A MAJOR POLYPEPTIDE OF CHLOROPLAST MEMBRANES OF *CHLAMYDOMONAS REINHARDI*

Evidence for Synthesis in the Cytoplasm as a Soluble Component

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ABSTRACT

Electrophoresis of thylakoid membrane polypeptides from *Chlamydomonas reinhardi* revealed two major polypeptide fractions. But electrophoresis of the total protein of green cells showed that these membrane polypeptides were not major components of the cell. However, a polypeptide fraction whose characteristics are those of fraction *c* (a designation used for reference in this paper), one of the two major polypeptides of thylakoid membranes, was resolved in the electrophoretic pattern of total protein of green cells. This polypeptide could not be detected in dark-grown, etiolated cells. Synthesis of the polypeptide occurred during greening of etiolated cells exposed to light. When chloramphenicol (final concentration, 200 μg/ml) was added to the medium during greening to inhibit chloroplastic protein synthesis, synthesis of chlorophyll and formation of thylakoid membranes were also inhibited to an extent resulting in levels of chlorophyll and membranes 20–25% of those found in control cells. However, synthesis of fraction *c* was not affected by the drug. This polypeptide appeared in the soluble fraction of the cell under these conditions, indicating that this protein was synthesized in the cytoplasm as a soluble component. When normally greening cells were transferred from light to dark, synthesis of the major membrane polypeptides decreased. Also, it was found that synthesis of both subunits of ribulose 1,5-diphosphate carboxylase was inhibited by chloramphenicol, and that synthesis of this enzyme stopped when cells were transferred from light to dark.

INTRODUCTION

Cells of the *y-1* strain of *Chlamydomonas reinhardi*, a unicellular green alga, are capable of growth for several days in the absence of light, but under these conditions fail to synthesize chlorophyll and assemble thylakoid membranes within the chloroplast (1, 2). The membranes are diluted among the daughter cells, and after growth for 4–5 days in the dark in liquid culture, the cellular contents of chlorophyll and thylakoid membranes are less than 5% of those in cells grown in continuous light (2). When etiolated cells are returned to light, chlorophyll, thylakoid membranes, and the functional activities of these membranes increase in parallel (3, 4).

Thylakoid membranes are assembled within the chloroplast from proteins synthesized both in the

cytoplasm and in the chloroplast (5, 6). Two major polypeptide fractions, as revealed by gel electrophoresis, are among those synthesized in the cytoplasm (5). These polypeptides must migrate into the chloroplast for membrane formation to occur. The mechanism by which this intracellular transport occurs is not known, but it is reasonable to assume that the membrane polypeptides are transported as soluble proteins. If synthesized in a soluble form, these polypeptides should appear in the soluble fraction of the cell when normal membrane assembly is prevented.

As described in this paper, inhibiting membrane formation by treating cells with chloramphenicol resulted in the accumulation of one of the major membrane polypeptides in the soluble fraction of the cell. The results also indicate that the enzyme ribulose 1,5-diphosphate carboxylase is synthesized in the chloroplast, and is the main product of protein synthesis in the organelle. Some proteins, including ribulose 1,5-diphosphate carboxylase, are synthesized very little or not at all when cells are transferred from light to dark.

METHODS

Handling of the Cells

Cells of *Chlamydomonas reinhardi y-1* were grown in light or dark as described before (2, 4). Etiolated cells, which contained 1 μg or less of chlorophyll per 10^7 cells after growth for 4 days in the dark, were suspended in fresh medium, supplemented with KH_2PO_4 (4), to a density of 6×10^6 cells/ml. 40-ml portions of the cell suspension were added to 500-ml Erlenmeyer flasks and exposed to light from white fluorescent lamps at an intensity of about 8000 lux at 25°C while on a rotating platform. At 0 hr, 4.4 ml of a solution of chloramphenicol (2 mg/ml) were added to treated cells, while an equal volume of distilled water was added to the control cells.

Preparation of Thylakoid Membranes

Thylakoid membranes were isolated as described previously (5), except that in some experiments the broken cell preparation (in 0.3 M sucrose, 25 mM Tris[1]-HCl, pH 7.6, and 1 mM $MgCl_2$) was incubated for 15 min at 4°C after adding pancreatic deoxyribonuclease to a final concentration of 10–20 μg/ml. This procedure was found to increase the yield of membranes and facilitate suspension of the crude membrane pellet after the first centrifugation step (5). In some experiments, the isolated membranes were suspended in either distilled water, 1.0 M Tris-HCl (pH 7.6), or 1 mM EDTA[1] (pH 8.0) and allowed to stand 30 min at 4°C. While in the wash solution, the membranes were further fragmented by passing the suspension through a French pressure cell at 5000 psi. The membranes were recovered by centrifuging the sample at 100,000 *g* for 40 min.

Cell Fractionation

In order to prepare samples of total cellular protein, total membrane protein, and soluble protein, cells were washed three times at 4°C with 0.3 M sucrose containing 10 mM Tris-HCl (pH 7.6) and broken by a passage through a chilled French pressure cell at 6000 psi. To a portion of the broken-cell preparation was added trichloroacetic acid to a final concentration of 10%. The ensuing precipitate provided a sample of total *cellular* protein. The remainder of the broken-cell preparation was centrifuged at 50,000 g_{av} for 15 min at 2°C. The pellet obtained provided a sample of total *membrane* protein. The 50,000 *g* supernatant fluid, which was free of chlorophyll-containing membranes, was then centrifuged for 3 hr at 120,000 g_{av} at 2°C. The supernatant fluid was removed, and trichloroacetic acid was added to a final concentration of 10%. The ensuing precipitate provided a sample of *soluble* protein. The precipitates, each containing 3–6 mg of protein, were washed with 5 ml of 5% trichloroacetic acid and finally with 2 ml of water. The samples were stored at −15°C if not used immediately.

Gel Electrophoresis

In preparation for electrophoresis, the sample of soluble protein was dispersed in water, and the suspension was made alkaline (pH 8–9) by adding a few drops of 2% Na_2CO_3. The volume of the sample was then measured, and an equal volume of a solution containing 0.2 M Tris acetate (pH 9.0), 0.7 mM EDTA, 1.0 M urea, and 4.0% sodium dodecyl sulfate was added. The final protein concentration was about 8 mg/ml.

Samples of total cellular protein were extracted with 90% acetone at room temperature so as to remove lipids. The insoluble protein was recovered by centrifugation at 1000 *g* for 5 min and then dissolved in a solution containing 0.1 M Tris acetate (pH 9.0), 0.35 mM EDTA, 0.5 M urea, and 2% sodium dodecyl sulfate to a final protein concentration of about 8 mg/ml.

Samples of membranes were extracted with 90% acetone at room temperature. After sedimenting the insoluble protein at 1000 *g* for 5 min, the acetone extract was removed and several grains of NaCl

[1] *Abbreviations used:* Tris, tris(hydroxymethyl) aminomethane; EDTA, ethylenediaminetetraacetate; DCI, 2,6-dichloroindophenol; CAP, chloramphenicol.

were added to the extract to bring any protein remaining in the extract out of solution. The extract was then added to the extracted residue, and the sample was again centrifuged. This step was found necessary for the recovery of all membrane protein, particularly from membranes washed with EDTA. The final, combined precipitates of membrane protein were dissolved in the same solution as given above for total cellular protein except that the concentration of sodium dodecyl sulfate was 1% and the final protein concentration was about 5 mg/ml. Before electrophoresis, each sample was treated with 2-mercaptoethanol (5). The procedures for gel electrophoresis, densitometry, and the determination of radioactivity in the gels were described previously (5). During the experiments and preparations for electrophoresis, corresponding samples from control and chloramphenicol-treated cells were handled identically. Thus, the results are equivalent on a cell basis.

Preparation of Fraction I Protein

Green cells were washed two times with 25 mM Tris-HCl (pH 7.6) containing 25 mM $MgCl_2$ and broken with a French pressure cell at 6000 psi. The sample was centrifuged for 15 min at 20,000 *g* and portions of the supernatant fluid were layered over linear gradients, 32 ml in volume, of 0.15–0.60 M sucrose containing 25 mM Tris-HCl (pH 7.6) and 25 mM $MgCl_2$. The gradients were centrifuged for 6 hr at 26,000 rpm at 2°C in a SW 27 rotor, and then analyzed with an ISCO (Instrumentation Specialties Co., Lincoln, Neb.) gradient fractionator. The region of the gradients containing the Fraction I protein was collected, and the protein was precipitated by adding trichloroacetic acid to a final concentration of 10%. The sample was then treated as described above for the sample of soluble protein in preparation for electrophoresis.

Assays

Chlorophyll was extracted with 80% acetone and measured spectrophotometrically (7, 8). Photoreduction of 2,6-dichloroindophenol was determined as described previously (4). Protein was estimated by the method of Lowry et al. (9) after the dissolving of acetone-extracted samples in 0.1 N NaOH.

Materials

Acrylamide and *N,N'*-methylene-bis-acrylamide were obtained from Eastman Organic Chemicals, Rochester, N.Y.; the acrylamide was recrystallized from chloroform (10). Omnifluor and arginine-^{3}H (7.3 Ci/mmole) were purchased from New England Nuclear Corp., Boston, Mass. Triton-X100 was obtained from Beckman Instruments Inc., Fullerton, Calif. Chloramphenicol was provided by Parke-Davis. Deoxyribonuclease (Type I, ribonuclease-free) was obtained from Worthington Biochemical Corp., Freehold, N.J. Urea solutions were prepared fresh and passed through a mixed bed resin (Amberlite MB-1) before use. Concentrated stock solutions of urea, sucrose, and Tris (Sigma Chemical Co., St. Louis, Mo.) buffers were passed through a Millipore filter (Millipore Corp., Bedford, Mass.) after preparation. All other chemicals were of reagent grade.

RESULTS

Membrane Polypeptides

When etiolated cells of *Chlamydomonas reinhardi y-1* were suspended in fresh growth medium and exposed to light, chlorophyll and thylakoid membranes within the chloroplast increased rapidly after a slow phase of about 3 hr. In work described previously (5), these membranes were isolated and the polypeptide components were

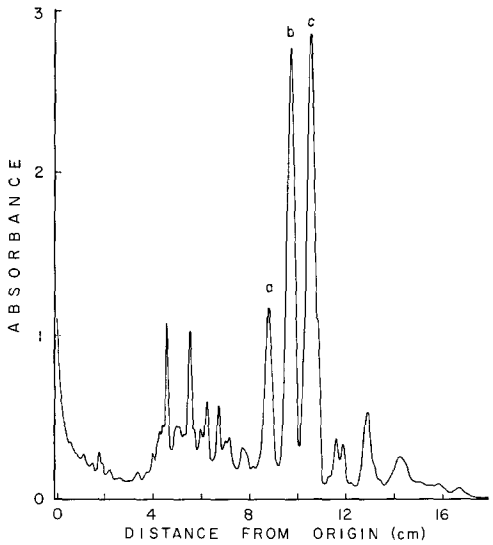

FIGURE 1 Electrophoretic analysis of the polypeptides of thylakoid membranes prepared from *C. reinhardi*. Membranes were isolated as before (5), washed with 1.0 M Tris-HCl, pH 7.6, extracted with 90% acetone, and prepared for electrophoresis as described under Methods. About 50 μg of the polypeptides (in a volume of 10 μl) were applied to the gel column and subjected to electrophoresis in the presence of sodium dodecyl sulfate (5) at 1.8 v/cm for 30 min and 6 v/cm for 7 hr. The gel was stained with Coomassie blue (5) and scanned at 563 mμ. The letters *a*, *b*, and *c* indicate fractions referred to in this paper.

examined by gel electrophoresis. Two major polypeptide fractions were resolved. Subsequently, the isolation procedure was extended to include a wash with 1.0 M Tris-HCl, pH 7.6, or with 1 mM EDTA, pH 8.0, conditions which reportedly extract some loosely bound proteins (11, 12). Fig. 1 shows the pattern of polypeptides from membranes washed with 1.0 M Tris-HCl. When compared to the pattern for membranes not washed after isolation (e.g., Fig. 2 B), the pattern in the washed sample showed only slight decreases in the amounts of some of the minor fractions. A pattern nearly identical to that shown in Fig. 1 was obtained after membranes were washed with 1 mM EDTA. The isolation procedure (5) apparently was sufficient for preparing membranes nearly free of extraneous proteins. In Fig. 1, the two prominent fractions marked *b* and *c* have molecular weights of 24,000 and 21,000, respectively (5), and together account for about 40% of the total protein stain on the gel. The assignments of the letters *a*, *b*, and *c* to the three fractions shown in Fig. 1 are for reference in this paper.

Identification of Membrane Polypeptides in Total Cellular Protein

Although electron microscopy revealed a large amount of thylakoid membrane material in green cells (2, 13), electrophoresis of total cellular protein did not reveal the membrane polypeptides as major protein components of these cells. Yet for subsequent experiments, it was necessary to establish that these polypeptides could be detected after electrophoresis of total protein. In Fig. 2, the pattern for total cellular protein (Fig. 2 A) is compared to that for the protein of isolated thylakoid membranes (Fig. 2 B). Several fractions (arrows) were resolved in the total protein sample which corresponded to the major polypeptides of the membranes. Whereas fractions containing *a* and *c* (see Fig. 1) appeared as discrete peaks in the total protein pattern, *b* appeared as a shoulder on the trailing side of another, larger fraction. The alignment of the patterns was confirmed by electrophoresis of a mixture of the membrane protein and the total protein.

So as to determine definitively whether the fractions in the total protein sample indicated by arrows in Fig. 2 were membrane components, the electrophoretic pattern for total protein of green cells was compared to that for yellow cells. Since yellow cells contain little thylakoid membranes (2), a specific difference in the amounts of the membrane polypeptides might be revealed by such a comparison. As shown in Fig. 3, the pattern of protein from green cells (solid line) showed a peak corresponding to membrane fraction *c* (at 10 cm from the origin, also indicated

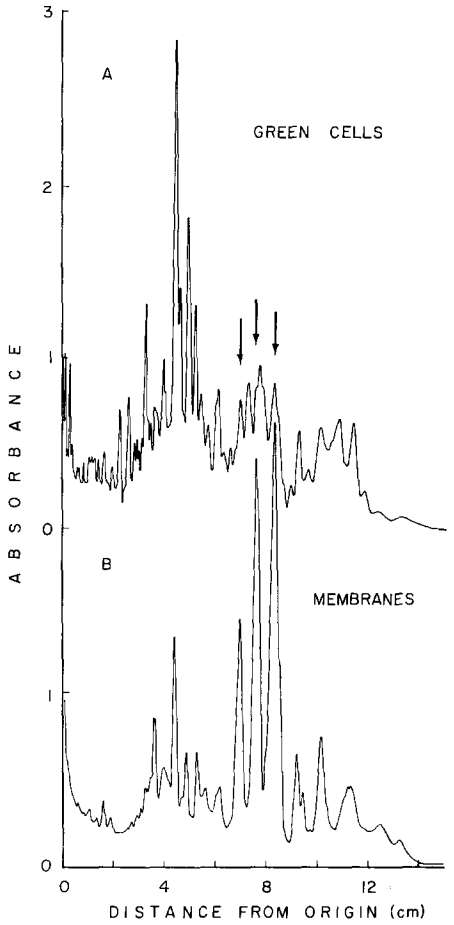

FIGURE 2 Comparison of the electrophoretic patterns for total polypeptides of green cells (Fig. 2 A) and for thylakoid membrane polypeptides (Fig. 2 B). Total protein was obtained, as described under Methods, from *C. reinhardi* cells grown 2 days in continuous light. Thylakoid membranes were isolated (5) from green cells. The two samples were extracted with 90% acetone and were prepared for electrophoresis as described under Methods. Portions of the samples containing about 50 μg of the polypeptides were subjected to electrophoresis on companion gel columns at 1.8 v/cm for 30 min and 7.2 v/cm for 4 hr. The arrows show the position, in the total polypeptide pattern, of the membrane fractions marked *a*, *b*, and *c* in Fig. 1. The alignment of the patterns was checked by scanning a gel containing a mixture of the two samples.

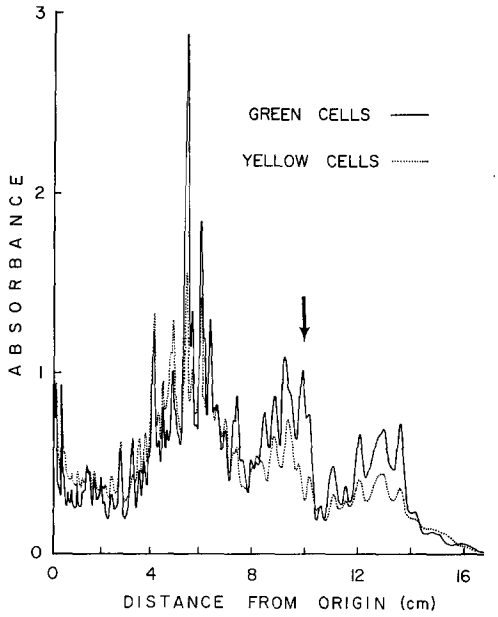

FIGURE 3 Comparision of the electrophoretic patterns for total polypeptides of green and yellow cells. Total protein from about 6×10^7 cells was obtained for each sample and treated in preparation for electrophoresis as described under Methods. Polypeptides in a volume of 10 μl were subjected to electrophoresis on each gel column at 1.8 v/cm for 30 min and 6 v/cm for 6.75 hr. The total area under the trace for green cells was about 35% greater than that for yellow cells, which is approximately the difference in protein content per cell between green and yellow cells (4, 6).

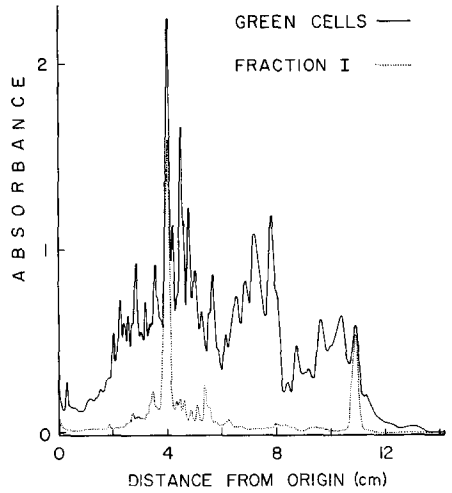

FIGURE 4 Comparision of the electrophoretic patterns for total polypeptides of green cells and of Fraction I protein. Total protein of green cells and Fraction I protein were obtained as described under Methods. The samples were treated as described for total protein, except that Fraction I protein was not extracted with acetone. Polypeptides in a volume of 10 μl were subjected to electrophoresis on each gel column at 1.8 v/cm for 30 min and 7.2 v/cm for 4.5 hr. The alignment of the patterns was checked by scanning a gel containing a mixture of the two samples.

by arrow), but no corresponding peak was observed in the pattern of protein from yellow cells (dotted line). The two small peaks in this region of the pattern for yellow cells were present in the pattern for green cells as shoulders on either side of the peak for fraction *c*. A fraction containing membrane fraction *a* (at 8.5 cm from the origin) was also present in the pattern for green cells but not in that for yellow cells. Membrane fraction *b* was again less well resolved.

Identification of the Subunits of Ribulose 1,5-Diphosphate Carboxylase in the Total Protein Sample

With the exception of the region from 8 to 10.5 cm from the origin, the patterns shown in Fig. 3 were *qualitatively* similar although some fractions were present in different amounts in the two types of cells. The main polypeptide in both green and yellow cells migrated at a rate corresponding to a molecular weight of about 55,000 (the peak at 5.5 cm from the origin in Fig. 3). This is the size of the large subunit of ribulose 1,5-diphosphate carboxylase (14, 15). Also, the fraction at 13.5 cm contained polypeptides the size of the small subunit of this enzyme (14). This identification was supported by comparing the electrophoretic pattern for total protein of green cells with that of Fraction I protein, which is primarily ribulose 1,5-diphosphate carboxylase (16). As shown in Fig. 4, the prominent polypeptide in the sample from green cells migrated at the same rate as the large subunit of this enzyme (to 4 cm from the origin in this experiment). The identification of the small subunit (at 11 cm) in the total protein pattern was also confirmed. The results shown in Fig. 3, in agreement with measurements of enzymatic activity (2), indicated that the level of this enzyme in green cells was about two times that in the yellow cells.

Effects of Chloramphenicol on Formation of Membrane and Synthesis of Polypeptides

Since it was possible to resolve a major polypeptide of thylakoid membranes and the two sub-

units of ribulose 1,5-diphosphate carboxylase by gel electrophoresis of total cellular protein, experiments were done in order to find out how these proteins are affected when chloroplastic protein synthesis is inhibited. As was found earlier (5), the two major polypeptide fractions of the thylakoid membranes are synthesized in the cytoplasm and therefore synthesis of these proteins should not be affected when protein synthesis is inhibited in the chloroplast. However, if membrane assembly were concomitantly inhibited, these proteins might accumulate in a nonmembrane fraction.

Chloramphenicol is an inhibitor of protein synthesis on chloroplastic ribosomes (17–20) and, as a result, of chloroplast development (4, 21, 22). When etiolated *Chlamydomonas* cells were exposed to light in the presence of 200 μg of chloramphenicol/ml, the increase in chlorophyll was strongly inhibited compared to control cells (Fig. 5).

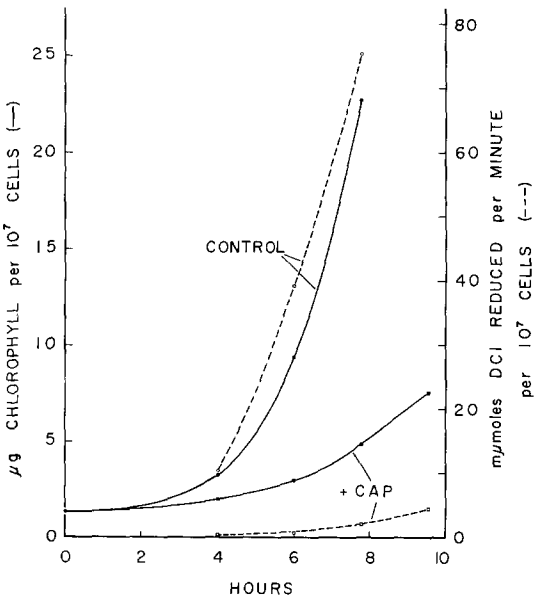

FIGURE 5 Effects of chloramphenicol at 200 μg/ml on the increases in chlorophyll and Hill reaction activity during greening of etiolated *C. reinhardi*. Yellow cells were suspended in fresh medium to 4×10^6 cells/ml and exposed to 8000 lux from white fluorescent lamps at 25°C. Each 50 ml portion of the suspension (in 500-ml flasks) received 5.5 ml of either chloramphenicol (2 mg/ml) or water at 0 hr. At the times indicated, control and chloramphenicol-treated samples were assayed for the ability to photoreduce DCI as described previously (4). Chlorophyll was measured spectrophotometrically (7) in 80% acetone extracts (——), chlorophyll; (----), photoreduction of DCI.

Furthermore, photoreduction of 2,6-dichloroindophenol was barely detectable in chloramphenicol-treated cells. Previous data indicated that proteins necessary for this activity of the membrane were synthesized on chloroplastic ribosomes (4). Examining cells with the electron microscope revealed an amount of thylakoid membranes in chloramphenicol-treated cells approximately in proportion to the amount of chlorophyll present (J. K. Hoober and G. E. Palade, unpublished results).

The magnitude of the chloramphenicol effect shown in Fig. 5 was dependent upon the time of addition of the drug. Results similar to that in Fig. 5 were obtained when chloramphenicol was added during the 1st hr of light exposure. If the drug was added after 3–4 hr of exposure to light, when the cells were actively making chlorophyll, no inhibition was observed until about 3 hr later, even though a clear effect was seen on chloroplastic ribosomes in less than 15 min under similar conditions (18). These results suggest that chloramphenicol affects chlorophyll synthesis indirectly, possibly by inhibiting the synthesis of catalytic proteins needed for chlorophyll biosynthesis.[2]

Since chloramphenicol does not affect cytoplasmic protein synthesis (17, 18), it can be assumed that the major membrane polypeptides are still synthesized in the presence of this drug. This was tested by the following experiment. Chloramphenicol was added to the culture medium to a concentration of 200 μg/ml at the time yellow cells were exposed to light. Arginine-^{3}H was then added at 5 hr, i.e. after control cells had begun to actively make thylakoid membranes, and 90 min later control and chloramphenicol-treated cells were collected. Total cellular protein samples were subjected to electrophoresis, and the patterns of radioactivity were determined.

[2] The effects of different concentrations of chloramphenicol should be emphasized. As found previously (4), chloramphenicol at 20–25 μg/ml caused only a small inhibition of chlorophyll synthesis. It was estimated that chloroplastic protein synthesis was inhibited about 70% under these conditions, and thus sufficient synthesis of enzymes possibly occurred to allow nearly normal rates of chlorophyll synthesis. Chloramphenicol at 200 μg/ml produced a maximal level of inhibition of protein synthesis for this drug (4), and also produced the effects on greening shown in Fig. 5. However, it is also possible that at this concentration the drug inhibited chlorophyll synthesis by means other than via its effects on protein synthesis.

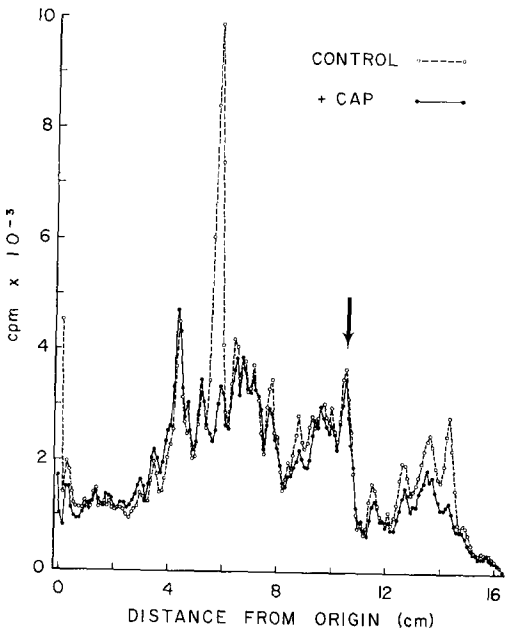

FIGURE 6 Electrophoretic analysis of the incorporation of arginine-^{3}H into polypeptides during greening of *C. reinhardi* cells incubated with or without chloramphenicol (200 μg/ml). Yellow cells were suspended in fresh medium to 6 × 10^6 cells/ml. Each 40 ml portion (in 500-ml flasks) received 4.4 ml of either chloramphenicol (2 mg/ml) or water at 0 hr. After 5 hr in light, arginine-^{3}H was added to each flask to a final concentration of 1 μCi per ml, and 90 min later the cells were collected, washed with 0.3 M sucrose containing 10 mM Tris-HCl, pH 7.6, and broken in the same buffer. Total protein samples were prepared for electrophoresis as described under Methods, and portions of the samples containing about 160 μg of the polypeptides in a volume of 20 μl were applied to companion gel columns and subjected to electrophoresis at 2 v/cm for 20 min and 6 v/cm for 7 hr. After staining, the gels were cut into 1-mm sections, each of which was incubated at 55°C overnight with 0.1 ml of 30% H_2O_2. After cooling, 10 ml of a solution containing toluene, Triton-X100, and Omnifluor (5) were added to each, and the radioactivity was determined. The arrow indicates the position in the gel of membrane fraction *c*.

Fig. 6 shows, firstly, that the labeling pattern for control cells (dashed line) was related to the pattern of protein stain for green cells (Figs. 2 and 3). Thus, many proteins are synthesized during the greening period, and not only membrane proteins. Secondly, it is apparent that chloramphenicol had no *general* inhibitory effect on protein synthesis (solid line). In particular, no significant inhibition was observed for the incorporation of arginine-^{3}H into the fractions which contain the major membrane polypeptides (from 9 to 11 cm from the origin), supporting the conclusion that these polypeptides are synthesized in the cytoplasm (5). However, incorporation of arginine-^{3}H into the fractions containing the large (at 5.8 cm) and the small (at 14.2 cm) subunits of ribulose 1,5-diphosphate carboxylase was inhibited. Thus, both subunits of this enzyme of the chloroplast are apparently made on chloroplastic ribosomes.

Although electron microscopy indicated little membrane formation in chloramphenicol-treated cells, the relative amounts of the membrane polypeptides in membrane were determined as a further test of the amount of the thylakoid membrane present in these cells. A total membrane fraction was, therefore, prepared by centrifuging the broken-cell preparations at 50,000 g_{av} for 15 min.[3] Electrophoresis of the protein of these fractions produced the patterns of stain shown in Fig. 7 A. Compared to the control, only small amounts of the major polypeptides of thylakoid membranes were present in the membrane fraction from chloramphenicol-treated cells. In the same experiment, the polypeptides were labeled with arginine-^{3}H as described above for the experiment shown in Fig. 6. Fig. 7 B shows the radioactivity recovered in polypeptides in the membrane fraction. (Since the top portion of the gel contained little radioactivity and no significant difference was observed between the two samples in the region 0–3 cm, as in Fig. 6, the top portion of the gels was not fractionated in this and subsequent experiments.) In control cells a large amount of radioactive fractions *a*, *b*, and *c* was incorporated into membranes. The pattern of radioactivity for the control sample (dashed line) was similar to that found previously for isolated thylakoid membranes (5). Labeled subunits of ribulose 1,5-diphosphate carboxylase were also present, possibly sedimenting in association with membranes (23). However, little radioactivity was found in these fractions from chloramphenicol-treated cells (solid line). In control cells, fraction

[3] Although this sample would be contaminated by other cellular membranes, a total membrane fraction was prepared to enable a quantitative evaluation of the amount of thylakoid membranes present. From the electrophoretic patterns, it is apparent that thylakoid membranes are the predominant constituent of this fraction in control cells.

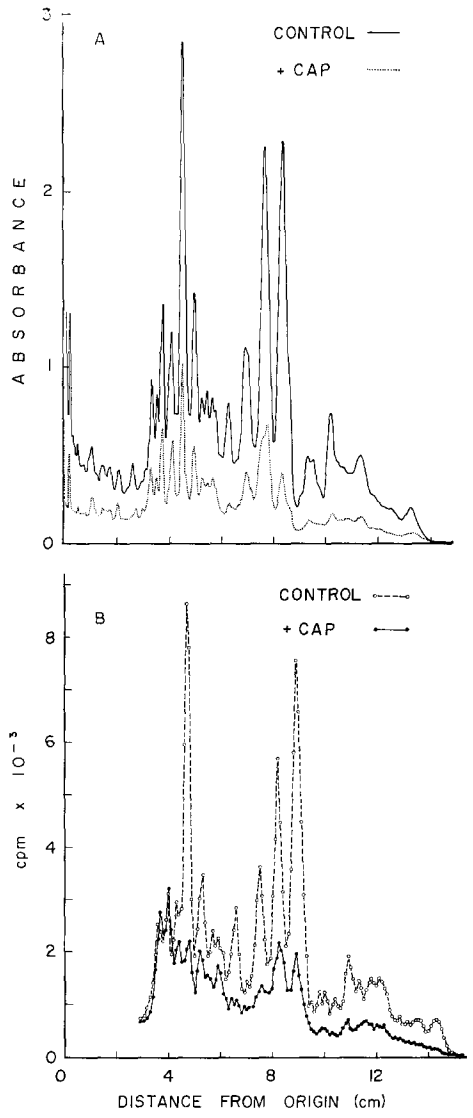

FIGURE 7 Patterns of protein stain (Fig. 7 A) and radioactivity (Fig. 7 B) after electrophoresis of polypeptides in the total membrane fraction from cells incubated with or without chloramphenicol (200 μg/ml). The experimental conditions were as described under Fig. 6. Arginine-^{3}H was added at 5 hr to 1 μCi/ml, and 2 hr later the cells were collected, broken, and centrifuged for 15 min at 50,000 g_{av} at 2°C. The pellet fractions were prepared for electrophoresis as described under Methods, and the polypeptides were subjected to electrophoresis at 1.8 v/cm for 30 min and 6 v/cm for 6 hr. After staining, the gels were scanned at 563 mμ or sectioned for a determination of radioactivity as described under Fig. 6.

c contained more label than fraction *b*, but the labeling, albeit low, was reversed in chloramphenicol-treated cells. Thus, relatively more of fraction *b* had entered membrane material than did fraction *c* in the treated cells. The amounts of fraction *c* in membranes, estimated from results as shown in Fig. 7, were comparable to the amounts of chlorophyll present. In these experiments, chloramphenicol-treated cells contained 20–25% of the chlorophyll found in control cells (e.g., Fig. 5).

In control cells, newly synthesized polypeptides were continuously incorporated into growing membranes (Fig. 7 B). However, in chloramphenicol-treated cells fewer polypeptides were incorporated into thylakoid membranes. If in treated cells the membrane polypeptides continue to be synthesized in the cytoplasm at a rate higher than the rate of incorporation into thylakoid membranes, and if these polypeptides are synthesized in a soluble form and are in transit through the cell sap or chloroplast, then they should be detectable among the proteins of the soluble fraction under these experimental conditions. As a test of this assumption, the broken-cell preparations were centrifuged for 3 hr at 120,000 g_{av} so as to sediment particulate material. The protein in the supernatant fluid was then subjected to electrophoresis. Fig. 8 shows the patterns of protein stain (Fig. 8 A) and of radioactivity (Fig. 8 B) for the soluble polypeptides from control and chloramphenicol-treated cells. The control gel contained a small amount of radioactivity (dashed line) in the region of the major membrane polypeptides (8–10 cm from the origin). But in the sample from chloramphenicol-treated cells a much greater amount of radioactivity was found at 9.2 cm (arrow). This peak of radioactivity was in the position expected for membrane fraction *c*. Since there was no significant difference in this region in radioactivity from the total protein samples (Fig. 6), the results shown in Figs. 7 and 8 suggest that in the chloramphenicol-treated cells fraction *c* was accumulating in the soluble fraction instead of entering the membranes, as occurred in control cells. In one experiment, the difference in this region between the amounts of radioactivity in the samples of soluble protein from control and treated cells was about 3800 cpm. The difference in an equivalent amount of the membrane fraction was about 4600 cpm. Thus, approximately 80% of the amount missing from membranes in

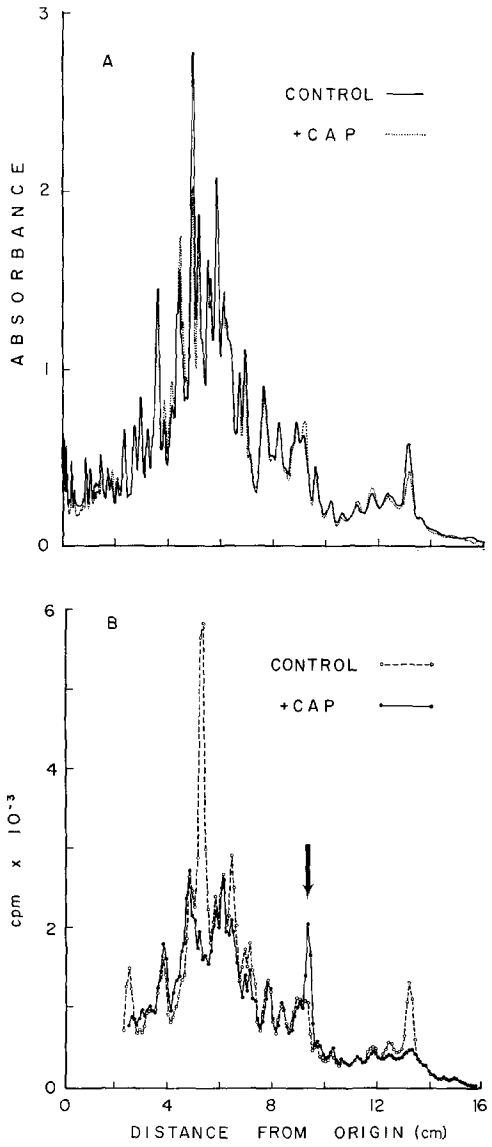

FIGURE 8 Patterns of protein stain (Fig. 8 A) and radioactivity (Fig. 8 B) after electrophoresis of soluble polypeptides from cells incubated with or without chloramphenicol (200 μg/ml). The experimental conditions were as described under Fig. 6. Arginine-^{3}H was added at 5 hr to 1 μCi/ml, and 2 hr later the cells were collected, broken, and centrifuged for 3 hr at 120,000 g_{av} at 2°C. Protein in the supernatant fluids was prepared for electrophoresis as described in Methods. Portions of the samples containing 90 or 180 μg of the polypeptides were applied to companion gel columns and subjected to electrophoresis at 1.8 v/cm for 30 min and 6 v/cm for 6.5 hr. After staining, gels containing the smaller loads were scanned, while gels containing the higher loads were sectioned to determine the radioactivity pattern as described under Fig. 6. The chlorophyll level increased during the experiment from 0.7 μg/10^7 cells at 0 hr to 1.2 μg and 6.8 μg of chlorophyll/10^7 cells in chloramphenicol-treated and control cells, respectively.

chloramphenicol-treated cells was recovered in the soluble fraction. Only a small difference in the protein stain was found at this position in these short-term experiments (Fig. 8 A).

The amount of this polypeptide, characteristic of fraction *c*, in the soluble fraction was related to the degree of inhibition of membrane formation. When chloramphenicol was added after 3 hr of exposure to light, near the end of the slow phase of greening (Fig. 5), chlorophyll synthesis was inhibited during the time period of the experiments to a lesser extent than when the drug was added at 0 hr, as described above. In such experiments, after 7 hr of light exposure the treated cells contained 65–75% of the amount of chlorophyll contained in control cells. Arginine-^{3}H was added at 5 hr, and 2 hr later the cells were collected and the soluble fractions were prepared. Electrophoresis of the soluble protein revealed only a slight increase, compared to Fig. 8, in radioactivity in the position of fraction *c* for treated cells over that for control cells. Also, only a correspondingly small decrease in radioactivity in this polypeptide was observed in the membrane fraction from such treated cells when compared to this fraction from control cells. Since less of this polypeptide fraction was recovered in the soluble fraction and more in the membrane fraction from cells in which membrane formation was inhibited less, these experiments provided further evidence that the soluble polypeptide is related to the membrane, and is fraction *c* of thylakoid membranes. Also, these results show that the appearance of the polypeptide in the soluble fraction was not simply the result of solubilization of a component of membranes assembled in the presence of the drug.

Attempts to chase the soluble protein into membrane after removing chloramphenicol were unsuccessful, since the cells recovered from the treatment only after a lag period of several hours, a period deemed too long to provide a clear result.

These experiments have not revealed the fate of membrane fractions *a* and *b*. Minor membrane polypeptides were present at concentrations too low to detect by the procedure used.

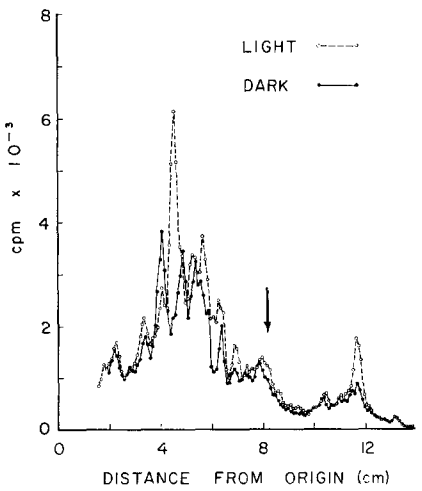

FIGURE 9 Electrophoretic analysis of the incorporation of arginine-^{3}H into soluble polypeptides of greening *C. reinhardi* cells incubated 1 hr in light or dark. Yellow cells were suspended to 6 × 10^6 cells/ml in fresh medium and exposed to light as described under Fig. 6, except that chloramphenicol was not added. After 6 hr of greening, one flask was wrapped with foil and placed in the dark. Arginine-^{3}H was added to a concentration of 1 μCi/ml to both the flask in the dark and to an equivalent flask kept in the light. 1 hr later, the cells were collected and the soluble fractions were prepared for electrophoresis as described under Methods. Portions of the samples containing about 160 μg of the polypeptides were applied to companion gel columns and subjected to electrophoresis at 1.8 v/cm for 20 min and 6 v/cm for 6 hr. Radioactivity was determined as described in Fig. 6. Chlorophyll levels at the end of the experiment were: 12.4 μg/10^7 cells (no increase during the labeling period) and 16.3 μg/10^7 cells for cells placed in the dark and kept in light, respectively.

Effect of Light on Protein Synthesis

Since treating cells with chloramphenicol caused concomitant inhibition of chlorophyll synthesis and accumulation of the fraction *c* polypeptide in the soluble fraction of the cell, a relationship between this polypeptide and chlorophyll was suggested. In these cells, chlorophyll synthesis can also be stopped by turning off the light (3). Thus, a result similar to Fig. 8 might be expected if cells were placed in the dark instead of being treated with chloramphenicol. Fig. 9 shows the results obtained from an experiment in which cells, after 6 hr of greening, were placed in the dark. Arginine-^{3}H was then added, and incorporation of the amino acid into soluble proteins by cells in the dark was compared after electrophoresis to that of an identical sample of cells kept in the light. No increase in radioactivity was observed for samples labeled in the dark in the region of the gel to which fraction *c* would migrate (arrow). Total membrane fractions (50,000 *g* pellet) were examined in order to determine whether labeled polypeptides were present in membranes. Fig. 10 shows, however, that a marked reduction of labeling of the membrane polypeptides occurred in cells transferred to the dark. Thus, apparently the synthesis of these major polypeptides as well as chlorophyll decreases in the dark. This agrees with results shown in Fig. 3, since after several days in the dark, the membrane polypeptides could not be observed in samples of total protein of yellow cells.

In confirmation of results reported recently by Schor et al. (24), no labeling of ribulose 1,5-diphosphate carboxylase occurred during the 1st hr after cells were transferred to the dark. Darkness was as effective as chloramphenicol in stopping synthesis of this enzyme. Lack of synthesis of other proteins was also detected (e.g., fractions near 6 cm from the origin in Fig. 9). Yet the synthesis of most soluble proteins appeared unaffected. These results provide further evidence that protein

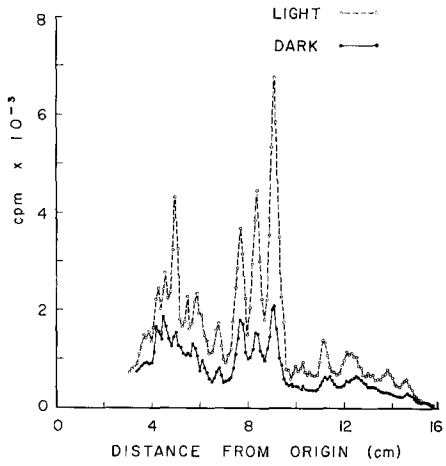

FIGURE 10 Electrophoretic analysis of the incorporation of arginine-^{3}H into total membrane polypeptides of greening *C. reinhardi* cells incubated 1 hr in light or dark. Experimental conditions were as for Fig. 9. Membrane fractions were prepared as in Fig. 7, and the polypeptides were subjected to electrophoresis at 1.8 v/cm for 30 min and 6 v/cm for 6.5 hr. Radioactivity was determined as described under Fig. 6.

synthesis does not occur in the chloroplast immediately after cells are transferred to the dark. But the extent of protein synthesis in the cytoplasm, as evidenced by the extent of labeling of soluble proteins (Fig. 9), was considerably more than was expected from data reported by Ohad et al. (3) for the incorporation of labeled acetate into protein in a similar type of experiment.

DISCUSSION

The array of thylakoid membranes within the chloroplast is a prominent structural feature in *C. reinhardi* cells grown in light. These membranes contain two main polypeptide components which, at present, can only be identified by electrophoresis. Thus, to determine the fate of these polypeptides, it was necessary to establish that they could be detected after electrophoresis of total protein of the cells. The main membrane polypeptides are not prominent components of the cells, but Figs. 2 and 3 show that they can be detected.

Since chlorophyll is a constituent of the thylakoid membranes (25), determining the chlorophyll content during greening provided an approximation of the amount of membranes present. When cells were treated with a relatively high level of chloramphenicol (200 μg/ml), an inhibitor of protein synthesis in the chloroplast (17–20), chlorophyll synthesis and membrane formation were inhibited. But previous results, obtained at a lower chloramphenicol concentration (25 μg/ml), showed that membrane assembly continued in spite of extensive inhibition of synthesis of membrane proteins in the chloroplast. The marked decrease in membrane production at the higher concentration (200 μg/ml) was possibly the result of effects of chloramphenicol on the synthesis of membrane lipids or on the production of enzymes involved in the synthesis of chlorophyll.[2]

When chlorophyll synthesis was inhibited in the presence of chloramphenicol, polypeptides which are synthesized in the cytoplasm (5) and whose characteristics are those of fraction *c* polypeptides of thylakoid membranes appeared in the soluble fraction of the cell (Fig. 8). Conversely, when the synthesis of these polypeptides was inhibited by cycloheximide, an abrupt halt in the accumulation of chlorophyll and membranes resulted (4). Eytan and Ohad (6) showed, however, that prior treatment of *C. reinhardi* with chloramphenicol under conditions similar to those described in this paper relieved the inhibition of chlorophyll synthesis by cycloheximide. Pretreatment of *Euglena* cells with chloramphenicol also produced similar results (26). Thus, treatment with chloramphenicol apparently allowed the accumulation of proteins, made on cytoplasmic ribosomes, which are needed for chlorophyll accumulation. As Fig. 8 shows, the only soluble[4] polypeptide that accumulated in *C. reinhardi* during treatment with chloramphenicol was the fraction corresponding to membrane fraction *c*. Concomitantly, a decrease in the amount of fraction *c* in membranes was found in chloramphenicol-treated cells comparable to the decrease in the amount of chlorophyll in these cells. This evidence, albeit circumstantial, suggests a relationship between these two membrane components. Since the synthesis of chlorophyll appears confined to the chloroplast (27, 28), assembly of the membrane can, therefore, occur only in the chloroplast.

The fate of the other major polypeptide, fraction *b*, during treatment with chloramphenicol is less clear than that of fraction *c*. Relatively more of fraction *b* than of fraction *c* entered membrane material in the treated cells (Fig. 7), but this amount was still small compared to that in control cells. Yet, none of fraction *b* was observed in the soluble fraction of these cells. In the presence of chloramphenicol a decrease in the synthesis of this polypeptide possibly occurred.

Although fraction *c* appears to be an integral part of the thylakoid membranes, its fate when cells are treated with chloramphenicol resembles that of the inner mitochondrial membrane appendage, the coupling factor F_1, in yeast cells treated with this drug. F_1, also synthesized in the cytoplasm, accumulated as a soluble protein when mitochondrial development was inhibited (29). This suggested that some proteins do not attach to the membrane unless the proper sites have been prepared for them. A coupling factor from chloroplasts, CF_1, whose subunits have a molecular weight of 62,000, has been purified (30). This chloroplast coupling factor also appears to be synthesized in the chloroplast (31). It, therefore, is distinct from the major polypeptides shown in Fig. 1.

[4] It should be emphasized that in this context "soluble" refers to character and not location within the cell, since these experiments cannot indicate whether at the time of cell disruption the polypeptide was in the cytosol, in the chloroplast matrix, or in both.

On the basis of the effects of chloramphenicol, the site of synthesis in *C. reinhardi* of both subunits of ribulose 1,5-diphosphate carboxylase is the chloroplast. This conclusion was previously reached by Margulies (20, 32) and Smillie et al. (33) for bean and *Euglena* cells, respectively. Several reports have suggested the involvement of cytoplasmic ribosomes in the synthesis of this chloroplastic enzyme because of the inhibitory effects of cycloheximide on its synthesis (34, 35). However, there is some evidence that suggests the messenger RNA for this enzyme is made in the nucleus. Several mutations affecting this enzyme in tomato plants are located on nuclear chromosomes (36). Also, rifampicin, which inhibited chloroplast RNA synthesis in *C. reinhardi* (37), did not affect its synthesis (35). Rather than inhibiting translation of the messenger RNA for this enzyme, cycloheximide might interfere with the production or transfer of RNA from the nucleus to the chloroplast.

Results shown in Figs. 9 and 10 suggest a role for light in the regulation of protein synthesis in *C. reinhardi*. As observed by Schor et al. (24), protein synthesis stopped abruptly in the chloroplast when cells were placed in the dark. This would explain the absence of label in the subunits of the enzyme ribulose 1,5-diphosphate carboxylase. Nevertheless, activities in the chloroplast must resume within a short time since ribulose 1,5-diphosphate carboxylase (2) and chloroplastic ribosomes (18) and DNA (38) are synthesized when the cells remain in the dark.

The synthesis in the cytoplasm of the main polypeptides of the thylakoid membranes also apparently is regulated by light. Although in the dark the rate of synthesis of these polypeptides did not decrease as rapidly as that of ribulose 1,5-diphosphate carboxylase, as judged from the amount of arginine-^{3}H incorporated into these polypeptides (Fig. 10), synthesis of these components eventually decreased to a rate resulting in a level too low to detect in yellow cells. The mechanism of this regulation of protein synthesis by light is not known.

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