Genetic Transcription and Translation Specifying Chloroplast Components in *Chlamydomonas reinhardi**

J. J. Armstrong,† S. J. Surzycki,‡ B. Moll,‡ and R. P. Levine§

ABSTRACT: Genetic transcriptional and translational steps involved in the synthesis of certain components of the photosynthetic electron transport chain and of two enzymes of the reductive pentose phosphate cycle of the unicellular green alga, *Chlamydomonas reinhardi*, were studied in synchronous cultures. Spectinomycin and chloramphenicol were used to inhibit translation on 70S chloroplast ribosomes and cycloheximide on 80S cytoplasmic ribosomes. Rifampicin, an inhibitor of the chloroplast-located, DNA-dependent RNA polymerase, was used to inhibit chloroplast DNA transcription. In synchronous cultures the rate of photosynthesis and the amount of each of the chloroplast components monitored undergo characteristic increases during the light period of each cycle. Exposure of cells to chloramphenicol or spectinomycin during one light period prevents the normal increase in photosynthetic rate and in the amounts of the chloroplast *c*-type and *b*-type cytochromes (cytochromes 553 and 563). Neither inhibitor affects the normal increase in amounts of chlorophyll, ferredoxin, and ferredoxin–NADP reductase. Cycloheximide also blocks normal increases in photosynthetic rates and in amounts of cytochromes 553 and 563. Rifampicin prevents normal increases in photosynthetic rates and in cytochromes 553 and 563, but it does not block increases in chlorophyll, ferredoxin, and ferredoxin–NADP reductase. We conclude from experiments with rifampicin that chloroplast DNA possesses information required for the synthesis of cytochromes 553 and 563 but that the synthesis of ferredoxin and ferredoxin–NADP reductase does not. We conclude from experiments with inhibitors of translation that translational steps on both chloroplast and cytoplasmic ribosomes are required to synthesize cytochromes 553 and 563 but that only cytoplasmic ribosomes are required for the synthesis of chlorophyll, ferredoxin, and ferredoxin–NADP reductase. Neither spectinomycin nor rifampicin affects the synthesis of phosphoribulokinase. However, its synthesis is abolished by cycloheximide. We conclude from these results that the synthesis of phosphoribulokinase does not depend on transcriptional and translational events that occur in the chloroplast but that the 80S cytoplasmic ribosomes participate in its translation. Rifampicin has no effect on the synthesis of ribulose 1,5-diphosphate carboxylase whereas both spectinomycin and cycloheximide inhibit its synthesis. We conclude from these results that chloroplast DNA transcription is not required for the synthesis of this enzyme, and that its translation entails the function of both 70S chloroplast ribosomes and 80S cytoplasmic ribosomes.

It has become increasingly evident that the biogenesis of organelles such as chloroplasts and mitochondria is neither under the total genetic control of the organelles themselves nor completely dominated by the genetic apparatus of the rest of the cell. Instead there appears to be an intricate interaction between genetic systems that involve, on the one hand, nuclear DNA and cytoplasmic ribosomes and, on the other, organelle DNA and organelle ribosomes (Kirk and Tilney-Bassett, 1967; Levine and Goodenough, 1970; Roodyn and Wilkie, 1968). In this paper we describe the results of an endeavor to determine the sites of genetic transcription and translation for the specification of certain components of the photosynthetic electron transport chain and for two enzymes of the reductive pentose phosphate cycle of the unicellular green alga *Chlamydomonas reinhardi*.

In designing experiments that would localize the genetic determinants of chloroplast structure and function, four general kinds of approaches can be taken. A first approach is made possible by the existence of mutant strains whose ability to carry out chloroplast protein synthesis is disrupted by mutation (Fork and Heber, 1968; Goodenough and Levine, 1970; Levine and Paszewski, 1970; Schiff and Epstein, 1965; Shumway and Weier, 1967; Togasaki and Levine, 1970; von Wettstein, 1961). The difficulty with this approach is that it assumes that the only effect of the gene mutation is on chloroplast protein synthesis and that all the other lesions observed result from the defective protein synthesis. Such an assumption must be carefully proven before any interpretations that utilize mutant strains are credible.

In the second approach, normal cells are grown for several generations in the presence of antibiotics that specifically inhibit genetic transcription or translation in either the chloroplast or the nucleus and cytoplasm, and one asks which chloroplast components are altered or lost as growth proceeds. Such long-term growth experiments are often difficult to interpret, for a given effect arising in the presence of an inhibitor may result from a lesion that is far removed from the primary effect of the inhibitor of protein synthesis. However, long-term growth experiments are often the best source of information regarding the genetic control over the formation of complex chloroplast structures such as ribosomes and membrane (Ben-Shaul and Ophir, 1970; U. W. Goodenough, unpublished data).

In the third approach, one takes cells whose ability to

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form a normal chloroplast has been experimentally disrupted; the disruptive influence is removed, and one monitors the "recovery" of the chloroplast to its normal, functional state. One can then use the same spectrum of protein-synthesis inhibitors and ask which aspects of the recovery process depend on chloroplastic *vs.* extrachloroplastic genetic activities (Graham *et al.*, 1970; Ingle, 1968; Kirk, 1968; Margulies, 1966; Smillie *et al.*, 1967). The major drawback to this approach is that the initial, disrupted state is often a complex one, involving primary, secondary, and higher order lesions, so that when the disruptive influence is removed, a complex recovery process occurs. In order to interpret this kind of experiment adequately, the initial disrupted state and the sequence of events that occurs during recovery must be thoroughly understood.

The fourth approach is described in this paper. Cells of *C. reinhardi* are synchronized by a light–dark cycle such that the amounts and activities of the chloroplast components increase at a time when the number of ribosomes per cell is not increasing and the cells are not dividing. The influence of inhibitors of transcription and translation on these increases is then monitored. There are several advantages to this approach over the others described: normal, fully green, and photosynthetically active cells are used; a disruptive experimental procedure is not employed; the cells in the culture are all in the same physiological state at a given time; and the cells are exposed to a given inhibitor for only a few hours.

Methods

Organisms and Culture Conditions. A substrain of wild-type strain 137c, mating-type plus, of *C. reinhardi* suitable for growth in synchronous cultures was selected. This strain releases its daughter cells from their mother wall very soon after cytokinesis. Cells were grown in minimal medium (Sueoka, 1960) in which the buffer was Tris-HCl and in which $MgCl_2$ was replaced with $MgSO_4$. Synchronous cultures were obtained by using a 12 hr light/12 hr dark cycle according to a modification of the procedure described by Kates and Jones (1964a). Light was provided to the cultures from below and from two sides by a combination of white and daylight fluorescent lamps. The light intensity was 5000 lux for all experiments except those employing rifampicin, in which it was necessary to increase the intensity to 8000 lux because of the absorption of some of the light by the inhibitor. The illumination in this instance was from below with tungsten reflector flood lamps. At the intensity used, the rifampicin-treated cultures achieve the same amount of illumination as do control cultures at 5000 lux. The temperature of the cultures was maintained at 21°.

All experiments were carried out during the third light period after inoculation (3L) at cell concentrations of *ca.* 1×10^6 cells/ml. Cell number increased to *ca.* $2.8–3.0 \times 10^6$ cells/ml during the cell division in the following dark period. It is seen with the microscope that in this division half of the cells divide to four and half to two. With the exception of cycloheximide, inhibitors were added during the dark period at 50 min before the onset of the light period. Cycloheximide was added after the onset of synthesis of the components being studied. The time at which the light period began is designated zero time.

Antibiotics. Rifampicin, obtained from Mann Research Laboratories, was dissolved in sterile, unbuffered 0.01 M KH_2PO_4, pH 5.4, to give a concentration of 15 mg/ml. A final concentration of 250 μg/ml was used in each experiment. An equal volume of sterile 0.01 M KH_2PO_4 was added to the control flask.

Spectinomycin (a gift from the Upjohn Co.) was dissolved in distilled water to give a solution containing 5 mg/ml. It was sterilized by filtration through a sterile Millipore membrane. The final concentration of spectinomycin in the culture was 3 μg/ml, and an equal volume of sterile, distilled water was added to the control flask.

Chloramphenicol (Sigma Chemical Co.) was dissolved in sterile distilled water to give a concentration of 2 mg/ml. A final concentration of 100 μg/ml was used in the experiments, and an equal volume of sterile distilled water was added to the control culture.

Cycloheximide (Sigma Chemical Co.) was dissolved in distilled water to give a concentration of 1.5 mg/ml. The solution was sterilized by filtration through a Millipore membrane. A final concentration of 1 μg/ml was used in the synchronous culture, and an equal volume of sterile distilled water was added to the control flask.

Preparation of Ferredoxin and Ferredoxin–NADP Reductase. Amounts of ferredoxin and ferredoxin–NADP reductase were estimated using extracts obtained from acetone powders of the cells. Samples containing *ca.* 1×10^9 cells were collected by centrifugation at 13,000*g* for 1 min and washed twice by resuspending the pellet of cells in 0.002 M phosphate buffer, pH 7.0, and centrifuging at 12,100*g* for 1 min. The pellet of cells was resuspended in a final volume of 3.5 ml of 0.002 M phosphate buffer, pH 7.0, and used for the preparation of the acetone powder. All subsequent steps were carried out in a cold room maintained at 4°.

The acetone powder was prepared by adding 3.5 ml of cells suspended in phosphate buffer to 16 ml of acetone which had been cooled to −30°. The cells were added over a 30-sec period while stirring vigorously. The tube which had contained the cells was rinsed with 0.5 ml of the 0.002 M phosphate buffer, pH 7.0, and this was added to the acetone mixture (final concentration of acetone, 80%). The acetone mixture was allowed to stir for 5 min from the time of addition of the cells. The white precipitate was then collected by centrifugation at 1000*g* for 1 min. The acetone was discarded, and the pellet was dried by a stream of air until it was freed from the sides of the tube. The dry pellet was resuspended in 2 ml of 0.01 M phosphate buffer, pH 7.0, for 15 min and the suspension was centrifuged as before. The supernatants were combined and assayed for ferredoxin and ferredoxin–NADP reductase.

Assay for Ferredoxin. Ferredoxin was assayed by measuring the photoreduction of NADP by chloroplast fragments of *C. reinhardi* (Gorman and Levine, 1966). The extract was substituted for the purified ferredoxin that is normally used in this assay. The reaction was measured using amounts of extract containing rate-limiting concentrations of ferredoxin. Chloroplast fragments used in the assay were prepared by ultrasonic disruption of the cells (Levine and Gorman, 1966). They were washed an additional time in order to be certain to remove any residual ferredoxin. No photoreduction of NADP was obtained in the absence of the extract.

Assay for Ferredoxin–NADP Reductase. Ferredoxin–NADP reductase was assayed by following the reduction of DPIP at 600 nm according to the method of Avron and Jagendorf (1956).

Preparation of Cytochromes 553 and 563. Cytochromes 553 and 563 were extracted from acetone powders of the cells prepared according to the method described above. The pellet

remaining after the removal of ferredoxin and ferredoxin–NADP reductase was treated according to the procedure of de Petrocellis *et al.* (1970). It was extracted with 1.5 ml of 0.1 M Tris-HCl buffer, pH 7.5, containing 1% Triton x-100 and 1% sodium deoxycholate. The mixture was centrifuged at 1000*g* for 10 min. The supernate was retained, and the pellet was reextracted twice with the Tris buffer–Triton–deoxycholate solution for 15 min, using first 1 ml and then 0.5 ml of the solution. The supernatants were combined and used for the determination of cytochromes. The final pellets were preserved in order to determine whether they were free of cytochromes. The phosphate buffer extract containing the ferredoxin and ferredoxin–NADP reductase was also examined to determine that it was free of cytochromes.

Cytochrome Determinations. Reduced minus oxidized difference spectra were recorded on a Cary Model 14 recording spectrophotometer at 25°. For the determination of cytochrome 553, 0.8 ml of extract was placed in each of two cuvets having a 1-cm light path. The cytochrome in one cuvet was reduced by adding 4 μmoles of ascorbate, pH 6.0, and oxidized by adding 4 μmoles of hypochlorite (de Petrocellis *et al.*, 1970) to the other cuvet. The amount of cytochrome present was calculated from the absorbance difference at 553 nm using the molar extinction coefficient of 2.5×10^4 given by Boardman and Anderson (1967).

Cytochrome 563 cannot be reduced by ascorbate, but it is reduced by dithionite (James and Leech, 1964). For the determination of cytochrome 563, 4 μmoles of ascorbate, pH 6.0, was added to one cuvet, and an equivalent volume of water plus a few grains of dithionite were added to the other. The amount of cytochrome 563 was calculated from the absorbance difference at 563 nm using the molar extinction coefficient of 2.0×10^4 given by Boardman and Anderson (1967).

Preparation of Crude Extracts for Enzyme Assay. Aliquots of 300 ml were removed from the synchronous cultures and concentrated by centrifugation at 13,000*g* for 1 min. The cells were washed twice and resuspended in 0.05 M Tris-HCl buffer, pH 7.5. All further operations were carried out at 0–4°. The pellet of washed cells was resuspended in 2 ml of the Tris buffer. For the assay of phosphoribulokinase, the Tris buffer was 0.01 M for dithiothreitol, and it contained 18 mg/ml of crystalline bovine serum albumin. A 0.1-ml sample of cells was taken for the determination of cell number. Crude extracts were prepared by the sonic disruption of cells using a Mullard 20 kc ultrasonic disintegrator operated at full power for two 90-sec intervals. The probe was cooled in ice between the two intervals. For the preparation of crude extract for the assay of RuDP carboxylase activity, the sonicated preparation was centrifuged at 480*g* for 5 min to remove whole cells. The resulting supernatant was centrifuged at 27,000*g* for 30 min, and the supernatant from this centrifugation was used for the assay of RuDP carboxylase activity. The supernatant resulting from an additional centrifugation at 141,000*g* for 45 min was used for the assay of phosphoribulokinase activity.

Enzyme Assays. Reaction mixtures contained crude extract equivalent to a known number of cells (usually between 1.4×10^5 and 1.9×10^5) and protein equivalent to 0.05 to 0.2 mg in a volume of 1.0 ml. The assays were carried out at room temperature (21–24°).

Phosphoribulokinase activity, corrected for ATPase activity, was assayed spectrophotometrically according to the procedure of Racker (1957) using ribose 5-phosphate and spinach phosphoriboisomerase (obtained from the Sigma Chemical Co.). The phosphoriboisomerase had no detectable phosphoribulokinase activity in the amount used, and the substitution of water for ribose 5-phosphate produced no significant increase in the rate of NADH oxidation. ATPase activity was determined prior to phosphoribulokinase activity by adding crude extract to a reaction mixture lacking ribose 5-phosphate and recording any loss in absorbance at 340 nm for 1.5 min. The ribose 5-phosphate was then added and the rate of the reaction recorded. The amount of ATPase activity was subtracted to give phosphoribulokinase activity.

RuDP carboxylase activity was assayed using [^{14}C]$NaHCO_3$ according to the method of Levine and Togasaki (1965).

Cell Number, Chlorophyll, and Protein. Cell numbers were determined with the aid of a hemacytometer. Chlorophyll was determined by a modification (Arnon, 1949) of the method of Mackinney (1941).

Samples (10 ml), taken from the synchronous cultures, were centrifuged at 1000*g* for 5 min. The supernatant from a given sample was discarded, and the pellet was resuspended in 2 ml of water. The suspension was transferred to a 10-ml volumetric flask and 8 ml of acetone was added. The extraction of chlorophyll was carried out for 3 min in the dark. Protein was determined by either the biuret reaction (Gornall *et al.*, 1949) or the Folin phenol reaction (Lowry *et al.*, 1951).

Oxygen Evolution and Respiration. A Yellow Springs Instrument Co. oxygen monitor (Model 53) and a Clark-type electrode were used to measure oxygen evolution and respiration. A cell sample of 60 ml from a synchronous culture was centrifuged at 1000*g* for 5 min and resuspended in 20 ml of Tris–acetate–phosphate medium (Gorman and Levine, 1965) containing 0.0025 M $NaHCO_3$. The measurements were made at 25° in a thermostated lucite vessel. For photosynthetic oxygen evolution the light intensity was 24,000 lux.

Results

In all of the results presented here, cell number is used as the reference for the amount of a given cell component or activity present in a synchronous culture at the time a sample is taken. It is pertinent to point out that cell number is the *only constant* during the light period of synchronous growth; all other references that are often used (Schor *et al.*, 1970a), such as chlorophyll or total protein, are on the increase during the light period along with photosynthesis, in preparation for the cell division that occurs in the subsequent dark period.

Effectiveness of Inhibitors. Four inhibitors were used in these experiments, and all have been shown by others to be effective against RNA or protein synthesis in *C. reinhardi* at the concentrations we have used. *Rifampicin*, an inhibitor of transcription (Hartmann *et al.*, 1967), acts specifically on the chloroplast-located, DNA-dependent RNA polymerase of *C. reinhardi* (Surzycki, 1969), and effectively abolishes chloroplast RNA synthesis at 250 μg/ml (Surzycki, 1969). *Chloramphenicol* is an effective inhibitor of translation on 70S chloroplast ribosomes of higher plants (Ellis, 1969; Goffeau and Brachet, 1965; Spencer, 1965) and algae (Anderson and Smillie, 1966; Hoober and Blobel, 1969); at 100 μg/ml it blocks an amino acid incorporation in *C. reinhardi* that can be attributed to the chloroplast activity (Hoober and Blobel, 1969), and it is apparently without effect on 80S ribosomes of *C. reinhardi* (Hoober and Blobel, 1969). *Spectinomycin* also inhibits translation on 70S chloroplast

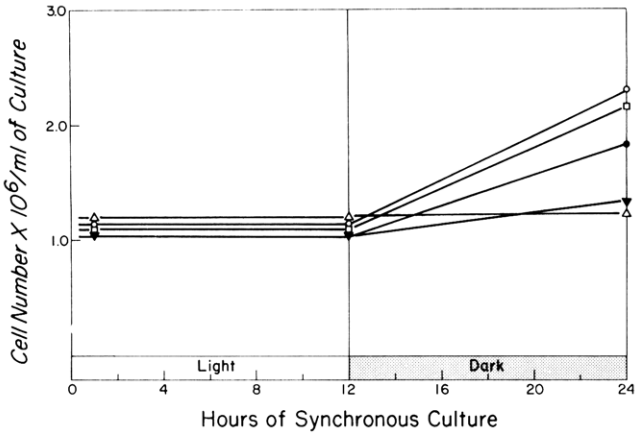

FIGURE 1: Effects of spectinomycin, chloramphenicol, rifampicin, and cycloheximide on cell division in synchronous cultures of *C. reinhardi*: ○, control; ●, 3 μg/ml of spectinomycin; ▲, 100 μg/ml of chloramphenicol; □, 250 μg/ml of rifampicin; △, 1 μg/ml of cycloheximide. Spectinomycin, chloramphenicol, and rifampicin were added 50 min before zero time; cycloheximide was added at 3 hr after zero time.

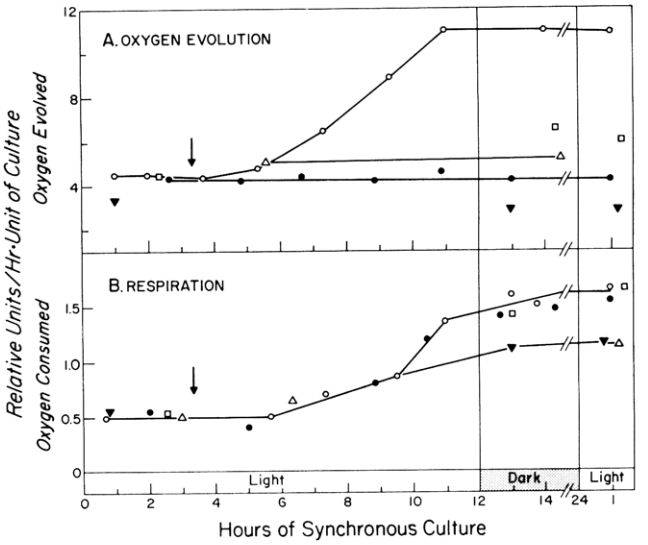

FIGURE 2: Effects of spectinomycin, chloramphenicol, rifampicin, and cycloheximide on the increase in (A) photosynthetic oxygen evolution and (B) respiration in synchronous cultures of *C. reinhardi*. Samples were taken and prepared as described in Methods. The rates of oxygen evolution shown are corrected for the respiratory rate. Experimental conditions and symbols are the same as described for Figure 1. Cycloheximide was added at the time indicated by the arrow.

ribosomes of higher plants (Ellis, 1970). In *C. reinhardi*, spectinomycin binds to the 70S chloroplast ribosomes but not to the 80S cytoplasmic ribosomes (Burton, 1970), and 3 μg/ml of spectinomycin appears to inhibit the formation of nascent polypeptide chains on *C. reinhardi* 70S ribosomes without affecting their formation on 80S ribosomes (Schor *et al.*, 1970b). *Cycloheximide* inhibits protein synthesis on cytoplasmic, 80S ribosomes (Siegal and Sisler, 1964); in *C. reinhardi*, 1 μg/ml of cycloheximide blocks an amino acid incorporation that can be attributed to the cytoplasmic activity (Hoober *et al.*, 1969); 70S ribosomes are apparently immune to cycloheximide, including the chloroplast ribosomes of *C. reinhardi* (Hoober and Blobel, 1969).

Cell Division. Cell division occurs in synchronously grown cultures between 14 and 16 hr (during the dark period). The extent of cell division following the third light period of synchronous growth in control cultures is usually about two- to threefold. Figure 1 shows the extent of cell division in untreated cells and in cells exposed to spectinomycin, chloramphenicol, cycloheximide, and rifampicin. Cell division in the presence of spectinomycin and rifampicin is very similar to that in the control culture. Cell division in the presence of chloramphenicol is visibly affected, indicating that the presence of this inhibitor may have an additional effect on the cells that is unrelated to the inhibition of protein synthesis on 70S ribosomes. Cycloheximide, as expected (U. W. Goodenough, unpublished data), inhibits cell division; however, the cells are still motile at the end of the experiment.

Oxygen Evolution and Respiration. Photosynthetic and respiratory activities increase in a stepwise fashion (Kates and Jones, 1964b; Surzycki *et al.*, 1970) during synchronous growth in *C. reinhardi* (Figure 2) as do all of the photosynthetic components studied to date. It should be noted that these increases occur at a time when no major increases are taking place in the amount of chloroplast or cytoplasmic species of ribosomal RNA (S. J. Surzycki and P. J. Hastings, unpublished data); thus the increases are presumed to represent net protein synthesis which is not a reflection of an increased number of ribosomes in the cell during the life cycle.

Neither spectinomycin nor rifampicin prevents the increase in the respiratory capacity of the cells. There is some inhibition with chloramphenicol and cycloheximide; however, respiratory capacity does increase about 2 times. On the other hand, each of the inhibitors markedly affects the increase in the capacity to evolve oxygen by photosynthesis. On this basis, it seems safe to conclude that the effect of the antibiotics on photosynthetic oxygen evolution is a specific one in that it does not reflect some generalized deleterious effect on the synchronously growing cells.

Figure 2 shows the capacity of the control and of the antibiotic-treated cells to evolve oxygen by photosynthesis and to respire during the course of the 12-hr light period of synchronous growth. The photosynthetic capacity of the control cells increases about threefold from 4 to 11 hr after the beginning of the light period. The respiratory capacity of these cells also increases threefold but during the 10th to 14th hr. The increase in respiration always continues for about 2 hr into the dark period.

Cells treated with spectinomycin do not increase their capacity to evolve oxygen by photosynthesis during the course of the experiment (Figure 2A). The ability of these cells to respire increases in parallel with control cultures (Figure 2B).

Chloramphenicol-treated cells do not increase their capacity to evolve oxygen by photosynthesis; in fact, the rate in the initial cell sample is slightly lower than in the control (Figure 2A), indicating that the antibiotic may have some immediate effect on the photosynthetic process itself which is unrelated to its effect on protein synthesis. Chloramphenicol-treated cells increase their capacity to respire only to a slightly lesser extent than do the control cells (Figure 2B).

The effect of rifampicin on photosynthetic oxygen evolution is not as dramatic as the effect of the other antibiotics (Figure 2A). During the light period, rifampicin-treated cells increase their capacity to evolve oxygen from 1.5 to 1.8 times as compared to a threefold increase in the control culture.

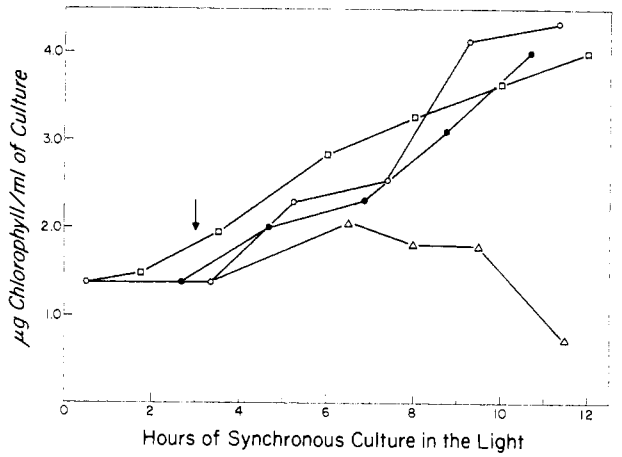

FIGURE 3: Effects of spectinomycin, rifampicin, and cycloheximide on the increase in chlorophyll in synchronous cultures of *C. reinhardi*. Chlorophyll per milliliter of culture was determined as described in Methods. Experimental conditions and symbols are the same as described for Figure 1. Arrow as in Figure 2.

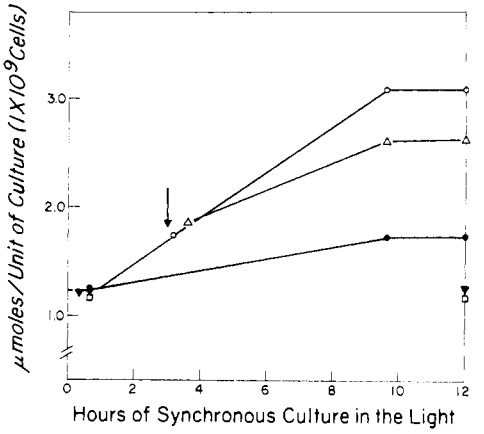

FIGURE 5: The effects of spectinomycin, chloramphenicol, rifampicin, and cycloheximide on the increase of cytochrome 553 in synchronous cultures of *C. reinhardi*. Experimental conditions and symbols are the same as described for Figure 1. Samples were taken at zero time, 9.5, and 12 hr, and extracts were prepared and assayed as described in Methods. Arrow as in Figure 2.

The rifampicin-treated cells increase their respiratory capacity exactly as do cells in the control culture (Figure 2B).

Cycloheximide-treated cells increase their capacity to respire to the same extent as chloramphenicol-treated cells and to a slightly lesser extent than control cells (Figure 2B), but the capacity of these cells to evolve oxygen shows no increase (Figure 2A).

Specific Chloroplast Components. The experiments described in the following sections are concerned with the effects of inhibitors on the synthesis of specific chloroplast components that participate in photosynthetic electron transport or the photoreduction of carbon *via* the reductive pentose phosphate cycle. In all of these experiments, synchronous cultures were divided into two equal parts: the appropriate inhibitor was added to one, and the other served as a control. Cells from the control culture were sampled in parallel with the antibiotic-treated cells in every experiment. Since all of the cell components being studied increase from between the 1st and the 12th hr, samples were routinely taken at zero time, 9.5, and 12 hr. All of the inhibitors except cycloheximide were added to synchronous cultures in the dark 50 min before the onset of the third light period (3L); cycloheximide was added to the culture just prior to the time of synthesis of the cell components being studied to minimize possible secondary effects produced by this inhibitor (Ashwell and Work, 1968). At the beginning and at the end of each experiment, cell number, respiratory rates, and rates of photosynthetic oxygen evolution were measured to ensure that the cultures were properly synchronized and that the inhibitors were acting in the expected fashion.

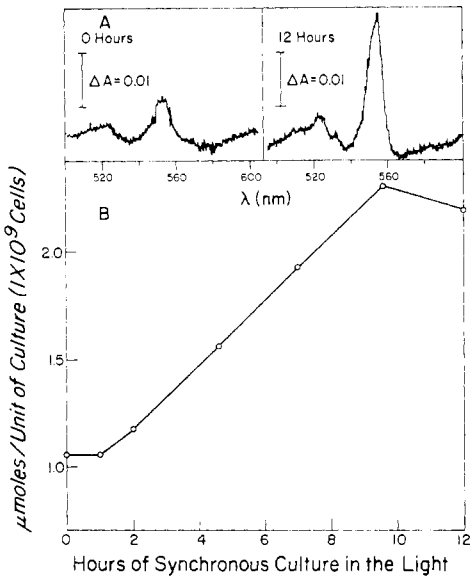

FIGURE 4: The increase in amount of cytochrome 553 in an untreated synchronous culture of *C. reinhardi*. (A) Typical ascorbate minus hypochlorite difference spectra of extracts of samples taken at zero time and 12 hr from synchronous cultures. (B) The amount of cytochrome 553 in samples taken at intervals throughout the light period. Extracts were prepared and assayed as described in Methods. Each sample contained *ca.* 1×10^9 cells, and the amount of cytochrome present is plotted in μmoles/unit of culture where a unit is 1×10^9 cells.

Chlorophyll. The synthesis of chlorophyll in synchronous cultures occurs from about the third to the tenth hour in the light period.

Figure 3 shows the extent of increase of chlorophyll in a control culture and in cultures treated with spectinomycin, rifampicin, and cycloheximide. Only cycloheximide appears to inhibit chlorophyll synthesis during synchronous growth.

Cytochrome 553. The amount of cytochrome 553 in synchronous cultures increases during the light period from the third to ninth hour. Figure 4A presents typical ascorbate-reduced minus hypochlorite-oxidized difference spectra of the samples (*ca.* 1×10^9 cells/ml) taken from a synchronous culture at zero time and at 12 hr. Figure 4B presents the amount of cytochrome 553 in samples taken from the same synchronous culture at zero time and at regular intervals throughout the light period. The increase in cytochrome 553 occurs between the first and ninth hour of synchronous growth. The extent of increase of this cytochrome is about threefold and usually corresponds to the magnitude of the subsequent cell division.

Figure 5 shows the extent of increase of cytochrome 553 in a control culture and in cultures treated with spectinomycin, chloramphenicol, rifampicin, and cycloheximide. The extent of the increase of cytochrome 553 is essentially nil

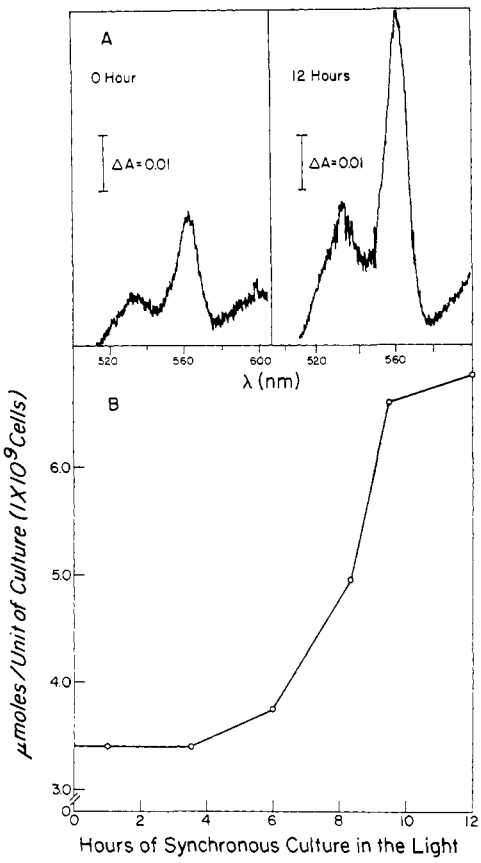

FIGURE 6: The increase in amount of cytochrome 563 in an untreated synchronous culture of *C. reinhardi*. (A) Typical dithionite minus ascorbate difference spectra of extracts of samples taken from synchronous cultures at zero time and 12 hr. (B) The amount of cytochrome 563 per unit of culture extracted from samples taken at intervals throughout the light period. Extracts were prepared and assayed as described in Methods.

when spectinomycin, chloramphenicol, or rifampicin are added to the culture. The addition of cycloheximide to the culture has only a slight effect on the expected increase in cytochrome 553.

Cytochrome 563. Cytochrome 563 increases in amount in control cultures during the fourth to the ninth hour of the light period. Figure 6A presents typical dithionite minus ascorbate difference spectra of the samples (*ca.* 1 × 10^9 cells/ml) taken from a synchronous culture at zero time and at 12 hr, and Figure 6B presents the amount of cytochrome 563 in samples taken from the same synchronous culture at zero time and at regular intervals throughout the light period. The onset of the increase in cytochrome 563 appears to commence later than the onset of the increases in cytochrome 553 and chlorophyll.

Figure 7 shows the extent of increase of cytochrome 563 in a control culture and in cultures treated with inhibitors. As in the case of cytochrome 553, the extent of increase in cytochrome 563 is essentially nil during the light period of synchronous growth when spectinomycin, chloramphenicol, or rifampicin are present. However, unlike cytochrome 553, the increase in cytochrome 563 is markedly inhibited by cycloheximide.

Ferredoxin. Ferredoxin increases from the third to the tenth hour after the onset of the light period in synchronous cultures. The extent of its increase is about threefold in

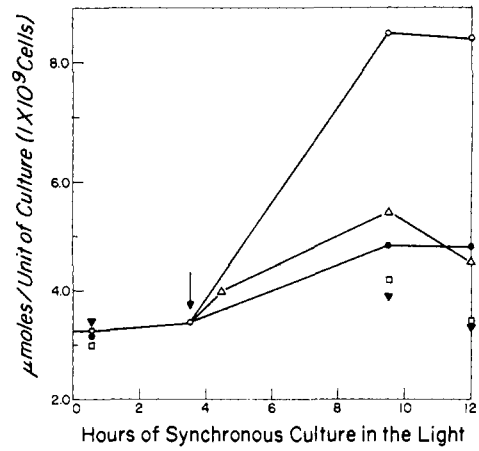

FIGURE 7: Effects of spectinomycin, chloramphenicol, rifampicin, and cycloheximide on the increase of cytochrome 563 in synchronous cultures of *C. reinhardi*. Experimental conditions and symbols are the same as described for Figure 1. Samples containing *ca.* 1 × 10^9 cells were taken at zero time, 9.5, and 12 hr. Extracts were prepared and assayed as described in Methods. Arrow as in Figure 2.

control cultures during the third light period. Figure 8A shows the amount of ferredoxin in samples taken from a control culture at regular intervals throughout one light period of synchronous growth, and Figure 8B presents the extent of increase of ferredoxin in cells from a control culture and in cells from cultures treated with the various inhibitors.

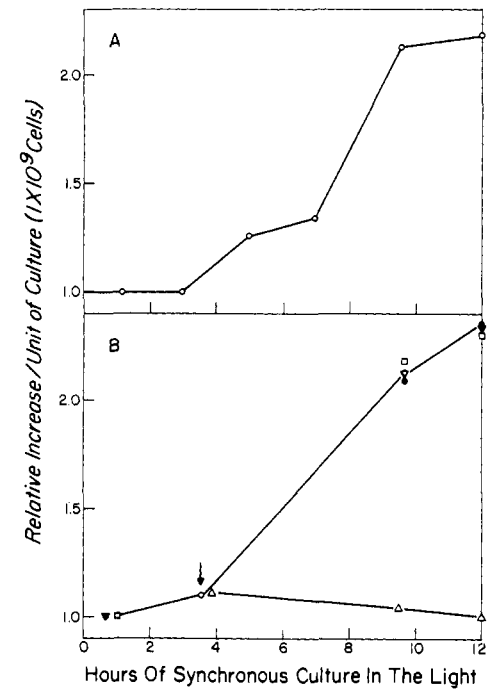

FIGURE 8: The increase of ferredoxin in synchronous cultures of *C. reinhardi*. (A) The extent of increase of ferredoxin in untreated synchronous cultures. Samples (*ca.* 1 × 10^9 cells) were taken at intervals throughout the light period of synchronous growth. Extracts were prepared and assayed as described in Methods, and the relative increase in ferredoxin per unit of culture is plotted. (B) Effects of spectinomycin, chloramphenicol, rifampicin, and cycloheximide on the increase of ferredoxin during the light period of synchronous growth. Experimental conditions and symbols are the same as described for Figure 1. Arrow as in Figure 2.

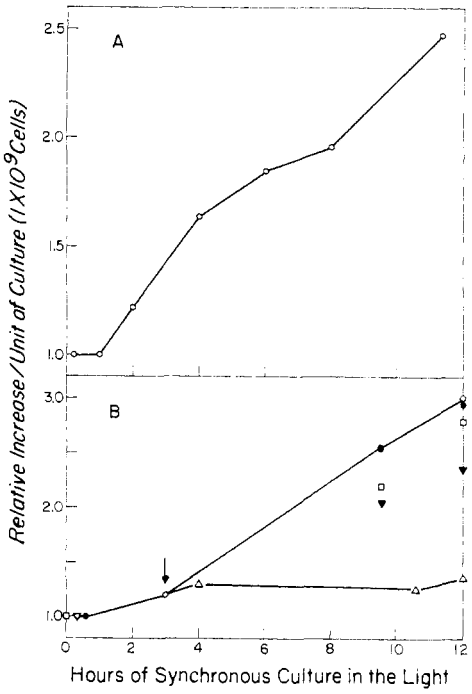

FIGURE 9: The extent of increase of ferredoxin–NADP reductase in synchronous cultures of *C. reinhardi*. (A) The amount of increase of ferredoxin–NADP reductase in untreated synchronous cultures. Samples containing *ca.* 1×10^9 cells were taken at intervals throughout the light period of synchronous growth. Extracts were prepared and assayed as described in Methods, and the relative increase in ferredoxin–NADP reductase per unit of culture is plotted. (B) The effects of spectinomycin, chloramphenicol, rifampicin, and cycloheximide on the increase of ferredoxin–NADP reductase during the light period of synchronous growth. Experimental conditions and symbols are the same as described for Figure 1. Arrow as in Figure 2.

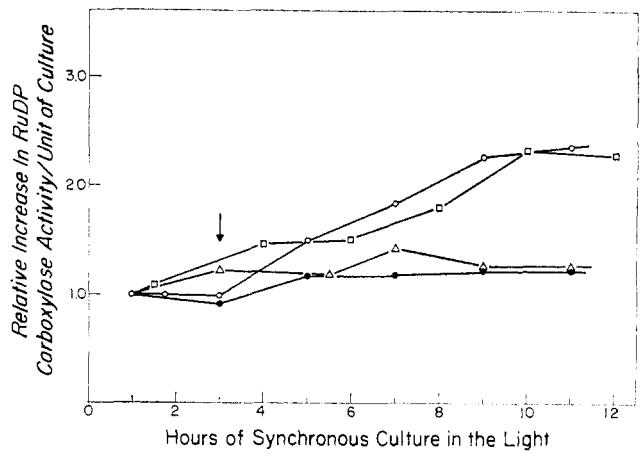

FIGURE 10: Effects of spectinomycin, cycloheximide, and rifampicin on the increase in ribulose 1,5-diphosphate carboxylase activity during the light period of synchronous growth of *C. reinhardi*: ○, control; ●, 3 μg/ml of spectinomycin; △, 1 μg/ml of cycloheximide; □, 250 μg/ml of rifampicin. Spectinomycin and rifampicin were added 50 min before zero time. Cycloheximide was added at the time indicated by the arrow. Arrow as in Figure 2.

Spectinomycin, chloramphenicol, and rifampicin do not prevent the increase in ferredoxin; however, cycloheximide does.

Ferredoxin–NADP Reductase. The flavoprotein ferredoxin–NADP reductase has been shown to be tightly bound to the chloroplast membranes of certain organisms (Davenport, 1963; Keister *et al.*, 1960), but in *C. reinhardi* it appears to be easily solubilized, for we find only a residual amount of this component associated with the detergent-treated extracts that contain other membrane-bound chloroplast components (de Petrocellis *et al.*, 1970). In spinach, the enzyme has been shown to be specific for NADPH (Avron and Jagendorf, 1956), and the enzyme also appears to be specific for NADPH in *C. reinhardi:* in the present experiments, we found that the rates obtained with both NADH and NADPH in the reaction mixture are equal to the combined rate from assays using NADH and NADPH separately. It therefore seems unlikely that the NADH-specific diaphorase interferes with our assay of the NADPH-specific enzyme.

In control cultures, ferredoxin–NADP reductase increases from the first to twelfth hour in the light. Figure 9A shows the amount of enzyme in samples taken from synchronous cultures at regular intervals during the light period, and Figure 9B presents the amount of enzyme in control cultures and cultures treated with the various inhibitors. As in the case of ferredoxin, the amount of ferredoxin–NADP reductase increases in a manner similar to the control culture when spectinomycin, chloramphenicol, and rifampicin are added to the synchronous cultures; however, there was absolutely no increase in the presence of cycloheximide.

RuDP Carboxylase. The activity of RuDP carboxylase increases approximately twofold from about the third to the ninth hour during the light period of synchronous growth. Figure 10 shows that rifampicin has no effect on this increase whereas spectinomycin and cycloheximide both inhibit it completely.

Phosphoribulokinase. Phosphoribulokinase activity increases during the light period of synchronous growth from the first to the ninth hour. A 2.5- to 3-fold increase in activity is commonly observed (Figure 11). Neither spectinomycin nor rifampicin affects this increase in activity (Figure 11). However, the addition of cycloheximide to cells in which the increase in enzyme activity is already in progress results in an immediate cessation of the increase in activity (Figure 12).

Discussion

The experiments reported in this paper permit certain conclusions to be drawn regarding the roles played by the nuclear and chloroplast DNA and by cytoplasmic and chloroplast ribosomes of *C. reinhardi* in determining several cellular processes and syntheses. For nonphotosynthetic parameters it can be concluded that cell division does not require genetic events that occur in the chloroplast, for neither rifampicin nor spectinomycin inhibit the normal increase in cell number during synchronous growth. However, translational events on cytoplasmic ribosomes are essential for cell division since cycloheximide prevents the expected increase in cell number. Respiration is unaffected by rifampicin and spectinomycin in the experiments reported here, suggesting that the mitochondrial protein-synthesizing system is immune to the concentrations of the inhibitors that we have used (Surzycki *et al.*, 1970). Chloramphenicol interferes to some extent with the normal increase in respiratory activity and cell division, indicating that this inhibition is less specific for chloroplast ribosomes at the concentration used than is spectinomycin.

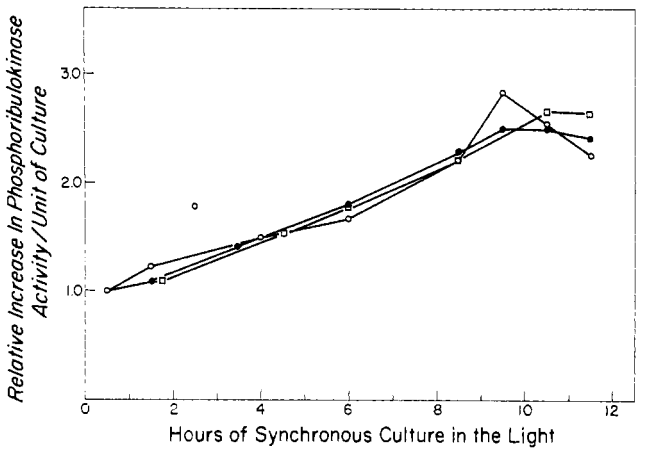

FIGURE 11: Effects of spectinomycin and rifampicin on the increase in phosphoribulokinase activity during the light period of synchronous growth of *C. reinhardi*. Experimental conditions and symbols are the same as described for Figure 10. The relatively high enzyme activity seen in the control at 3.5 hr has been observed in two independent experiments.

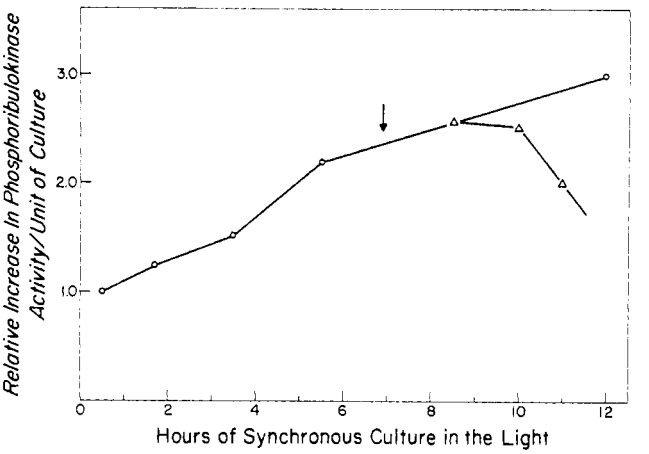

FIGURE 12: Effect of cycloheximide on the increase in phosphoribulokinase activity during the light period of synchronous growth of *C. reinhardi*. Experimental conditions and symbols are the same as described for Figure 10 except that 2 rather than 1 μg of cycloheximide was used. Arrow as in Figure 2.

The synthesis of protein associated with chlorophyll or of enzymes responsible for chlorophyll synthesis requires translational events on cytoplasmic ribosomes but not on chloroplast ribosomes, since chlorophyll synthesis is inhibited by cycloheximide but not by either spectinomycin or chloramphenicol. This finding is in accord with the observation (Hoober *et al.*, 1969) that chlorophyll synthesis in regreening experiments is inhibited by cycloheximide but not by chloramphenicol in *C. reinhardi*. Experiments on the effects of these inhibitors on regreening *Euglena* and higher plants have thus far produced conflicting results (reviewed in Levine and Goodenough, 1970; see also Graham *et al.*, 1970). Chloroplast DNA does not appear to contain information required for chlorophyll synthesis in *C. reinhardi* since rifampicin is ineffective in blocking the expected increase in chlorophyll during synchronous growth. It should be pointed out that negative rifampicin data are inconclusive since no information is available on the lifetime of chloroplast messenger RNA's.

In contrast to the results with chlorophyll, rifampicin does block the expected increases in cytochrome 553 and 563, indicating that a transcriptional step on chloroplast DNA is essential for the synthesis of these cytochromes. Since rifampicin only partially blocks the expected increase in photosynthetic oxygen evolution, it is concluded that the cytochromes are present in the chloroplast in excess, so that a block in their synthesis does not fully inhibit the chloroplast's photosynthetic capacity when measured during a single cell cycle. Inhibitors of translation on chloroplast ribosomes also block the normal course of cytochrome synthesis. Our finding that the chloroplast participates in the synthesis of these cytochromes relates to recent findings that have been made with *ac-20*, the mutant strain of *C. reinhardi* described in the introduction: both cytochromes are present in reduced amounts in the mixotrophically grown *ac-20* cells that contain few ribosomes, and both cytochromes increase in amount following the increase in chloroplast ribosomes that occurs after the cells are transferred from mixotrophic to phototrophic growth conditions.

Our experiments further suggest that the synthesis of cytochrome 563 may not be an exclusive activity of the chloroplast ribosomes, for cycloheximide also produces a marked inhibition of its increase in synchronous cultures. A similar conclusion has been reached by Smillie *et al.* (1967) for *Euglena*. It is pertinent to note in this regard that the protein moiety of yeast mitochondrial cytochrome *c* is determined by a nuclear structural gene (Sherman, 1964; Sherman *et al.*, 1968) and synthesized on cytoplasmic ribosomes (Gonzàles-Cadavid and Cambell, 1967; Kadenbach, 1967). Considering that several enzymatically controlled steps are involved in the biosynthesis of the heme portion of cytochromes, a requirement for more than one translational site for the construction of cytochrome 563 does not seem unexpected. On the other hand, we are sufficiently concerned about the possibility that nonspecific inhibitory effects are produced by cycloheximide (as discussed by Ashwell and Work, 1968) to state that all conclusions based on cycloheximide data are necessarily tentative.

We can be even less definite about the role of cytoplasmic ribosomes in the synthesis of cytochrome 553. Cycloheximide produces only a partial inhibition of the synthesis of this cytochrome in synchronous cultures, a result that we do not know how to interpret. It should be mentioned that a mutant strain of *C. reinhardi*, *ac-206*, has been studied in which cytochrome 553 is undetectable (Gorman and Levine, 1966) although cytochromes 563 and a second chloroplast *b*-type cytochrome, cytochrome 559, are present (Gorman and Levine, 1966; Levine, 1969). The *ac-206* gene is inherited in a Mendelian fashion and is thus assumed, although not proven, to be nuclear (Levine and Goodenough, 1970). Thus, there is some indication that nuclear and chloroplast genetic information and cytoplasmic and chloroplast sites of protein synthesis may share the duties of specifying cytochrome 553, but more concrete information is clearly needed on the possible nuclear and cytoplasmic participation in the synthesis of this cytochrome. Meanwhile, the chloroplast role seems quite well established by the experiments reported in this paper.

The picture presented by ferredoxin and ferredoxin–NADP reductase is much less intricate. The synthesis of these soluble chloroplast proteins does not appear to depend on information in chloroplast DNA nor does their synthesis occur on chloroplast ribosomes in *C. reinhardi*. This last result con-

flicts with results of Smillie *et al.* (1967) who report an inhibition of ferredoxin–NADP reductase synthesis by chloramphenicol in long-term experiments with *Euglena*. The inhibition by cycloheximide of the expected increase in these proteins implicates the cytoplasmic ribosomes as the site of their synthesis. It is assumed that the information for the synthesis of these proteins resides in nuclear DNA. Independent evidence in support of the finding that chloroplast ribosomes are not the site for the synthesis of these components comes again from studies with the *ac-20* strain. In spite of the dramatic reduction in the level of chloroplast ribosomes in this strain, the amounts of ferredoxin and ferredoxin–NADP reductase are the same as in the wild-type strain (Levine and Paszewski, 1970).

The nuclear–cytoplasmic system also appears to be uniquely involved in the synthesis of the enzyme phosphoribulokinase. This finding is supported by two independent observations. First, in *ac-20* the level of phosphoribulokinase activity does not differ significantly from the level of activity in wild-type cells (Togasaki and Levine, 1970); thus chloroplast ribosomes do not appear to participate in the synthesis of the enzyme. Second, another mutant strain of *C. reinhardi*, F-60, that lacks phosphoribulokinase activity shows classical Mendelian inheritance (Moll and Levine, 1970; B. Moll, unpublished data); this suggests that at least one nuclear gene controls the synthesis of the enzyme.

The findings reported here indicate that the genetic specification of RuDP carboxylase is more complex. The fact that rifampicin does not affect its increase in synchronous cultures probably indicates that its synthesis does not require transcriptional events on chloroplast DNA. We therefore assume that nuclear DNA contains the necessary information for the synthesis of this enzyme. No gene mutations are yet known that directly affect the synthesis of this protein in *C. reinhardi;* however, Anderson *et al.* (1970) have characterized several strains of tomato that lack RuDP carboxylase activity and that carry gene mutations that are inherited in a Mendelian fashion. Thus there is apparently nuclear control over the synthesis of this enzyme in higher plants as well as in *C. reinhardi*.

Both chloroplast and cytoplasmic ribosomes appear to be required for RuDP carboxylase synthesis in *C. reinhardi*, for inhibitors of protein synthesis that act on both the 80S cytoplasmic ribosomes and the 70S chloroplast ribosomes prevent the expected increase in enzyme activity in synchronous cultures. These findings are in accord with recent studies of Togasaki on the *ac-20* mutant strain. Following the transfer of mixotrophically grown *ac-20* cells to phototrophic growth conditions there occurs a dramatic increase in the amount and also in the activity of RuDP carboxylase (Togasaki and Levine, 1970); R. K. Togasaki (unpublished data) has shown that these increases are inhibited by both chloramphenicol and cycloheximide. Graham *et al.* (1970) and Smillie *et al.* (1967) have published data that in some cases differ from those reported here. In all the organisms they have studied chloramphenicol inhibits the synthesis of RuDP carboxylase in regreening systems; however, conflicting data are presented for the effects of cycloheximide. The authors report no inhibition in *Euglena*, only a partial inhibition in sorghum, and a more marked inhibition in maize (the symbols in Figure 2 of Graham *et al.*, 1970, have been reversed for 0.5 μg/ml of cycloheximide and 2 μg/ml of cycloheximide; D. Graham, private communication). Thus the cytoplasmic role in the synthesis of RuDP carboxylase in other organisms is not yet clearly established.

RuDP carboxylase is a large protein with a molecular weight of some 500,000 (see Ridley *et al.*, 1967, for references). In spinach it is made up of two types of subunit, one having a molecular weight of 55,000 and the other having a molecular weight of 12,000 (Rutner, 1970); the native enzyme is thought to be an association of 8 of the heavy subunits and 8–10 of the light subunits (Rutner, 1970). It thus does not seem surprising that the genetic specification of such a complex protein should be under complex control at the translational level. Indeed, it is tempting to suggest that the polypeptides of one subunit are formed on cytoplasmic ribosomes and that those of the other subunit are formed on chloroplast ribosomes.

In conclusion, our results indicate that one can envisage four possible systems of transcription and translation for chloroplast components: (a) chloroplast transcription–chloroplast translation; (b) chloroplast transcription–cytoplasmic translation; (c) nuclear transcription–chloroplast translation; and (d) nuclear transcription–cytoplasmic translation. In addition to these four basic combinations, it is possible that complicated proteins may require elements transcribed from both chloroplast and nuclear DNA; it is also possible that information for different portions of their structure may be translated on both chloroplast and cytoplasmic ribosomes. In fact, some proteins may require the functioning of all four systems for their synthesis. As described above, cytochromes 553 and 563 may be examples of components whose synthesis depends on an interaction of genetic functions by all four systems. On the other hand, chlorophyll, ferredoxin, ferredoxin–NADP reductase, and phosphoribulokinase appear to be determined exclusively by a system of nuclear transcription and cytoplasmic translation. The most complex picture is presented by RuDP carboxylase, whose synthesis is apparently specified by nuclear transcription and both chloroplast and cytoplasmic translation.

Acknowledgment

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Added in Proof

Evidence for the differential synthesis of RuDP carboxylase has recently been reported (see Criddle *et al.*, 1970).

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