

KINETICS AND REGULATION OF SYNTHESIS OF THE
MAJOR POLYPEPTIDES OF THYLAKOID
MEMBRANES IN *CHLAMYDOMONAS*
REINHARDTII *y-1* AT ELEVATED TEMPERATURES

J. KENNETH HOOBER and WILLIAM J. STEGEMAN

From the Department of Biochemistry, Temple University
School of Medicine, Philadelphia, Pennsylvania 19140

ABSTRACT

Etiolated cells of *Chlamydomonas reinhardtii y-1* exhibit rapid and linear initial kinetics of greening when exposed to light at 38°C. The initial rate of chlorophyll accumulation under these conditions is greater than the maximal rate during greening at 25°C. Synthesis of the major polypeptides of thylakoid membranes within intact cells was assayed during greening by the incorporation of [³H]leucine and the subsequent electrophoresis of total cellular protein on polyacrylamide gels in the presence of sodium dodecyl sulfate. At 38°C the major membrane polypeptides (about 28,000 and 24,000 daltons in mass) were synthesized at a linear rate after exposure of the cells to light, with no evidence of a lag period. A 1–2 h preincubation in the dark at the higher temperature was necessary to achieve linear initial kinetics. Actinomycin D inhibited synthesis of the membrane polypeptides if added at the beginning of a 2 h dark preincubation, but not when added near the end. These results suggested that transcription of the messenger RNA for the membrane polypeptides occurred during the dark period at 38°C. But the major membrane polypeptides were not made by *y-1* cells in the dark. The wavelengths of light most effective in eliciting production of the membrane polypeptides were the same as those allowing chlorophyll synthesis. In contrast, wild type cells, which are capable of chlorophyll synthesis in the dark, also make the membrane polypeptides in the dark. The data indicate that at elevated temperatures synthesis of the major thylakoid membrane polypeptides is controlled at a posttranscriptional step, and that this reaction normally proceeds only under conditions which permit reduction of protochlorophyllide.

Greening of etiolated plant cells is characterized by the development of photosynthetic competency. The elemental process in the course of this development is the formation of thylakoid membranes within chloroplasts in response to light. Thylakoid membranes, which convert light energy

into chemical energy, constitute the major system of membranes within light-grown plant cells. Although these membranes are located within the chloroplast, their formation requires the participation of synthetic systems in the cytoplasmic matrix as well as in the chloroplast. Chlorophyll and

other membrane lipids are synthesized within the chloroplast (8, 11, 42, 43), but the membrane proteins are synthesized both inside and outside the organelle (12, 17, 30). Indeed, as work on the effects in vivo of inhibitors of protein synthesis (10, 13, 17, 21, 30) and with isolated chloroplasts (4, 9) has shown, the major integral polypeptides of this membrane are synthesized outside the chloroplast on cytoplasmic ribosomes.

A central question concerning the assembly of thylakoid membranes is the mechanism(s) by which light controls the process. A reaction that is known to directly involve light is the reduction of protochlorophyllide to chlorophyllide (28, 31, 39, 47). But how this reaction influences synthesis of a number of other membrane components is not known. Of particular interest is the manner by which synthesis of chlorophyll controls synthesis of the major membrane polypeptides.

To examine the control by light of the synthesis of the membrane polypeptides, it is advantageous to study a system which responds rapidly to light. Etiolated cells of the *y-1* strain of *Chlamydomonas reinhardtii* exhibit relatively rapid rates of greening as compared to several other algae and higher plants (19). At 25°C, an initial lag of about 2 h in duration occurs, after which the process proceeds rapidly until the full complement of chlorophyll (20–30 $\mu\text{g}/10^7$ cells) is achieved in about 8 h (20, 21). However, as described in this report, at 38°C greening begins with no lag, provided the cells are preincubated in the dark for 1–2 h before exposure to light. Under these conditions, synthesis of both chlorophyll and of the major membrane polypeptides exhibits linear kinetics during the first hour in the light, with initial rates of chlorophyll synthesis that are greater than the maximal rate at 25°C. The results reveal that a mechanism exists to control the synthesis of the membrane polypeptides at a posttranscriptional stage.

MATERIALS AND METHODS

Greening Experiments

Cells of *Chlamydomonas reinhardtii y-1* were grown in the dark for 4 days at 25°C as described before (20). Etiolated cells, containing 1–2 μg of chlorophyll/ 10^7 cells, were suspended at a density of 6×10^6 cells/milliliter in fresh medium supplemented with KH_2PO_4 (20). For routine experiments, 4.0-ml portions of the cell suspension were placed into 50-ml beakers wrapped with black vinyl tape. The beakers were capped with clear plastic dishes, placed in a constant temperature water-bath equipped with a rotating platform (Model G-86,

New Brunswick Scientific Co., Inc., New Brunswick, N. J.), and illuminated with 200-W incandescent lamps mounted above the bath. Incident light intensity, measured after the light passed through a 7-cm water filter, was about 8×10^4 erg/cm²/s.

Labeling of Cells

L-[4,5-³H]leucine (50–60 Ci/mmol, 1 mCi/ml) (Amersham/Searle Corp., Arlington Heights, Ill.) was mixed with L-[¹H]leucine (15 mM in 0.2 M KH_2PO_4) in a ratio of 1:0.1 (vol/vol). To 4.0 ml of a cell suspension was added 0.1 ml of this mixture, resulting in a final concentration of leucine of about 3×10^{-5} M with a sp act of 0.7 Ci/mmol. At the end of the labeling period, the cell suspensions were each mixed with 1.0 ml of 50% (wt/vol) trichloroacetic acid on ice. The samples were centrifuged, and the pellets were washed two times with 5% trichloroacetic acid and finally with H_2O . Pellets were stored frozen until analyzed by electrophoresis.

Gel Electrophoresis

The procedure described previously (17, 18) for electrophoresis in the presence of sodium dodecyl sulfate (SDS) was followed. A well-drained pellet containing 2.4×10^7 cells (about 0.7 mg of protein) was suspended in 0.1 ml of 0.1 M Tris-acetate (pH 9.0) containing 2% (wt/vol) SDS, 0.01% (wt/vol) ethylenediaminetetraacetic acid (EDTA), and 0.5 M urea. To the resulting suspension was added 3 μl of 2-mercaptoethanol, and the tube was flushed with N_2 and sealed with a serum stopper. The sample was heated for 2 min at 70°C to achieve solubilization and then incubated for 30 min at 40°C. A vol of 30 μl was applied to gels which, after staining, were sliced for determination of the pattern of radioactivity. Each 1-mm slice was digested in 0.1 ml of 30% H_2O_2 at 55°–60°C overnight in 8-ml vials (Research Products International Corp., Elk Grove Village, Ill.) and counted in 5 ml of a mixture containing 750 ml toluene, 250 ml of Triton X-100, 40 ml of H_2O and 5 g of Omnifluor (New England Nuclear Corp., Boston, Mass.).

Preparation of the Major Thylakoid Membrane Polypeptides

Thylakoid membranes were purified as described previously (17). The purified membranes were dispersed in 8 M urea, and after standing on ice for 30 min were pelleted by centrifugation at 100,000 g_{av} for 2 h. The urea-washed membranes were extracted two times with 80% acetone, and the residue was taken nearly to dryness under a stream of N_2 . The protein was dissolved in a vol of 8% (wt/vol) SDS, containing 0.1 M Tris-acetate (pH 9.0), 0.5 M urea, and 0.01% EDTA, sufficient to provide a detergent: protein ratio of 2.5:1 (mg/mg). The solubilized protein was treated with 2-mercaptoethanol and subjected to electrophoresis in the presence of SDS on 10% polyacrylamide gels. The sample volume on

each gel was 20 μ l. After electrophoresis, the positions of the major polypeptide fractions (*b* and *c*, see Fig. 1) were located by fixing the gels overnight in isopropanol-acetic acid-H₂O (25:10:65, vol/vol/vol) (14) followed by a 30–60 min treatment with fixative containing 0.02% Coomassie Blue. The bands containing fractions *b* and *c* were cut out of the gels, rinsed with H₂O, and crushed by passage through a syringe. Protein was extracted into several vol of 0.1 M NH₄HCO₃ containing 0.1% SDS for 3 h at 50°C. The pH was adjusted, if necessary, to 8.5–9 with NH₃. The extraction procedure was repeated for 2 h. Gel particles were removed by filtration, and the extract was lyophilized. The powder was dissolved in a small volume of water, and the protein was precipitated by adding acetone to 80% (vol/vol).

Amino Acid Analysis

Protein samples were hydrolyzed with 6 N HCl in sealed, evacuated vials. Methionine and cystine (half) were estimated after hydrolysis of proteins oxidized with performic acid (37). Tryptophan was determined with 90%–95% recovery after hydrolysis with 3 N *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)-indole (29). The amino acids were analyzed on a single column with a Beckman Model 119 automatic amino acid analyzer (Beckman Instruments, inc., Spincro Div., Palo Alto, Calif.) equipped with an Infotronics integrator (Columbia Scientific Industries Corp., Austin, Texas).

Analytical Procedures

Chlorophyll was measured spectrophotometrically in 80% acetone extracts of the cells (2, 48). For measuring protein, cells were extracted with 80% acetone and washed two times with acetone. Pellets were dried under reduced pressure and then dissolved in 2% SDS. Protein was estimated by the biuret method (16) with bovine serum albumin in 2% SDS as the standard. Assay mixtures were centrifuged at room temperature before the absorbance was measured.

RESULTS

Analysis of the Major Polypeptides of Thylakoid Membranes of *C. reinhardtii* by SDS-Electrophoresis

An analysis of the composition of thylakoid membranes is necessary in order to interpret data on the synthesis of membrane components. Thylakoid membranes, purified from *C. reinhardtii* and then washed with 8 M urea, contain protein and chlorophyll in a ratio of 4:7 (mg/mg) (22). When the protein in these membranes was subjected to electrophoresis in the presence of SDS, the results shown in Fig. 1 were obtained. The two major polypeptide fractions, noted previously and desig-

nated *b* and *c* for reference (18), remained with the membranes after the treatment (22). The electrophoretic mobilities of these polypeptides, relative to those of a number of standard proteins (listed in the legend for Fig. 1), indicated that polypeptides in fractions *b* and *c* have a mass of $28,000 \pm 1,000$ and $24,000 \pm 1,000$ daltons, respectively. It was established, by plotting mobility vs. gel concentration (3), that *b* and *c* have the same free electrophoretic mobility in SDS as the standard proteins and therefore exhibit no anomalous behavior during electrophoresis.

These thylakoid preparations, washed with urea, have a less complex composition than those described by Chua et al. (5, 7). However, to determine whether *b* and *c* could be subdivided on the basis of size, the protein load on the gels was

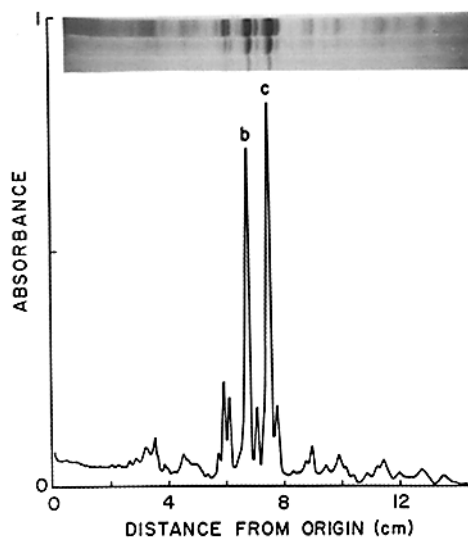


FIGURE 1 Electrophoresis of polypeptides of purified thylakoid membranes. Before analysis, the membranes were washed with 8 M urea and extracted with 90% acetone. The protein was dissolved and subjected to electrophoresis on 10% polyacrylamide gels as described in Materials and Methods. The three gels illustrated contained 3, 10, and 35 μ g of protein. The gel with the intermediate load was scanned to provide the pattern of absorbance. The following standard proteins (and their mass in daltons) were subjected to electrophoresis on companion gels to determine the mass of the major membrane polypeptides: bovine serum albumin (67,000), ovalbumin (43,000) deoxyribonuclease I (31,000), chymotrypsinogen (25,700), β -lactoglobulin (18,400), myoglobin (17,200), and the large and small subunits (55,000 and 16,500, respectively [25]) of ribulosebiphosphate carboxylase.

decreased to heighten the resolution. Fig. 1 shows that both *b* and *c* migrated as single components even at the lowest amount of protein on the gel. As an indication of the resolving ability of the gel, the polypeptides in fraction *c* and in the minor fraction on the leading edge of *c* differ in mass by about 1,200 daltons. We conclude that the major fractions contain polypeptides that are either homogeneous in mass or differ by less than 500 daltons. However, an analysis of the degree of homogeneity *in type* is hampered by the difficulty in analyzing these fractions by a method not involving SDS. Therefore, it is possible that each fraction contains more than one type of polypeptide.

The amino acid compositions of these two fractions are shown in Table I. The minimum mol wt of the polypeptides, calculated from the composition, were 26,000 for *b* and 23,700 for *c*, in good agreement with the values obtained by electrophoresis. Also listed in Table I are the estimated residues of each amino acid per molecule based on the sizes of the polypeptides, as determined by electrophoresis. Although the evidence for homogeneity is equivocal, this means of expressing the composition is instructive in emphasizing the re-

markable similarity in the compositions of the two fractions.

Labeling of Polypeptides during Greening

Because of the high and nearly equal number of leucine residues in *b* and *c* (Table I), conditions were developed that enabled labeling of cellular proteins with [³H]leucine. At 25°C no significant labeling occurred when the amino acid was added at tracer levels (2.5 μCi/ml; 5 × 10⁻⁸ M in the medium).¹ But labeling of protein did occur when the extracellular concentration of [³H]leucine was increased 10-fold and the temperature of the incubation was raised to 38°C. The inefficient entry of leucine into the cells suggested that no specific transport system exists on the plasma membrane for this amino acid and that leucine enters these cells primarily by simple passive diffusion. This suggestion was supported by the observation that the level of labeling was relatively insensitive to the addition of unlabeled leucine. Thus, after the addition of sufficient L-[¹H]leucine to increase the extracellular concentration of leucine from 5 × 10⁻⁷ M to 3 × 10⁻⁴ M, the incorporation of [³H]leucine was still one-third the level obtained at the lower concentration (and higher specific radioactivity).²

In contrast to results previously obtained with [³H]arginine (18, 21), the incorporation of [³H]leucine produced a pattern of radioactivity for total cellular protein in which peaks corresponding to *b* and *c* were prominent and well resolved (Fig. 2 A). The use of [³H]leucine to assay synthesis of the major polypeptides of the thylakoid membranes provided the opportunity to perform the analyses on total protein, thus making isolation of

TABLE I
Amino Acid Composition of Chloroplast Membrane Fractions *b* and *c*

Amino acid	Fraction <i>b</i>		Fraction <i>c</i>	
	Residues per 100 residues	Residues per molecule*	Residues per 100 residues	Residues per molecule*
Gly	12.03	31	12.50	28
Ala	11.75	30	11.12	25
Leu	10.18	26	11.10	25
Asx	9.10	24	9.18	20
Glx	8.79	23	8.96	20
Pro	6.41	17	7.49	17
Phe	6.11	16	6.76	15
Thr	5.92	15	4.67	10
Lys	5.82	15	4.72	10
Val	5.04	13	4.24	9
Ser	4.74	12	3.72	8
Ile	3.36	9	3.84	9
Tyr	3.26	9	3.37	8
Arg	2.96	8	3.10	7
Met	1.61	4	1.80	4
Trp	1.43	4	1.72	4
His	1.06	3	1.33	3
Cys (half)	0.42	1	0.46	1
Total		260		223
% polar amino acids		38		35

* Estimated on the basis of a mass of 28,000 daltons for *b* and of 24,000 daltons for *c*.

¹ The suggestion (45) that [³H]leucine was selectively incorporated into products of mitochondrial protein synthesis was in error, as described elsewhere (46).

² If leucine entered the cells by simple passive diffusion, increasing the extracellular concentration by the addition of unlabeled leucine should proportionally increase the total influx, without decreasing the amount of [³H]leucine entering the cells. The data indicate that this condition does not strictly hold. However, we have found that extracellular leucine at 10⁻³ M did not significantly increase the amount of free leucine within the cells. Therefore, the results may be explained by assuming a rate of entry of leucine into the cells that is too low to permit equilibration of the intracellular pool with extracellular leucine. Apparently, there is no transport mechanism for which labeled and unlabeled leucine molecules compete.

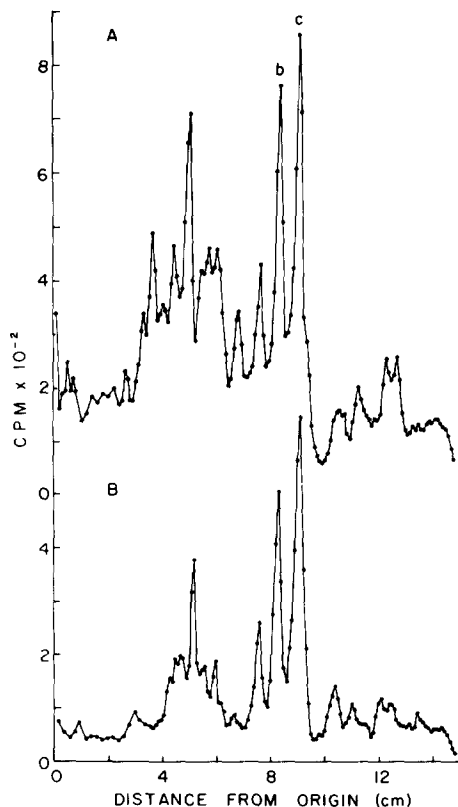


FIGURE 2 Patterns of radioactivity after electrophoresis of (A) total protein of greening *C. reinhardtii* *y-1* cells and of (B) protein in the membrane fraction from labeled cells. Etiolated cells were incubated in the dark at 38°C for 2 h. [³H]leucine was added, and the samples were illuminated for 1 h with incandescent light as described in Materials and Methods. The membrane fraction was prepared as described previously (18) after breaking the cells by sonication (21). Electrophoresis was performed with 8% polyacrylamide gels.

membranes unnecessary and obviating difficulties in interpretation of results when membrane formation was inhibited.

Kinetics of Greening at 38°C

The rate of greening of etiolated cells was affected by the temperature of the culture, with a greater maximal rate of chlorophyll synthesis achieved as the temperature was raised toward 38°C. If the cells were exposed to light at the same time the temperature was increased, the initial slow phase of chlorophyll synthesis decreased from 2 h at 25°C to about 30 min at 38°C. However, it was found that at temperatures above 35°C the initial rate of chlorophyll synthesis was enhanced if first the cells were incubated in the

dark for 1–2 h. Fig. 3 shows the results of an experiment in which etiolated cells were incubated in the dark at 38°C for 2 h and then exposed to light. Chlorophyll synthesis began immediately and proceeded at a linear rate during the hour of illumination. The initial rate of synthesis achieved in this type of experiment (ca. 6 μg of chlorophyll/ 10^7 cells/h) exceeded the maximal rate obtained at 25°C (4–5 μg of chlorophyll/ 10^7 cells/h).

In the same type of experiment as that shown in Fig. 3, the kinetics of synthesis of *b* and *c* were determined. [³H]leucine was added at the time cells were exposed to light. Aliquots of the culture were removed at intervals thereafter, and total cellular protein was subjected to electrophoresis. Fig. 4 shows patterns of radioactivity for the middle portions of the gels, which contained the major membrane polypeptides, i.e., 6–9 cm from the origin, as in Fig. 2. Each pattern was plotted on a scale corresponding to the time that sample was exposed to light. Therefore, if the incorporation of [³H]leucine was linear with time, identical patterns relative to each other should be obtained. Fig. 4 illustrates the fact that synthesis of the major membrane polypeptides did proceed at approximately a linear rate during the hour of illumination. Furthermore, there was no detectable lag in the incorporation of [³H]leucine into *b* and *c* when light was given; the sample taken at 5 min of illumination contained fully the amount of radio-

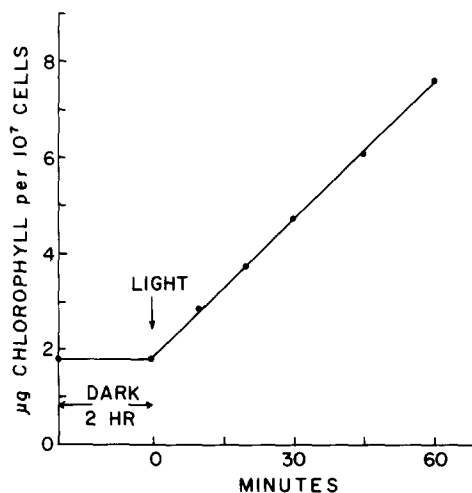


FIGURE 3 Kinetics of chlorophyll synthesis of *C. reinhardtii* *y-1* cells at 38°C. Etiolated cells were incubated in the dark at 38°C for 2 h and then exposed to incandescent light. Chlorophyll in portions of the cell suspension was extracted with 80% acetone and measured spectrophotometrically (48).

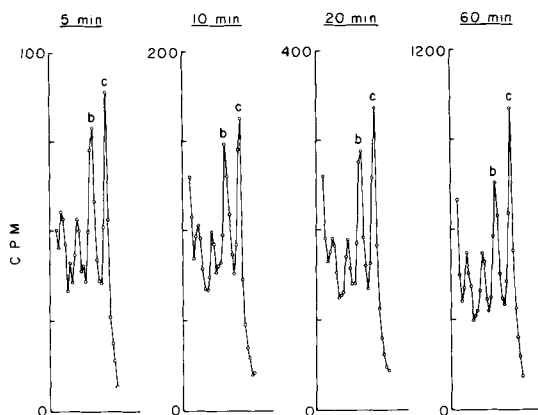


FIGURE 4 Kinetics of synthesis of *b* and *c* during greening of etiolated cells of *C. reinhardtii y-1* at 38°C. Experimental conditions were as described under Fig. 3. [³H]leucine was added at the time the cells were exposed to light. At each of the times indicated, a portion of the cell suspension was removed for analysis by electrophoresis. Only the radioactivity patterns for the middle portion of each gel (refer to Fig. 2) are shown. The data for each sample were plotted on ordinates corresponding to the length of time cells were in the light.

activity in *b* and *c* expected for an immediate attainment of the maximal rate of synthesis of these polypeptides.

Because of the immediate appearance of labeled *b* and *c* when cells were exposed to light, it was necessary to determine whether these polypeptides were made in the dark at the higher temperatures. Fig. 5 shows a comparison of patterns obtained with [³H]leucine for cells labeled in the light, after a 2-h incubation in the dark, and for cells labeled during the second hour in the dark. No production of *b* and *c* was observed in cells labeled in the dark, but synthesis of another polypeptide (Fig. 5, arrow), slightly smaller in size than *b*, was detected. This polypeptide was not produced to a significant extent during the first hour but was made during the second hour in the dark. Extending the dark period to 3 h, and labeling cells during the third hour, did not cause any further change from the pattern obtained during the second hour. Synthesis of this polypeptide could not be detected in the dark at 25°C. The relationship, if any, between synthesis of this polypeptide and that of *b* and *c* is not known.

Effects of Inhibitors of Protein Synthesis

The effects of selective inhibitors on the synthesis of *b* and *c* at 38°C were examined. Chloram-

phenicol (200 μg/ml) or streptomycin (100 μg/ml), a more effective inhibitor of chloroplast ribosomes than chloramphenicol *in vitro* (6), had no effect on the synthesis of these polypeptides.

The results obtained when cytoplasmic protein synthesis was blocked with cycloheximide are shown in Fig. 6. In the treated cells, [³H]leucine was incorporated into several polypeptides whose synthesis was resistant to the action of cycloheximide. In these experiments, the pattern and level of incorporation of [³H]leucine in treated cells were independent of concentrations of cycloheximide between 5 and 25 μg/ml. But the presence of chloramphenicol in addition to cycloheximide abolished the incorporation of [³H]leucine. Similar results were obtained with mixtures of streptomycin and cycloheximide. Thus, the polypeptides whose synthesis was resistant to cycloheximide were apparently synthesized on chloroplast ribosomes. Since cycloheximide may inhibit RNA synthesis in *Chlamydomonas* cells in addition to protein synthesis on 82S cytoplasmic ribosomes (35),

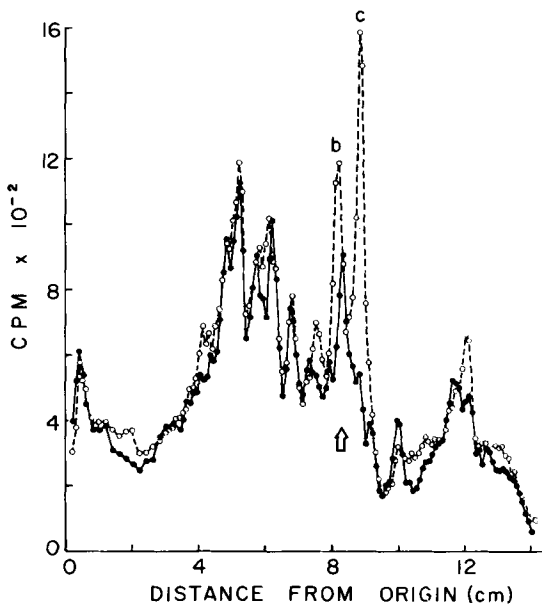


FIGURE 5 Comparison of the patterns of radioactivity after electrophoresis of total protein from *C. reinhardtii y-1* cells labeled in the dark or light. Etiolated cells were incubated in the dark at 39.5°C. [³H]leucine was added after 1 h of incubation to one sample, which remained in the dark for an additional hour. The second sample received [³H]leucine after 2 h in the dark and then was exposed to light for an additional hour. Electrophoresis was performed on 8% polyacrylamide gels. (●—●), cells labeled in dark; (○---○), cells labeled in light.

the polypeptides that are synthesized in the presence of the inhibitor may not be made to the same extent as in untreated cells. However, for this work the important consideration is the *pattern* of labeled polypeptides.

Fig. 6 illustrates that cycloheximide blocked the

synthesis of *b*. But after electrophoresis through an 8% polyacrylamide gel, radioactivity was found in the position of *c*. However, after electrophoresis through an 11% polyacrylamide gel, this labeled material migrated more nearly at the rate at which fraction *b* moved through the gels (Fig. 6,

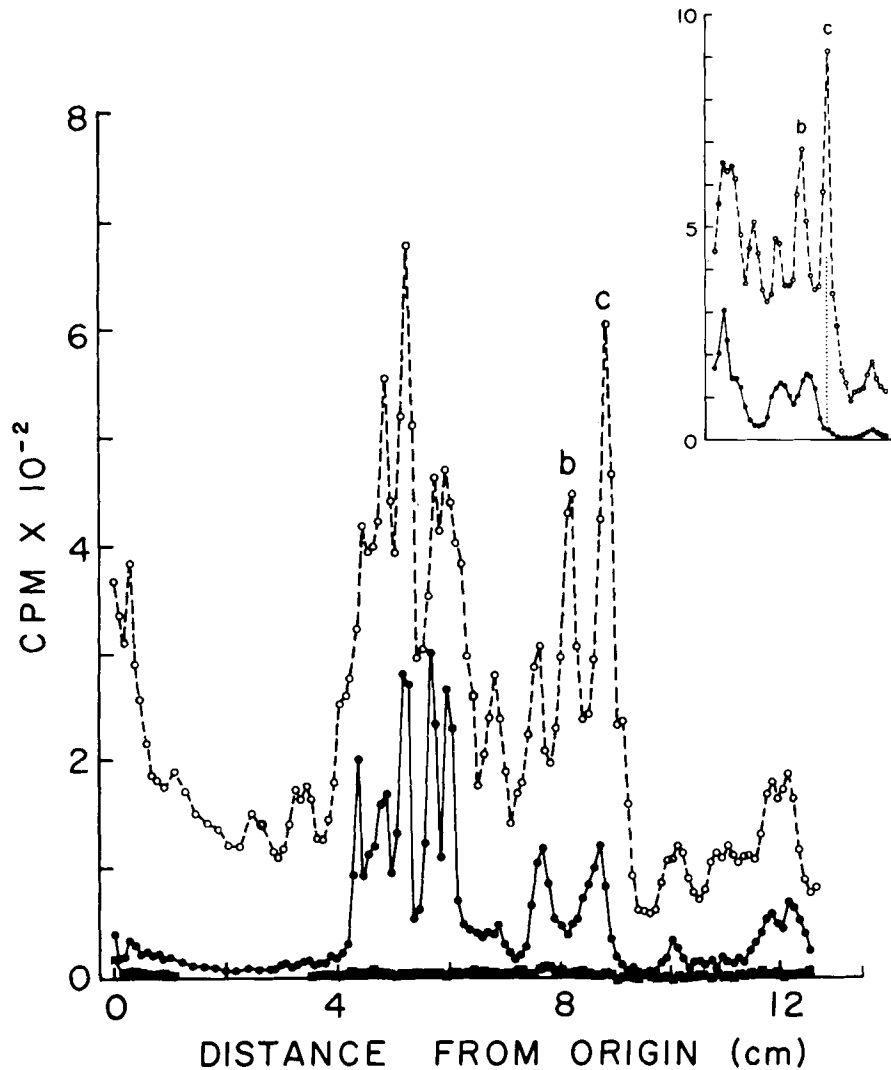


FIGURE 6 Effects of cycloheximide and of cycloheximide plus chloramphenicol on the incorporation of [^3H]leucine into protein of *C. reinhardtii* *y-1* cells after exposure to light. Etiolated cells were incubated in the dark at 38°C for 2 h. To one 4-ml portion of cells was added 0.4 ml chloramphenicol (2 mg/ml) 20 min before exposure to light. To this portion and to a second was added 0.1 ml cycloheximide (1 mg/ml) 5 min before light. A third portion served as a control. [^3H]leucine was added at the time cells were exposed to incandescent light, and the incubations were continued for another hour. Patterns of radioactivity were determined after electrophoresis on 8% polyacrylamide gels. (○---○), control cells; (●—●), cycloheximide-treated cells; (■—■), cells treated with cycloheximide plus chloramphenicol. The inset shows patterns of radioactivity after electrophoresis of control (○---○) and cycloheximide-treated (●—●) samples on 11% gels.

inset). Since the change in acrylamide concentration did not affect the relative migrations of fractions *b* and *c*, we concluded that the radioactivity found for cycloheximide-treated cells in the position of *b* and *c* was contained in another, unrelated polypeptide. Therefore, cycloheximide effectively blocked synthesis of both *b* and *c*, confirming that these polypeptides are synthesized on cytoplasmic ribosomes (17).

Effect of Actinomycin D

The linear initial kinetics of the synthesis of *b* and *c* suggested that messenger RNA for these polypeptides was synthesized in the dark at 38°C and was available when the cells were exposed to light. This suggestion was tested by determining the effects of actinomycin D on the greening process. At a concentration of 30 µg/ml in the culture medium, actinomycin D lowered the rate of incorporation of [³H]adenine into total RNA³ in treated cells in the dark to 18% of the rate in control cells. Upon exposure to light, the rate of [³H]adenine incorporation decreased further to less than 10% of the rate in control cells.

The effect of actinomycin D on the synthesis of chlorophyll was dependent on the time the drug was added to the cells (Table II). Chlorophyll synthesis was strongly inhibited in cells that were incubated in the dark for 120 min with the drug before illumination. However, if added 10 min before light, actinomycin D caused only a small decrease in chlorophyll synthesis.

The effects of actinomycin D on the synthesis of *b* and *c* are shown in Fig. 7. When actinomycin D was present during a 120-min preincubation in the dark, little if any *b* or *c* was synthesized during a subsequent hour in light. But if actinomycin D was added just 10 min before the cells were exposed to light, there was no inhibition of the synthesis of *b* and *c*. Yet the latter treatment did lower the rate of synthesis of other polypeptides.

In experiments similar to those in which actinomycin D was used, rifampicin (200 µg/ml) had no effect on the synthesis of *b* and *c*.

Spectral Requirements of Production of *b* and *c*

Chlorophyll synthesis is a light-dependent process in *C. reinhardtii* *y-1*, with light energy appar-

³ RNA fractions were prepared by a procedure similar to that described by Fleck and Munro (15).

TABLE II
Effect of Actinomycin D on the Synthesis of Chlorophyll in Greening *C. reinhardtii*

Actinomycin D		
Concentration	Length of treatment before light	Increase in chlorophyll
µg/ml	min	µg/10 ⁷ cells
0		4.1
20	120	0.7
50	120	0.3
50	10	3.1

Actinomycin D was added either at the start of a 2 h incubation in the dark at 38°C or 10 min before light was given. The values for chlorophyll are the increases during an hour of exposure to light above an initial value of 1.7 µg/10⁷ cells.

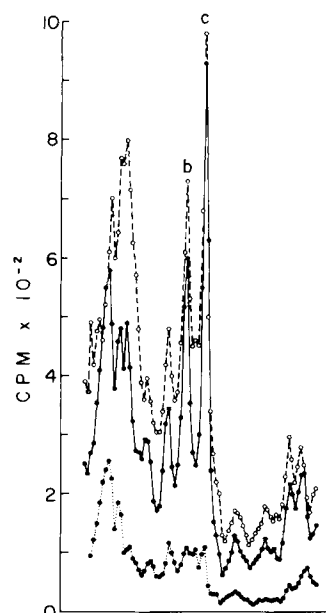


FIGURE 7 Effect of time of addition of actinomycin D (30 µg/ml) on the synthesis of *b* and *c*. Etiolated cells were incubated in the dark at 38°C for 2 h. Actinomycin D was added to separate samples at the beginning of the 38°C incubation or 10 min before exposure to light. [³H]leucine was added at the time cells were exposed to light, and the incubations were continued for another hour. The total cellular protein was subjected to electrophoresis on 11% polyacrylamide gels. The pattern of radioactivity for the region of the gels containing *b* and *c* are shown. (O---O), control cells; (●—●), actinomycin D added 10 min before exposure to light; (●···●), actinomycin D added 2 h before exposure to light.

ently involved in the photoreduction of protochlorophyllide (32, 33). The effect of the wavelength of light on the synthesis of chlorophyll and of polypeptide fractions *b* and *c* was studied to determine the maximum in the region of 600–700 nm for these processes at 38°C. Etiolated cells were incubated in the dark for 2 h at 38°C, then [³H]leucine was added, and the cells were exposed to light transmitted by filters for another hour. After electrophoresis of each sample, the increase in the amount of radioactivity in the position of *c* over the level for cells kept in the dark was determined. As Fig. 8 shows, the maximum amount of *c* was synthesized in cells exposed to light of 650 nm. Synthesis of *b* followed a similar pattern, but because of the incorporation of [³H]leucine into polypeptides nearly the same size as *b* in the dark (see Fig. 5), a quantitative estimation of the amount of *b* made in the light was not attempted. These results show that synthesis of the major polypeptides of the thylakoid membrane responds to the same wavelengths of light as does the synthesis of chlorophyll.

Synthesis of Fractions *b* and *c* in Wild Type Cells

Cells of wild type *C. reinhardtii* (strain 137c) do not need light to synthesize chlorophyll, and so remain green when grown in the dark (24, 49). To determine whether synthesis of *b* and *c* is dependent upon a direct involvement of light or, rather, upon the synthesis of chlorophyll, wild type cells were tested for their ability to make *b* and *c* in light or dark at 38°C. Under conditions identical to those of experiments described for *C. reinhardtii* *y-1* cells, wild type cells were labeled with [³H]leucine during the second hour in the dark or during an hour in the light after a 2 h incubation in the dark. As Fig. 9 shows, *b* and *c* were synthesized in both cases, although the extent of synthesis was greater in cells incubated in the light. The pattern of radioactivity for wild type cells labeled in the light was essentially identical to that for *y-1* cells.

DISCUSSION

Although *Chlamydomonas* cells are usually grown at 21°–25°C, the growth rate is not diminished by increasing the temperature to 33°–35°C, conditions used to select conditional mutant strains (5, 23, 34). Above a temperature of 35°C, the rate of growth falls sharply, until no significant increase in

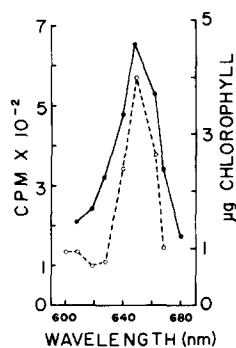


FIGURE 8 Effect of light of different wavelengths on the synthesis of chlorophyll and of fraction *c*. Cells were exposed to light transmitted by interference filters (3-cavity, 11 nm half-width, Ditic Optics, Marlboro, Mass.) for 1 h at 38°C. The intensity of incident light was 1.4×10^8 erg/cm²/s. (○---○), chlorophyll synthesized by 10^7 cells; (●—●), radioactivity as [³H]leucine incorporated into fraction *c* during the hour of illumination.

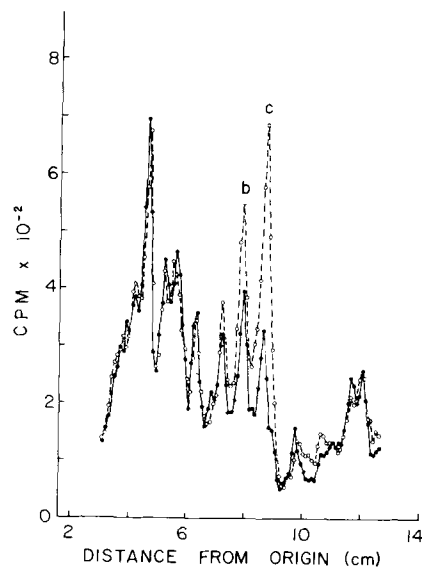


FIGURE 9 Comparison of the patterns of radioactivity after electrophoresis of total protein of wild type cells labeled in the dark or light. *C. reinhardtii* cells, strain 137c, *mt*⁺ (kindly provided by Dr. N. W. Gillham) were grown in the dark for 3 days, suspended to 6×10^6 cells/ml and incubated in the dark at 38°C. After 75 min at 38°C, [³H]leucine was added to one portion of the cell suspension, and the incubation was continued in the dark for another hour. [³H]leucine was added to a second portion after 135 min in the dark, and the sample was exposed to incandescent light for 1 h. Electrophoresis was carried out on 8% polyacrylamide gels. (●—●), cells labeled in the dark; (○---○), cells labeled in the light.

cell number occurs at 40°C (our unpublished results). However, if the pH of the medium is maintained near neutrality, the cells continue to synthesize protein at 38°–40°C with no diminution in rate over the time span of the experiments described here.

The rate of greening of etiolated *C. reinhardtii* *y-1* cells is more rapid at 38°C than at 25°C. Moreover, linear initial kinetics of chlorophyll synthesis, with rates of 18–20 µg chlorophyll/mg protein/h, were achieved by first allowing the cells to remain in the dark for 1–2 h at the higher temperature before exposure to light. This rate of greening is approx. 1.5-fold greater than the maximal rate at 25°C and permits the algal cells to achieve the green-cell level of chlorophyll in about 3 h. Alberte et al. (1) developed conditions under which etiolated seedlings of jack bean exhibit linear kinetics of chlorophyll synthesis during greening. Chlorophyll accumulated in these higher plant seedlings at a rate of about 1 µg chlorophyll/mg protein/h, assuming that roughly 5% of the fresh weight mass of the leaves was protein (36). The most rapidly greening portions of etiolated barley leaves synthesized chlorophyll at a rate only slightly higher than that found with bean leaves (44).

Of significant interest was the discovery that, in etiolated *C. reinhardtii* cells incubated in the dark at 38°C for 2 h, an immediate and linear response occurred in the synthesis of the major polypeptides of thylakoid membranes upon exposure to light. The absence of a lag in the synthesis of *b* and *c* suggested that messenger RNA for these polypeptides already was present when cells were exposed to light. In bacteria, translation may begin before transcription of messenger RNA is completed (26, 27, 38). But even in a situation with such coupling of events, a lag of ca. 3 min in duration occurs at 37°C between the times of addition of an inducer and the appearance of the induced protein (27, 40, 41). It has not been established whether the genes for *b* and *c* in *Chlamydomonas* are part of nuclear or chloroplast DNA, but for translation to occur on cytoplasmic ribosomes, the messenger RNA must exit the responsible organelle. Because of this obvious physical separation of transcription and translation, it is expected that a lag of at least several minutes would prevail before initiation of the synthesis of the membrane polypeptides, if derepression of these genes required light for greening at 38°C. The observations that actinomycin D did not inhi-

bit synthesis of *b* and *c*, if added 10 min before light was given, also support the suggestion that messenger RNA for the polypeptides was synthesized in the dark at 38°C.

The implication of these results is that production of *b* and *c* at elevated temperatures is controlled at a posttranscriptional step. The spectral requirement for the production of these polypeptides coincides with that for chlorophyll synthesis, suggesting that synthesis of chlorophyll in some manner controls synthesis of the polypeptides. This suggestion is supported by the observation that wild type cells, which synthesize chlorophyll in the dark (24, 49), also are capable of making *b* and *c* in the dark. However, since the site of synthesis of chlorophyll is within chloroplasts (42, 43), a mechanism by which synthesis of *b* and *c* on cytoplasmic ribosomes can be controlled by chlorophyll or its precursors is not obvious. Moreover, it is not known whether *b* and *c* are primary products of translation or whether they are derived from a larger precursor.

Evidence was presented earlier (13, 21, 22) for regulation of the synthesis of *b* and *c* at the level of transcription. At 25°C the lag in the synthesis of these polypeptides and the increase in *b* and *c* in parallel with chlorophyll are consistent with a transcriptional control mechanism. But this mechanism apparently becomes nonfunctional as the temperature is raised, thereby revealing an additional site of control after transcription.

The authors express their gratitude to Mrs. Ruth Millington for her skillful assistance.

This work was supported by National Science Foundation grant no. BMS-71-01550.

Received for publication 10 October 1975, and in revised form 19 February 1976.

REFERENCES

1. ALBERTE, R. S., E. L. FISCUS, and A. W. NAYLOR. 1975. The effects of water stress on the development of the photosynthetic apparatus in greening leaves. *Plant Physiol.* **55**:317–321.
2. ARNON, D. 1949. Copper enzymes in isolated chloroplasts. Polyphenol oxidases in *Beta vulgaris*. *Plant Physiol.* **24**:1–15.
3. BANKER, G. A., and C. W. COTMAN. 1972. Measurement of free electrophoretic mobility and retardation coefficient of protein-sodium dodecyl sulfate complexes by gel electrophoresis. A method to validate molecular weight estimates. *J. Biol. Chem.* **247**:5856–5861.

4. BOTTOMLEY, W., D. SPENCER, and P. R. WHITFIELD. 1974. Protein synthesis in isolated spinach chloroplasts: comparison of light-driven and ATP-driven synthesis. *Arch. Biochem. Biophys.* **164**:106-117.
5. CHUA, N.-H., and P. BENNOUN. 1975. Thylakoid membrane polypeptides of *Chlamydomonas reinhardtii*: wild-type and mutant strains deficient in photosystem II reaction center. *Proc. Nat. Acad. Sci. U. S. A.* **72**:2175-2179.
6. CHUA, N.-H., G. BLOBEL, and P. SIEKEVITZ. 1973. Isolation of cytoplasmic and chloroplast ribosomes and their dissociation into active subunits from *Chlamydomonas reinhardtii*. *J. Cell Biol.* **57**:798-814.
7. CHUA, N.-H., K. MATLIN, and P. BENNOUN. 1975. A chlorophyll-protein complex lacking in photosystem I mutants of *Chlamydomonas reinhardtii*. *J. Cell Biol.* **67**:361-377.
8. DOUCE, R. 1974. Site of biosynthesis of galactolipids in spinach chloroplasts. *Science (Wash. D. C.)* **183**:852-853.
9. EAGLESHAM, A. R. J., and R. J. ELLIS. 1974. Protein synthesis in chloroplasts. II. Light-driven synthesis of membrane proteins by isolated pea chloroplasts. *Biochim. Biophys. Acta.* **335**:396-407.
10. ELLIS, R. J. 1975. Inhibition of chloroplast protein synthesis by lincomycin and 2-(4-methyl-2,6-dinitroanilino)-N-methylpropionamide. *Phytochemistry (Oxf.)* **14**:89-93.
11. ERNST-FONBERG, M. L., and K. BLOCH. 1971. A chloroplast-associated fatty acid synthetase system in *Euglena*. *Arch. Biochem. Biophys.* **143**:392-400.
12. EYTAN, G., and I. OHAD. 1970. Biosynthesis of chloroplast membranes. VI. Cooperation between cytoplasmic and chloroplast ribosomes in the synthesis of photosynthetic lamellar proteins during the greening process in a mutant of *Chlamydomonas reinhardtii* y-1. *J. Biol. Chem.* **245**:4297-4307.
13. EYTAN, G., and I. OHAD. 1972. Biogenesis of chloroplast membranes. VIII. Modulation of chloroplast lamellae composition and function induced by discontinuous illumination and inhibition of ribonucleic acid and protein synthesis during greening of *Chlamydomonas reinhardtii* y-1 mutant cells. *J. Biol. Chem.* **247**:122-129.
14. FAIRBANKS, G., T. L. STECK, and D. F. H. WALLACH. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* **10**:2606-2617.
15. FLECK, A., and H. N. MUNRO. 1962. The precision of ultraviolet absorption measurements in the Schmidt-Thannhauser procedure for nucleic acid estimation. *Biochim. Biophys. Acta.* **55**:571-583.
16. GORNALL, A. G., C. J. BARDAWILL, and M. M. DAVID. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**:751-766.
17. HOOBER, J. K. 1970. Sites of synthesis of chloroplast membrane polypeptides in *Chlamydomonas reinhardtii* y-1. *J. Biol. Chem.* **245**:4327-4334.
18. HOOBER, J. K. 1972. A major polypeptide of chloroplast membranes of *Chlamydomonas reinhardtii*. Evidence for synthesis in the cytoplasm as a soluble component. *J. Cell Biol.* **52**:84-96.
19. HOOBER, J. K. 1976. Synthesis of the major polypeptides of thylakoid membranes in *Chlamydomonas reinhardtii* y-1 in response to light. In *Biogenesis and Turnover of Membrane Molecules*. J. S. Cook, editor. Raven Press, New York. In press.
20. HOOBER, J. K., P. SIEKEVITZ, and G. E. PALADE. 1969. Formation of chloroplast membranes in *Chlamydomonas reinhardtii* y-1. Effects of inhibitors of protein synthesis. *J. Biol. Chem.* **244**:2621-2631.
21. HOOBER, J. K., and W. J. STEGEMAN. 1973. Control of the synthesis of a major polypeptide of chloroplast membranes in *Chlamydomonas reinhardtii*. *J. Cell Biol.* **56**:1-12.
22. HOOBER, J. K., and W. J. STEGEMAN. 1975. Regulation of chloroplast membrane synthesis. In *Genetics and Biogenesis of Mitochondria and Chloroplasts*. C. W. Birky, Jr., P. S. Perlman, and T. J. Byers, editors. Ohio State University Press, Columbus, Ohio. 225-251.
23. HOWELL, S. H., and J. A. NALIBOFF. 1973. Conditional mutants in *Chlamydomonas reinhardtii* blocked in the vegetative cell cycle. I. An analysis of cell cycle block points. *J. Cell Biol.* **57**:760-772.
24. HUDOCK, G. A., and R. P. LEVINE. 1964. Regulation of photosynthesis in *Chlamydomonas reinhardtii*. *Plant Physiol.* **39**:889-897.
25. IWANU, V., N.-H. CHUA, and P. SIEKEVITZ. 1974. The purification and some properties of ribulosebiphosphate carboxylase and of its subunits from the green alga *Chlamydomonas reinhardtii*. *Biochim. Biophys. Acta.* **358**:329-340.
26. KAEMPFER, R. O. R., and B. MAGASANIK. 1967. Mechanism of β -galactosidase induction in *Escherichia coli*. *J. Mol. Biol.* **27**:475-494.
27. KEPES, A. 1969. Transcription and translation in the lactose operon of *Escherichia coli* studied by *in vivo* kinetics. *Prog. Biophys. Mol. Biol.* **19**:201-236.
28. KOSKI, V. M., C. S. FRENCH, and J. H. C. SMITH. 1951. The action spectrum for the transformation of protochlorophyll to chlorophyll *a* in normal and albino corn seedlings. *Arch. Biochem. Biophys.* **31**:1-17.
29. LIU, T.-Y., and Y. H. CHANG. 1971. Hydrolysis of proteins with p -toluenesulfonic acid. Determination of tryptophan. *J. Biol. Chem.* **246**:2842-2848.
30. MACHOLD, O., and O. AURICH. 1972. Sites of synthesis of chloroplast lamellar proteins in *Vicia faba*. *Biochim. Biophys. Acta.* **281**:103-112.
31. MATHIS, P., and K. SAUER. 1972. Circular dichroism studies on the structure and the photochemistry of protochlorophyllide and chlorophyllide holo-

- chrome. *Biochim. Biophys. Acta.* **267**:498-511.
32. MATSUDA, Y., T. KIKUCHI, and M. R. ISHIDA. 1971. Studies on chloroplast development in *Chlamydomonas reinhardtii*. I. Effect of brief illumination on chlorophyll synthesis. *Plant Cell Physiol.* **12**:127-135.
 33. McLEOD, G. C., G. A. HUDOCK, and R. P. LEVINE. 1963. The relation between pigment concentration and photosynthetic capacity in a mutant of *Chlamydomonas reinhardtii*. In *Photosynthetic Mechanisms in Green Plants*. National Academy of Sciences-National Research Council. Washington, D. C. Publ. no. 1145, 400-408.
 34. McMAHON, D. 1971. The isolation of mutants conditionally defective in protein synthesis in *Chlamydomonas reinhardtii*. *Mol. Gen. Genet.* **112**:80-86.
 35. McMAHON, D. 1975. Cycloheximide is not a specific inhibitor of protein synthesis *in vivo*. *Plant Physiol.* **55**:815-821.
 36. MEGO, J. L., and A. T. JAGENDORF. 1961. Effect of light on growth of Black Valentine bean plastids. *Biochim. Biophys. Acta.* **53**:237-254.
 37. MOORE, S. 1963. On the determination of cystine as cysteic acid. *J. Biol. Chem.* **238**:235-237.
 38. MORSE, D. E., R. D. MOSTELLER, and C. YANOF-SKY. 1969. Dynamics of synthesis, translation, and degradation of *trp* operon messenger RNA in *E. coli*. *Cold Spring Harbor Symp. Quant. Biol.* **34**:725-740.
 39. NIELSEN, O. F., and A. KAHN. 1973. Kinetics and quantum yield of photoconversion of protochlorophyll(ide) to chlorophyll(ide) *a*. *Biochim. Biophys. Acta.* **292**:117-129.
 40. PARDEE, A. B., and L. S. PRESTIDGE. 1961. The initial kinetics of enzyme induction. *Biochim. Biophys. Acta.* **49**:77-88.
 41. PIOVANT, M., and C. LAZDUNSKI. 1975. Different cyclic adenosine 3',5'-monophosphate requirements for induction of β -galactosidase and tryptophanase. Effect of osmotic pressure on intracellular cyclic adenosine 3',5'-monophosphate concentrations. *Biochemistry.* **14**:1821-1825.
 42. REBEIZ, C. A., J. C. CRANE, C. NISHIJIMA, and C. C. REBEIZ. 1973. Biosynthesis and accumulation of microgram quantities of chlorophyll by developing chloroplasts *in vitro*. *Plant Physiol.* **51**:660-666.
 43. REBEIZ, C. A., K. R. MATTHEIS, B. B. SMITH, C. C. REBEIZ, and D. F. DAYTON. 1975. Chloroplast biogenesis. Biosynthesis and accumulation of protochlorophyll by isolated etioplasts and developing chloroplasts. *Arch. Biochem. Biophys.* **171**:549-567.
 44. ROBERTSON, D., and W. M. LAETSCH. 1974. Structure and function of developing barley plastids. *Plant Physiol.* **54**:148-159.
 45. STEGEMAN, W. J., and J. K. HOOBER. 1974. Mitochondrial protein synthesis in *Chlamydomonas reinhardtii* y-1. Polypeptide products of mitochondrial transcription and translation *in vivo* as revealed by selective labeling with [³H]leucine. *J. Biol. Chem.* **249**:6866-6873.
 46. STEGEMAN, W. J., and J. K. HOOBER. 1975. Induction of synthesis of bacterial protein by excretory product of the alga *Chlamydomonas reinhardtii* y-1. *Nature (Lond.)*. **257**:244-246.
 47. THORNE, S. W. 1971. The greening of etiolated bean leaves. I. The initial photoconversion process. *Biochim. Biophys. Acta.* **226**:113-127.
 48. VERNON, L. P. 1960. Spectrophotometric determination of chlorophylls and pheophytins in plant extracts. *Anal. Chem.* **32**:1144-1150.
 49. WANG, W.-Y., W. L. WANG, J. E. BOYNTON, and N. W. GILLHAM. 1974. Genetic control of chlorophyll biosynthesis in *Chlamydomonas*. Analysis of mutants at two loci mediating the conversion of protoporphyrin-IX to magnesium protoporphyrin. *J. Cell Biol.* **63**:806-823.