CONTROL OF THE SYNTHESIS OF A MAJOR POLYPEPTIDE OF CHLOROPLAST MEMBRANES IN *CHLAMYDOMONAS REINHARDI*

J. KENNETH HOOBER and WILLIAM J. STEGEMAN

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

ABSTRACT

The regulation of the synthesis of one of the major polypeptides of chloroplast membranes in *Chlamydomonas reinhardi y-1* has been studied in order to determine what factors are involved in the control mechanism. The polypeptide is synthesized in the cytoplasm and previously was designated as c (J. K Hoober. 1972. *J. Cell Biol.* 52:84). Under normal conditions the synthesis of polypeptide c appears to be coupled to the synthesis of chlorophyll. When greening cells are illuminated through a light filter opaque below 675 mμ, the conversion of protochlorophyllide to chlorophyllide is blocked. Although this elimination of light below 675 mμ does not affect, in the main, protein synthesis in the chloroplast and cytoplasm, synthesis of polypeptide c is inhibited. Also, control cells synthesize neither chlorophyll nor polypeptide c in the dark. However, when cells are treated with chloramphenicol, an inhibitor of chloroplast protein synthesis, the synthesis of polypeptide c occurs in the absence of light required for chlorophyll synthesis. Chlorophyll per se does not appear to be required for synthesis of polypeptide c, since treating cells with hemin, maleate, or malonate causes an inhibition of the synthesis of chlorophyll but not of polypeptide c. The results of these experiments are discussed in terms of a proposed mechanism by which synthesis of polypeptide c is regulated at the transcriptional level by a precursor of chlorophyll, and this regulation is mediated by a protein or proteins synthesized within the chloroplast.

INTRODUCTION

Thylakoid membranes of *Chlamydomonas reinhardi* are assembled from lipids apparently synthesized within the chloroplast (1, 2) and from polypeptides synthesized both inside and outside the organelle (3–5) The two major polypeptides of these membranes, as revealed by gel electrophoresis (3), are among those synthesized on cytoplasmic ribosomes. For convenience of reference, these polypeptides, having molecular weights of about 24,000 and 21,000, were previously designated b and c, respectively (5). Polypeptide c accumulates in the soluble fraction of the cell when chlorophyll and

membrane formation are inhibited in the presence of chloramphenicol, an inhibitor of protein synthesis on chloroplast ribosomes (6–8), and it was suggested that this polypeptide is synthesized in the cytoplasm as a soluble component (5).

Several observations indicated that synthesis of polypeptide c, and perhaps other thylakoid membrane polypeptides, is under specific control. Polypeptide c cannot be detected in dark-grown etiolated cells of *C. reinhardi y-1* (5). Also, a sharp reduction in the rate of synthesis of this polypeptide occurs after cells are transferred to the dark (5).

Under these conditions, the cells are able to synthesize at least a small amount of protochlorophyllide but cannot convert this porphyrin to chlorophyll (9, 10). Eytan and Ohad (11) suggested that conversion of protochlorophyllide to chlorophyll, which apparently occurs in the chloroplast (12–14), affects synthesis of the major polypeptides of thylakoid membranes at the transcriptional level. However, the data available have not permitted an elucidation of the mechanism of this control.

Among the possible mechanisms of control of the synthesis of membrane polypeptides, three seemed particularly open to investigation with the techniques available. First, it is possible that a general synthetic or metabolic event in the chloroplast is required for continued synthesis of polypeptide c in the cytoplasm and that, in the dark, insufficient products of photosynthesis are available to support this synthesis. Second, it is possible that translation of the mRNA for polypeptide c is under specific control. Or third, as Eytan and Ohad suggested (11), control is possibly exerted at the level of the synthesis of the mRNA for this polypeptide. In support of the first possibility, the rate of synthesis of ribulose-1,5-diphosphate carboxylase, the main product of chloroplast protein synthesis (5), falls to a low level immediately after cells are transferred to the dark (5, 15). However, cells seem capable of adapting to the dark, since in prolonged darkness synthesis of the carboxylase (10) and ribosomes (6) occurs in the chloroplast. Yet the major polypeptides of thylakoid membranes apparently are not synthesized even in prolonged darkness (5). Thus, some type of specific regulatory mechanism must operate to inhibit the synthesis of these polypeptides when chlorophyll and thylakoid membranes are not made. Resumption of chlorophyll synthesis when cells are exposed to light releases this inhibition, allowing synthesis of these polypeptides and assembly of thylakoid membranes. The data in this paper support the possibility that a precursor of chlorophyll regulates transcription of the mRNA for polypeptide c, and that this regulation is mediated by a protein or proteins synthesized within the chloroplast.

METHODS

Greening Experiments

CELLS: Cells of *Chlamydomonas reinhardi y-1* were grown in the dark for 3–4 days as described previously (10, 16). Etiolated cells were suspended at a density of 4×10^6 cells/ml in fresh medium supplemented with KH_2PO_4 (16).

LIGHT FILTERS: In experiments in which filters were used, the cell suspensions in Erlenmeyer flasks were placed within a box divided into two compartments by a center partition. A 6-inch square window was cut on each end of the box. Air was circulated through the box by directing air from a fan through baffles to dissipate heat produced by the lamps. The temperature of the cultures was maintained during the experiments at 26–28°C The cells were agitated by magnetic stirring bars and were illuminated through the windows of the box by light from 100-watt incandescent lamps. The intensity of the light in the absence of light filters was adjusted to approximately 4000 lux at the position of the cells. The filters, 6 mm thick, were positioned over the windows. Three types of sharp-cut light filters were used, which transmitted light above wavelengths of 590, 610, and 675 mμ (Corning glass numbers 2434, 2418, and 2030, respectively, from Corning Glass Works, Corning, N. Y.).

Since light of 640–650 mμ is required for conversion of protochlorophyllide to chlorophyll (17, 18), the effects of the light filters on chlorophyll synthesis were tested. Fig. 1 shows the results of an experiment in which filters were positioned over the windows of the
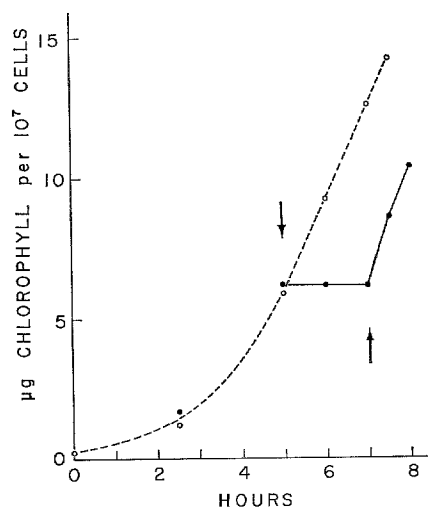

FIGURE 1 Effects of light filters on the increase of chlorophyll during greening of etiolated *C. reinhardi.* Cells grown for 4 days in the dark were suspended in fresh medium to 4×10^6 cells/ml and exposed to 4000 lux from incandescent lamps as described in Methods. At 5 hr, light filters were inserted (↓) into the light beams to provide light above 675 mμ to one culture and above 590 mμ to the control culture. 2 hr later, the filters were removed (↑). Chlorophyll in portions of the cultures was measured spectrophotometrically (21).

box after 5 hr of exposure to light, and then were removed 2 hr later. Synthesis of chlorophyll continued at an unimpaired rate in cells illuminated with light above 590 mμ (dashed curve). However, the amount of chlorophyll did not increase in cells illuminated through the filter which transmitted light above 675 mμ (solid curve). When this filter was removed, synthesis of chlorophyll resumed. Therefore, by filtering out light below 675 mμ, a situation was established in which synthesis of chlorophyll was prevented, but sufficient red light (above 675 mμ) was still provided to allow cells to perform photosynthesis (19, 20). In another experiment, the effect of the filter which transmitted light above 610 mμ was the same as that which transmitted light above 590 mμ. In greening experiments not involving light filters, the cells were exposed to light from white fluorescent lamps as described previously (16).

Assay for Chlorophyll

Chlorophyll was measured spectrophotometrically in 80% acetone extracts of the cells (21). In experiments in which hemin was added to the cultures, hemin taken up by the cells interfered slightly with the determination of chlorophyll. To correct for this interference, the absorbance of the acetone extracts at 700 mμ was measured, and the absorbance of hemin at 652 mμ was estimated from the shape of the spectrum of hemin alone in 80% acetone. The actual correction was 5–10% of the uncorrected absorbance of the acetone extracts of the cells The validity of this correction was checked by measuring the absorbance of the acetone extract of the cells at 665 mμ, where the absorbance of chlorophyll a is at a maximum (22) and the interference by hemin was less than 4%.

Preparation of Samples for Electrophoresis

At the end of the experiments, cells were collected by centrifugation at 1000 g for 3 min. The cells were washed twice with 20 mM Tris-HCl, pH 7.6, at 2°C, suspended in the Tris-HCl buffer to a density of 3–4 $\times$ 10^7 cells/ml, and broken by sonication for 20 sec at a power output of 40–45 watts from a Model W185 Sonifier Cell Disrupter (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.). Trichloroacetic acid was added to portions of the broken-cell preparations to a final concentration of 10% (w/v). The ensuing precipitates provided samples of total cellular protein. In some experiments, portions of the broken-cell preparations were centrifuged in an SW-50L rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 204,000 g_{av} for 2 hr at 2°C. Trichloroacetic acid was added to the supernatant fluids, and the ensuing precipitates provided samples of soluble proteins. The precipitates obtained with trichloroacetic acid were collected by centrifugation at 1000 g for 3 min, washed with 2–3 ml of water, and stored at −15°C until used. The 204,000 g particulate fractions, containing total membrane protein, were also stored at −15°C

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, densitometry, and the determination of radioactivity in the gels were carried out as described previously (3, 5).

Interpretation of the Electrophoretic Patterns

GENERAL: Fig. 2 illustrates patterns of protein stain and of radioactivity obtained after electrophoresis of total protein from cells greening 7 hr in light. Arginine-^{3}H was present during the last hour of exposure to light. The pattern of radioactivity was, in general, similar to the pattern of protein stain, and, although complex, the patterns were entirely reproducible from experiment to experiment for the same type of sample. The presence of the total protein on the gel facilitated the interpretation of the results. In these experiments it was possible to judge whether any polypeptide, if sufficiently resolved in the pattern of radioactivity, was synthesized by determining the relative amount of label in this polypeptide. Synthesis of the bulk of the polypeptides, therefore, served as a type of internal control. In addition, the amount of arginine-^{3}H incorporated into the subunits of ribulose-1,5-diphosphate carboxylase provided a convenient indicator of the activity of protein synthesis in the chloroplast (5). The most prominent peak in both patterns in Fig. 2 (at 5.2 cm from the origin, indicated by L) corresponds to the large subunits of this enzyme (5), while the small subunits are represented by the peak at 12.9 cm (indicated by S)

REFERENCE TO POLYPEPTIDE c: In Fig. 2 a significant peak of radioactivity is present at 9.2 cm from the origin in the position, indicated by the vertical dotted line, where only a relatively small peak is present in the pattern of protein stain. The fraction represented by this peak contains the polypeptide c of thylakoid membranes, as determined previously (5). This peak of radioactivity was markedly reduced in size, relative to the general pattern, when synthesis of this polypeptide was inhibited. For clarity of the interpretations, the results in this paper are presented as comparisons of the patterns for protein from experimental cells with those from control cells. Since the experiments were concerned primarily with control of the synthesis of polypeptide c, on subsequent figures showing electrophoretic patterns attention should be focused on the peak in the pattern indicated by the vertical dotted line marked c.

Materials

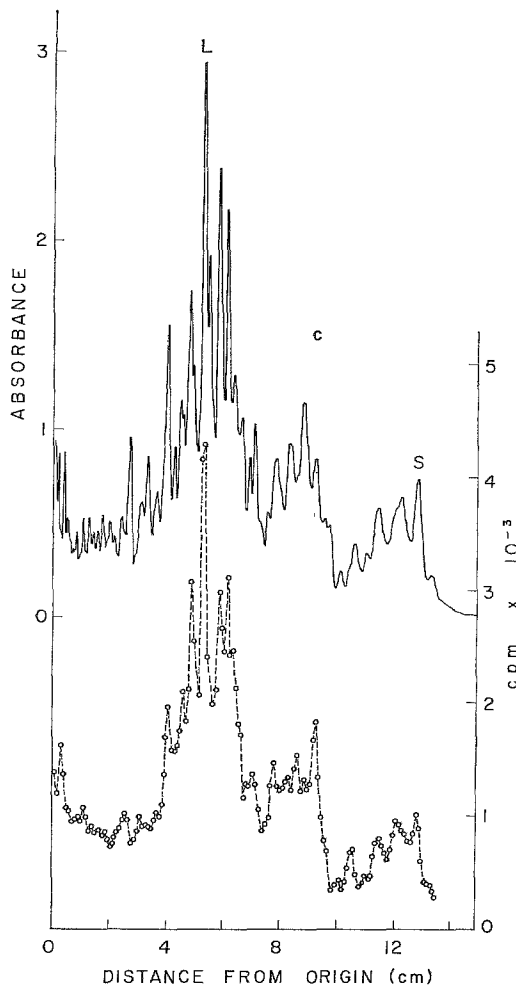L-Arginine-^{3}H (26.4 Ci/mmole) was purchased from New England Nuclear Corp., Boston, Mass.

Figure 2   A comparison of the pattern of protein stain with the pattern of radioactivity after electrophoresis of total protein of *C. reinhardi*. Etiolated cells were suspended in fresh medium to 4 × 10⁶ cells/ml and exposed to light. After 5 hr of light exposure, arginine-^{3}H was added to the culture medium to 1 μCi/ml. 1 hr later, cells were collected and total cellular protein was subjected to electrophoresis at 2 v/cm for 20 min and 6 v/cm for 6.5 hr. The vertical dotted line indicates the position in the patterns of polypeptide *c*.

Hemin (equine, two times crystallized) was purchased from Schwarz/Mann, Orangeburg, N. Y. A 10 mM solution of hemin was prepared by adding NH₄OH (final concentration about 40 mM) to a suspension of hemin in water, excess NH₃ was removed under vacuum (water aspirator), and the pH of the solution was adjusted to 7.5–8 with NaH₂PO₄. Chloramphenicol was provided by Parke, Davis & Co.,

Detroit, Mich. The disodium salt of malonic acid was obtained from Eastman Organic Chemicals, Rochester, N. Y. Maleic acid was obtained from Matheson Coleman & Bell, East Rutherford, N. J., solutions were neutralized with NaOH. Reagents for electrophoresis and determination of radioactivity were described previously (3, 5).

RESULTS

Effects of Light

Synthesis of the major polypeptides of thylakoid membranes was inhibited when cells of *C. reinhardi* *y-1* were transferred from light to dark, but resumed at normal rates upon subsequent exposure to light (5, 11). These polypeptides were not detected in dark-grown, etiolated cells, but were present in fully green cells (5). Initially, experiments were run to test two mechanisms which might account for these observations. It was postulated that (*a*) the inhibition of the synthesis of polypeptide *c* was the result, directly or indirectly, of insufficient products of photosynthesis in the dark, or that (*b*) there was a direct requirement for light in the synthesis of polypeptide *c*.

As described in Methods, illumination of the cells with light above 675 mμ provided a situation which resembled the dark in that chlorophyll was not produced, but yet light for photosynthesis was still available. Therefore, to test the first of the above possibilities, an experiment was done to see if synthesis of polypeptide *c* occurred in cells illuminated with light above 675 mμ Etiolated cells were exposed to light from incandescent lamps, and light filters were positioned in the light beams after 5 hr of greening. Arginine-^{3}H was added to the culture medium 30 min later, and the cells were allowed to incorporate the labeled amino acid for 1 hr. Cells were then broken, and portions of the broken-cell samples were centrifuged to obtain soluble and particulate fractions. Proteins in the total, soluble, and particulate fractions were subjected to electrophoresis.

Fig. 3 A shows the scan of a gel stained with Coomassie blue containing total protein of control cells. The vertical dotted lines indicate the positions in the patterns of polypeptide *c* as well as the other major thylakoid membrane polypeptide, *b*. The fraction marked *c* contains a mixture of polypeptides (5), and in these experiments the amount of polypeptide *c* that accumulated during the 5–7 hr of greening was not sufficient to cause a de-
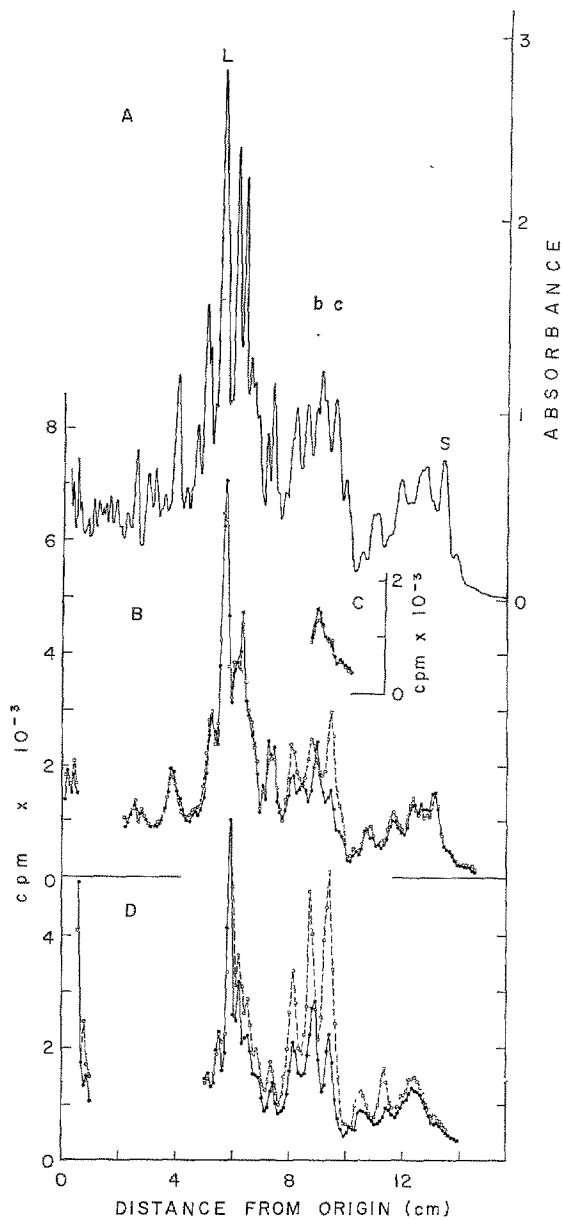
FIGURE 3 Patterns of protein stain and radioactivity after electrophoresis of polypeptides of *C. reinhardi*. The experiment was as described under Fig. 1. 30 min after the light filters were in place, arginine-³H was added to the cultures to 1 μCi/ml. 1 hr later, the cells were washed, broken, and centrifuged as described in Methods. Polypeptides in the total, soluble, and particulate fractions were subjected to electrophoresis at 2 v/cm for 20 min and 6 v/cm for 6.5 hr. After staining, a gel containing total protein was scanned (3) to provide the trace shown in Fig. 3 A. Other gels were cut

tectable increase in protein stain. However, during greening sufficient arginine-³H was incorporated into polypeptide c to produce a significant increase in this peak in the pattern of radioactivity. Fig. 3 B shows patterns of radioactivity obtained with gels containing total cellular protein A peak of radioactivity corresponding to polypeptide c is present in the pattern of protein from cells illuminated with light above 590 mμ, but this peak is markedly reduced in the pattern for cells illuminated above 675 mμ. The amounts of labeled c in the particulate fractions (Fig. 3 D) were similar to that found previously when cells were transferred to the dark (5). No polypeptide c was detected in the soluble fraction (Fig 3 C). Thus, filtering out light below 675 mμ had the same inhibitory effect on the synthesis of c as did placing cells in the dark. Yet, in cells illuminated with light above 675 mμ, the rate of protein synthesis, and presumably also of photosynthesis, in the chloroplast was not impaired, as shown by the normal level of labeling of the subunits of ribulose-1,5-diphosphate carboxylase at 5.6 cm (peak marked L) and at 13.3 cm (peak marked S). Therefore, the inhibition of synthesis of c, in cells not making chlorophyll during the period of labeling, was not the result of lowering the level of high-energy compounds or the rate of synthesis of proteins within the chloroplast.

The patterns of radioactivity for the particulate fractions (Fig. 3 D) illustrate an observation made during the course of these experiments. The prominent peak of radioactivity at 5.6 cm is at the position expected for the large subunits of the carboxylase. However, a commensurate peak at 13 3 cm, representing the small subunits of this enzyme, was not present in the patterns for the particulate fractions. Both subunits should have been present in these fractions if any of the enzyme,

into 1-mm sections, each of which was digested overnight at 55°C with 0.1 ml 30% H_2O_2. After cooling, 10 ml of a solution containing toluene, Triton X-100, and Omnifluor (3) were added to each, and the radioactivity was determined. Figs. 3 B, 3 C, and 3 D are portions of the radioactivity patterns for polypeptides in the total, soluble, and particulate samples, respectively. O—O, control cells illuminated with light above 590 mμ; ●——●, cells illuminated above 675 mμ. The vertical dotted lines indicate the positions in patterns of the two major polypeptides of thylakoid membranes, b and c. The validity of the alignment of the patterns was determined previously (5).

which has a molecular weight of near 560,000 (23, 24), had sedimented during preparation of the particulate fractions. The results of these experiments suggest that large subunits alone were sedimenting, possibly in association with the membranes.

Effects of Chloramphenicol

Since polypeptide c is synthesized on cytoplasmic ribosomes (3, 5), chloramphenicol does not inhibit its synthesis. It has been shown previously that, although chloramphenicol caused an inhibition in the synthesis of chlorophyll, cells treated with this drug synthesized polypeptide c to the same extent as untreated cells (5). Therefore, an experiment was run to see if chloramphenicol would allow synthesis of c under conditions in which chlorophyll synthesis was inhibited by filtering out light below 675 mμ. The design of this experiment was the same as for the one shown in Fig. 3. Chloramphenicol was added to the medium at the time the cells were exposed to light. After 4.5 hr, chloramphenicol-treated cells were illuminated with light above 675 mμ while control cells received light above 590 mμ. At 5 hr, i.e. 30 min after the filters were in place, arginine-³H was added to the cultures. The cells were collected 1 hr later, broken, and centrifuged to obtain soluble and particulate fractions. The proteins in the total, soluble, and particulate fractions were subjected to electrophoresis. In Fig. 4 A, the radioactivity patterns for total cellular protein show that cells, treated with chloramphenicol and illuminated with light above 675 mμ during the period of labeling, synthesized at least as much polypeptide c as did the control cells illuminated with light above 590 mμ. Portions of the radioactivity patterns for soluble protein are shown in Fig. 4 B. Most of the polypeptide c was found in the soluble fraction of the cell, and the distribution of c between the soluble and the particulate fractions (not shown) was similar to that described previously for cells treated with chloramphenicol while illuminated with white light (5).

In another experiment, cells were treated with chloramphenicol in white light for 5 hr and then were transferred to the dark. After 30 min, arginine-³H was added to the cultures and the cells were labeled while in the dark. Samples of total protein were subjected to electrophoresis. As found previously (5), the rate of synthesis of polypeptide c markedly decreased in control cells after transfer
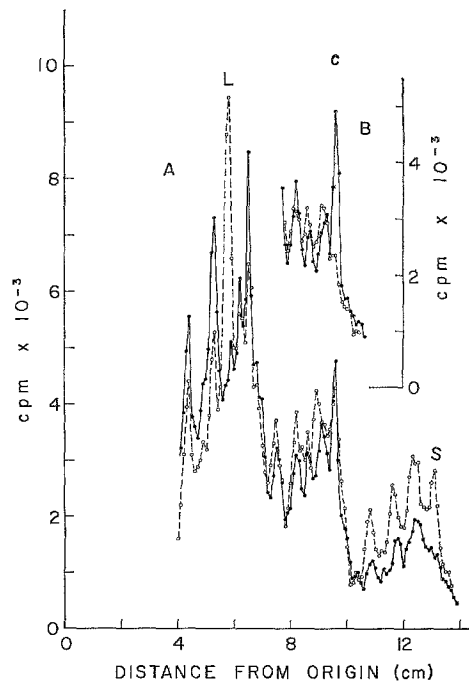

FIGURE 4 Effects of chloramphenicol (200 μg/ml) on the synthesis of polypeptide c. Cells grown 4 days in the dark were suspended in fresh medium to 4 × 10⁶ cells/ml. Each 60-ml portion of the suspension (in 500-ml flasks) received 6.6 ml of either chloramphenicol (2 mg/ml) or water at the time the cells were exposed to light from incandescent lamps. After 4.5 hr, the light filters were inserted in the light beams. Arginine-³H was added 30 min later to the cultures to 1 μCi/ml. After 1 hr of labeling, the cells were washed, broken, and centrifuged as described in Methods. Polypeptides were subjected to electrophoresis and the radioactivity in sections of the gels was determined as described under Fig. 3. Figs. 4 A and 4 B are portions of the patterns for polypeptides in total and soluble samples, respectively. O—O, control cells illuminated with light above 590 mμ, no chloramphenicol; ●——●, cells illuminated with light above 675 mμ in the presence of chloramphenicol. The vertical dotted line indicates the position in the patterns of polypeptide c.

to the dark, but chloramphenicol-treated cells continued to synthesize c, and the pattern of radioactivity for the treated cells was the same as that shown for the chloramphenicol-treated cells in Fig. 4. Therefore, these experiments with chloramphenicol show that light is not required directly for the synthesis of polypeptide c Thus, both initial postulates indicated above were eliminated.

The difference between the results shown in

Figs. 3 and 4 suggested that protein synthesis in the chloroplast in some way affects synthesis of a polypeptide in the cytoplasm. If protein synthesis in the chloroplast is responsible for making an inhibitor of the synthesis of polypeptide c, etiolated cells treated with chloramphenicol should begin synthesis of c without any exposure to light. To test this prediction, etiolated cells were treated with chloramphenicol and labeled with arginine-^{3}H in the dark Total cellular protein was then subjected to electrophoresis Fig 5 shows that a polypeptide, which after electrophoresis was in the position expected for polypeptide c, was synthesized to a much greater extent in chloramphenicol-treated cells than in control cells. Thus, chloramphenicol allowed the cells to resume synthesis of this polypeptide without the involvement of light. Since previous work (5, 11) has indicated that polypeptide c is not synthesized normally by y-1 cells in the dark, the level of radioactivity found for control cells in the position of c may provide a baseline for
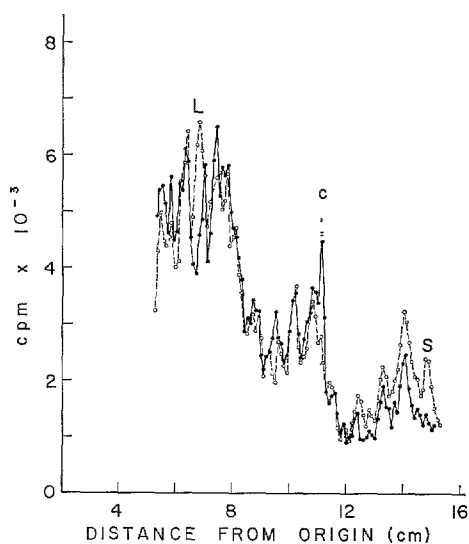

FIGURE 5 Effect on the synthesis of polypeptide c of incubating etiolated cells with chloramphenicol (200 μg/ml) in continuous darkness. Cells grown 4 days in the dark were suspended to 4×10^6 cells/ml in fresh medium and incubated in the dark with or without chloramphenicol. After 6 hr, arginine-^{3}H was added to control cultures to 2 μCi/ml and to treated cultures to 1 μCi/ml, and the cells were labeled for 1 hr. Total protein samples were subjected to electrophoresis and the radioactivity in sections of the gel was determined as described under Fig 3 O—O, control cells; ●——●, cells treated with chloramphenicol.

determining the extent of synthesis of polypeptide c. The patterns of protein stain for these cells were the same as the pattern for etiolated cells found previously (5).

Inhibitors of Chlorophyll Synthesis

Protein synthesis in the chloroplast could be involved in the regulation of synthesis of polypeptide c if a protein, synthesized in the chloroplast, controls the synthesis of the mRNA for c. The decline in the synthesis of c in control cells when the required wavelengths of light for chlorophyll synthesis are not provided would then be the result of an inhibition of further synthesis of this mRNA and degradation of the existing mRNA for c. However, this mechanism implies that the activity of this regulatory protein is controlled by other factors. Figs. 1 and 3 show that light between 590 and 675 mμ affects synthesis of both chlorophyll and polypeptide c. Since light in this wavelength range functions to convert protochlorophyllide to chlorophyllide (17, 18), the activity of the regulatory protein is possibly coupled to the synthesis of chlorophyll, with protochlorophyllide, or an immediate precursor, acting as a "corepressor," or with chlorophyll(ide) acting as an "inducer."

In attempts to test these possibilities, cells were treated with inhibitors of chlorophyll synthesis. Hemin, which inhibits δ-aminolevulinic acid synthetase (25–27), the first step in chlorophyll synthesis (28), was an effective inhibitor of chlorophyll synthesis in $C.$ $reinhardi$ Fig. 6 shows a greening experiment in which hemin was added to the culture medium after 5.5 hr of light exposure. Since hemin strongly absorbs light below 640 mμ, both control and hemin-treated cells were illuminated, after hemin was added, through filters opaque below 610 mμ in an attempt to nearly equalize light impinging on the cells. The rate of greening in control cells was not affected by the filter. The addition of hemin, however, inhibited the rate of synthesis of chlorophyll to about 15% of that found in control cells.

An experiment identical with that shown in Fig. 6 was run to test the effect of hemin on the synthesis of polypeptide c. Arginine-^{3}H was added 30 min after the addition of hemin, and the cells were allowed to incorporate the labeled amino acid for 1 hr Cells were then broken, and samples of total, soluble, and particulate fractions were prepared. Fig. 7 shows the patterns of radioactivity for these samples after electrophoresis. The pat-

terns for the total protein samples (Fig. 7 A) show that polypeptide c was synthesized in the hemin-treated cells at least to the extent that it was in control cells. This polypeptide was distributed between the soluble and particulate fractions (Figs. 7 B and 7 C). Since membrane formation requires chlorophyll (10, 16, 29), in the experiments with hemin membrane formation was likely depressed, and the appearance of c in the soluble fraction of the cells was expected (Fig. 7 B). But the reason for the high level of c in the particulate fraction is not known. Although hemin absorbed some of the light incident on the culture, and possibly lowered the rate of chlorophyll synthesis as a result, the effects of hemin cannot be ascribed solely to this effect. If hemin affected cells only by absorbing light, the results of this experiment with respect to
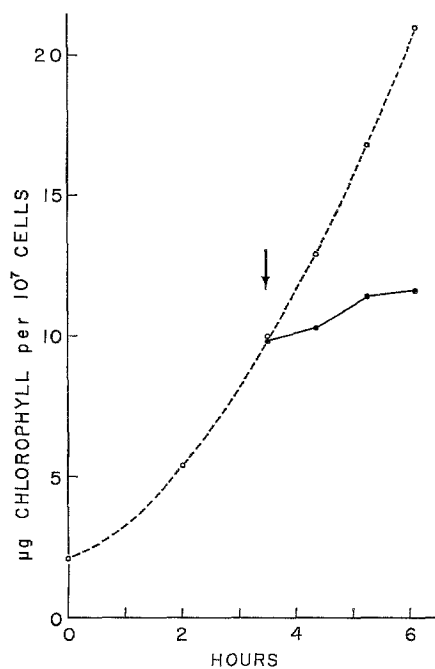

FIGURE 6   Effect of hemin (0.5 mM) on the increase of chlorophyll during greening of etiolated *C. reinhardi.* Cells grown 3 days in the dark were suspended to 4 × 10^6 cells/ml in fresh medium and exposed to light from incandescent lamps. Hemin was added to one culture at 3.5 hr, and an equal volume of water was added to the control culture. Filters transmitting light above 610 mμ were placed in the light beams at the time hemin was added. Chlorophyll was estimated spectrophotometrically in portions of the cultures as described in Methods.
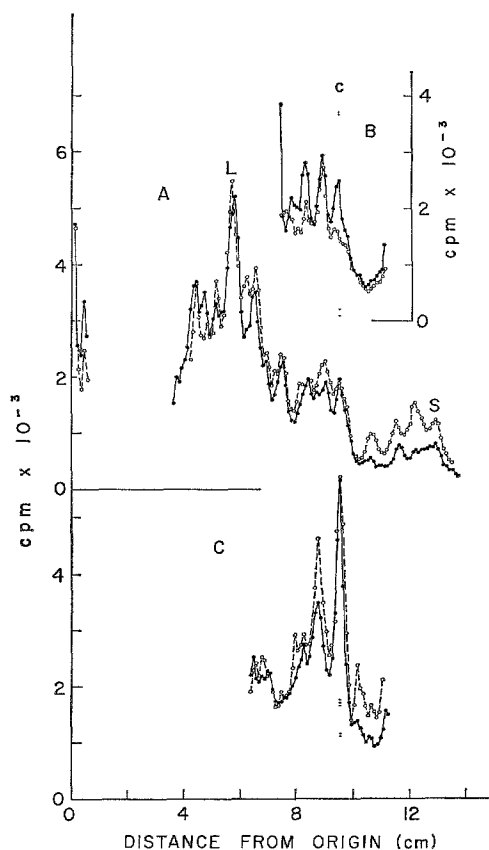

FIGURE 7   Effect of hemin (0.5 mM) on the synthesis of polypeptide c. The experiment was as described under Fig. 6. 30 min after adding hemin to the cultures, arginine-^{3}H was added to 1 μCi/ml, and the cells were labeled for 1 hr. Polypeptides in the total, soluble, and particulate samples were subjected to electrophoresis and the radioactivity was determined as described under Fig. 3. O—O, control cells; ●——●, hemin-treated cells.

the synthesis of polypeptide c should have been similar to that shown in Fig. 3.

Among a series of other compounds[1] tested, maleic and malonic acids at concentrations of 80–100 mM were found to cause an 80–85% inhibi-

[1] Other compounds tested as inhibitors of chlorophyll synthesis were either toxic to the cells at levels which caused a reduction in the synthesis of chlorophyll (aminotriazole [30, 31], ethionine [32], and hydroxylamine [33]) or had no or little effect on chlorophyll synthesis (nitrofurfuraldehyde [34], nitrofurazone [34], threonine [32] and levulinic acid [35]).

tion in the synthesis of chlorophyll in *C. reinhardi* when added at the time the cells were exposed to light. Although maleate is an inhibitor of many reactions (see 36), the growth of at least some plants does not seem to be sensitive to maleate (37, 38). In preliminary experiments, maleate did not significantly inhibit protein synthesis in *C. reinhardi*. Fig. 8 shows the pattern of radioactivity for total cellular protein obtained after labeling maleate-treated cells with arginine-[3]H. The pattern was similar to that obtained for control cells, with a significant peak of radioactivity present in the position of polypeptide *c*, indicated by the vertical dotted line. Therefore, although chlorophyll synthesis was strongly inhibited in these cells, polypeptide *c* was synthesized at a significant rate relative to the synthesis of other proteins in the cells. Results with malonate, which probably inhibited porphyrin synthesis indirectly (see 39), were the same as those with maleate for the synthesis of polypeptide *c* (data not shown).

DISCUSSION

The experiments described in this paper were designed to study the control of the synthesis of polypeptide *c*, one of the major polypeptides of thylakoid membranes in *C. reinhardi y-1*. Evidence has been presented (3, 5) that this polypeptide is synthesized on cytoplasmic ribosomes, but the present experiments show that events within the chloroplast are involved in the regulation of the synthesis of this polypeptide. Table I summarizes the results of the experiments described here and in a previous paper (5) Three possible mechanisms for control of the synthesis of polypeptide *c* were considered in light of the results shown in Table I. First, a general metabolic process in the chloroplast might be required for synthesis of polypeptide *c* in the cytoplasm. This possibility was considered because, when cells were transferred from light to dark, the synthesis of polypeptide *c* was inhibited (Table I, line 2). At the same time the rate of protein synthesis in the chloroplast, as indicated by synthesis of the subunits of ribulose-1,5-diphosphate carboxylase, decreased, presumably the result of a lack of photosynthesis in the dark. However, a metabolic control of this type was ruled out by the results shown in Table I, lines 3 and 6. During prolonged darkness (Table I, line 3), protein synthesis in the chloroplast recovered, as indicated by the synthesis of the carboxylase, but synthesis of polypeptide *c* re-
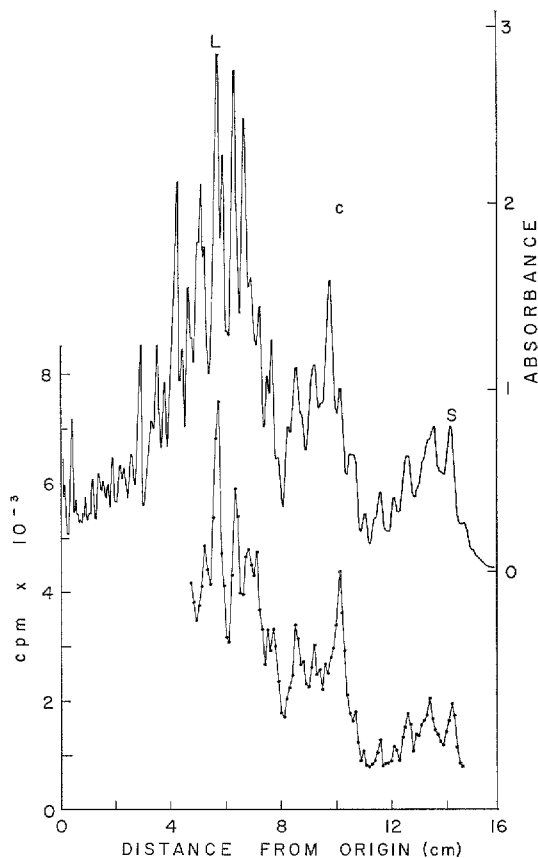

FIGURE 8 Effect of maleate (0.1 M) on the synthesis of polypeptide *c*. Cells grown 4 days in the dark were suspended to 4×10^6 cells/ml in fresh medium and exposed to 8000 lux from white fluorescent lamps. Maleate was added at the time the cells were exposed to light. At 5.5 hr, arginine-^{3}H was added to the cultures to 1 μCi/ml, and the cells were labeled for 1 hr. Total protein samples were subjected to electrophoresis and the radioactivity was determined as described under Fig. 3. The vertical dotted line indicates the position in the patterns of protein stain and radioactivity of polypeptide *c*.

mained inhibited. Also, when the illumination was changed during greening from white light to light above 675 mμ (Table I, line 6), photosynthesis was allowed to continue, but synthesis of polypeptide *c* was again inhibited. In the presence of light above 675 mμ, general metabolism in the chloroplast should not have been drastically altered. Furthermore, chloramphenicol strongly inhibited protein synthesis in the chloroplast without affecting the synthesis of polypeptide *c* (Table I,

TABLE I

Summary of Results

Before labeling	During labeling	Chlorophyll	Polypeptide c	Ribulose 1,5-diphosphate carboxylase*	Reference
Group I: Effects of light					
1. Light	Light	+	+	+	Ref. 5, Fig. 6.
2. Light	Dark	−	−	−	Ref. 5, Figs. 9, 10
3. Dark	Dark	−	−	+	Fig. 5
4. Light	Light > 590 mμ	+	+	+	Fig. 3
5. Light	Light > 610 mμ	+	+	+	Fig. 7
6. Light	Light > 675 mμ	−	−	+	Fig. 3
Group II: Effects of chloramphenicol (CAP)					
7. Light + CAP	Light + CAP	−	+	−	Ref. 5, Fig. 6
8. Light + CAP	Dark + CAP	−	+	−	Text
9. Dark + CAP	Dark + CAP	−	+	−	Fig. 5
10. Light + CAP	Light > 675 mμ + CAP	−	+	−	Fig. 4
Group III: Inhibitors of chlorophyll synthesis					
11. Light	Light > 610 mμ + hemin	−	+	+	Figs. 6, 7
12. Light + maleate	Light + maleate	−	+	+	Fig. 8
13. Light + malonate	Light + malonate	−	+	+	Text

* Synthesis of ribulose-1,5-diphosphate carboxylase was used as an indicator of the activity of chloroplast protein synthesis.

In each experiment etiolated cells were exposed to the conditions indicated under "Before labeling" for 4–5 hr. The cells were then transferred to the conditions indicated under "During labeling." Arginine-³H was added 30 min after the transfer. Synthesis of the components is indicated by a (+), and inhibition of synthesis is indicated by a (−).

lines 7–10). Therefore, synthesis of polypeptide c does not appear to be linked to general metabolism in the chloroplast.

The results of experiments with light (Table I, lines 1–6) indicated that a specific rather than a general mechanism operates to control the synthesis of polypeptide c. Moreover, the effects of light implicated the involvement of chlorophyll synthesis in this control mechanism. In each instance when light required for conversion of protochlorophyllide to chlorophyllide was not provided (Table I, lines 2, 3, and 6), synthesis of polypeptide c was inhibited. Thus, a second possibility that was considered was the inhibition of translation of the mRNA for polypeptide c. This possibility merited consideration, since inhibition of the synthesis of c was selective, and synthesis of other proteins in the cytoplasm was not similarly affected by the various treatments. In the absence of light necessary for chlorophyll synthesis, precursors might accumulate which selectively affect translation of this mRNA.

However, this possibility also seems unlikely, since in the presence of chloramphenicol polypeptide c was synthesized regardless of whether light for chlorophyll synthesis was available (Table I, lines 7–10). Although chloramphenicol caused an inhibition of the synthesis of chlorophyll (4, 5), this inhibition was not complete and, in the absence of light below 675 mμ (Table I, line 10), precursors still should have accumulated, although perhaps more slowly, to an extent sufficient to cause inhibition of translation. Also, the chloroplast envelope is apparently not permeable to precursors of chlorophyll beyond δ-aminolevulinic acid (14, 40), and these precursors therefore should not be present in the cytoplasm of the cells. This does not preclude the possibility that other metabolites could act as translational inhibitors, and chloramphenicol inhibits synthesis of the enzymes involved in the production of the metabolites.

Chloramphenicol, by inhibiting protein synthesis in the chloroplast, seemed to uncouple the

synthesis of polypeptide c from the synthesis of chlorophyll. The results suggested that chloramphenicol inhibited the synthesis of a protein which normally acts as an inhibitor of the synthesis of polypeptide c when chlorophyll cannot be made. This protein, therefore, might have the properties of a regulatory protein. Since, in bacterial systems, regulatory proteins are known to operate at the transcriptional level in the synthesis of specific proteins (41–43), synthesis of polypeptide c also may be regulated at this level.

Therefore, a third possible control mechanism that was considered involved regulation of the synthesis of the mRNA for polypeptide c. Eytan and Ohad (11) have suggested that the synthesis of the L protein fraction, which probably includes polypeptide c, is controlled at the transcriptional level by the conversion of protochlorophyllide to chlorophyll. The results described in this paper are consonant with this proposal and suggest a mechanism by which the control is exerted A protein synthesized in the chloroplast apparently is required for inhibition of the synthesis of polypeptide c. However, normally the inhibition is observed only when photoconversion of protochlorophyllide to chlorophyllide is prevented. Therefore, inhibition of the synthesis of polypeptide c can be relieved by light, which converts protochlorophyllide to chlorophyllide, or by treatment of the cells with chloramphenicol, which inhibits synthesis of the inhibitor protein. These results are consistent with the hypothesis that a regulatory protein, which inhibits synthesis of the mRNA for polypeptide c, is synthesized on chloroplast ribosomes and that the activity of this regulatory protein is controlled by the conversion of protochlorophyllide to chlorophyll. This protein is apparently active as an inhibitor when chlorophyll cannot be made, and is inactive when cells are making chlorophyll. Thus, either chlorophyll acts as an inducer of the synthesis of polypeptide c, or conversely, a precursor of chlorophyll acts as a type of corepressor The data indicate that chlorophyll is not an inducer, since treating cells with hemin, maleate, or malonate (Table I, lines 11–13) caused an inhibition of chlorophyll synthesis but not of synthesis of polypeptide c. In fact, cells treated with these compounds generally showed enhanced synthesis of c. However, eliminating light below 675 mμ blocked mechanism regulating the synthesis of polypeptide synthesis of chlorophyll at one of the last steps, and also caused an inhibition of the synthesis of poly-

peptide c (Table I, line 6). Under these conditions precursors of chlorophyll could accumulate, and, since protochlorophyllide is the end product of this biosynthetic pathway in the dark (14, 28), these results are consistent with the suggestion that this porphyrin acts by means analogous to that of a corepressor. Protochlorophyllide is present in etiolated C. $reinhardi$ y-1 (44, 45), and Matsuda et al. (45) estimate that 3–6 $\times$ 10^6 molecules of protochlorophyllide are present per cell during growth in the dark.

Since chloramphenicol inhibits chloroplast ribosomes (6–8), and since the pathway for the synthesis of chlorophyll, including the conversion of protochlorophyllide to chlorophyllide, appears to be entirely within the chloroplast (12, 13), the data in this paper imply that transcription of the mRNA for polypeptide c occurs in the chloroplast. However, we have no direct evidence to rule out the possibility that the mRNA for polypeptide c is transcribed on nuclear DNA[2].

Evidence obtained from experiments utilizing chloramphenicol must be interpreted with care, since this drug at high concentrations may affect plant cells by means in addition to its effects on chloroplast protein synthesis (48, 49). In the experiments described in this paper, whether a particular polypeptide was synthesized in chloramphenicol-treated cells was determined with reference to an external control (the incorporation of arginine-^{3}H into polypeptides of untreated cells) as well as an internal control (incorporation of arginine-^{3}H into other polypeptides of the same cells). The effectiveness of the drug in inhibiting chloroplast protein synthesis was indicated by the inhibition of the synthesis of the subunits of ribulose-1,5-diphosphate carboxylase. Thus, these controls served to eliminate possible ambiguities in the conclusions arrived at with respect to the synthesis of polypeptide c.

It should be noted that the evidence for a c was obtained from experiments done with cells of the y-1 strain of C. $reinhardi$. Whether this mechanism operates in cells of the wild-type strain has

---

[2] Rifampicin (250 μg/ml) did not inhibit synthesis of polypeptide c in our experiments. Although Surzycki (46) has presented evidence that this antibiotic inhibits transcription of chloroplast ribosomal RNA, Bottomley et al. (47) found that rifampicin did not inhibit RNA synthesis in isolated chloroplasts from several higher plants. Our results with rifampicin, therefore, remain inconclusive.

not been determined. In contrast to cells of the y-1 strain, wild-type cells are capable of synthesizing chlorophyll and of producing thylakoid membranes in the dark (9). Therefore, if a regulatory protein which controls the production of polypeptide c is present in wild-type cells, it should exist largely in an inactive form even in the dark. As in y-1 cells, such a protein might function, with its activity regulated by the level of precursors of chlorophyll, to coordinate the production of membrane polypeptides with the production of chlorophyll.

REFERENCES

1. GOLDBERG, I., and I. OHAD. 1970. *J. Cell Biol.* **44:**572.
2. SIREVÅG, R., and R. P. LEVINE. 1972. *J. Biol. Chem.* **247:**2586.
3. HOOBER, J. K. 1970. *J. Biol. Chem.* **245:**4327.
4. EYTAN, G., and I. OHAD. 1970. *J. Biol. Chem.* **245:**4297.
5. HOOBER, J. K. 1972. *J. Cell Biol.* **52:**84.
6. HOOBER, J. K., and G. BLOBEL. 1969. *J. Mol. Biol.* **41:**121.
7. ELLIS, R. J. 1969. *Science (Wash. D. C.).* **163:**477.
8. BOULTER, D. 1970. *Annu. Rev. Plant Physiol.* **21:**91.
9. SAGER, R., and G. E. PALADE. 1954. *Exp Cell Res.* **7:**584.
10. OHAD, I., P. SIEKEVITZ, and G. E. PALADE. 1967. *J. Cell Biol.* **35:**521.
11. EYTAN, G., and I. OHAD. 1972. *J. Biol. Chem.* **247:**122.
12. REBEIZ, C. A., and P. A. CASTELFRANCO. 1971. *Plant Physiol.* **47:**24.
13. WELLBURN, F. A M., and A. R. WELLBURN. 1971. *Biochem. Biophys. Res. Commun.* **45:**747.
14. KIRK, J. T. O., and R. A. E. TILNEY-BASSETT. 1967. The Plastids. W. H. Freeman and Co. Ltd., London. 368.
15. SCHOR, S. L , P. SIEKEVITZ, and G. E. PALADE. 1970. *J. Cell Biol.* **47**(2, Pt. 2):182 a (Abstr.).
16. HOOBER, J. K., P. SIEKEVITZ, and G. E. PALADE. 1969. *J. Biol. Chem.* **244:**2621.
17. FRANK, S. R. 1946. *J. Gen. Physiol.* **29:**157.
18. KOSKI, V. M., C. S. FRENCH, and J. H. C. SMITH. 1951. *Arch. Biochem. Biophys* **31:**1.
19. JOLIOT, P., A. JOLIOT, and B. KOK 1968. *Biochim. Biophys. Acta.* **153:**635.
20. BOARDMAN, N K. 1968. *Adv. Enzymol. Relat. Areas Mol. Biol.* **30:**1.
21. ARNON, D. I. 1949. *Plant Physiol.* **24:**1.
22. VERNON, L. P. 1960. *Anal. Chem.* **32:**1144.
23. PAULSEN, J. M., and M. D. LANE. 1966. *Biochemistry.* **5:**2350.
24. RUTNER, A. C., and M. D. LANE. 1967. *Biochem. Biophys. Res. Commun.* **28:**531.
25. BURNHAM, B. F., and J. LASCELLES. 1963 *Biochem. J.* **87:**462.
26. WARNICK, G. R., and B. F. BURNHAM. 1971. *J. Biol. Chem.* **246:**6880.
27. PORRA, R. J., and E. A. IRVING. 1972. *Arch. Biochem. Biophys.* **148:**37.
28. BOGORAD, L. 1966. *In* The Chlorophylls. L. P. Vernon and G. R. Seely, editors. Academic Press Inc., New York. 481.
29. OHAD, I., P. SIEKEVITZ, and G. E. PALADE. 1967. *J. Cell Biol.* **35:**553.
30. WOLF, F. T. 1960. *Nature (Lond.).* **188:**164.
31. BURNS, E. R., G. A. BUCHANAN, and M. C. CARTER. 1971. *Plant Physiol.* **47:**144.
32. GIBSON, K. D., A. NEUBERGER, and G. H. TAIT. 1962. *Biochem. J.* **83:**550.
33. PLUSCEC, J., and L. BOGORAD. 1970. *Biochemistry.* **9:**4736.
34. McCALLA, D. R. 1965. *J. Protozool.* **12:**34.
35. NANDI, D. L., and D. SHEMIN. 1968. *J. Biol. Chem.* **243:**1236.
36. WEBB, J. L. 1966. Enzyme and Metabolic Inhibitors. Academic Press Inc., New York **3:**285.
37. GREULACH, V. A. 1953. *Science (Wash. D. C.).* **117:**601.
38. PARUPS, E. V., I. HOFFMAN, and H. V. MORLEY. 1962. *Can. J. Biochem. Physiol.* **40:**1159.
39. WEBB, J. L. 1966. Enzyme and Metabolic Inhibitors. Academic Press Inc., New York. **2:**1.
40. CARELL, E. F., and J. S. KAHN. 1964. *Arch. Biochem. Biophys.* **108:**1.
41. EPSTEIN, W., and J. R. BECKWITH. 1968. *Annu. Rev. Biochem.* **37:**411.
42. MARTIN, R. G. 1969. *Annu. Rev. Genet.* **3:**181.
43. CHADWICK, P., V. PIRROTTA, R. STEINBERG, N. HOPKINS, and M. PTASHNE. 1970. *Cold Spring Harbor Symp. Quant. Biol.* **35:**283.
44. SAGER, R. 1961. *Carnegie Inst. Wash. Year Book.* **60:**374.
45. MATSUDA, Y., T. KIKUCHI, and M. R. ISHIDA. 1971. *Plant Cell Physiol.* **12:**127.
46. SURZYCKI, S. J. 1969. *Proc. Natl. Acad. Sci. U. S. A.* **63:**1327.
47. BOTTOMLEY, W., D. SPENCER, A. M. WHEELER, and P. R. WHITFELD. 1971. *Arch. Biochem. Biophys.* **143:**269.
48. ARMSTRONG, J. J , S. J. SURZYCKI, B. MOLL, and R. P. LEVINE. 1971. *Biochemistry.* **10:**692.
49. ELLIS, R. J , and M. R. HARTLEY. 1971. *Nat. New Biol.* **233:**193.