. $\times 43,000$; inset: $\times 52,000$.



washed in 0.1 M phosphate buffer, pH 7.3, and resuspended in the same buffer to a final concentration of 2.4×10^7 cells/ml. The difference in absorbancy between 554 and 540 m μ was recorded with a double-beam spectrophotometer while the cells were kept either unexposed or exposed from one side to red light (680 m μ). The actinic light was saturating, in that the response was proportional to cell concentration. The results were expressed as the increase in OD units of the absorbancy difference (554 - 540 m μ) caused by illumination with red light. The increases were calculated from an average of five to seven responses for each time point whenever these responses were stable for 10-15 sec and were reproducible. The total content of cytochrome *f* of aliquots taken at zero time and after 8 hr of illumination was determined in acetone-extracted cells as described (22). Chlorophyll concentrations were measured in 80% acetone extracts (23).

Chemical Determinations

For the estimation of protein, lipid, and RNA, the Schneider procedure (24) was followed: whole cells or homogenates were precipitated, and the precipitates washed five times with 10% TCA,¹ and finally resuspended in cold 5% TCA. For nucleic acids extraction, an aliquot was heated to 90° for 20 min, and the extract was used to determine RNA by the orcinol procedure (25) and RNA radioactivity by ³²P counting. When the amount of starch in the sample exceeded by far the amount of RNA (weight ratio ≥ 5), the orcinol method was inaccurate. Since determinations of RNA radioactivity can be affected by polyphosphate contamination, the original cold TCA precipitate was tested for the presence of polyphosphate (26) by hydrolysis in 1 N H₂SO₄ at 100° for 20 min, followed by extraction of the inorganic phosphate produced with molybdate reagent in isobutanol-benzene. It was found that the polyphosphate contamination, as measured by this method, accounted for 10% or less of the total ³²P content of the extract.

Lipid was extracted from the TCA precipitate with chloroform-methanol (2/1 by volume). The organic phase was removed and washed with an equal volume of 0.1 N HCl so as to precipitate traces of extracted protein (27). Aliquots of the lipid extract were used to measure total phosphorus (28), and radioactivity incorporated as ³²P, ¹⁴C (when acetate-¹⁴C was used), or ³⁵S. Neutral lipid was separated by drying in vacuo an aliquot of the extract, redissolving the residue in chloroform and passing the solution through a column of activated silicic acid suspended in chloroform (29). The neutral lipid fraction thus obtained contained also pigments.

¹ The abbreviations used in this paper are the same as in reference 22.

Aliquots of extracted lipids labeled with ¹⁴C were chromatographed on two-dimensional silicic acid-impregnated paper as described (22, 30): radioautograms of the chromatographs were obtained on no-screen Kodak films. For quantitation, the known spots (cf. 22) from chromatograms of lipid extracted from a known amount of cells were cut out of the paper, extracted three times with 0.5 N HCl-methanol, the extracts combined, and aliquots counted. Three extractions were necessary for us to remove essentially all the radioactivity.

Protein was measured by the procedure of Lowry et al. (31), and protein radioactivity was determined by measuring the amount of ¹⁴C incorporated, from acetate-¹⁴C or leucine-¹⁴C, into a lipid-, starch-, and nucleic acid-free TCA precipitate. The starch content was estimated as described earlier (22).

Specimens for Electron Microscopy and Estimation of Membrane Indices

These performed as described in the preceding paper (22), except that measurements were done on prints at final magnification of 40,000 and on an average of 900 cm² of chloroplast area for each time point.

For radioautography, cells were incubated in the growth medium either in the dark or light for different periods of time, then washed and resuspended in the same medium without acetate-¹H and pulsed with acetate-³H for 5 min. The incorporation was stopped by dilution with acetate-¹H followed by washing (~5 min), fixation with OsO₄, and embedding, as described (22). Thin sections (~600 Å) were covered with Ilford L-4 emulsion and developed after a 65-day exposure following the procedure of Caro and van Tubergen (32). Electron micrographs were taken at original magnification of 12,000 and enlarged photographically to 40,000. Areas from micrographs of individual cells corresponding to nuclei, cytoplasm (including mitochondria), chloroplast matrix, and lamellae, starch grains, and pyrenoids were cut, weighed, and the number of grains in each fraction counted. The results are expressed as grains per fraction weight for each subcellular component investigated. A total of 600-700 grains were counted for each time point.

All chemicals used were of analytical grade. ¹⁴C- and ³H-precursors were purchased from Radiochemical Center, Amersham, England, and ³⁵SO₄²⁻, and carrier-free ³²PO₄³⁻ from Volk Radiochemical Company, Skokie, Ill.

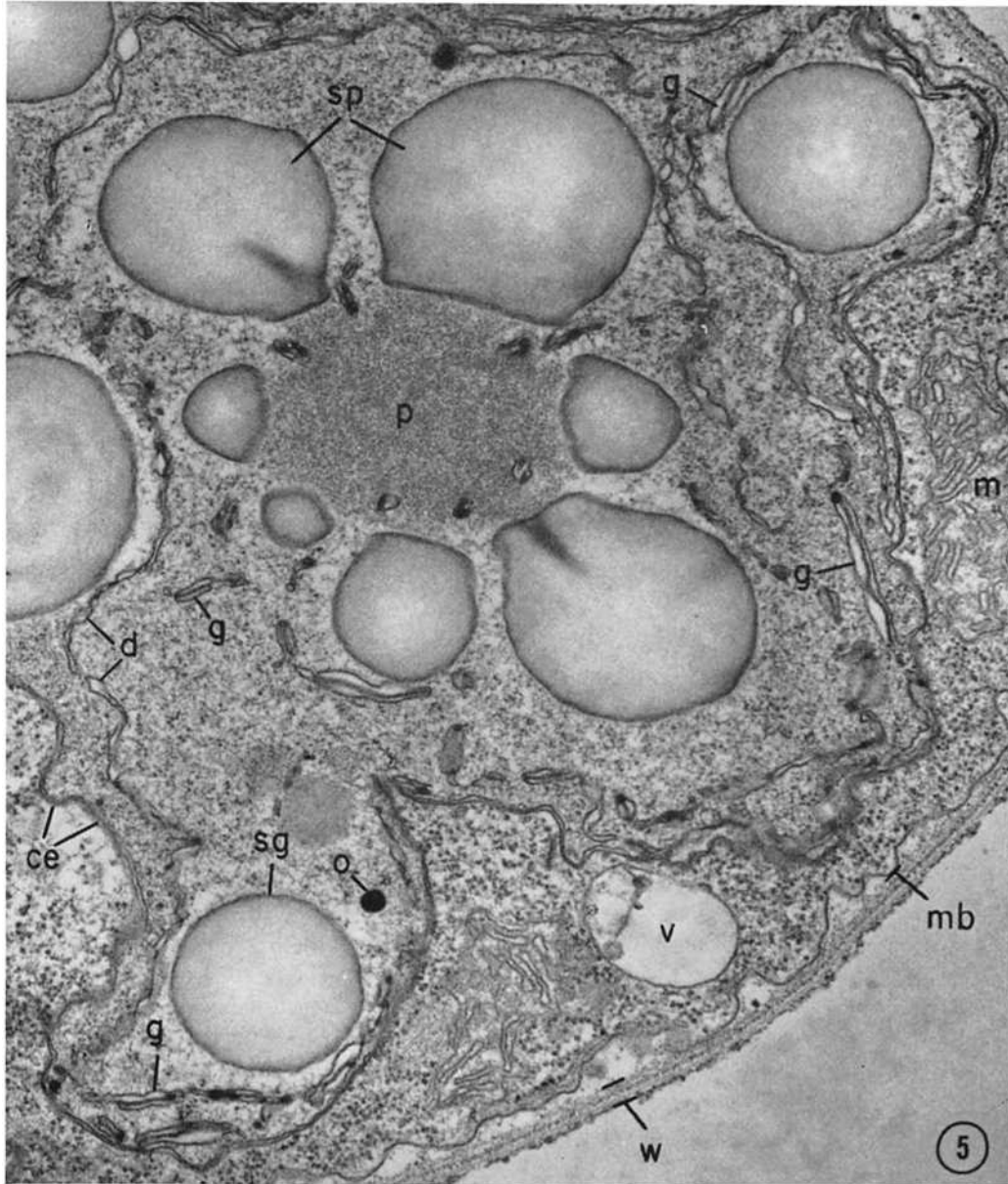
RESULTS

Chlorophyll Synthesis

When chyd cells were exposed to continuous illumination, a rapid synthesis of chlorophyll oc-

curred and followed a course that can be resolved into: (a) an initial lag period defined as the time necessary to reach a constant rate of synthesis, and (b) a period of synthesis at constant rate. The

length of the lag period varied from 2.5 to 4.5 hr, the rate of synthesis gradually increasing during this phase. This lag period can be considerably shortened (to ~45 min) by starting with chyd



FIGURES 5 to 7 Cells exposed to light for 4.5 hr. Chlorophyll concentration: $4.5 \mu\text{g}/10^7$ cells.

FIGURE 5 Section through the base of the plastid showing a small pyrenoid (*p*) surrounded by large plane-convex starch plates (*sp*). In addition to long, irregular disc profiles (*d*) (some of which have the appearance of strings of vesicles connected by dense bands), the plastid contains a number of minimal grana (*g*) each formed by two discs fused side to side and frequently dilated. $\times 36,000$.

cells having larger amounts of chlorophyll (greater than $1 \mu\text{g}$ chlorophyll/ 10^7 cells) or by varying the cell concentration and illumination intensity (22). The linear phase of chlorophyll synthesis continued for 4–5 hr, by which time the levels of chlorophyll per cell were 70–80% of those of logarithmically grown chyl cells (Fig. 1). Further chlorophyll synthesis continued then at a decreasing rate for 2–4 hr, and finally leveled off at 95–100% of the content of chyl cells. It was found that the cells divide in the culture only 2–4 hr after this final chlorophyll concentration is reached.

Morphological Changes during Greening

The main morphological changes induced by illumination were confined to the chloroplast, the other cellular organs being much less affected. The general morphology of the dark-grown cells has already been described in the preceding paper (22). Concomitantly with the synthesis of chlorophyll, there was an increase in the number of membrane profiles present within the chloroplast (Figs. 2–13), and a change in their pattern of organization. The mixture of many circular and few elongated profiles which represents the disc remnants of a typical chyl cell chloroplast (Figs. 2 and 3) was progressively replaced by elongated profiles of considerable length, indicating the gradual transformation of vesicles and tubular networks into regular discs (Fig. 4). Some of the latter were bent over in “U”-shaped profiles. The tips of these elongating profiles as well as the bending points of “U”-shaped profiles were frequently marked by small, globular, or irregular masses of high density (Figs. 4, 6, and 7) which in three dimensions correspond to focal thickenings of the disc rims. After 3–4.5 hr of illumination, many elongated profiles appeared interrupted by dense bands or masses (Figs. 4–6) which could represent either the elimination of residual fenestrae in a disc remnant, or the end-to-end fusion of small discs. Although elongating profiles were regularly found in the immediate vicinity of the chloroplast enve-

lope (Figs. 4–7), there was no indication that new discs form by budding from, or invagination of, the inner (dislike) membrane of the envelope.

After 5 hr of light exposure, the growing discs showed a marked tendency to align in parallel and to form pairs in which they appeared either separated by a variable interspace (Figs. 8 and 9), or closely apposed so as to form minimal, two-disc grana (Fig. 5). Examples of transition from one type of relationship to the other were frequently encountered at this time (Figs. 7–9).

Grana formation became evident at chlorophyll concentrations $>5\text{--}6 \mu\text{g}/10^7$ cells (cf. Fig. 20). Pairing without extensive fusion prevailed up to 5 hr (Figs. 8 and 9), but by 6.5 hr most discs appeared stacked and fused into grana (Fig. 12). Once produced and paired in the light, the discs could proceed to form grana in the dark (Figs. 10 and 11). After 9–10 hr of illumination, the disc system of the chloroplast approached or reached the appearance it has in typical chyl cells (Figs. 13 and 14).

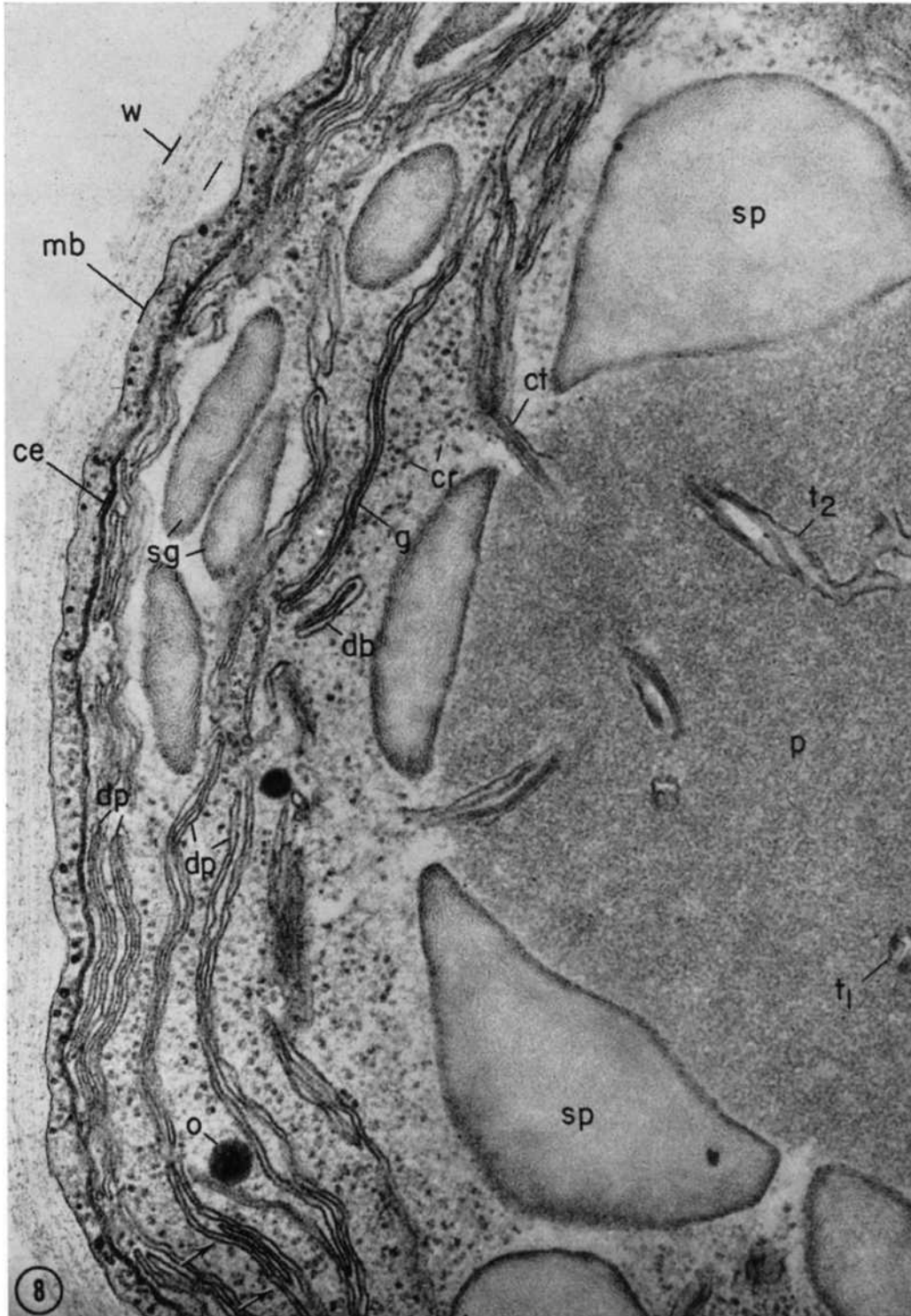
During greening, the discs began by increasing in size in two dimensions and then fused side-to-side with their neighbors. In the process, many of them bent over and fused once (Fig. 11) or even twice (Fig. 18), but most of them remained isolated, flattened sacs whose intradisc space did not communicate with that of its neighbors. Occasionally, however, appearances were found which could be explained only by bending over, and which suggested that branching or rim-to-side fusion or side infolding (Figs. 15–17) might occur during disc biogenesis.

Throughout the greening process, connections between the tubular system of the pyrenoid and the developing discs and grana were maintained. In fact, it was during greening that the clearest examples of grana-tubule continuity and of tubule ridges-grana partitions continuity (Fig. 19) were encountered.

During the latter stages of the greening process, the formation of membranes keeps pace somewhat

FIGURES 6 and 7 Small fields along the anterior rim of the plastid cup illustrating: the increase in number and size of discs (*d*); the frequent bending (*db*), which affects long as well as short discs; the presence of dense, irregular masses along the disc rims the high frequency of disc profiles with partially obliterated lumina (arrows); and the presence of a few minimal grana (*g*). Fig. 7 suggests that disc growth, pairing, and final fusion can occur concomitantly on successive segments of the same discs. *d'* marks an obliquely sectioned disc, and *ee* the chloroplast envelope. Fig. 6: $\times 72,000$. Fig. 7: $\times 60,000$.

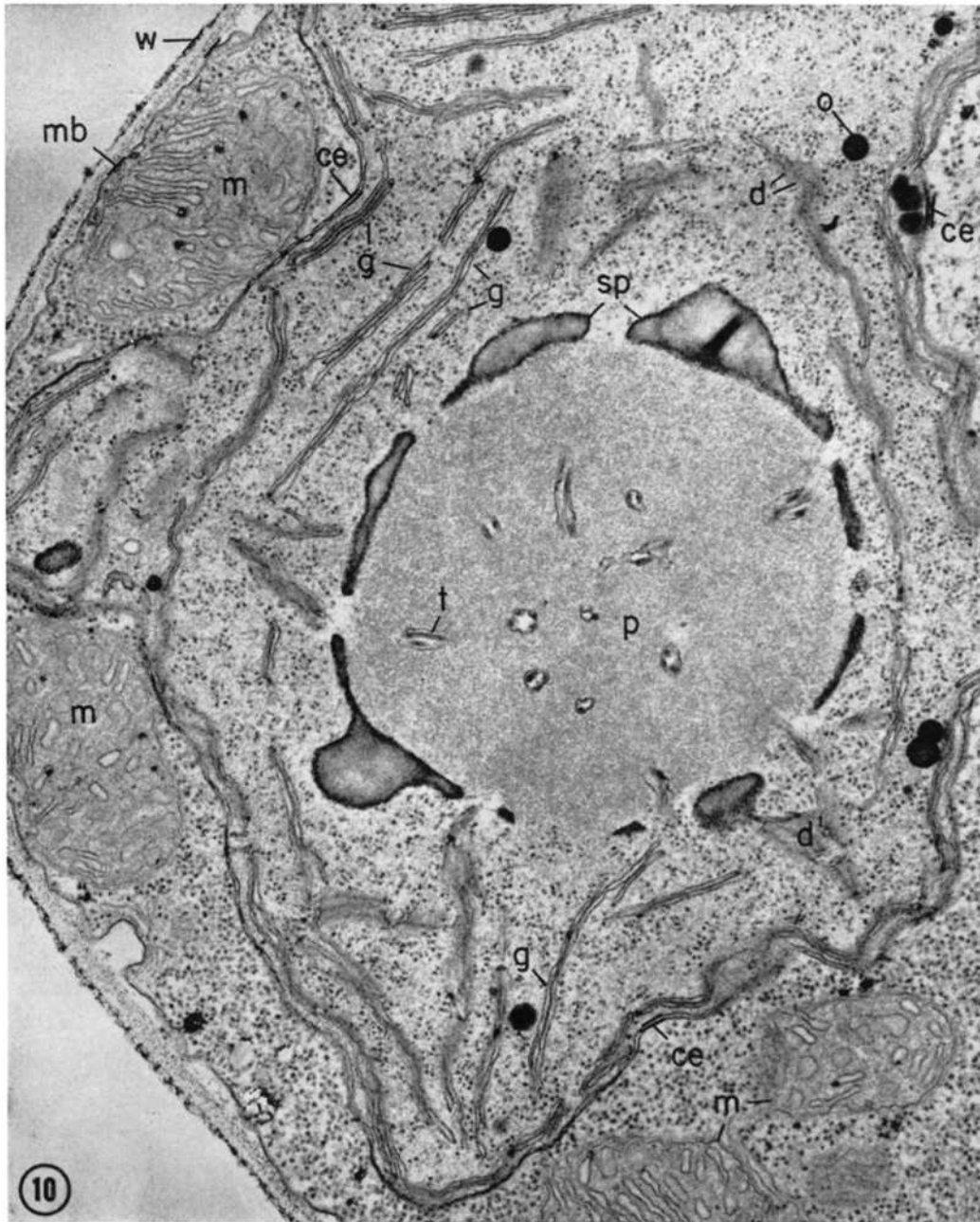




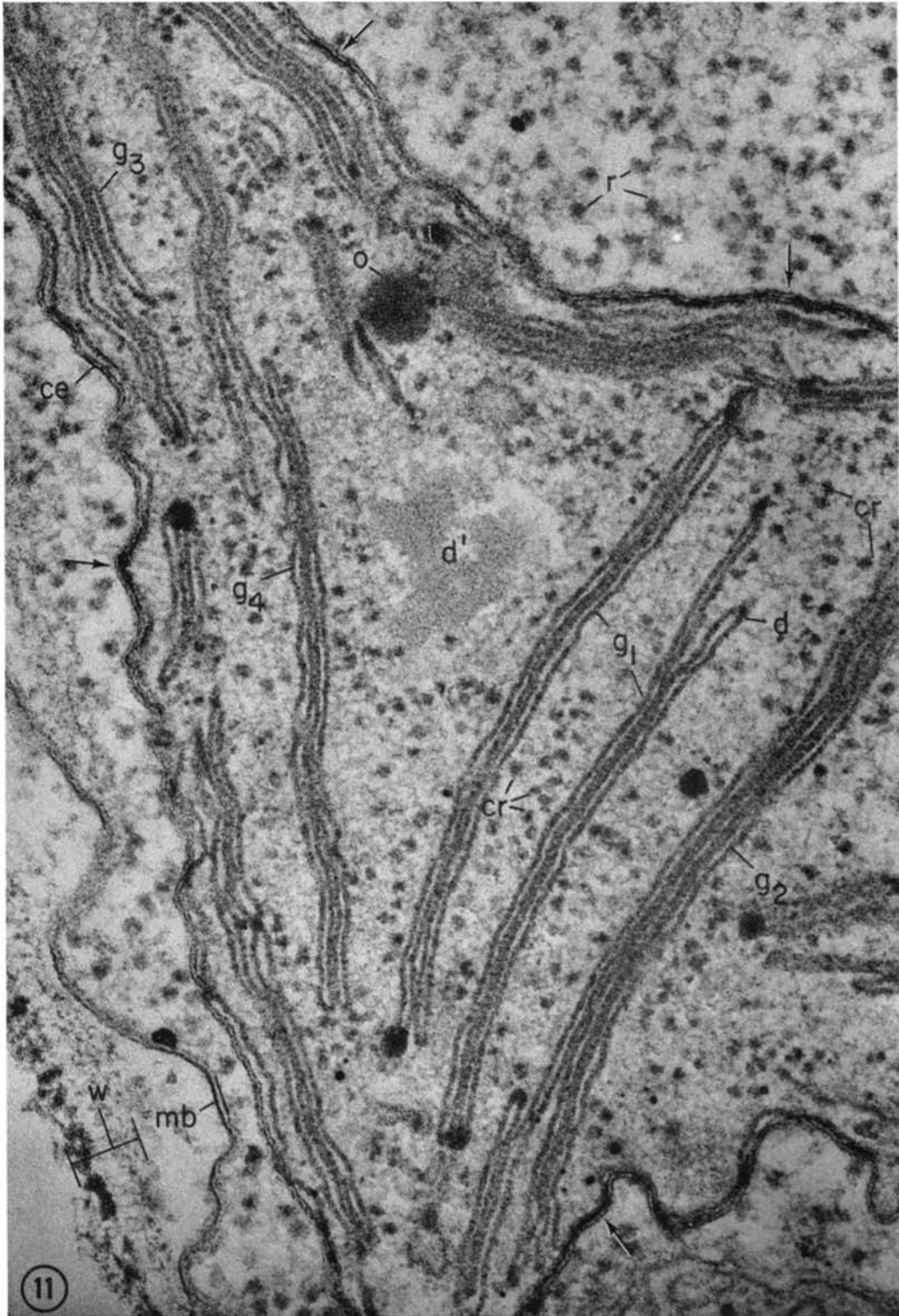
FIGURES 8 and 9 Cells after 5 hr of exposure to light. Chlorophyll concentration: $5.3 \mu\text{g}/10^7$ cells Fig. 8 shows a small field at the periphery of the pyrenoid (*p*) and Fig. 9 an even smaller one at the anterior rim of the chloroplast cup. Both figures illustrate the increase in number and length of disc profiles (*d*), the extensive disc pairing (*dp*), the still limited extent of disc fusion into minimal grana (*g*), and the sharp in-



crease in the ribosome (*cr*) population of the chloroplast. The arrows in Fig. 8 point out a stack of three stacked, but not yet completely fused discs. A clear example of transition from peripheral to central type of completely fused discs. A clear example of transition from peripheral to central type of pyrenoid tubule appears at t_2 . A normally sectioned peripheral tubule appears at t_1 . Fig. 8: $\times 60,000$. Fig. 9: $\times 96,000$.



FIGURES 10 and 11 Cells exposed to light for 5 hr, then incubated in the dark for 2 additional hr. Chlorophyll concentration: $5.3 \mu\text{g}/10^7$ cells, i.e., no increase over level reached after 5 hr of illumination. There is no further increase in disc number and size, but fusion of paired discs has progressed to the point at which nonfused, paired discs are rarely seen in the posterior (Fig. 10) as well as the anterior (Fig. 11) part of the chloroplast. FIGURE 11 shows: minimal (two-disc) grana at g_1 ; a three-disc granum at g_2 (note that the left disc is bent over and fused to itself at the lower end of this granum); an imperfectly fused three-disc granum at g_3 , and a disc connecting two minimal grana at g_4 . At d' appears the grazed surface of a disc. In Fig. 10, all grana are marked g , irrespective of the number of discs they contain. Note in Fig. 11 the frequent, focal fusion (arrows) of the two membranes of the chloroplast envelope. Note also in Fig. 10 the thin deeply stained plates of apparently newly synthesized starch (sp) around the pyrenoid. Both figures demonstrate high concentration of chloroplast ribosomes (cr). Fig. 10: $\times 36,000$. Fig. 11: $\times 120,000$.



For Legend, see page 564.

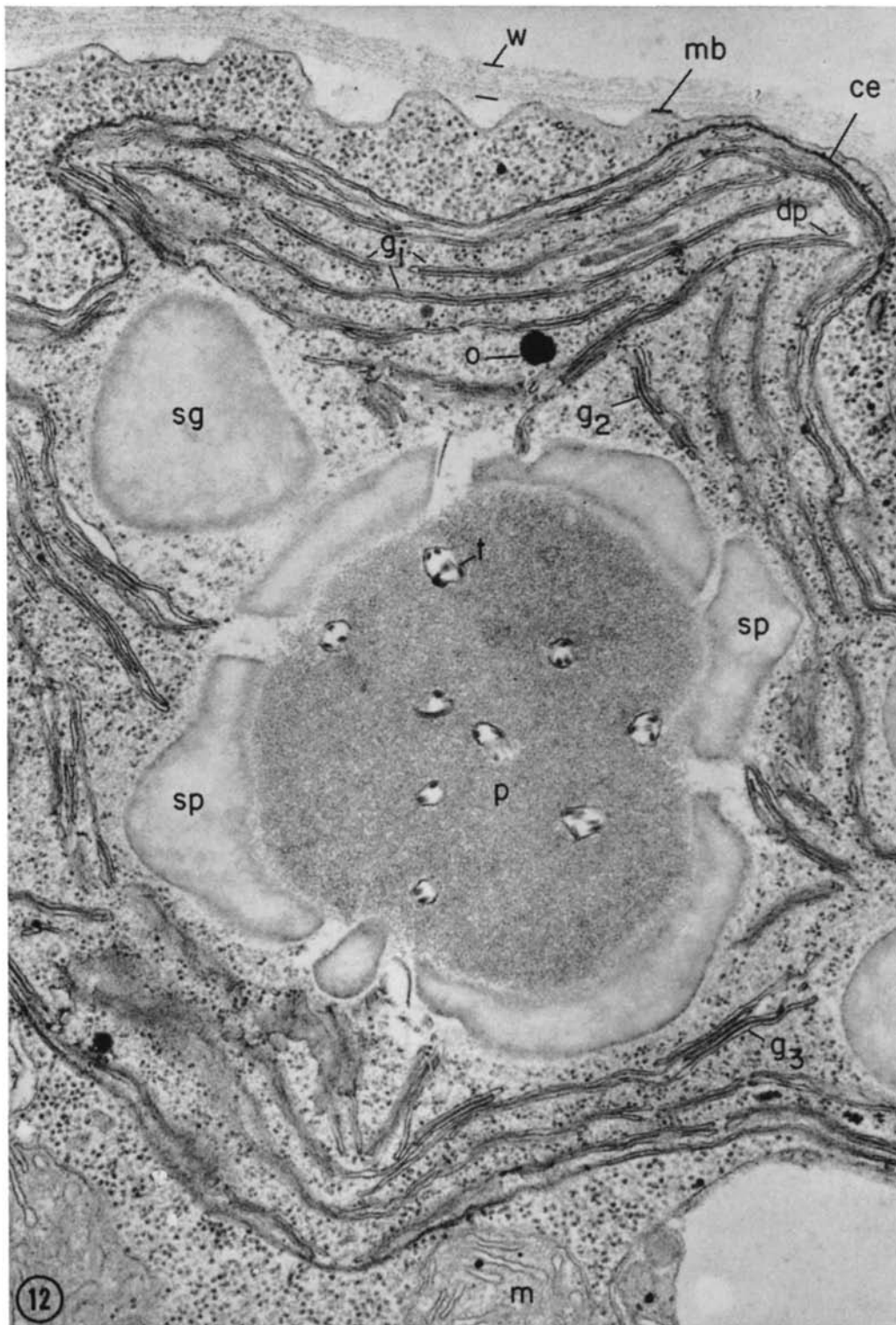
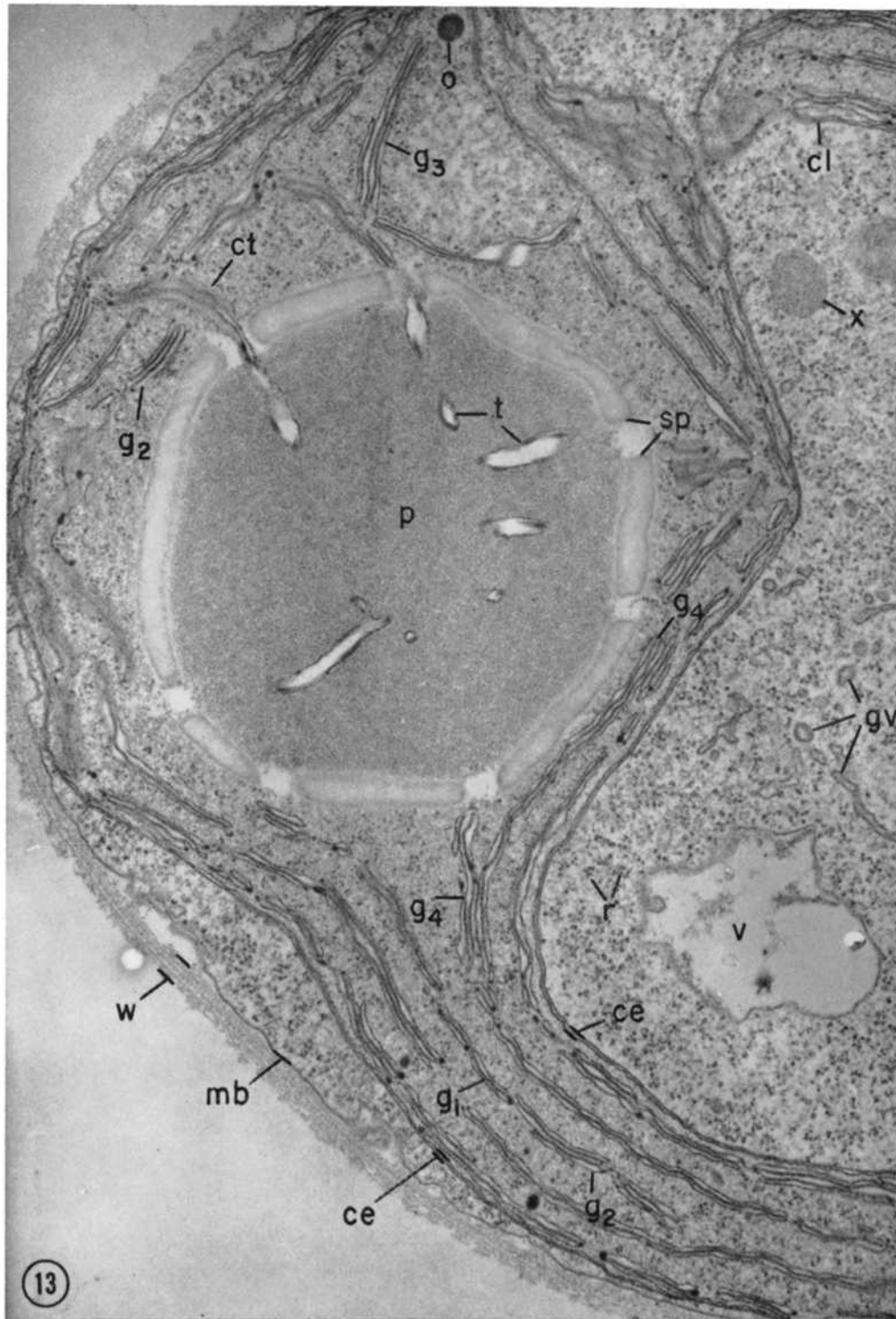


FIGURE 12 Cell exposed to light for 6.5 hr. Chlorophyll concentration: $8.8 \mu\text{g}/10^7$ cells. Section through the base of the chloroplast cup showing a further increase in disc number and in grana number and size: g_1 , g_2 , and g_3 mark grana with two, three, and four discs, respectively. Paired, nonfused discs (dp) are only occasionally seen. $\times 42,000$.



FIGURES 13 and 14 Cells exposed to light for 9 hr. Chlorophyll concentration: $17 \mu\text{g}/10^7$ cells.

FIGURE 13 Section through the base of the chloroplast cup, showing a large pyrenoid (*p*) surrounded by thin, plane starch plates (*sp*), an isolated profile of a chloroplast lobe (*cl*), and part of the side wall of the plastid cup (in the lower right corner). There is a general increase in the size and regularity of the grana which range from two (*g₁*) to five (*g₄*) discs. *gv*, Golgi vesicles; *x*, body already mentioned in the legend of Fig. 5 in the companion paper. $\times 31,000$.

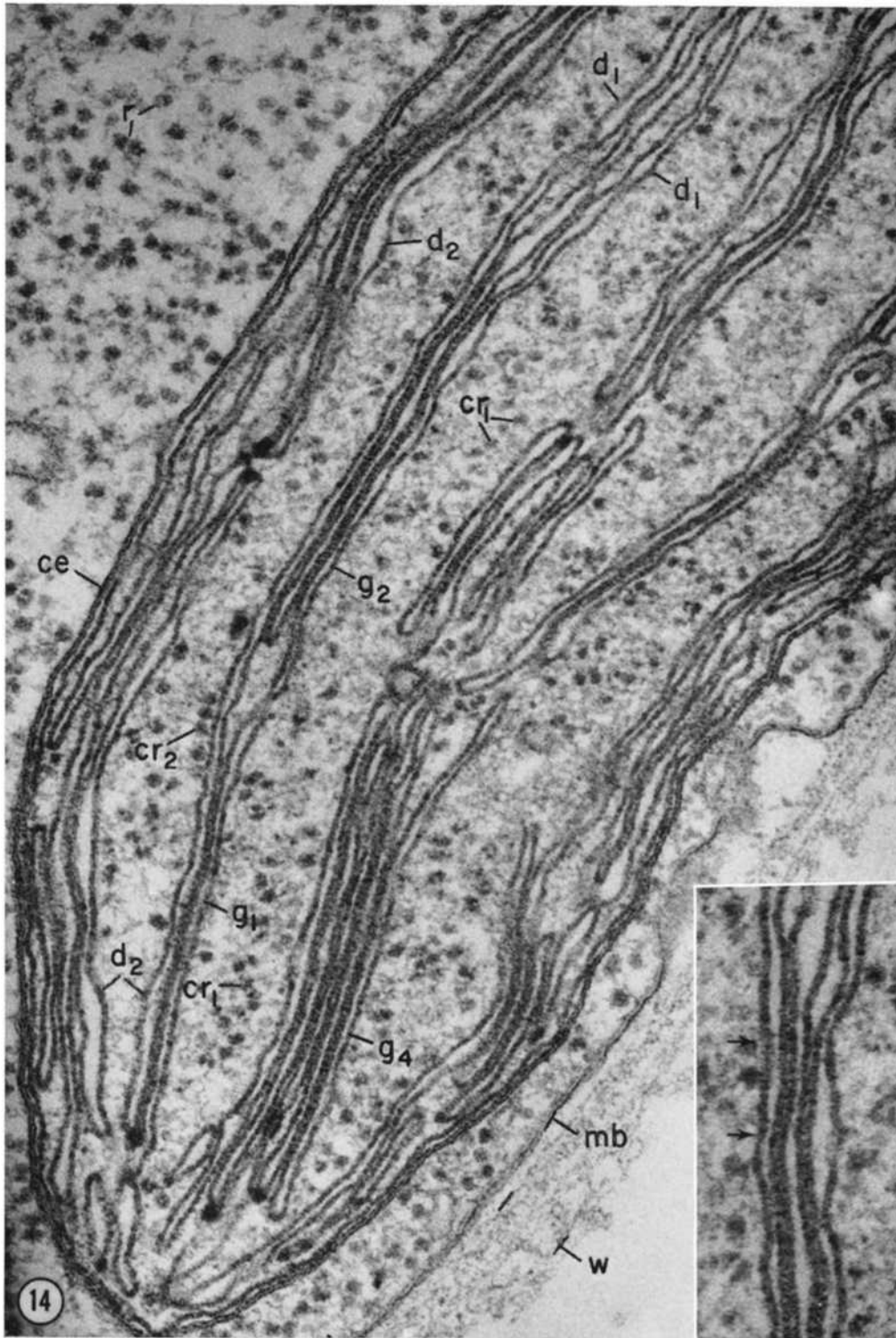


FIGURE 14 Anterior rim of the chloroplast cup. The micrograph illustrates the extensive persisting variation in the size of grana which ranges from two (g_1) to five (g_4) discs, and the frequent, irregular dilation of discs not yet fused into grana (d_1) or marginally located in grana (d_2). Note between g_1 and g_2 transition from a two-disc to a three-disc granum by intercalation of an additional disc. The ribosome population of the chloroplast is still high. Some particles occur in clusters (cr_1); others appear to be aligned on disc membranes (cr_2). The inset shows at a higher magnification part of g_2 to illustrate the suggestion of periodicity in phase (arrows) in the fusing membranes of adjacent discs. Fig. 14: $\times 90,000$; inset: $\times 180,000$.

with chlorophyll synthesis (Fig. 20), but there is no lag in membrane formation, as there is in chlorophyll synthesis, during the early stages of the greening. The apparent linearity of membrane formation with time (Fig. 20) could be due to the persistence of a small number of chloroplast vesicles in the chyd cells. However, the fusion of membranes into grana is distinctively delayed, and, thus, there is no correlation between chlorophyll synthesis and photosynthetic activity, on the one hand, and grana formation on the other (Figs. 1 and 20).

The degree of organization of the eyespot appeared to increase with the progress of the greening process, but the cell population was not homogeneous in this respect.

Along with the formation of the chloroplast lamellar system, the population of cytoplasmic ribosomes as well as the ribosome-like particles in the chloroplast increased, while the size and number of starch granules decreased. The size of the starch plates around the pyrenoid decreased also, but in such a way as to preserve the contact with the pyrenoid core (Figs. 8, 9, and 12). The population of ribosome-like particles of the chloroplast reached its higher concentration between 5 and 7 hr of illumination (Figs. 8 and 12).

Enzymatic Changes

During the regeneration of the lamellar structures of the chloroplast (Fig. 20), photosynthetic activity expressed as photoreduction of DCI (Hill reaction) (Fig. 1), PPN reductase activity (Fig. 21), and oxygen evolution (Fig. 22) was reestablished as soon as measurable amounts of chlorophyll were present. At the same time, only relatively small changes occurred in the already present RuDP carboxylase and FDPase activities (Table I). The activity of NADP-linked G-3-P dehydrogenase (Table I) increased in both the dark control and illuminated chyd cells, but with relatively large variations from one batch of cells to another. Although the total amount of cytochrome *f* remained practically constant during the greening period, the fraction becoming oxidized upon excitation with red light (680 m μ) increased parallel to chlorophyll formation (Fig. 23). While the PPN reductase activity and oxygen evolution followed closely the chlorophyll synthesis (Figs. 21 and 22), the specific activity (per mg chlorophyll) of the Hill reaction was not always constant. In a few experiments, it was initially high and de-

creased only slightly when the chlorophyll level reached about 80% of the normal level. In other experiments, in which the rate of chlorophyll synthesis was low, the specific activity of the Hill reaction was somewhat lower at the beginning (1st 2-4 hr), increasing subsequently to the usual range of values (1.5-2.0 m μ moles DCI/ μ g chlorophyll). Also, it was noted that when aliquots were removed from a greening culture during the lag period of chlorophyll synthesis and tested in light and dark for their oxygen consumption on acetate, this rate was higher when examined in the dark, and furthermore, the ratio, O₂ consumption (light)/O₂ consumption (dark) constantly decreased during the greening. This decrease was due, at least in part, to increasing oxygen evolution in the light, although net O₂ evolution was not yet detectable during this lag period (Fig. 22). The significance of the changes in enzyme concentration noted in Table I is not known, but it should be pointed out once again (cf. reference 22) that these enzymes involved in photosynthesis were already present in rather large amounts in the chyd cells, and that even the large increase in dehydrogenase activity (Table I) occurred somewhat in the dark, and in the light this change was much less than that of photosynthetic activity (Figs. 1, 21-23).

Changes under Discontinuous Greening (Alternating Light and Dark Exposure)

When chyd cells were incubated for different periods of alternating light and dark, chlorophyll synthesis stopped immediately upon transfer to the dark, but resumed again upon reexposure to light (Fig. 24), at a rate comparable to that reached at the moment light was shut off. Also, when the Hill reaction was tested, no significant influence of the dark period on the activity was observed (Fig. 25). It has been pointed out that after onset of illumination, the respiration rate on acetate was increased when tested in the dark. These rates were 1.5-two times higher after 2-3 hr of illumination, and remained constant for cells kept further in the dark for 1.5-2.5 hr. At the morphological level, it was observed that no further increase in the amount of membranes was detectable when chyd cells exposed to light were transferred back to the dark. However, it was noticed that discs which had already reached the level of stack formation in the light fused to form grana when further incubated

in the dark (compare Figs. 8 and 9 with Figs. 10 and 11).

Macromolecular Synthesis

Macromolecular syntheses during the greening process were followed by incorporations of $^{32}\text{PO}_4^{\equiv}$, $^{35}\text{SO}_4^{\equiv}$, -acetate ^{14}C and leucine ^{14}C into lipid, protein, and RNA. The pattern of incorporation into protein was the same whether acetate- ^{14}C or leucine- ^{14}C was used, and the pattern of incorporation into lipid was similar for ^{32}P or acetate- ^{14}C . When cells were labeled with ^{32}P or ^{35}S during the last generation in the dark before harvesting, and then exposed for a 6- to 8-hr period to light or dark in unlabeled media, no significant change was observed in the total radioactivity per cell of the lipid, RNA, and protein fractions. Thus, the incorporation of radioactive precursors into these macromolecules can be considered as the result of net syntheses in light or dark, and not mere turnover.

Rapid incorporation of radioactive precursors into protein (Fig. 26), total and neutral lipids (Fig. 27), phospholipids (Fig. 28), galactolipids (Table II), and RNA (Fig. 29) occurred in chyd cells kept in the dark or exposed to light, but all incorporation rates were about twice as high in the light. The time course of incorporation of ^{35}S into sulfolipid was found to be similar to that of ^{32}P incorporation into phospholipid. For chyd cells exposed to light, rates of incorporation increased as a function of time, as in the case of chlorophyll synthesis, while in the dark control, incorporation proceeded at a low constant rate.

In experiments involving alternating light and dark exposures, the response of macromolecular syntheses varied apparently in parallel to the rate of chlorophyll synthesis. After a short period of

exposure to light, and while chlorophyll synthesis was still in the lag phase, transfer to the dark (light suppression) did not affect lipid, protein, or RNA syntheses, which proceeded at a rate equivalent to that of the dark control. After longer exposure to light, however, when chlorophyll synthesis had already reached a linear phase, light suppression causes a significant drop in the rates of macromolecular syntheses. Incorporation even stopped for periods of 2-3 hr, then started again at approximately the rate of the dark control (Figs. 26-29).

Starch Degradation

A possible source of precursors (and energy) for the extensive biosynthetic processes that occur in chyd cells is the large amount of starch, up to 200 $\mu\text{g}/10^7$ cells, which the mutant can accumulate in the dark, especially in the stationary phase of growth (22, 33). When chyd cells were exposed to light, a rapid degradation of starch occurred (Fig. 30) and proceeded from the beginning at a constant rate. This process was strictly dependent upon continuous illumination (Fig. 30), and in all cases, about 40% of the initial starch (40-80 $\mu\text{g}/10^7$ cells) had been degraded before the linear increase in chlorophyll synthesis was achieved. Since there might be a correlation between starch degradation and changes in the length of the lag period of chlorophyll synthesis, mentioned earlier, this phenomenon was looked at more closely. For a given batch of chyd cells, variations in cell concentration (from 1.3×10^6 to 1.5×10^7 cells/ml), and changes in illumination intensity (from ~ 300 to ~ 800 ftc) had a marked effect on the length of the lag phase, but did not influence the rates of chlorophyll synthesis once constant rates were reached (22). Furthermore, neither the length of the lag phase nor the chlorophyll content showed

FIGURES 15-18 Unusual grana formation during the greening process.

FIGURES 15 and 16 show evidence of continuity of the intradisc space among more than two adjacent discs of a granum (three in Fig. 15, four in the imperfect granum in Fig. 16). Such appearances indicate that discs can either branch or fuse side to end, or undergo side infolding to establish continuity of their intradisc spaces. FIGURE 17 apparently illustrates an early stage in such a process. In all three figures, the arrows indicate the sites of unusual disc-to-disc continuity. FIGURE 18 shows an imperfect granum which seems to consist of five discs. Closer inspection shows that it has only three discs (d_1 - d_3) and that the disc marked d_2 bends twice in opposite directions. The course and bending points of its profile marked by arrows. Fig. 15: $\times 120,000$. Fig. 16: $\times 56,000$. Fig. 17: $\times 170,000$. Fig. 18: $\times 100,000$.

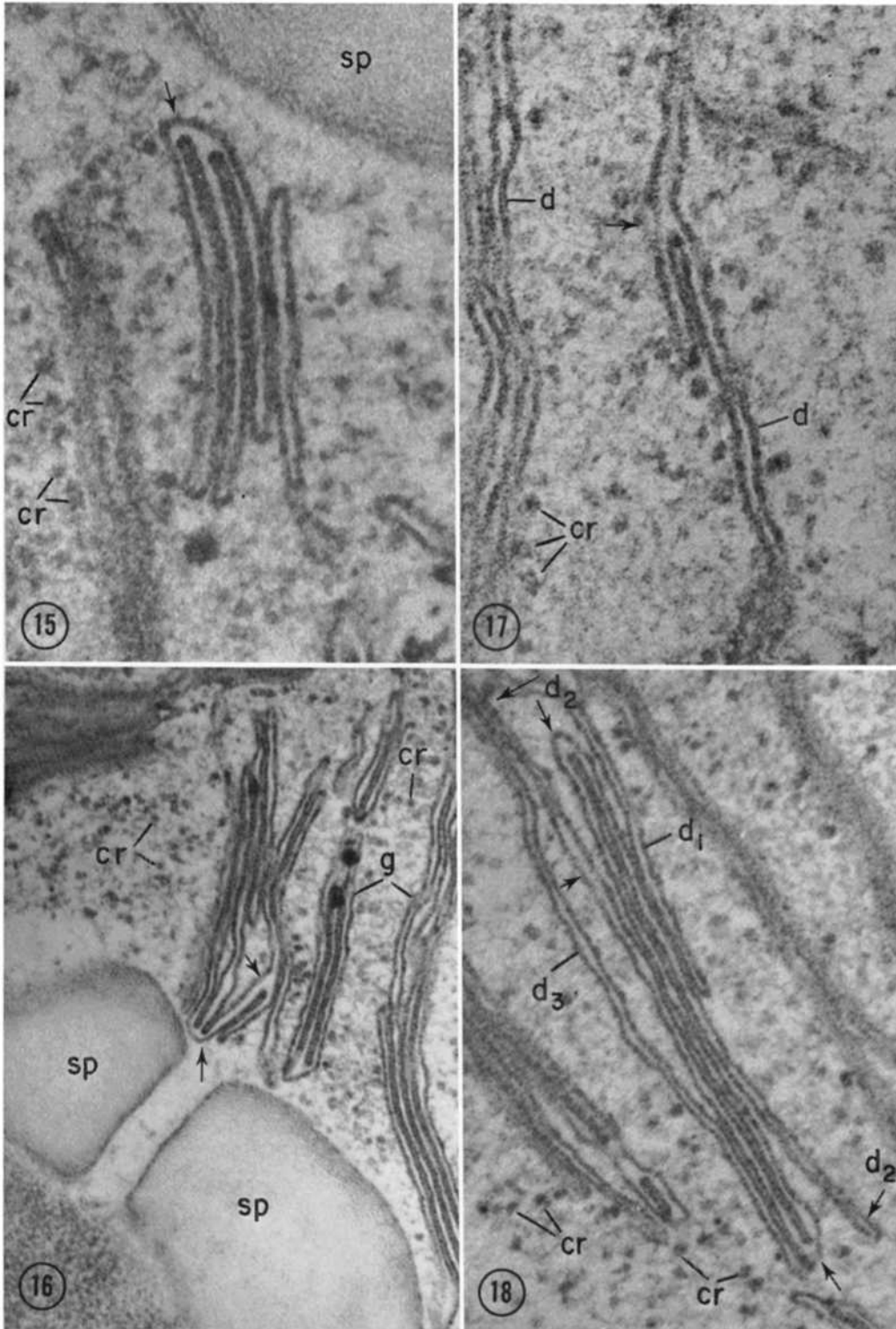




FIGURE 19 Periphery of the pyrenoid (*p*) in a greening cell after 9 hr of light exposure. The micrograph shows developing grana with two, three, four, and five discs (*g*₁-*g*₄). The granum marked *g*₄ is in clear continuity with the large (peripheral type) pyrenoid tubule, *t*₄. Most intradisc spaces are continuous with the tubular lumen, and three of the fused partitions of the granum can be followed into three ridges (or fused infoldings) of the tubule wall. The fourth partition stops at the arrow. $\times 53,000$.

any correlation with the initial rate of starch degradation; in other words, no matter what the length of the lag, the rate of starch breakdown was about the same. However, a correlation was found between the amount of chlorophyll synthesized *de novo* and the amount of starch degraded. The ratio: chlorophyll synthesized/starch degraded, was proportional to the initial chlorophyll concentration, i.e., to the number of previous generations in the dark (Fig. 31).

Radioautography

The experiments described have demonstrated that acetate-¹⁴C is rapidly incorporated by the *Chlamydomonas* mutant. The acetate pool of these cells is rather small since ¹⁴C incorporation is immediately stopped by dilution or washing with acetate-¹²C. Hence, short pulses with radioactive acetate can be obtained for radioautography ex-

periments. For such experiments, cells grown for six to seven generations in the dark in the semi-continuous culture apparatus were incubated in the dark (control cells) or light (greening cells) at a final concentration of 3.6×10^6 cells/ml. Aliquots from each incubate were pulsed for 5 min with acetate-³H (490 $\mu\text{C}/\mu\text{mole}$, 0.5 $\mu\text{mole}/\text{ml}$) at 2.25, 3.75, and 6.25 hr from the beginning of the experiment. Control cells were labeled in the dark and greening cells in the light. At the end of the pulse, samples were fixed and processed for electron microscopic radioautography as described under Methods, while the rest of the aliquot was analyzed for the distribution of radioactivity in protein, nucleic acid, lipid, starch, and residue fractions prepared as indicated in Table III. The results (Table III and Fig. 32) indicate that ~ 65 and $\sim 80\%$ of the radioautographic grains were found over the chloroplast, after 2.25

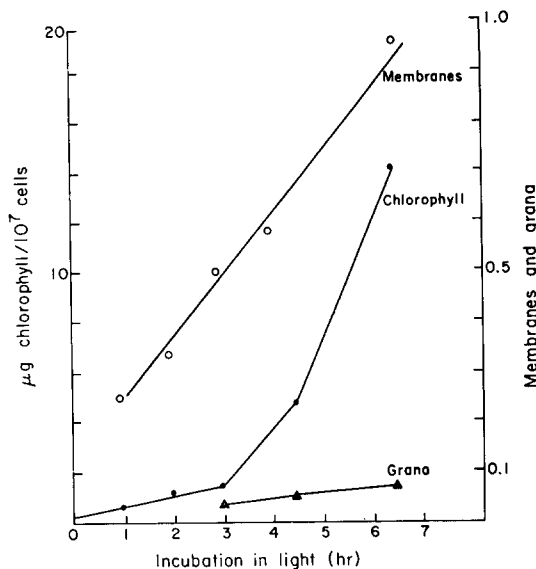


FIGURE 20 Synthesis of chlorophyll and increase in amount of chloroplast membranes. Dark circles, chlorophyll; open circles, membrane index; triangles, grana index. Chyd grown in batch-type culture for 6 days, resuspended in fresh medium at a final concentration of 3.3×10^6 cells/ml, and exposed to light. Samples were taken at different times for chlorophyll measurements and electron microscopy (see Methods).

and 3.75 hr of illumination, respectively, as compared with only $\sim 40\%$ in the 6.25-hr dark control.

DISCUSSION

When chyd cells were exposed to light, no cell division was observed during the first 6–9 hr, even at cell concentrations compatible with logarithmic growth, although a 100–120%² increase in cell count was expected since this interval is the equivalent of at least one generation time for chyl cells. It seems that during the greening process, the pace of nuclear division is geared to that of plastid division, and the latter divides in the light only

² It was previously observed that the generation time for chyd cells is 18–20 hr (22, 33). In the greening experiments mentioned above, an increase of only about 10% in the cell count of the dark control was observed; this is much less than the 30–50% increase expected for the 6–9-hr duration of the experiment. It is possible that this reduction in growth rate was due to the density of cell suspension used in these experiments (three to six times higher than that obtained in the logarithmic phase of growth).

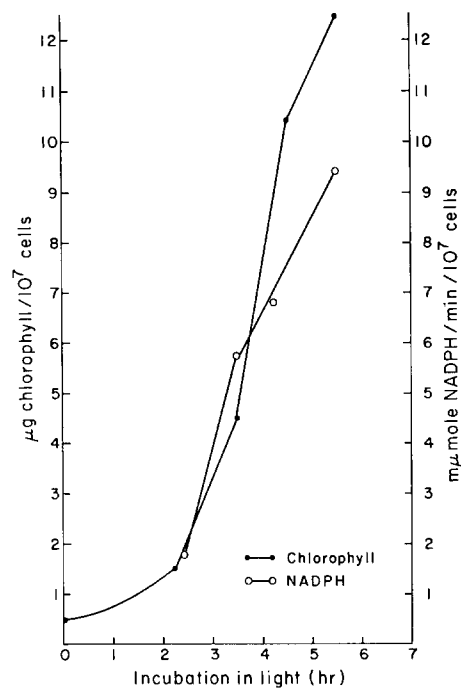


FIGURE 21 Photosynthetic pyridine nucleotide reductase activity during the greening process. Dark circles, chlorophyll; open circles, enzyme activity. Chyd cells grown for six generations in batch culture, resuspended in fresh medium at a final concentration of 1.1×10^7 cells/ml, and exposed to light. Other conditions, as described under Methods.

after reaching full or nearly full chyl characteristics. In this respect, the etiolated *Chlamydomonas* mutant differs from etiolated *Euglena* cells, which multiply while greening and for which a special medium is required to prevent division (5). Extensive cell growth without cell division can also be excluded, since no significant change in cell size was observed in greening *Chlamydomonas* cells. Moreover, the radioautographic experiments showed that most of the macromolecules produced in the process are synthesized in, or become part of, the chloroplast. Hence, it can be concluded that practically all the biosynthetic and morphogenetic activities of the greening cell are related to chloroplast differentiation.

Morphological Changes

Structurally, the growth of the chloroplast disc system in the *Chlamydomonas* mutant differs markedly from that described in many higher plants (1–4, 9, 34) and algae (5–8), in which the

TABLE I
Changes in the Activities of Various Soluble Photosynthetic Enzymes during Incubation in Light and Dark of the Dark-Grown Mutant
 Assays were performed as described previously (22). The homogenate contained $1-2 \times 10^6$ cells for the NADP-linked G-3-P dehydrogenase-, $4-8 \times 10^7$ cells for the FDPase-, and $1-4 \times 10^7$ cells for the RuDP carboxylase assays. The dark-grown cells were prepared as described in the text.

Enzyme	Incubation	Experiment	
		1	2
NADP-linked G-3-P dehydrogenase	0 hr	0.071 μ mole NADPH/1'/ 10^7 cells	0.102 μ mole NADPH/1'/ 10^7 cells*
	6.5 hr-dark	0.121	0.196 †
	7.5 hr-light	0.138	0.306 ‡
pH 8.8 FDPase	0 hr	0.19 μ mole $P_i/30'/10^7$ cells	0.24 μ mole $P_i/30'/10^7$ cells
	6.5 hr-dark	0.15	0.17
	7.5 hr-light	0.16	0.16
RuDP carboxylase	0 hr	4.38 μ moles $CO_2/10'/10^7$ cells	12.1 μ moles $CO_2/10'/10^7$ cells
	6.5 hr-dark	5.43	14.6
	7.5 hr-light	5.77	14.6

* 0.83 μ mole NADPH/1'/mg protein.

† 1.56 μ moles NADPH/1'/mg protein.

‡ 1.98 μ moles NADPH/1'/mg protein.

|| 16.4 μ moles $CO_2/10'/10^7$ cells after 20 generations in the light.

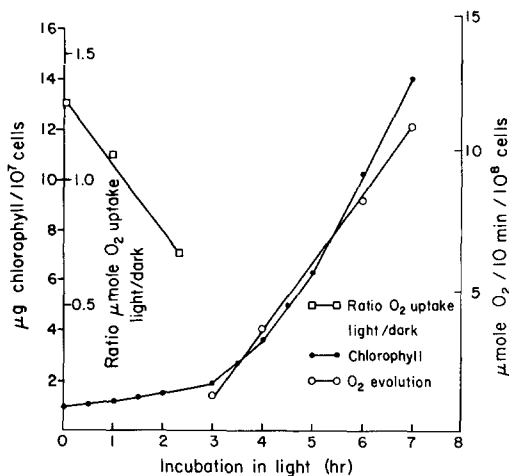


FIGURE 22 Respiration (left) and oxygen evolution (right) during the greening process. Black circles, chlorophyll; open circles, oxygen evolution; open squares, ratio O₂ uptake, light/dark. Cells were taken at different times (left) from the incubation mixture, washed, and resuspended in 3 ml of growth medium at a final concentration of 7×10^7 cells/ml. Their respiration (with KOH in the central well) was measured in a Warburg at 28° in light (500 ftc) and dark. Results are plotted as a ratio of O₂ uptake in light versus dark as a function of time. Initial respiration rate in the dark was 6.7 $\mu\text{moles O}_2/10'/10^8$ cells. Dark-grown cells (right) (six generations in semicontinuous culture) were incubated in the light at a final concentration of 10^7 cells/ml; samples of 10^8 cells in 4 ml were incubated in the light and dark in Warburg vessels in a mixture of 0.07 M KHCO₃ and 0.13 M NaHCO₃ (pH 8.4), and oxygen evolution measured at 28°.

primordia of the developing membranes are formed by an invagination of the inner membrane of the plastid envelope (6, 34); in the *Chlamydomonas* mutant this phenomenon was never observed. In chyd cells, remnants of the chloroplast disc system are always present in the form of a few irregular vesicles, tubular networks, and fenestrated cisternae as well as in the form of a tubular system of the pyrenoid with its extra-pyrenoidal extensions (22). All these remnants may act as primordia during the greening process. In the early stages of greening, some of the apparent elongation of vesicular remnants may be accounted for by their flattening and/or fusion, but further growth must be due to the formation of new membranes. The increase in membrane amount could be achieved by the appearance of new centers of assembly, or by the insertion of molecules or molecular aggregates into preexisting membranes. In the latter case, insertion could occur at many sites distributed at random over the membrane surface, or be localized at specific sites, possibly in connection with the peripheral extensions of the pyrenoid tubular system or with the dense, irregular masses found at the periphery of developing discs. These dense masses, which were observed at all time points during the greening process, are similar in density to the "osmiophilic globules" described in the chloroplast of higher plants, but differ in size, shape, and location, being preferentially situated along the rims of elongated, unpaired, and especially paired and stacked discs. The os-

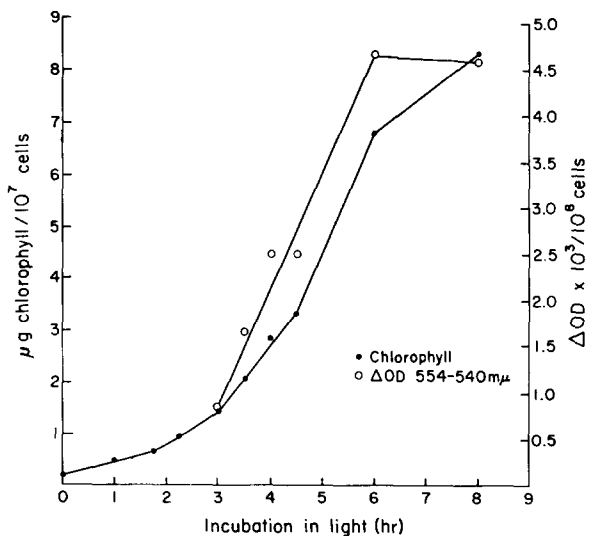


FIGURE 23 Photooxidation of cytochrome *f* induced by red light (680 m μ) excitation of whole cells during the greening process. Open circles, photooxidation of cytochrome *f*; dark circles, chlorophyll. Cells grown in the dark for six generations in semicontinuous culture (reaching finally the stationary phase) were suspended in fresh medium at a final concentration of 7.3×10^6 cells/ml and exposed to light. Samples were taken at different times, and chlorophyll content and photooxidation of cytochrome *f* tested, as described in Methods.

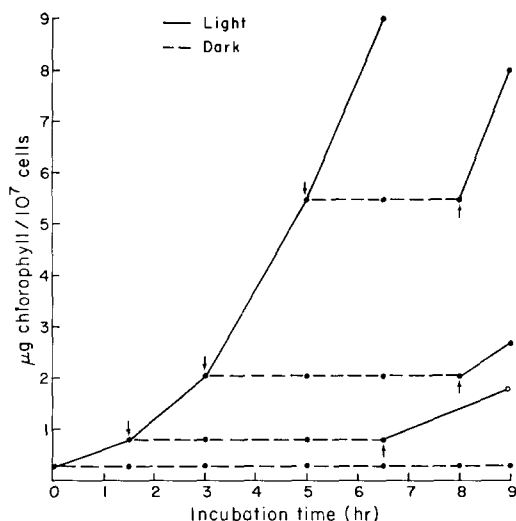


FIGURE 24 Synthesis of chlorophyll during discontinuous greening experiment. Cells were grown for 6 days in batch culture, washed, and resuspended in fresh medium at a final concentration of 1.5×10^7 cells/ml. Other conditions as described in Methods. Full line, cells exposed to light; dotted line, cells transferred to dark. Arrows (\downarrow) indicate transfer from light to dark. Arrows (\uparrow) indicate transfer from dark to light.

miophilic globules of higher plant chloroplasts are free in the matrix. Osmiophilic globules have been shown to contain galactolipids and plastoquinones, but not chlorophyll or β carotenoids (35).

The distribution of radioautographic grains over the chloroplast suggests that there are no specific centers of membrane growth: the grains appear to be distributed at random over the discs throughout the greening process, in keeping with the assumption that the addition of new material can occur at all points on their surface. But these findings cannot be considered conclusive because the radioautographic resolution obtained was not better than 0.2μ , and the precursor used was not exclusively incorporated into membrane components. Hence, these experiments should be repeated with a more suitable precursor, a shorter labeling pulse, and a better resolution before a definitive answer be given to this question.

Biochemical Changes

The series of biochemical events initiated upon illumination, includes: degradation of reserve

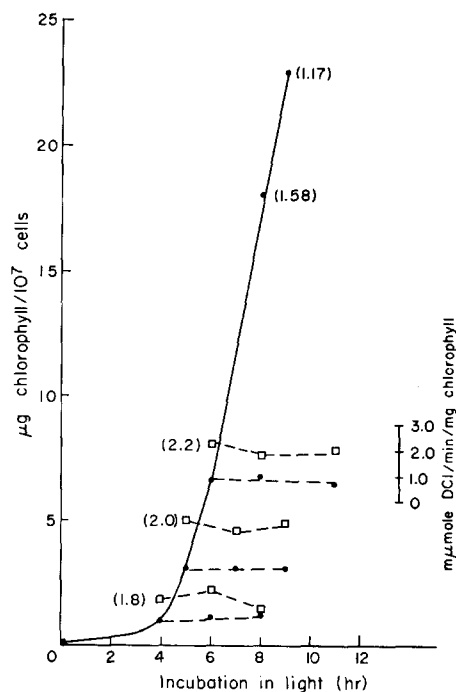


FIGURE 25 Hill reaction and chlorophyll concentration determined in cell homogenates during discontinuous greening. Same experimental conditions as in Fig. 20. \bullet — \bullet , chlorophyll content of cells exposed to light; \bullet — \bullet , chlorophyll content of cells transferred to the dark; \square — \square , Hill reaction of cells transferred to dark. Number in parenthesis, Hill reaction activity (μ mole DCI/min/mg chlorophyll). Scale on right indicates magnitude of changes in Hill reaction at the three different times tested.

starch, synthesis of chlorophyll, and other membrane building blocks, as well as synthesis of RNA and ribosomes.

STARCH DEGRADATION: The light-induced and light-dependent starch degradation that precedes and accompanies greening seems to be a characteristic feature of the *Chlamydomonas* mutant which deserves further inquiry. Light induced but not light-dependent starch breakdown has been reported during plastid morphogenesis (36). In chyl cells, greening occurs in the presence of an internal carbon source, a situation in which enhancement of photomorphogenetic processes has been reported in higher plants (37); yet chloroplast development in etiolated *Euglena* is actually repressed by substrates to which the algae have previously been adapted (38), while certain strains of *Chlorella* cells are bleached when grown

on media rich in glucose even in the presence of light (7).

CHLOROPHYLL SYNTHESIS: The lag in chlorophyll synthesis may depend at least in part, upon the replenishment of a metabolite pool as suggested by Sisler and Klein (39) who observed that, in etiolated bean seedlings, chlorophyll synthesis proceeds without a lag until the 6th day of development, an increasing lag appearing thereafter. A similar suggestion comes from Virgin (40) who, working with 8-day etiolated wheat leaves, has shown that under certain conditions the lag can be abolished, the rates of chlorophyll synthesis recorded after a variable period of preillumination (from 2 min–3 hr) being maximal and equal, if an appropriate dark period be interposed. This finding may be correlated with the fact that in higher plants during photomorphogenesis, starch degradation is initially light-induced but can continue thereafter in the dark (36). This interpretation does not exclude, however, the possibility that the lag represents time needed for the light-induced synthesis of an mRNA involved in the production of membrane proteins which, in turn, controls chlorophyll synthesis. In *Chlamydomonas* *y* the lag is relatively

short and can be further shortened under optimal conditions of greening. Moreover, the rate of chlorophyll synthesis is not increased upon re-exposure to light after an alternating light-dark treatment. Hence, light exposure does not seem to trigger, in this case, the synthesis of a molecular species the production of which can continue in the dark.

SYNTHESIS OF MACROMOLECULAR CONSTITUENTS OF THE CHLOROPLAST: The incorporation of isotopes into the protein, lipid, and RNA of the whole cell apparently reflects net synthesis since it was found that exchange and, therefore, turnover were practically absent during the time of an experiment. Furthermore, for reasons already mentioned, almost all biosynthetic activity appears restricted to the chloroplast after 2–3 hr from the onset of illumination.

All components tested, i.e., protein, phospholipid, galactolipids, sulfolipid, neutral lipid, and RNA, are synthesized at an increased rate during the greening process. The rate of acetate-¹⁴C incorporation into the monogalactosylglyceride was twice that into the digalactosylglyceride, the two accounting together for ~40% of the total counts incorporated into the lipid

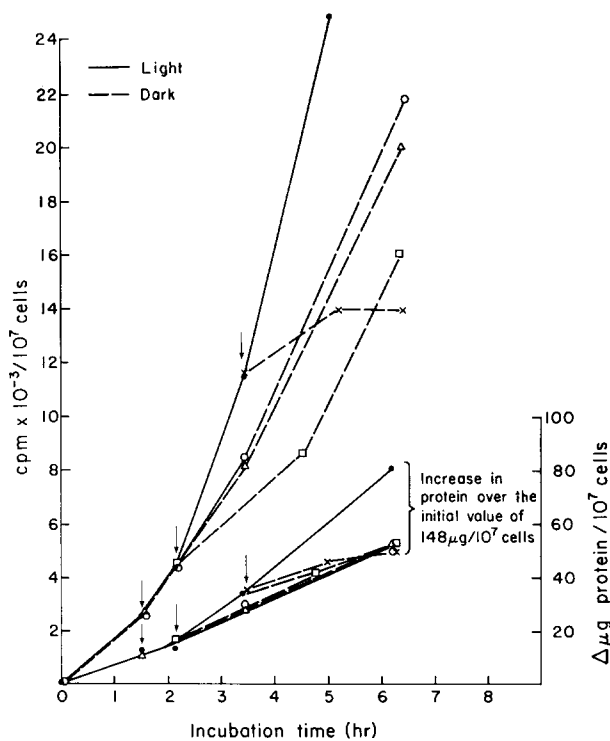


FIGURE 26 Increase in protein content and incorporation of acetate-¹⁴C into protein during a discontinuous greening process. Cells grown in the dark in batch culture for nine generations were incubated at a final concentration of 7×10^6 cells/ml in fresh growth medium with acetate-¹⁴C ($6 \mu\text{c}/\mu\text{mole}$) replacing acetate-¹²C in the medium. Solid lines, incubation in light; dashed lines, incubation in the dark; black circles, continuous light; open circles, dark control; open triangles, cells exposed to light for 1.5 hr and then transferred to the dark; open squares, cells exposed to light for 2 hr and then transferred to the dark; crosses, cells exposed to light 3.3 hr and then transferred to the dark. Arrows indicate transfer from light to dark. Lower curves show increases in the protein content over the initial level of $148 \mu\text{g}/10^7$ cells.

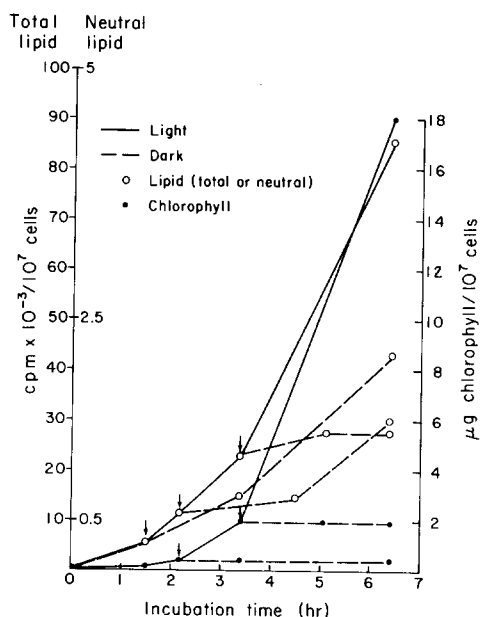


FIGURE 27 Synthesis of chlorophyll and incorporation of acetate- ^{14}C into total and neutral lipid during a discontinuous greening process. Same experimental conditions as in Fig. 26. Solid lines, incubation in continuous light; dotted lines, incubation in the dark; black circles, chlorophyll; open circles, incorporation of acetate- ^{14}C into lipids (both neutral and total). Arrows indicate transfer from light to dark.

fraction. These results are in agreement with data available on higher plants, *Euglena* and *Chlorella*, in which galactolipids, known to be localized mainly in chloroplast membranes, were found to increase in amount during greening (12-14; cf. also 30, 41). The difference in the rates of incorporation of ^{14}C -labeled compounds between chyd control cells and chyd cells exposed to light can be regarded as a minimal value, since dilution of acetate- ^{14}C and leucine- ^{14}C with the corresponding ^{12}C -compounds derived from the breakdown products of starch is expected. Only the rate of dilution would be different for different compounds according to the labeled metabolite used and the pathway of interconversion to or from acetate- ^{14}C in the medium and hexose- ^{12}C derived from the starch. Thus, with ^{32}P a marked difference in the rates of incorporation into lipid was immediately observed between chyd and chyl cells, while with ^{14}C -precursors a difference became apparent only after 1-2 hr of illumination, in agreement with the

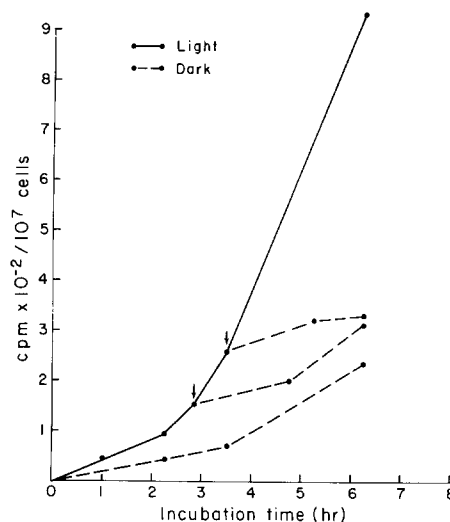


FIGURE 28 Incorporation of ^{32}P into phospholipid during a discontinuous greening process. Chyd cells were grown in batch culture for 7 days, resuspended in fresh medium containing ^{32}P (sp. act. about $0.4 \mu\text{c}/\text{mmole}$) at a final concentration of 5×10^6 cells/ml, and incubated in continuous light (●—●) or in the dark (●---●). Arrows indicate transfer from light to dark.

assumption that the starch, which is degraded from the onset of illumination, is a major contributor to the internal metabolite pool and thus dilutes the supply of external ^{14}C -precursors. The methods used do not permit an estimate of the concentrations of the enzymes involved in lipid synthesis, but the findings show clearly that these enzymes are present in chyd cells.³ The synthesis of RNA parallels that of protein and lipid. Part of this RNA is probably associated with synthesis of new ribosomes which increase in number during the greening process in both cytoplasm and chloroplast.⁴

³ Preliminary observations on the distribution of ^{14}C (derived from acetate) among galactolipid split products showed that the incorporation is equally distributed in the fatty acid and galactosylglycerol moieties, indicating that the whole molecule is synthesized de novo.

⁴ The small dense particles present in the chloroplast may be chloroplast ribosomes. They appear smaller and less dense than the cytoplasmic ribosomes (22). When ribosomes isolated from the whole cells at Mg^{++} concentrations from 2-10 mM were sedimented on sucrose gradients, two populations were obtained (about 70-80 S and 40-60 S). It is possible that the lighter peak represents the large subunit of chloroplast ribosomes, since this peak was enriched when

TABLE II

Initial Incorporation of Acetate-¹⁴C into Total and Various Isolated Lipids by Chyd Cells Exposed to Light or Dark

Cells were grown for 5 days in a semicontinuous culture apparatus and resuspended in a final concentration of 1.1×10^7 cells/ml in fresh medium containing 1.1 μ mole acetate-¹⁴C/ml (9.1 μ c/ μ mole) for further incubation as noted below. Chlorophyll content after 5 hr of incubation in light was 1.6 μ g/ 10^7 cells. MG = monogalactosylglyceride; DG = digalactosylglyceride; SL = sulfolipid; PC = phosphatidylcholine.

Lipid	cpm $\times 10^{-3}/10^7$ cells					
	2 hr		3.5 hr		5 hr	
	Light	Dark	Light	Dark	Light	Dark
Total	1200	800	4200	2850	7500	3900
MG	100	25	920	110	1920	230
DG	80	65	490	380	1020	550
SL	35	25	310	170	440	220
PC	50	10	200	76	210	120

As already mentioned, the transfer of chyd cells to light apparently causes a drastic shift in the general metabolism of the cell, leading from

ribosomes were prepared from chloroplast-rich fractions. In this respect, they appear to be similar to chloroplast ribosomes prepared from *Euglena* and higher plants (42, 43). The concentration of these light particles relative to cytoplasmic ribosomes (the heavy peak) in chyd cells is about the same as in chyl cells. Although their number increases during the greening, as indicated both morphologically and by incorporation experiments, their initial concentration in chyd cells appears to be sufficient to allow chloroplast protein synthesis when this is induced by light. Such a possibility is suggested by the results of preliminary experiments in which chyd cells were incubated for 8 hr in the light or dark, both in the presence of leucine-¹⁴C. Ribosomes were isolated from each of these two-cell suspensions and put on sucrose density gradients. When the specific radioactivity was measured of each of the peaks of both light- and dark-incubated cells, the highest value found was for the light peak of the light-exposed cells, the actual values (CpM/OD_{260m μ} for the peak tubes) being 24 and 14 for the heavy peaks, and 28 and 9 for the light peaks of chyl and chyd cells, respectively. Moreover, leucine-¹⁴C incorporation into the ribosomes of the light peak was extensively reduced in the presence of chloramphenicol, although, under these conditions, chlorophyll synthesis still proceeded at 40–60% of the control rate. A partial inhibition of protein synthesis in *Chlamydomonas* by chloramphenicol was also reported by Hudock et al. (8). These results are in agreement with the idea that preexisting ribosomes

synthesis of all cell components to synthesis of chloroplast material. This shift becomes evident, however, only after a sufficient period of illumination. For example, when cells in the lag phase of chlorophyll synthesis were put back in the dark, the rate of macromolecular synthesis returned to the rate of the dark control within 1–2 hr; when cells in the linear phase of chlorophyll synthesis were transferred back to the dark, incorporation into all macromolecules either stopped or was slowed considerably. If synthesis of both chloroplast and cytoplasmic components were proceeding concomitantly, at this time one would have expected a reduction in rate but not a cessation of incorporation. The findings cannot be attributed to a lack of carbon or energy sources since in all experiments utilizable acetate is present in the medium. It is also improbable that an energy deficiency develops in the cell as a result of differences in substrate and cofactor concentration in the pools of the two major cytoplasmic compartments involved (mitochondria and chloroplast). Light-exposed cells show increased respiration when transferred to darkness (i.e., increased mitochondrial activity) and remain at this high level of respiratory ac-

may support that protein synthesis necessary for initial membrane formation. Further synthesis of ribosomes could explain the increase in protein synthesis found during greening experiments as compared with the dark control or with cells exposed to light in the presence of chloramphenicol (44).

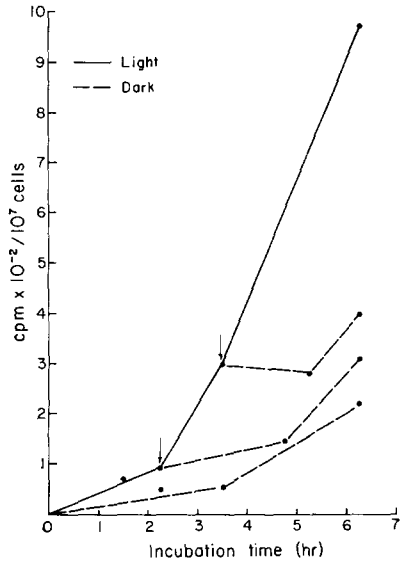


FIGURE 29 Incorporation of ^{32}P into RNA during a discontinuous greening process. Same experimental conditions as in Fig. 28. Solid lines, cells incubated in continuous light; dotted lines, cells incubated in the dark; arrows indicate transfer from light to dark.

tivity for at least 2 hr. The initial increase occurs concomitantly with a rise in the concentration of metabolites derived from the starch degraded during the preceding light exposure.

The *Chlamydomonas* mutant behaves in a different way from dark-grown *Euglena* which, during induction of chloroplast formation, synthesized protein only after a long lag (20 hr). During this lag, RNA appears to be synthesized from the start (15), galactolipids are synthesized at a constant rate (12), while the synthesis of sulfolipids follows closely the chlorophyll synthesis curve.

Membrane Assembly

REGULATORY ROLE OF CHLOROPHYLL: A reasonable assumption is that in cases in which the differentiation of a proplastid into a chloroplast is involved, the greening process is controlled at the transcription level by the light-induced production of appropriate mRNA's. In *Chlamydomonas*, mRNA synthesis probably occurs during greening, but synthesis of chloroplast membrane constituents does not appear to be controlled

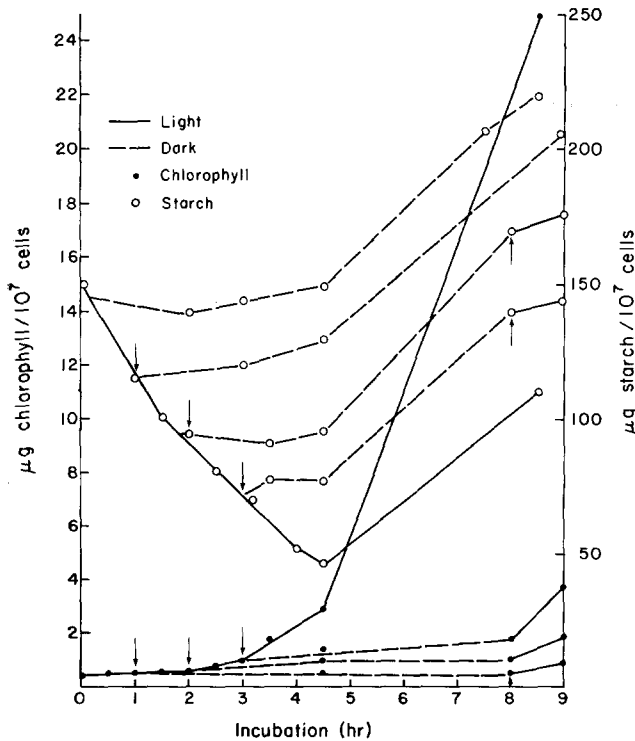


FIGURE 30 Degradation of insoluble starch during a discontinuous greening process. Chyd cells maintained for seven generations in logarithmic growth in a semicontinuous culture apparatus were incubated at a final concentration of 9.7×10^6 cells/ml. Solid lines, cells incubated in continuous light; dotted lines, cells incubated in the dark; arrows (\downarrow) indicate transfer from light to dark, and arrows (\uparrow) from dark to light; open circles, starch; black circles, chlorophyll.

at the same level. Our findings could be explained by assuming that chlorophyll molecules are needed to form a complex with certain membrane-specific proteins before the latter can be released from their site of synthesis on plastid ribosomes. Lack of chlorophyll would result in accumulation of such proteins on ribosomes and would eventually block their further synthesis. If one or more of these proteins were necessary for the construction of a specific membrane, membrane assembling would become geared to chlorophyll synthesis,⁵ and membrane formation could be controlled at the translation level.

FUNCTIONAL CHARACTERISTICS OF THE MEMBRANE: From the earliest time point tested, the membranes formed during the greening process showed a normal and almost constant photosynthetic electron-transport activity. Although fusion of paired discs to form a granum was a late event in the process, the activity of the Hill reaction, O₂ evolution, NADP-photoreduction, and cytochrome *f* photooxidation paralleled the increase in chlorophyll, indicating that both photosynthetic systems, PSI and PSII, were formed simultaneously. Moreover, their specific activity per chlorophyll unit was maximal, i.e. comparable to that of light-grown cells. This result implies that chlorophyll and other components of the electron-transfer system become part of the disc membranes prior to their fusion into grana, as suggested also by the results of discontinuous greening experiments in which fusion of discs occurred in the dark without increases in chlorophyll content or Hill activity. This fusion of discs does not appear to be essential for photosynthetic activity, although it might be of importance in the efficiency of quantum conversion.

TYPE OF ASSEMBLY: The formation of the chloroplast disc system can be envisaged as the result of one of the following processes: (a) *simultaneous or one-step assembly* of newly synthesized and preexisting components, or (b) *sequential or multi-step synthesis and assembly* of various components. The first scheme implies relatively constant composition, morphology, and specific biochemical activity of the membrane at any time during the greening process, while the

⁵ Studies on hemoglobin synthesis (45) suggest that the removal of nascent polypeptide chains from ribosomes may play a role in the control of protein synthesis.

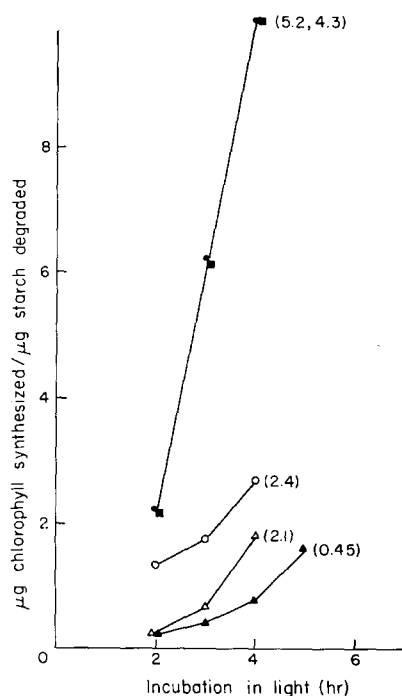


FIGURE 31 Ratio of chlorophyll synthesized to starch degraded as a function of time during greening of various batches of cells with different initial chlorophyll levels. Chyd cells grown in a semicontinuous culture apparatus for two, three, four, . . . seven generations in logarithmic phase, resuspended at a final concentration of 5×10^6 cells/ml, and exposed to light for different periods of time. Numbers in parentheses, initial chlorophyll levels ($\mu\text{g}/10^7$ cells): dark circles, 5.2; dark squares, 4.3; open circles, 2.4; open triangles, 2.1; dark triangles, 0.45. Starch degradation in these experiments was linear to 4.5 hr.

second calls for changes in composition and morphology and for an increase in specific photosynthetic activity during the process. In the latter case, a relative accumulation of one component over the others is implicit, while in the first scheme such an accumulation is, by definition, not necessary. It is, however, possible and may provide the actual basis for a reciprocal control mechanism by which lack, or accumulation, of one component would influence the rate of synthesis of the others. Within the limitations imposed by our assay procedures, the data presented in this work suggest that synthesis and assembly of chlorophyll, specific proteins, and specific lipids into disc membranes are synchronized and interdependent, for changes with time

TABLE III
Acetate-³H Pulse Labeling and Radioautography of the Mutant Cells during a Greening Process

For experimental conditions see text.

Incubation before pulse $\mu\text{g}/10^7$ cells	Distribution of radioactivity among various chemical fractions					Distribution of radioactive grains among different cellular compartments											
	Chlorophyll $\mu\text{g}/10^7$ cells	Total radioactivity/ 10^7 cells	% of total			Residue§	Chloroplasts			Remainder			Cytoplasm minus chloroplast				
			Soluble fraction†	Lipid	Starch		Membranes and matrix	Pyrenoid	Starch	Total	Nucleus						
6.25 hr, dark	0.7	4.7	27	11	24	38	5.2	24	12.0	3	7.9	19	46	8.4	13	4.2	41
2.25 hr, light	3.5	10.0	17	34	12	37	7.2	51	8.9	5	5.6	9	65	1.5	1	2.9	34
3.75 hr, light	7.0	8.4	27	38	10	25	12.0	58	12.2	9	9.2	9	76	3.0	1	2.9	23
6.25 hr, light	25.0	7.9	29	57	7	7	9.4	60	20.0	12	7.4	8	80	3.0	1	3.6	19

* Specific activity is defined as number of grains per unit weight of cellular compartment.

† The soluble fraction is a 2% Na_2CO_3 extract from an 80% acetone powder of whole cells and contains soluble proteins, RNA and DNA.

§ The residue is the Na_2CO_3 insoluble fraction from which the starch was removed by extensive digestion with hog pancreas amylase (22).

|| Most of the grains were located on the membranes; the matrix between and around them contributed only ~5-10% of the grains in the measured fractions, but accounted for a substantial part of the weight on the basis of which the specific activity was calculated. Therefore, their recorded specific activity is a minimal value.



FIGURE 32 Chyd cell exposed to light for 6.75 hr, labeled with acetate- ^3H , and processed for electron microscopic radioautography as indicated in the text. Most of the radioautographic grains (*ag*), namely 18 out of 22, occur over the chloroplast in association with grana (*g*), the matrix, and the pyrenoid (*p*). Of the four extrachloroplastic grains, one marks a Golgi complex (*dc*), another the cell wall (*w*), still another is found in the vicinity of a mitochondrion (*m*), and the last (along the upper margin of the figure) is of uncertain location. $\times 29,000$.

in the structure, composition, and specific activity of these membranes are relatively small. Hence, in this case, one-step assembly can be considered as a working hypothesis to be tested by further experimentation.

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