Formation of Chloroplast Membranes in Chlamydomonas reinhardi y-1

EFFECTS OF INHIBITORS OF PROTEIN SYNTHESIS*

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SUMMARY

During normal greening of the y-1 mutant of Chlamydomonas reinhardi, photoreductive activities with 2,6-dichlorophenol (DCI), an assay for Photosystem II, and with methyl red (ascorbate and DCI as electron donors), an assay for Photosystem I, increased in parallel with chlorophyll. Disc membranes formed approximately in parallel with chlorophyll synthesis and fused to form grana.

In the presence of 20 μg of chloramphenicol (CAP) per ml, the cells produced chlorophyll and membranes at 90% of the rate of control cells, but photoreductive activities were drastically reduced. Photoreduction of DCI and of methyl red in CAP-treated cells increased at rates only 35% and 50%, respectively, of those in control cells. Also, in CAP-treated cells the discs rarely fused to form grana. Inhibition of these activities was reversible upon removal of CAP.

Chlorophyll synthesis and disc formation in cells in the presence of cycloheximide (CHI) at 1 μg per ml occurred at about one-half of the rate of control cells, but photoreductive activity with DCI and disc fusion into grana was the same as in controls on a chlorophyll basis. At concentrations of CHI above 5 μg per ml, chlorophyll synthesis was completely inhibited.

The incorporation of 14C-leucine into total protein by whole cells was inhibited maximally 30 to 50% by CAP and maximally 40 to 50% by CHI; the effects of the drugs were additive.

The membranes formed in the presence of CAP could be repaired by transferring the cells to a medium containing CHI (10 μg per ml). During 2 hours of subsequent incubation in light, the existing membranes gained activity until, on a chlorophyll basis, they were at least as active as the membranes formed in control cells.

These results indicate that products of both the cytoplasmic and the chloroplastic protein-synthesizing systems are required for disc membrane production in the chloroplast of this alga.

Unlike the wild strain, the y-1 mutant of the unicellular green alga, Chlamydomonas reinhardi, cannot synthesize chlorophyll in the dark. As a consequence, no chlorophyll-containing membranes and hence no chloroplast discs are formed without light. Thus, after growing for six generations in the dark, the contents of chlorophyll and chloroplast discs of the mutant cell are about 2% and 5%, respectively, of those of the fully green alga. When exposed to light, the yellow, dark-grown cells undergo greening. During an interval of 8 to 10 hours, the only significant structural change is the progressive filling of the chloroplast with newly synthesized discs, while the predominant biochemical events are the synthesis and accumulation of chlorophyll and the development of photosynthetic activity.

Some components of the photosynthetic systems are present in the yellow cells (e.g. ferredoxin, cytochrome f, ribulose 1,5-diphosphate carboxylase, and yellow pigments) and increase only slightly in amount in relation to chlorophyll, since, by contrast with chlorophyll, the mutant cells can synthesize these components in the dark.

Since greening of C. reinhardtii y-1 involves, along with the rapid production of chlorophyll, the synthesis of certain chloroplast enzymes and of protein necessary for the structure of the membrane, it became of interest to find out what role is played by each class of the cell's ribosomes in this process. The alga has at least two distinct ribosome classes: chloroplastic (68 S particles) and cytoplasmic (80 S particles) (3, 5-7). The contribution of each of these systems could be determined by exposing greening cells to inhibitors of protein synthesis which affect preferentially one type of ribosome. Over the last several years data have accumulated indicating that chloramphenicol is a specific inhibitor of synthesis by chloroplast ribosomes (8-12), whereas cycloheximide is a specific inhibitor of synthesis by plant cytoplasmic ribosomes (13-15).

Hudock et al. (16) treated greening cells of a C. reinhardtii mutant similar to y-1 with CAP,† and showed that low concentrations of the drug do not affect chlorophyll synthesis but do inhibit the development of the cells' ability to evolve oxygen. Our results extended the findings of Hudock et al. (16) and suggest that both ribosomal systems participate in the synthesis of proteins required by the greening process. Furthermore, a substantial flow of macromolecular material from cytoplasm to mitochondria is suggested by the data presented in this report.

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1 The abbreviations used are: CAP, chloramphenicol; DCI, 2,6-dichlorophenol; CHI, cycloheximide.
OOG pmnlo of 3-(3,4-dichlorophenyl)-1,1-dimethylurea completely N-nil portion of the suspension (in 500-ml flasks) received 1.0 ml 8,600 lux from white fluorescent lamps on a rotary shaker. Each assay was about 17,000 lux from a tungsten lamp. Addition of chlorophyll to control samples, inhibited light-dependent DC1 reduction. Chlorophyll was measured spectrophotometrically (22) in acetone extracts of the broken cell suspension. Activity contained in a final volume of 3.0 ml: 30 nmol of sodium phosphate (pH 7.0), containing 0.005 M KCl, suspended in the phosphate-KCl buffer to a volume of 5.0 ml, and broken in a chilled French pressure cell at 6,000 psi. Assay mixtures for Hill reaction activity contained in a final volume of 3.0 ml: 3;0 nmol of sodium phosphate (pH 6.7), 15 nmol of KCl, 0.1 nmol of DC1, and 0.05 to 0.4 ml of the broken cell preparation. Light intensity for the assay was about 17,000 lux from a tungsten lamp. Addition of 0.06 nmol of 3-(3,4-dichlorophenyl)-1,1-dimethylurea completely inhibited light-dependent DC1 reduction. Chlorophyll was measured spectrophotometrically (22) in acetone extracts. Chlorophyll, control samples; chlorophyll, CAP-treated samples; Hill reaction, control samples; Hill reaction, CAP-treated samples.

METHODS

Handling of Cells—C. reinhardi y-1, a strain derived from the mutant strain 4y (1), was grown in a defined liquid medium as described in (3). After growth for 5 days in the dark, at cell densities not exceeding 1.5 x 10^6 cells per ml, the cells were collected by centrifugation at 1500 x g for 5 min and suspended to a density of 4 x 10^6 cells per ml in fresh culture medium to which 2 mcq of KIIPO 4 were added per liter of medium. Cell counts were made in duplicate with a hemocytometer. Fifty-milliliter samples of this suspension were put in 500-ml Erlenmeyer flasks, mounted on a rotating platform, and exposed to light from white fluorescent lamps at an intensity of 8000 to 8600 lux. With added phosphate, the pH of the medium increased from 6.4 to 7.6 during greening; without it, the final pH became nearly 9. For each assay, the cells in each flask were centrifuged at 2000 x g for 3 min at 4°C, washed once with cold 0.01 M sodium phosphate buffer, pH 7.0, in 0.005 M KCl, and suspended in the phosphate-KCl buffer to a final volume of 5.0 ml. The suspension was passed once through a chilled French pressure cell at 6000 psi, which broke open all cells as ascertained by light and electron microscopy (3). Aliquots of the broken cell suspension were immediately assayed for light-dependent electron transport activities.

Assays for Light-dependent Electron Transport—The photo-reduction of 2,6-dichlorophenol, the Hill reaction (17), was assayed as described in (3) with a light intensity of about 17,000 lux (approximately 40% saturating) from a tungsten lamp. The activity was corrected for the small amount of dye reduced in an identical cuvette kept in the dark. The light-induced reduction of DC1 was completely inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

The photoreduction of methyl red was assayed in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea essentially according to Vernon, Shaw, and Ke (18). The cuvettes were exposed to light, and after each 30-sec interval the remaining unreduced methyl red was measured at 430 nm (ε = 14.1 x 10^3), against a blank of the reaction mixture in which the methyl red was completely reduced by addition of dithionite.

Amino Acid Incorporation—Yellow cells were suspended to 6 x 10^6 cells per ml of the buffered medium as described above, and 25-ml portions of the suspension were put into 250-ml Erlenmeyer flasks. After about 5 hours in the light, 14C-lucine was added (details for each experiment are given in Table 1). Ten-milliliter aliquots of the suspension were removed at 30 and 60 min thereafter, and the cells were precipitated by adding 1 ml of 100% (w/v) trichloracetic acid. Precipitates freed of nucleic acid and lipid (19) were dissolved in 0.05 N NaOH. Aliquots were measured for protein (20) and for radioactivity by scintillation counting with 10 ml of Bray's solution (21).

Analyses—Four milliliters of reagent grade acetone were added to a 1.0-ml aliquot of broken cells suspension and the mixture was centrifuged after standing on ice for 1 to 5 hours. Chlorophyll in the 80% acetone extract was measured by the procedures of Arnon (22) or Vernon (23). The precipitate was dissolved in 0.1 N NaOH and the protein was assayed (20) with crystallized bovine serum albumin as the standard. The protein content per 10^6 cells was found to average 0.32 mg of protein per yellow cells and 0.55 mg of protein for cells after 7 hours in light.

Electron Microscopy—Whole cells were fixed in 1% OsO4 in 0.1 M phosphate buffer (pH 7.4), dehydrated in graded alcohols, and embedded in Epon. Sections were prepared and electron microscopy was performed as described previously (3).

Chemicals—Chloramphenicol was a gift from Parke Davis; 3-(3,4-dichlorophenyl)-1,1-dimethylurea was a gift from E. I. du Pont de Nemours and Company. Cytochrome c (Actidione) was obtained from Nutritional Biochemicals; 2,6-dichlorophenol was supplied by Eastman; and methyl red, sodium salt, was purchased from Mann.

RESULTS

Normal Greening—When etiolated y-1 cells were exposed to light, chlorophyll and the Hill reaction initially increased slowly. The duration of this slow phase of greening seemed to correlate inversely with the amount of chlorophyll present at the start and with the effective light intensity in the bulk of the culture (4). Light-dependent electron transport activities increased in parallel with chlorophyll, irrespective of the electron acceptor used: DC1 (Fig. 1), methyl red (Fig. 2), NADH (see Reference 4), or cytochrome c (3). Under the conditions used, photoreduction of DC1 is an assay for Photosystem II (24-26); photoreduction of methyl red in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea and an electron donor measures Photosystem I.

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Effects of C.4P—The addition of C.4P (final concentration, 10 \( \mu g \) per ml) to a suspension of yellow cells just before light exposure did not impair chlorophyll synthesis, but reduced the rate of development of the Hill reaction to about 53% of the control value. When C.4P was added to 20 \( \mu g \) per ml, the rate of increase in this activity was only 35% of the control value (Fig. 1), and the rate of development of methyl red photoreduction decreased to 50% of the control value (Fig. 2). Chlorophyll synthesis proceeded at a rate only 10% lower than in controls. However, at higher concentrations of C.4P (200 \( \mu g \) per ml) chlorophyll synthesis was drastically reduced to about 12% of the control value and the activity of the Hill reaction became barely detectable.

Comparable results with a similar mutant of \( C. reinhardtii \) were obtained by Hudson et al. (16) who also observed that C.4P had little or no effect on the development of \( O_2 \) evolution when added after 2-hour light exposure. Fig. 3 shows, however, that C.4P was still effective when added at 4 hours, but that the effect required more than 1 hour to become observable. This is a longer interval than that allowed by Hudson et al. (16) in their experiments. Eventually the rate of increase in activity of the Hill reaction reached the same low level irrespective of the time of addition (4 hours or 0 hour).

Electron Microscopy of C.4P-treated Cells—During normal greening, the increase in disc membranes per chloroplast approximates rather closely the increase in chlorophyll per cell (4). Low concentrations of C.4P (20 \( \mu g \) per ml) do not seem to affect the production of chloroplast discs, since electron micrographs of cells greening in the presence of the drug showed the same, or nearly the same, amount of disc membranes as the controls (Fig. 4, a and b). Disc fusion into grana was, however, noticeably affected. In control cells, after 7- to 8-hour light exposure, most of the chloroplast discs were involved in the formation of grana among which two to three disc units prevailed, although units up to five discs were occasionally encountered (Fig. 4a). In cells greening in the presence of 20 \( \mu g \) of C.4P per ml, grana formation was curtailed to an extent that varied from one experiment to another apparently in correlation with the degree of inhibition of photoreductive activity. For instance, at ~60% inhibition there was extensive disc pairing but practically no fusion (Fig. 4b); at >30% inhibition, two to three disc grana occurred, generally at lower frequency than in controls.

At 200 \( \mu g \) of C.4P per ml, the discs were reduced in number and showed limited pairing and no fusion, even after 9-hour light exposure. In addition, many discs were focally reduced to a single dense layer, an appearance often seen at much earlier stages (3 to 4.5 hours) in normal greening.

Effect of Light Intensity—The results indicate that production of disc membranes continues in the presence of C.4P but that these membranes have low photoreductive activity. This conclusion was supported by light saturation experiments which showed (Fig. 5) that the same relative difference existed between C.4P-treated and control cells at all light intensities examined. A plot of the inverse of activity against the inverse of light intensity (29) indicated that one-half maximal reaction velocity was reached in both cases at the same light intensity. Since maximal velocity was less in C.4P-treated cells, their membranes appeared to contain less active proteins than those of control cells. No effect on the Hill reaction was observed when C.4P (220 \( \mu g \) per ml) was added directly to the reaction mixture.

A comparison of the spectrum of a preparation of broken cells obtained after 7 hours of greening in the presence of C.4P (20 \( \mu g \) per ml) with the spectrum of the corresponding control showed slight differences in the red region. There was slightly less chlorophyll \( a \left( \Theta_{\text{max}} = 678 \text{ nm} \right) \), which is the sum of a 668 nm component and a 680 to 683 nm component (30) in relation to chlorophyll \( b \left( \Theta_{\text{max}} = 650 \text{ to } 651 \text{ nm} \right) \) in C.4P-treated than in control cells. The ratio chlorophyll \( a \) to chlorophyll \( b \), calculated by Vernon's procedure on acetone extracts (28), was 2.06 in the C.4P-treated cells and 2.54 in the control cells. Hill reaction
Fig. 4. a, part of the chloroplast of a Chlamydomonas reinhardi y-1 mutant after 7-hour greening. Wherever normally sectioned, the chloroplast discs appear fused into very long grana of two to three discs (g2 and g3); unfused discs (arrows) are rare and appear to cross from one granum to another. The chloroplast stroma contains numerous ribosomes (r) and a number of osmophilic granules (o). The pyrenoid is marked py, its starch plates sp, and its tubules t. The chloroplast envelope is seen at ce, mitochondria at m, the cell membrane at cm, and the cell wall at cw. × 42,000. b, part of the chloroplast of a C. reinhardi y-1 mutant after 7-hour greening in the presence of 20 μg of CAP per ml. Photoreductive activity was inhibited 60%. The chloroplast contains a large number of discs (d1), many paired (d2), but very few fused into grana (g2). Other notations as for a. A long "tongue" of cytoplasm interposed between two chloroplast lobes is marked cy. × 42,000.
activities in the CAP-treated and control samples were 1.8 and 3.6 μmoles of DC1 reduced per min per mg of chlorophyll, respectively. The significance of the altered chlorophyll a-chlorophyll b ratios with regard to the inhibited development of the Hill reaction remains unknown.

Effects of CHI—CHI affected the greening process differently from CAP. Added to a suspension of yellow cells at zero hour (the time of exposure to light), it inhibited in parallel and to the same extent chlorophyll synthesis and development of photosynthetic activity. At a concentration as low as 5 μg of CHI per ml the inhibition was complete; at 1 μg per ml the drug reduced both rates to less than half of the control values (Fig. 6).

The effect of CHI was immediate and reversible. When introduced at a concentration of 1 μg per ml at any time during the greening process, it caused a prompt and parallel reduction in the rates of chlorophyll synthesis and development of photosynthetic activity (Fig. 7). At higher concentrations, it stopped chlorophyll synthesis, but its removal, even after exposure to CHI at 20 μg per ml, was promptly followed by a resumption of chlorophyll synthesis at the rate reached before the introduction of the drug. In this respect, the situation was reminiscent of that found by Ohad, Siekevitz, and Palade (4) in experiments which involved light → dark → light transfers during the greening process.

Electron Microscopy of CHI-treated Cells—The electron microscopy of cells greening in the presence of CHI indicated that the amount of disc membrane present in their chloroplasts roughly paralleled the chlorophyll content per cell. For instance, cells with about half of the chlorophyll content of the controls have about half of the disc membranes of the latter (Fig. 8, a and b). By contrast with the situation described in CAP-treated cells, these membranes show, however, extensive fusion into grana containing two to four discs (Fig. 8b). Single and paired but unfused discs frequently showed focal reductions to a single dense layer, a characteristic appearance for earlier stages in normal disc formation.

Effects of CAP and CHI on Amino Acid Incorporation—Because of the diverse effects of CAP and CHI on greening of the
FIG. 8. a and b, *C. reinhardi* y-1 cells greened for 7 hours in the absence (a) or presence (b) of CHI (1 μg per ml). These limited but representative fields show that in the presence of CHI the frequency of newly formed discs is about half that found in controls. In both cases, however, there is extensive disc fusion which leads to the formation of two-disc and three-disc grana (g1, g2). Whatever unfused discs remain are seen to connect adjacent grana (d) or to represent incomplete fusion within a grana (d1). The broad, moderately dense bands at g in b represent obliquely sectioned discs or grana. Other notations as in Fig. 4. X 82,000.
alga, the effects of these drugs on protein synthesis by whole cells were tested. Maximal inhibition of $^{14}$C-leucine incorporation into total cell proteins by CAP was obtained at concentrations above 50 µg per ml. However, at the midpoint of greening, CAP alone inhibited maximally only 30 to 50% of the control value (Table I, Experiments 1 and 3). The antibiotic appeared to penetrate slowly into the cells. In the experiments shown, CAP was added at the concentrations indicated at zero hour to the greening cells, whereas $^{14}$C-leucine was introduced around 5 hours later. When the drug and the labeled amino acid were added together to the assay, no inhibition was observed up to 1 hour at CAP concentrations as high as 50 µg per ml. This result could be correlated with the lag in the CAP effect on Photosystem II (Fig. 3).

Experiment 2, Table I, shows that CHI alone inhibited the incorporation of $^{14}$C-leucine into total proteins in yellow cells more strongly in the dark (71%) than in greening cells in the light (42%). Since the effect of CHI is immediate (Fig. 7), in these experiments the drug was added only 5 min prior to adding the labeled amino acid to the cell culture. A concentration of 10 µg per ml was sufficient to achieve a maximal effect with CHI.

In several greening experiments, maximal inhibition of protein synthesis by CAP varied from 30 to 50%, while maximal inhibition by CHI ranged from 40 to 50%. However, in any experiment the effects of the two drugs were additive (Experiment 2, Table I); in the presence of both, protein synthesis was inhibited by 85 to 95%.

Repair of Deficient Membranes—Since disc membranes were apparently deficient in components necessary for photoreductive activity when formed in the presence of CAP, the Hill reaction was followed after transfer of the cells to a drug-free medium to determine whether the CAP effect was reversible and whether normal activity could be achieved after CAP removal. In a first type of experiment, the cells were removed from medium containing CAP (25 µg per ml) by centrifugation, suspended in fresh, drug-free medium, and returned to light. As shown in Fig. 9a, these cells continued to synthesize chlorophyll, and the rate of increase in their photoreductive activity promptly reached that of controls. However, since in this type of experiment new, active membranes were continuously synthesized, it was not possible to examine closely what happened to the membranes produced during the CAP treatment.

Therefore, in a second type of experiment, conditions were chosen in which no further membrane formation occurred (4). Fig. 9b shows results obtained when cells were transferred to fresh medium and placed in the dark. Production of chlorophyll and membranes ceased under these conditions, but the difference between control and CAP-treated cells remained the same, even up to 7 hours in the dark. Alternatively, after removing CAP, the cells were suspended in medium containing CHI (10 µg per ml) which, as shown above, inhibits further membrane formation. Fig. 9e shows that in the light, no significant increase in chlorophyll occurred during the next 2 hours, but the photoreductive activity in the CAP-treated cells reached a level equal to that of controls, even though the Hill reaction of the latter also increased by about 25% during the first 2 hours after transfer. On a chlorophyll basis, cells previously treated with CAP had higher activity after 2 hours in CHI than did control cells. Thus, under these latter conditions, the functionally deficient membranes produced in the presence of CAP reached full activity. The activity decreased after 2 hours in medium containing CHI, possibly as the result of a general toxic effect on the cells.

Additional experiments performed to explore in more detail the recovery of photoreductive activity upon transfer to CHI-containing medium showed that CAP-sensitive protein synthesis as well as light is necessary for the finishing touches of the greening process. In the dark or in the continued presence of CAP, CHI treatment brought only partial (from one-third to one-half) recovery of photoreductive activity. The requirement for light might be connected with the dependence of the process on energy produced by the normal photoreductive activities of the chloroplast.

Electron Microscopy of Cells Transferred from CAP to CHI—In cells greening for 7 hours in CAP (25 µg per ml) and subsequently for 2 hours in CHI (10 µg per ml) as in Fig. 9e, the long generally unfused discs characteristic of CAP-treated cells (Fig. 4b) formed short three to five disc grana extensively interconnected by intergranal discs (Fig. 10a), an appearance reminiscent in part of the situation found in fully greened chloroplasts. In controls exposed to light for 7 hours without CAP (see Fig. 4a)

### Table I

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Fig. 9. Effects of transferring cells to CAP-free medium under conditions which either permit or do not permit continued membrane formation. a, dark-grown cells were greened in the presence of CAP (26 μg per ml) as described in Fig. 1. After 6.5 hours in light, the cells were centrifuged, suspended in fresh medium, and returned to light. Hill reaction activity was measured at times shown as described in Fig. 1. b, procedure as in a except that cells were centrifuged in dark. c, procedure as in a except that cells were suspended in fresh medium containing CHI (10 μg per ml). Different batches of cells were used in a, b, and c; the larger amounts of chlorophyll formed, and of DCI reduced, in c can be explained by a shortening of the slow period of greening (3, 4).

and for 2 hours with CHI, the chloroplast discs had the same appearance (Fig. 10c). In both cases, the discs appeared flattened with the intradisc space reduced or nearly obliterated. The granal parts of the discs were more tightly packed than in controls and the intergranal segments showed focal reductions to a single dense layer like the unfused discs of cells greening from zero hour in the presence of CHI. In cells exposed in succession to CAP and CHI, the chloroplast of ~20% of the cells contained large (diameter, ~0.3 to 0.6 μ), globular masses of small interconnected vesicles which appeared to be in continuity with fused or unfused discs (Fig. 10b), the organization of which was in part similar to that of the prolamellar bodies of higher plant chloroplasts. Similar appearances occurred less frequently in controls greened for 7 hours without CAP and for 2 hours with CHI.

**DISCUSSION**

As previously shown (4), the major events in the greening process of the y-1 mutant are chlorophyll synthesis and disc membrane production. Since the mutant retains the ability of synthesizing in the dark all membrane lipids (except chlorophyll) and some of the protein components of the chloroplast photosystems (5), it could have been assumed that the proteins synthesized during the initial phase of greening were primarily "structural" membrane proteins. The results that we report indicate clearly, however, that in addition to proteins involved in the structure of disc membranes, the cell produces while greening certain protein (or proteins) which make disc membranes fully functional and promote their fusion into grana.

The finding that apparently two sets of proteins are required for the production of fully functional disc membranes and that the synthesis of each set shows a distinctive sensitivity to antibiotics leads to a number of interesting considerations which deserve to be discussed in some detail. Since a disc membrane which already contains proteins of the structural type can be made fully functional in a subsequent operation (Fig. 9), it follows that, contrary to the original assumption of Ohad et al. (4), these membranes are assembled in a multistep process like the other cellular membrane, that of the endoplasmic reticulum, the biosynthesis of which has been studied in some detail (31-33).

Recent results by I. Ohad (personal communication), concerning the photosynthetic activity of cytochrome f in developing chloroplast membranes of C. reinhardtii y-1, are also suggestive of a multistep formation of these membranes.
Fig. 10. a, part of the chloroplast of a *C. reinhardii* y-1 mutant after 9-hour greening of which 7 hours were in the presence of 25 μg of CAP per ml and 2 hours were in 10 μg of CHI per ml. The discs appear fused into short two to six grana (g2 to g5), extensively interconnected by unfused d's & s (d), and reminiscent by their shortness of the grana of the fully greened mutant. Within each granum, the discs appear tightly packed. The intradisc spaces are generally narrowed and, in places, nearly obliterated. The arrows point to focal reductions to a single dense layer in unfused discs. The cluster of vesicles at o is part of a structure similar in appearance to a prolamellar body. The tangle of filaments around f presumably represents chloroplast DNA. The other notations are as in Fig. 4a. × 85,000. b, same preparation as in a. The tightly meshed network of tubules is reminiscent of a prolamellar body. At arrows its elements are in continuity with usual chloroplast discs. × 30,000. c, part of the chloroplast of a *C. reinhardii* y-1 mutant after 9-hour greening, the last 2 hours in the presence of 10 μg of CHI per ml. As in a, the discs are fused into short two to four disc grana (g2 to g4) extensively interconnected by unfused elements (d), which show frequent focal reductions to a single dense layer (arrows). Other notations are as in Fig. 4a. × 85,000.
The existence of two distinct sets of disc membrane proteins could be connected in Chlamydomonas reinhardtii with the already established existence of two or more types of DNA, potentially two or more distinct genomes, one of which has been localized in the chloroplast of the wild strain (34-37), and also with the existence of at least two distinct ribosomal populations: one consisting of 88 S particles restricted to the chloroplast and the other comprised of 80 S units located in the cytoplasmic matrix (3, 5, 7). The evidence so far obtained suggests that these two ribosome populations exist in all chloroplast-bearing cells (see references in Reference 7). The etiolated C. reinhardtii y-1 cells also contain chloroplast DNA (35) and a relatively large population of chloroplastic ribosomes (4, 7) which increases during greening (4).

Evidence obtained on bacterial (38-41), chloroplastic (9-11), and mitochondrial (42, 43) systems has convincingly established the sensitivity of 70 S ribosomes to CAP. Conversely, evidence obtained on a variety of yeast and animal cell systems has shown the insensitivity to CAP of 80 S ribosomes (13, 47, 48). Data on the differential sensitivities of ribosomes in green plant cells are rather limited. The synthesis of nitrite reductase, a chloroplast enzyme (49), is inhibited by CAP (12), whereas the synthesis of nitrate reductase, a cytoplasmic enzyme (49), is not (12, 50). Synthesis of the latter is, however, sensitive to CHI (14, 50).

In our present work, both CAP and CHI were found necessary to inhibit completely protein synthesis in vivo in C. reinhardtii during greening, each drug alone inhibiting only about one-half of the total incorporation (Table 1). Finally, it was recently shown in this organism that under the influence of CAP chloroplastic monomeric ribosomes form polysomes, while the same type of conversion is incurred by cytoplasmic ribosomes in the presence of CHI (7). There is, therefore, enough evidence to support the assumption that, during the greening of y-1 cells, CAP inhibits protein synthesis on 68 S chloroplastic particles while CHI has the same effect on 80 S cytoplasmic units.

Our findings show that the two ribosomal systems can be experimentally uncoupled and that CHI inhibition of the cytoplasmic system permits the repair of functionally deficient discs (previously produced in the presence of CAP), and favors their concomitant fusion into short extensively interconnected granules of the type seen in fully greened plastids. The reasons for the structural peculiarities of these plastids (tight packing of discs, partial obliteration of intradisc spaces, and presence of "prolamellar" bodies) remain unknown.

The striking and prompt effect of CHI on chlorophyll synthesis remains unexplained. The data suggest that continued synthesis and accumulation are dependent on chlorophyll binding to protein, as might occur in the case of globin effects on heme production (52). The state at which this binding seems to occur is after protochlorophyllide synthesis. Gaassen and Bogorad (53) showed that CHI does not arrest the synthesis of protochlorophyllide in etiolated bean leaves. Also, Schopfer and Siegelman (54) have purified a protochlorophyllide-protein complex, which appears to be the active intermediate in the light-mediated reduction to chlorophyllide. In the absence of protein, feedback inhibition by free protochlorophyllide molecules upon their own synthesis may prevent further pigment accumulation (55). Although 3-amino leucine synthetase, the first enzyme in the biosynthetic sequence to chlorophyll, has a high turnover rate (56, 57), the CHI effect cannot be ascribed to an inhibition of synthesis of this enzyme. The promptness with which CHI inhibits chlorophyll synthesis, as well as the rapidity with which pigment synthesis is resumed when CHI is removed by washing, renders such an explanation unlikely (see also Reference 58). A mechanism based on the formation of chlorophyll-protein complexes could ensure dual control of disc membrane production either by stopping chlorophyll synthesis (as in the dark) or by inhibiting the synthesis of specific proteins (as in the case of CHI).

The conclusion that many chloroplast proteins are synthesized on cytoplasmic ribosomes (polysomes) is compatible with available genetic evidence. Many mutations in Chlamydomonas studied by Levine (59) and others are known which segregate as nuclear, chromosomal markers, and which cause the disappearance (or lack of function) of specific chloroplast membrane components involved in electron transport. The nuclear gene product could be transferred to the chloroplast either as mRNA or as protein. Transport of newly synthesized proteins from the cytoplasm to the chloroplast must be fast since autoradiographic experiments carried out during the greening of C. reinhardtii y-1 cells (4) indicate that after a 5-min [3H]acetate pulse (when ~50% of the total radioactivity is in proteins) 80% of the autoradiographic grains are already over the chloroplast.

The implication that a sizable part of chloroplast proteins is produced in the cytoplasm may appear paradoxical in view of the fact that the plastid has its own genome and its own apparatus of protein synthesis. However, data to this effect were also obtained in regard to chloroplasts in Euglena (15), and in regard to mitochondria in animal and yeast cells (60-63), all of which suggest that some of the membrane components of these organelles are made in the cytoplasm. In the case of C. reinhardtii y-1 mutant, and because of its unique characteristics (3, 4), our results suggest that proteins involved in the formation of chloroplast membranes are also synthesized in the cytoplasm. A somewhat similar conclusion concerning mitochondrial biogenesis has been reached by Henson et al. (63, 64); the model that they propose for this process may also apply to chloroplasts.

In both mitochondria and chloroplasts, the interplay of the two genomes and their associated ribosomal systems may render possible a coordination of genome activities during the life cycle of the cell, so that the duplication and development of these organelles are geared to that of the cell as a whole. In both cases also, the findings imply large scale transport of macromolecules, i.e. proteins and possibly mRNAs across a double membrane barrier, a process which fully deserves further investigations.

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Formation of Chloroplast Membranes in *Chlamydomonas reinhardtii* y-1: EFFECTS OF INHIBITORS OF PROTEIN SYNTHESIS

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