SYNTHESIS AND TURNOVER OF RIBULOSE BIPHOSPHATE CARBOXYLASE AND OF ITS SUBUNITS DURING THE CELL CYCLE OF *CHLAMYDOMONAS REINHARDTII*

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ABSTRACT

The chloroplast enzyme ribulose-1,5-bisphosphate (Ru-1,5-P_2) carboxylase (EC 4.1.1.39) is made up of two nonidentical subunits, one synthesized in the chloroplast and the other outside. Both of these subunits of the assembled enzyme are synthesized in a stepwise manner during the synchronous cell cycle of the green alga *Chlamydomonas reinhardtii*. The activity of this enzyme increases in the light and this increase is due to *de novo* protein synthesis as shown by the measurement of the amount of protein and by the pulse incorporation of radioactive arginine in the 18S enzyme peak in linear sucrose density gradients. During the dark phase of the cell cycle, there is little change in the enzymatic activity as well as in the amount of this enzyme. Pulse-labeling studies using radioactive arginine indicated that there is a slow but detectable rate of synthesis of the carboxylase and of its subunits in the dark. Ru-1,5-P_2 carboxylase, prelabeled with radioactive arginine throughout the entire light period, shows a similarly slow rate of degradation in the following dark period. This slow turnover of the enzyme in the dark accounts for the steady levels of carboxylase protein and of enzymatic activity during this period. A wide variety of inhibitors of protein synthesis by 70S and 80S ribosomes abolished the incorporation of [^{3}H]arginine into total Ru-1,5-P_2 carboxylase during short-term incubation. These results suggest a tight-coordinated control of the biosynthesis of the small and large subunits of the enzyme. This stringent control is further substantiated by the finding that both subunits are synthesized in synchrony with each other, that the ratio of radioactivity of the small to the large subunit remains constant throughout the entire light-dark cycle, and that the rates of synthesis and of degradation of both subunits are similar to that of the assembled enzyme.

The unicellular alga *Chlamydomonas reinhardtii* can be used as a model system in the studies of cellular events since it can be synchronized easily by alternating light-dark cycles (1). The time of replication in the cell cycle of nuclear and chloroplast DNA (2) as well as the changes in ribosomal RNA content (3) have been extensively examined.

The system, therefore, is particularly amenable to correlative studies on the periodic availability of genetic information and on the periodic activity of protein synthesis.

The purpose of the present investigation is to examine the synthesis of the chloroplast enzyme, ribulose bisphosphate (Ru-1,5-P_2) carboxylase

(EC 4.1.1.39), during the cell cycle of *C. reinhardtii*. This enzyme is of primary importance under conditions of autotrophic growth since it occupies a strategic location in the CO_2 fixation pathway. It is present in large amounts in the plant cell and can be isolated and purified quite readily (4–12).

A unique feature of Ru-1,5-P_2 carboxylase, relevant to the present study, is that the enzyme consists of two nonidentical subunits (8, 9, 11, 13–15) which are probably synthesized in different compartments of the cell, the large within the chloroplast and the small in the cytoplasm (9, 16–22). The regulation of the biosynthesis of such proteins is potentially complex and at present not understood. Therefore, a study of the pattern of biosynthesis of Ru-1,5-P_2 carboxylase during the cell cycle may help to elucidate the cooperation between the two protein-synthesizing systems in eukaryotes.

MATERIALS AND METHODS

Handling of the Cells

The wild-type strain (137c, mating type plus) of *C. reinhardtii* was used for all experiments. Cells were grown at 25°C in the minimal medium of Sager and Granick (23) as described by Ohad et al. (24), and synchronized by 12 h light (light intensity ~4,000 lx) and 12 h dark cycles (1). Experiments were carried out with cultures having a cell density of 1.0×10^6 cells/ml at the beginning of the light phase of the cell cycle. For each series of experiments, the changes in cell density, chlorophyll content, and protein concentration were monitored throughout the entire cell cycle. In the nomenclature, L-0, L-4 etc., and D-0, D-4 etc. refer to the length of time (hours) the cells were in the light (L) or in the dark (D).

Labeling of Cells with Radioactive Arginine

The rate of incorporation of radioactive precursor into the proteins of *C. reinhardtii* was followed by pulse labeling. At each time point 200 ml of culture were withdrawn from the main flask and incubated with L-[³H]arginine (24 nM, 0.5 µCi/ml culture) for 30 min, in the light or dark, in accordance with the period of the cell cycle. The incorporation was stopped by the addition of an equal volume of crushed ice to the culture medium. Cells were then harvested in the cold by centrifugation at 2,000 g for 5 min, washed twice with 50 mM Tris-HCl, pH 8.0, containing 5 mM $MgCl_2$ and 1 mM DTT (TMD buffer), and resuspended in 1 or 2 ml of the TMD buffer. Several time points, in both the light and the dark periods, were taken routinely from the same synchro-nized culture and the samples were processed simultaneously (see below).

To study the turnover of the Ru-1,5-P_2 carboxylase during the cell cycle, continuous labeling with [³H]- and [¹⁴C]arginine was employed. A synchronized culture (2 liters) was incubated with [¹⁴C]arginine (0.15–0.2 µCi/ml culture) throughout the entire light period. The following operations were carried out under sterile conditions. At L-12, cells were harvested, washed with minimal medium containing 10 mM arginine, and then with minimal medium alone, and finally resuspended in the latter medium. The final volume of the cell suspension was adjusted to obtain the original cell density at L-12. [³H]arginine (0.3–0.5 µCi/ml culture) was then added to the culture at D-0, and at each desired time point in the dark period 200 ml of the culture were withdrawn and processed as described above.

Fractionation of Ru-1,5-P_2 Carboxylase from Homogenate of C. reinhardtii

The harvested cells concentrated from 200 ml of the original cultures, obtained as described above, were resuspended in 1 or 2 ml of TMD buffer and sonicated with a Heat Systems-Ultrasonics sonifier, W 185, (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.), at setting no. 4 for three 1-min periods. Cell breakage was estimated to approach 100% as revealed by light microscope examination of the sonicate. Equal aliquots (0.5 ml) of the broken cell suspension were layered onto 10–30% linear sucrose density gradients containing the TMD buffer. The gradients were centrifuged at 4°C in the SB 283 rotor of the International centrifuge (B-60) at 40,000 rpm (180,000 $g_{average}$) for 15–16 h. Gradients were fractionated with an ISCO gradient fractionator as described before (11). Fractions containing the Ru-1,5-P_2 carboxylase peak were pooled and the pooled samples from the various time points of the cell cycle were brought to an identical volume with TMD buffer.

Gel Electrophoresis

Polyacrylamide gel electrophoresis of the pooled Ru-1,5-P_2 carboxylase samples from the sucrose density gradients was carried out in 5.5% disc gels according to Davis (25), at 0.5 mA per gel. SDS-polyacrylamide gel electrophoresis was performed on 11% disc gels as described by Laemmli (26), at a current of 0.2 mA per tube applied for 7–8 h at room temperature.

Radioactivity Measurements

Preparation of materials for counting varied according to the source of material and the isotope used. Aliquots from total cell homogenate and fractions from sucrose gradient were pipetted onto Whatman 3 MM filter paper disks, which were then sequentially washed with 5% cold TCA, 5% hot TCA, ethanol-ether (1:1), and ether, as described by Mans and Novelli (27). Radioac-

tivity was then measured in 10 ml of Liquifluor-toluene mixture in a Packard scintillation counter at +5°C.

Polyacrylamide gels were sliced and the 1-mm thick slices were placed into scintillation vials, followed by 5 ml of scintillation fluid (100 ml of Protosol, 20 ml of 4 N NH₄OH, 42 ml of Liquifluor in 1,000 ml of toluene) as described by Ward et al. (28). Vials were tightly closed and incubated for 48-50 h at 37°C with periodic shaking. After cooling, the samples were counted in a Packard scintillation counter at 4°C. When proteins were labeled with both ^{14}C and ^{3}H, the channels were set so that the ^{3}H channel contained 21-27% of ^{14}C counts, whereas the ^{14}C channel had essentially no ^{3}H counts. [^{3}H]albumin was used as a reference protein for establishing the recovery of radioactive counts from the polyacrylamide gels, which recovery was found to be ~85%.

Miscellaneous Determinations

Ru-1,5-P₂ carboxylase activity was measured by the fixation of NaH^{14}CO₃ into acid-soluble residue as described previously (10, 11). Radioactive amino acids in the acid-soluble pools were determined by two-dimensional thin layer chromatography according to Jones and Heathcote (29). These acid-soluble fractions were obtained by mixing an equal volume of cell homogenate with 10% cold TCA and removal of the TCA precipitate by centrifugation at 40,000 g for 20 min. Total radioactivity in the soluble fractions were determined as described (28). Protein was determined by the procedure of Lowry et al. (30) with bovine serum albumin as standard, and chlorophyll concentrations were measured according to Arnon (31). Cell density was determined by duplicate hemocytometer counting.

Materials used in these experiments were purchased as follows: [^{14}C](guanidino)-arginine (45 mCi/mmol), NaH^{14}CO₃ (57 mCi/mmol) and sucrose, ribonuclease-free grade, from Schwarz/Mann Div., Becton, Dickinson and Co., Orangeburg, N.Y.; [^{3}H](C₃)-arginine (27 Ci/mmol), Protosol, and Liquifluor from New England Nuclear, Boston, Mass.; TLC-cellulose plates from Eastman Kodak Co., Rochester, N.Y.; ribulose-1,5-bisphosphate (Ba salt), cycloheximide, chloramphenicol, and erythromycin from Sigma Chemical Co., St. Louis, Mo.; lincomycin and spectinomycin from Upjohn Co., Kalamazoo, Mich.; anisomycin from Chas. Pfizer & Co., Inc., New York; [^{3}H]albumin prepared in the laboratory (V. Iwanij, unpublished observations).

RESULTS

General Characteristics of Cell Cycle

Fig. 1 A and B illustrates some of the changes which occur during the cell cycle in the synchronized cultures of C. reinhardtii. There is no change in the cell number during the light period, and division takes place in the first half of the dark period, giving rise to approximately a fourfold increase in the cell number at the end of the cycle. These findings confirm earlier results (32). After a 3-4-h lag period, chlorophyll content per culture (or per cell) rapidly increases up to fourfold (Fig. 1 A), then remains stationary during the entire dark period. Net protein synthesis occurs approximately linearly in the light, resulting in a 3.5-fold increase in protein concentration (Fig. 1 B). However, no appreciable change occurs during the dark period (Fig. 1 B), as has been found previously (33, 34). The total enzymatic activity of Ru-1,5-P₂ carboxylase increases usually three- to fourfold in the light (21), appears to be constant during the first 6 h of the dark period, and declines slightly at the end of the dark period (Fig. 1 B).

Changes in Ru-1,5-P₂ Carboxylase during Cell Cycle

The observed increase in the enzymatic activity of the carboxylase may represent either an activation of previously existing enzyme or an increase in the net amounts of enzyme protein. To answer this question, two types of experiments were performed: the first, exploiting the unique sedimentation properties of the enzyme, measured the amount of enzyme, while the second measured the incorporation of radioactivity into it. The Ru-1,5-P₂ carboxylase complex has a weight of ~500,000 daltons and during centrifugation in a continuous sucrose density gradient sediments much faster than the other soluble proteins and forms a distinctive absorbance peak with a sedimentation coefficient of ~18S (Fig. 2). Virtually all (>95%) of the applied Ru-1,5-P₂ carboxylase activity can be recovered in that peak regardless of the time of the cell cycle from which the samples were taken. Therefore the protein content in the 18S peak was taken to represent the total, assembled Ru-1,5-P₂ carboxylase complex produced by the cell during vegetative development of the alga. Samples were taken at 3-4-h intervals during the entire 24-h cell cycle. Fig. 2, showing the results of a representative experiment, illustrates the increase in the area of the 18S peak corresponding to Ru-1,5-P₂ carboxylase protein content from the time L-0 (a) to L-7 (b), while no change in the peak area was noted between D-0 (c) and D-4 (d). No increase was noted from D-4 to D-12 (data not shown). The increase of the peak area corresponds to the increase of protein concentration of the sample recovered from that peak, from 16 μg/ml culture at L-0 to 54 μg at D-0 and 56 μg at D-6.
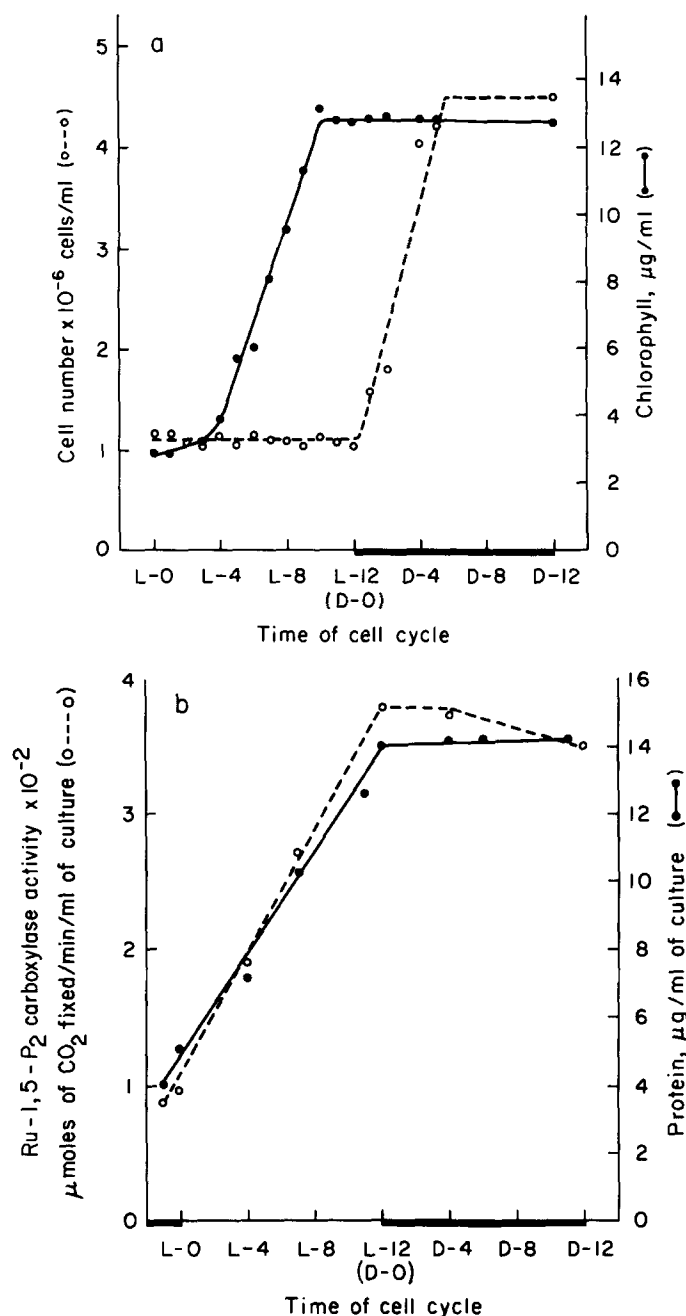
FIGURE 1   Changes in cell number, protein content, chlorophyll content, and Ru-1,5-P₂ carboxylase activity during the cell cycle of *C. reinhardtii*. (a) O---O, cell number; ●---●, chlorophyll content; (b) ●---●, protein content; O---O, Ru-1,5-P₂ carboxylase activity. Synchrony was performed and assays were done as described in Materials and Methods.

To eliminate interference by possibly contaminating proteins that cosedimented in the 18S region, polyacrylamide gel electrophoreses in the presence and absence of SDS were performed.

When Coomassie blue-stained gels of the total enzyme and of its subunits (see Materials and Methods), obtained from different time points of the cell cycle, were compared (Figs. 3 and 5), the
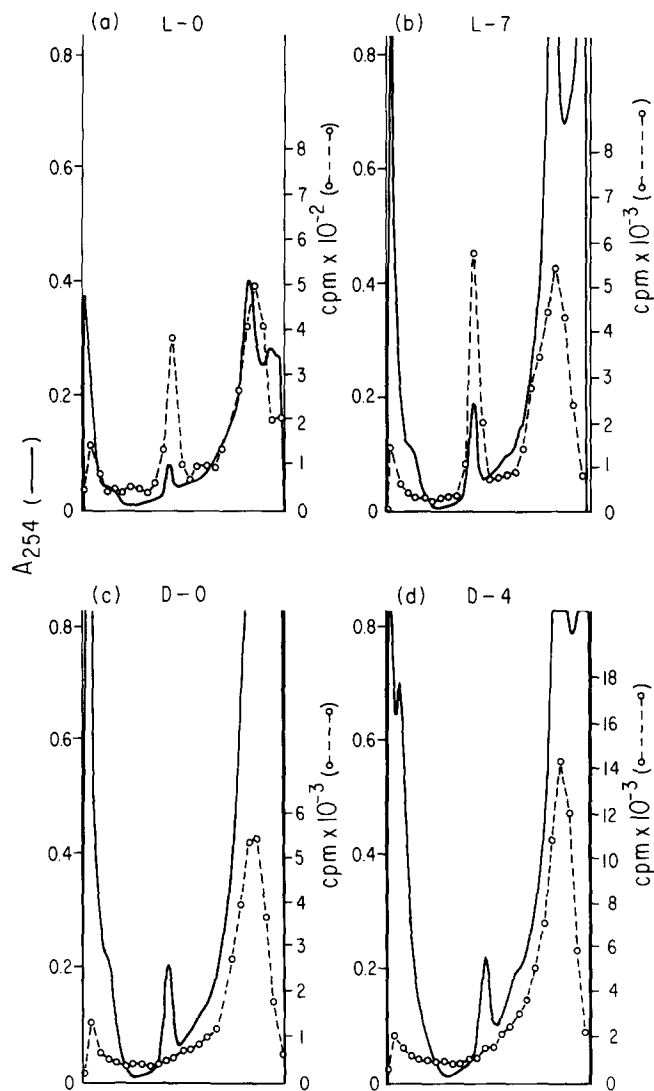
FIGURE 2   Sucrose gradient analyses of Ru-1,5-P$_2$ carboxylase pulse labeled for 30 min with [^{3}H]arginine at different times of the synchronous cell cycle. Preparation of cell homogenate and centrifugation conditions are described under Materials and Methods. Each gradient, containing equal aliquots of the cell culture, was fractionated into 25 fractions (containing 0.5 ml each). The optical density peak in the middle of the gradient ($\sim$18S) marks the position of the carboxylase. 100-μl aliquots from each fraction were used for radioactivity measurement according to Mans and Novelli (27). The horizontal arrow indicates the direction of centrifugation.

results were similar to those of the centrifugation experiments. Total Ru-1,5-P$_2$ carboxylase (Fig. 3) and both its subunits (Fig. 5) showed increases from L-0 (5 a) to L-4 (5 b) to L-7 (5 c) and again no significant change from D-0 (5 d) to D-4 (5e). Also, no changes were observed from D-4 to D-12 (data not shown). Therefore, the net amount of Ru-1,5-P$_2$ carboxylase increased during the light and remained unchanged during the dark period.

The second type of experiment involves the pulse labeling by [^{3}H]arginine of Ru-1,5-P$_2$ carboxylase. This incorporation rate increased during the light but dropped to a uniformly low level in the dark (Figs. 2–4). Fig. 2 shows the incorporation into the 18S peak of a sucrose density gradient, while Figs. 3 and 4 show the incorporation into the whole

enzyme after polyacrylamide gel electrophoresis. The rate of incorporation into the total proteins of *C. reinhardtii* remained at a high level in the dark period, except for D-0 (Fig. 4), indicating that the low level of incorporation into the Ru-1,5-P_2 carboxylase in the dark is not due to a lack or limitation of radioactive precursors. The ratio of incorporation into total enzyme compared to total protein is about 10% during the light period and falls to less than 1% during the dark period. These experiments indicate that the enzyme is synthesized periodically during the cell cycle. Under illumination, the amount of Ru-1,5-P_2 carboxylase increases due to *de novo* protein synthesis. With transition to the dark, the synthesis of Ru-1,5-P_2 carboxylase drops to a low level while its concentration, measured either chemically or enzymatically, remains constant.

Biosynthesis of Large and Small Subunits of Ru-1,5-P_2 Carboxylase during the Cell Cycle

Since the carboxylase is composed of two nonidentical subunits, it was of interest to examine the incorporation of [^{3}H]arginine into these subunits during the cell cycle of *C. reinhardtii*. Pulse-labeling studies done as above showed that the subunit incorporation behaved identically to that of total, assembled Ru-1,5-P_2 carboxylase. The rate of incorporation increases during the light period and drops drastically in the dark (Fig. 5). Furthermore, the ratio of radioactivity in the small compared to the large subunit (Table I) is essentially constant during the cell cycle of *C. reinhardtii*. This ratio of approximately 0.3 reflects the actual ratio of arginine content of small to large subunits as revealed by amino acid compositions of these polypeptides (11). Therefore, the syntheses of the large and small subunits of Ru-1,5-P_2 carboxylase are seemingly under rigid control and appear to be synchronized at least with respect to [^{3}H]arginine as a precursor. The results in Table I also suggest that there are no unequal pools of subunits which might arise as a result of nonsynchronous synthesis, e.g. the synthesis of small or large subunits in the dark and awaiting assembly into the whole enzyme in the light. To investigate such a possibility, cells were prelabeled with [^{3}H]arginine in the dark (to label any possible subunit pools), washed, and then incubated with [^{14}C]arginine in the light. The enzyme and its subunits were isolated and analyzed for radioactivities at various time points in the light period. The results in Table II show

that there is a large increase in the incorporation of ^{14}C precursor into both subunits in the light and a much smaller increase of ^{3}H radioactivity during the same period. The presence of the latter is due to the persistence of ^{3}H-radioactive acid-soluble pool, labeled in the dark and carried over into the light (see below and Fig. 7). If one of the subunits was synthesized in the dark and carried over in a pool to be assembled in the light, then the ratio of ^{3}H/^{14}C would not be the same for the two subunits. However, the ratio of ^{3}H/^{14}C of the whole enzyme is the same as those of its subunits at three different time points after the introduction of
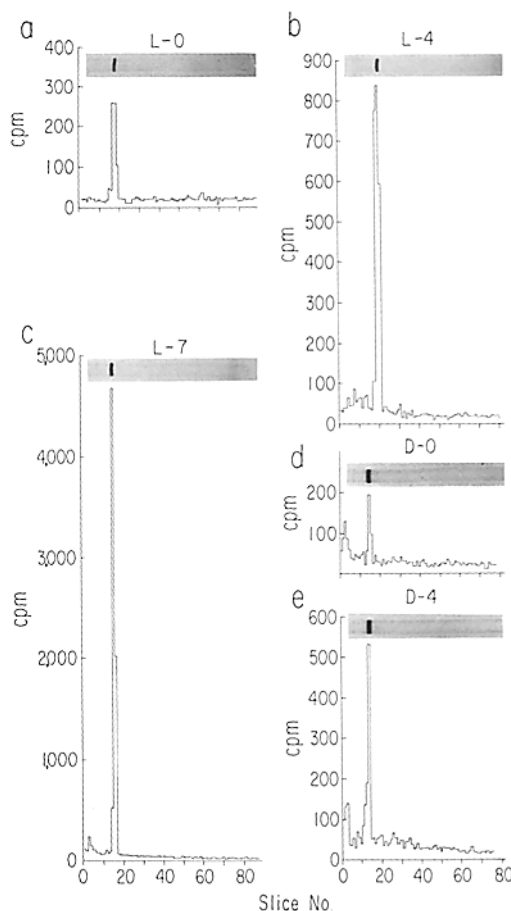
FIGURE 3 Polyacrylamide gel electrophoresis of ^{3}H-labeled Ru-1,5-P_2 carboxylase at different times of the cell cycle. ^{3}H-Labeled Ru-1,5-P_2 carboxylase at different time points was collected from the sucrose gradients shown in Fig. 2, with an additional time point at L-4. 100-μl aliquots of the enzyme preparation were applied to 5.5% polyacrylamide gel and electrophoresed and processed as given in Materials and Methods.
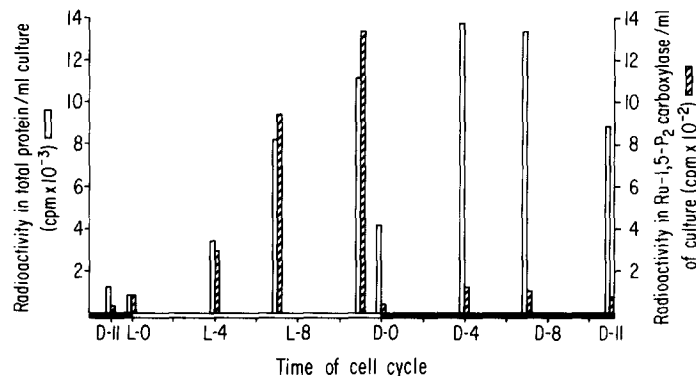
FIGURE 4 Variations in the rate of incorporation of [³H]arginine into total cell protein and into Ru-1,5-P₂ carboxylase of *C. reinhardtii* at different times of the cell cycle. Incorporation of [³H]arginine into total protein of *C. reinhardtii* during 30 min was obtained from counting equal aliquots of the homogenates by the filter paper method (see Materials and Methods) and then corrected for per milliliter of culture. Incorporation of [³H]arginine into Ru-1,5-P₂ carboxylase during 30 min was obtained by the summation of radioactivity found in the carboxylase band in the 5.5% polyacrylamide gels (as described in detail in Fig. 3) and then corrected for per milliliter of culture. The data were not corrected for efficiency of counting. Specific radioactivities (cpm × 10⁻⁴/mg protein) for the carboxylase were: 1.0 for an average of all the dark time points, and 3.8 for L-0 and 7.2 for L-7. The specific radioactivities for total cell protein were: 6.1 for the first D-11 time point, 9.4 for the second D-11 time point, with 17.8 for L-11.

[¹⁴C]arginine (Table II). This finding strongly suggests a synchronous synthesis of both subunits of Ru-1,5-P₂ carboxylase and indicates that the existence of unequal pools of subunits is improbable.

The Influence of Inhibitors of Protein Synthesis on Biosynthesis of Ru-1,5-P₂ Carboxylase

A large number of reports indicate a difference in the subcellular sites of biosynthesis of the large and small subunits of Ru-1,5-P₂ carboxylase (9, 16–22). Therefore, it was of interest to conduct experiments using compounds which inhibit specifically either chloroplast or cytoplasmic protein synthesis. In order to avoid possible nonspecific effects of inhibitors during long term incubation, synchronized cells at L-4, a time of vigorous protein synthesis (Fig. 1), were exposed to a variety of inhibitors for only a short period of time, 30 min. The results are summarized in Table III. Inhibitors of protein synthesis by 70S ribosomes (chloramphenicol, erythromycin, lincomycin, spectinomycin [35]) reduced by 90% the incorporation of [³H]arginine into the 18S Ru-1,5-P₂ carboxylase peak as compared to control (Table III), but not into the total soluble protein on top of

the sucrose gradient (results not shown). The inhibitors of protein synthesis by 80S ribosomes (cycloheximide and anisomycin [35]) drastically decreased incorporation both into the carboxylase and into total soluble protein (Table III). These results reaffirm those of previous workers (9, 21, 22) that the biosynthesis of Ru-1,5-P₂ carboxylase from *C. reinhardtii* is dependent on the activities of both 80S and 70S ribosomes.

Turnover of Ru-1,5-P₂ Carboxylase during the Cell Cycle

The previous experiments indicate that synthesis of Ru-1,5-P₂ carboxylase falls to a low level in the dark (Fig. 4) while enzymatic activity (Fig. 1 B) and protein concentration (Fig. 2) do not change significantly. These observations indicate a slow turnover of the enzyme in the dark. Accordingly, we investigated directly the turnover of Ru-1,5-P₂ carboxylase using a double-label technique. A synchronized population of cells was prelabeled with [¹⁴C]arginine for the entire light period. Cells were collected aseptically by centrifugation, washed, and then incubated with [³H]arginine in the dark. As was noted before, during the dark period the total protein and chlorophyll contents remain constant and therefore the cells are in a
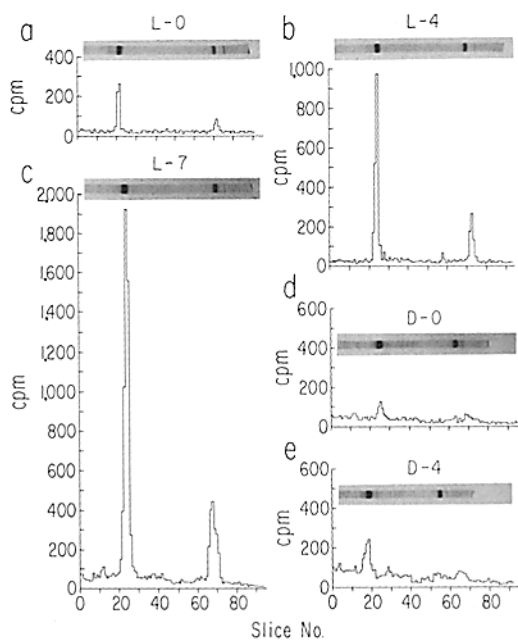
FIGURE 5 SDS-polyacrylamide gel electrophoresis of the subunits of ^{3}H-labeled Ru-1,5-P$_2$ carboxylase at different times of the cell cycle. Samples of Ru-1,5-P$_2$ carboxylase were obtained from sucrose gradient centrifugations as described in Fig. 2, with an additional time point at L-4. 100-μl aliquots of the enzyme were mixed with an equal volume of a solution containing 0.4 M Na$_2$CO$_3$, 4% SDS, and 20% β-mercaptoethanol. The resulting mixture was incubated in a boiling water bath for 2 min before being applied to an 11% SDS-polyacrylamide gel. Conditions for electrophoresis and subsequent processing of the gels for radioactivity measurements are described in Materials and Methods. The band at the left is the large subunit.

steady-state condition. The results are presented in Fig. 6 A for the whole enzyme and in 6 B for its subunits. Only about 10% of the initial ^{14}C label is lost during the first 11 h of the dark period, while an additional 15% is lost during the first 0.5 h of transition from dark to light. As a control, it was found that there was very little incorporation of ^{3}H-radioactivity during the dark period, mirroring the results in Figs. 2–5. These results verify that Ru-1,5-P$_2$ carboxylase is being turned over only slowly during the dark part of the cell cycle and both large and small subunits of the enzyme (Fig. 6 B) behave similarly to the total assembled enzyme (Fig. 6 A).

However, the results are complicated by the persistence of a ^{14}C-radioactive acid-soluble pool

TABLE I

Incorporation of [^{3}H]Arginine into Subunits of Ru-1,5-P$_2$ Carboxylase at Different Times of the Cell Cycle

Time of cell cycle	cpm in band		Ratio S/L
	L	S	
D-11	275	88	0.32
L-0	457	157	0.34
L-4	1,701	577	0.34
L-7	5,428	1,772	0.33
L-11	7,374	2,223	0.30
D-0	356	118	0.33
D-4	916	316	0.33

The incorporation experiments were performed as described in Materials and Methods and in legend to Fig. 2. Sucrose density gradients and gel electrophoresis were performed as in Figs. 2 and 5. Samples were excised from the subunit bands (see Fig. 5) and were counted as described in Materials and Methods.

L and S = large and small subunits, respectively.

TABLE II

Synchronized Synthesis of Large and Small Subunits of Ribulose-1,5-bisphosphate Carboxylase

Enzyme	^{14}C	^{3}H	^{3}H/^{14}C
W$_{L-4}$	25,165	6,351	0.24
L$_{L-4}$	18,570	4,801	0.26
S$_{L-4}$	5,560	1,447	0.26
W$_{L-7}$	58,880	8,585	0.15
L$_{L-7}$	46,780	7,113	0.15
S$_{L-7}$	13,660	1,816	0.13
W$_{L-11}$	66,995	8,093	0.12
L$_{L-11}$	56,330	6,908	0.12
S$_{L-11}$	16,210	2,040	0.13

Cells were incubated with [^{3}H]arginine (0.3 μCi/ml culture) at D-0 and kept in the dark for the entire 12-h dark period. They were then spun down and washed as given in Materials and Methods, resuspended in their original volume, and incubated at L-0 with [^{14}C]arginine (0.15 μCi/ml culture). Aliquots were removed at L-4, L-7, and L-11 and processed for the isolation of the enzyme and of its subunits, as described in Materials and Methods. The whole enzyme (W) was purified and counted from gel-electrophoretic bands while the subunits (L = large and S = small) were purified and counted from SDS-gel electrophoretic bands, as described in Materials and Methods and in legends to Figs. 4 and 5.

in the dark, after prelabeling in the light (Fig. 7 A). As shown in Fig. 7 A, there is a large increase in the ^{3}H-radioactivity of the acid-soluble pool during labeling in the dark, but despite the existence of the pool, little incorporation of the label from the ^{3}H-pool occurs in the dark (Fig. 6). If we assume a mixing of the ^{14}C- and ^{3}H acid-soluble pools, then very little incorporation into the enzyme from the ^{14}C pool should occur and hence little breakdown and reutilization of ^{14}C-radioactivity in the carboxylase would have taken place. However, it is possible that the [^{14}C]arginine derived from protein degradation does not mix with the exogenous [^{3}H]arginine.

Next, we attempted to examine whether any turnover of the carboxylase occurs in the light. This is more difficult to answer, for two reasons. First, since the radioactive label had to be presented to the cells in the synthetic light period, and then the cells had to be carried through the nonsynthetic dark period before they could be examined for turnover in the next light period, a radioactive acid-soluble pool was built up and persisted (Fig. 7); second, in this second light period the cells are not in a steady state, being in

the process of actively synthesizing enzyme (Fig. 2), incorporating radioactivity into it from the acid-soluble pool. The results so far are ambiguous and require further experimentation.

TABLE III

Effect of Inhibitors of Protein Synthesis on Incorporation of [^{3}H]Arginine into Ru-1,5-P$_2$ Carboxylase

Inhibitors	Concentration	cpm*	Percent of incorporation
None	—	890	100
Chloramphenicol	100 μg/ml	35	4
Erythromycin	50 μg/ml	140	16
Spectinomycin sulfate	50 μg/ml	155	17
Lincomycin hydrochloride	150 μg/ml	101	11
Cycloheximide	2 μg/ml	71	8
Anisomycin	0.5 mM	62	7

Cells at L-4 were incubated for 30 min in the light, as described in Materials and Methods.
* As summated from the Ru-1,5-P$_2$ carboxylase peak on sucrose density gradients, similar to those shown in Fig. 2.
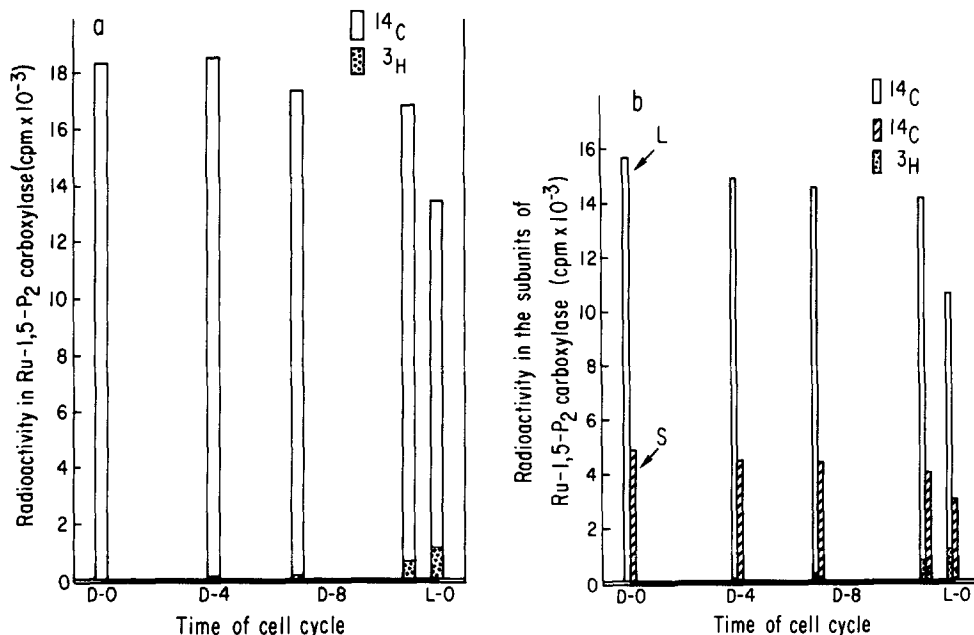

FIGURE 6 Turnover of Ru-1,5-P$_2$ carboxylase and of its subunits during the dark period of the cell cycle of *C. reinhardtii*. The labeling protocol is described under Materials and Methods. A, Changes in ^{14}C- and ^{3}H-radioactivity of the Ru-1,5-P$_2$ carboxylase gel bands, analyzed as given in Fig. 3. B, Changes in ^{14}C- and ^{3}H-radioactivity of the large (L) and small (S) subunits of Ru-1,5-P$_2$ carboxylase as revealed by 11% SDS-polyacrylamide gel, as analyzed in Fig. 5.
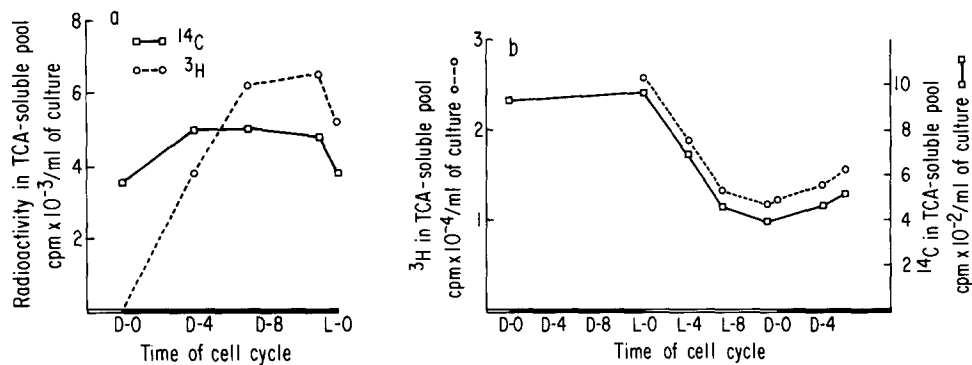
FIGURE 7   The appearance of radioactivity in the TCA-soluble pool during the cell cycle of *C. reinhardtii*. A, (O---O) represents the kinetics of the uptake of [³H]arginine into the TCA-soluble pool during continuous labeling of the cells in the dark. (□---□) represents the continuation of the ¹⁴C-radioactivity in the dark period after 12-h labeling in the light and removal of the label by washing of the cells at the beginning of the dark period. B, (O---O) represents the changes in TCA-soluble radioactivity in the light and dark after a 12-h labeling with [³H]arginine in the dark. (□---□) represents the changes in TCA-soluble radioactivity in the dark and light after a 12-h prelabeling with [¹⁴C]arginine in the light. In both cases, the cells were spun down and washed and incubation was continued during the subsequent 12-h dark, 12-h light, and 6-h dark periods. Determination of radioactivity in the acid-soluble fractions was done as described in Materials and Methods.

Estimation of Radioactive Pool in C. reinhardtii

Since fluctuations in the amino acid pool during the cell cycle are important in the studies of the protein biosynthesis and turnover, we investigated the fate of the labeled precursor in the TCA-soluble pool during the cell cycle. This pool was examined as part of the turnover experiment (Fig. 6). Cells were prelabeled with [¹⁴C]arginine during the entire light period, washed, and then [³H]arginine was introduced at the beginning of the following dark period. Samples were withdrawn at different time points during the dark period and examined for ¹⁴C- and ³H-radioactivity contents in the TCA-soluble fractions, as well as radioactivity in the carboxylase (Fig. 6). Fig. 7 A shows that the amount of ¹⁴C-radioactivity in the pool increased from D-0 to D-4, remained approximately constant in the next 7 h, and then declined at the time of transition from dark to light (L-0). Simultaneously, the uptake of [³H]arginine from the medium was rapid and reached a plateau after 6-7 h of continuous labeling. These results indicate that under the described experimental conditions, a large radioactive acid-soluble pool is maintained. Jones (32) noted an increase of total amino acids in the free pool during the dark as compared to the light. However, these higher concentrations of amino acids during the dark apparently do not

inhibit the uptake of the [³H]arginine from the medium, indicating that the amino acid pool of the algal cells is expandable.

We next studied the disappearance of the radioactive precursor from the TCA-soluble pool during the cell cycle. Cells were prelabeled with ¹⁴C- and [³H]arginine in the light and dark period, respectively, as described above and in Fig. 6. Samples were then taken in the second light-dark cycle and analyzed for radioactivity content in the free pool (Fig. 7 B). During the second light period, 50% of the radioactivity from the TCA-soluble fraction was utilized during the first 7 h of illumination and both the ³H label (which persisted for 12 h in the dark) and the ¹⁴C label (which persisted for 12 h in the light and 12 h in the dark) showed identical kinetics of disappearance. Upon transfer to the second dark period, the amount of radioactivity in the free pool began to increase, and at D-6, reached 60% of the radioactivity measured at L-0 (Fig. 7 B). This increase probably reflects a turnover of total cell protein in the dark.

Analyses of TCA-soluble material, obtained at D-4 and D-11 in the dark-turnover experiment (Fig. 6 and Fig. 7 A), and at L-7 and D-4 in the light-turnover experiment (see above and Fig. 7 B) were performed by thin-layer chromatography. They all gave the same results: high radioactivity in arginine, smaller amounts in citrulline, ornithine, and serine, glycine, and glutamic acid, with

arginine accounting for over 90% of this total in the dark-turnover experiment and ~50% in the light-turnover experiment. 50-80% of the radioactivity in the TCA-soluble fractions from these four time points can be accounted for by the above labeled amino acids, with the remainder being present in ninhydrin-negative compounds. The results indicate that under the described conditions of cell maintenance (see Materials and Methods) and labeling, arginine is found in high levels in the free pool. A similar observation was made by Strijkert et al. (36), who studied arginine metabolism by *C. reinhardtii*.

DISCUSSION

The chloroplast enzyme, Ru-1,5-P_2 carboxylase, like mitochondrial cytochrome oxidase and ATPase (37), is another example of an enzyme made up of subunits which are synthesized in different compartments of the cell. The large subunit is made by the 70S ribosomes in the chloroplast and the small subunit by the 80S ribosomes in the cytoplasm. The evidence for this is quite well documented: differential rates of in vivo synthesis of the subunits in tobacco leaves (17); occurrence in wheat (18) and bean (20) of nascent large subunits on 70S ribosomes and of nascent small subunits on 80S ribosomes as monitored by antibodies to each subunit; the identification of isolated chloroplasts as the in vitro site of synthesis of the large subunit (19); and the use of inhibitors of protein synthesis specific to either 70S or 80S ribosomes (38). We have used a variety of inhibitors, acting at different loci of the protein-synthesizing apparatus, and specific for either 70S or 80S ribosomes, taking care to incubate for short periods of time in order to eliminate possible side effects, and have shown that the biosynthesis of Ru-1,5-P_2 carboxylase from *C. reinhardtii* is dependent on both 70S and 80S ribosomes (Table I).

The biosynthesis of the enzyme and of its subunits during the cell cycle occurs mainly in the light (Figs. 1 B, 2-5). However, the effect of light is not direct, since both the wild-type cells (39) and Y-1 mutant (40, 41) can synthesize this enzyme in the dark when grown in a medium supplemented with acetate. The stepwise accumulation of Ru-1,5-P_2 carboxylase during the light period of the cell cycle has also been reported in *Chlorella* (42). However, this pattern of biosynthesis is not restricted to chloroplast-located enzymes since in synchronous *C. reinhardtii* cultures the activities of three cytoplasmic enzymes, alanine dehydrogenase

(33), ornithine transcarbamylase (33), and acid phosphatase (43), also increase in the light and remain constant in the dark. We have shown that in the case of Ru-1,5-P_2 carboxylase in *C. reinhardtii*, the stepwise effect is the result of *de novo* synthesis in the light and of little turnover in the dark (Fig. 6 A, B). Thus, the enzyme is seemingly apportioned to the daughter cells which arise in the dark.

The finding of a low turnover of the carboxylase in the dark is atypical since there seems to be active turnover of total cell proteins during this period, as indicated by high levels of pulse incorporation but no change in the net protein (Figs. 1 B, 4). A similar high rate of turnover of total cell protein in the dark has also been noted by Jones et al. (32). In differentiating barley seedlings, Peterson et al. (44) reported a slow degradation of the carboxylase in the dark with synthesis occurring in the light. On the other hand, Sitz et al. (45) found that the carboxylase of *Chlorella* is degraded fairly rapidly in the light in the presence of cycloheximide, with a half-life of approximately 4.4 h. However, this faster rate of degradation of carboxylase could be attributed to possibly damaging effects due to the prolonged presence of the inhibitor during the 8-h incubation.

As discussed above, there are three novel aspects of the biosynthesis of Ru-1,5-P_2 carboxylase which bear on its control: nonidentical subunits synthesized in the different compartments of the cell, restriction of their biosynthesis to the light part of the cell cycle, and a slow turnover of the enzyme and of its subunits in the dark. There are six pieces of evidence which suggest a stringent, co-ordinated control of the biosynthesis of the subunits: (*a*) both subunits are synthesized mostly in the light (Fig. 5); (*b*) both subunits have a low degree of turnover in the dark (Fig. 6 B); (*c*) inhibitors of protein synthesis by both 70S and 80S ribosomes abolish the in vivo incorporation into the whole enzyme (Table III), as has been noted before (9, 21, 46); (*d*) during the cell cycle the ratio of incorporation of [³H]arginine into the small and the large subunits remains constant (Table I); (*e*) no evidence could be found of the existence of a pool of nascent small subunits, possibly synthesized in the dark and awaiting the synthesis of large subunits in the light for final assembly into the protein (Table II); (*f*) enzyme activity (Fig. 1 B), enzyme amount (Fig. 2), and incorporation of precursor into whole enzyme (Figs. 3, 4) do not commence immediately after switch from light to dark, indicating the

absence of a pre-existing pool of nascent subunits awaiting assembly in the light. Points (e) and (f) strongly indicate that the syntheses of the subunits are always in step with one another. How the synthesis of the two subunits is controlled is at present unknown, though there have been some ideas brought forth (18). The control could conceivably be effected through the regulation of the synthesis of the large subunit during the dark part of the cell cycle since the incorporation of radioactive precursor into the total cell proteins in the dark is not altered by spectinomycin, a specific inhibitor of protein synthesis by 70S ribosomes (S. L. Schor, P. Siekevitz, and G. E. Palade, unpublished data).

The work presented here is mainly concerned with regulation of biosynthesis of Ru-1,5-P_2 carboxylase on the level of the synthesis of its subunit polypeptides. However, there is genetic evidence that the genes for the small subunit (47) and for the large subunit (48) are located in the nuclear and chloroplast DNAs, respectively. Therefore the duplication of the genes coding for the small and large subunits may be separated temporally since the nuclear DNA is duplicated in the dark and the chloroplast DNA is duplicated in the light (2, 49). Thus, a further regulation of the biosynthesis of Ru-1,5-P_2 carboxylase can be imposed at the transcriptional level.

REFERENCES

1. BERNSTEIN, E. 1964. Physiology of an obligate photoautotroph (*Chlamydomonas moewusii*). 1. Characteristics of synchronously and randomly reproducing cells and an hypothesis to explain their population curves. *J. Protozool.* **11**:56–74.
2. CHIANG, K. S., and N. SUEOKA. 1967. Replication of chloroplast DNA in *Chlamydomonas reinhardi* during vegetative cell cycle: its mode and regulation. *Proc. Natl. Acad. Sci. U. S. A.* **57**:1506–1513.
3. CATTOLICO, R. A., J. W. SENNER, and R. F. JONES. 1973. Changes in cytoplasmic and chloroplast ribosomal ribonucleic acid during the cell cycle of *Chlamydomonas reinhardtii*. *Arch. Biochem. Biophys.* **156**:58–65.
4. DORNER, R. W., A. KAHN, and S. G. WILDMAN. 1957. The proteins of green leaves. VII. Synthesis

and decay of the cytoplasmic proteins during the life of the tobacco leaf. *J. Biol. Chem.* **229**:945–952.
5. RACUSEN, D., and M. FOOTE. 1965. Protein synthesis in dark-grown bean leaves. *Can. J. Bot.* **43**:817–824.
6. KIERAS, F. J., and R. HASELKORN. 1968. Properties of ribulose-1,5-diphosphate carboxylase (carboxydismutase) from Chinese cabbage and photosynthetic microorganisms. *Plant Physiol.* **43**:1264–1270.
7. GOLDTHWAITE, J. J., and L. BOGORAD. 1971. A one-step method for the isolation and determination of leaf ribulose-1,5-diphosphate carboxylase. *Anal. Biochem.* **41**:57–66.
8. SUGIYAMA, T., T. ITO, and T. AKAZAWA. 1971. Subunit structure of ribulose 1,5-diphosphate carboxylase from *Chlorella ellipsoidea*. *Biochemistry*. **10**:3406–3411.
9. GIVAN, A. L., and CRIDDLE, R. S. 1972. Ribulosediphosphate carboxylase from *Chlamydomonas reinhardi*: purification, properties and its mode of synthesis in the cell. *Arch. Biochem. Biophys.* **149**:153–163.
10. BOYTON, J. E., N. W. GILLHAM, and J. F. CHABOT. 1972. Chloroplast ribosome deficient mutants in the green alga *Chlamydomonas reinhardi* and the question of chloroplast ribosome function. *J. Cell Sci.* **10**:267–305.
11. IWANIJ, V., N.-H. CHUA, and P. SIEKEVITZ. 1974. The purification and some properties of ribulosebisphosphate carboxylase and of its subunits from the green alga *Chlamydomonas reinhardtii*. *Biochim. Biophys. Acta*. **358**:329–340.
12. KAWASHIMA, N., and S. G. WILDMAN. 1970. Fraction I protein. *Annu. Rev. Plant Physiol.* **21**:325–358.
13. RUTNER, A. G., and M. D. LANE. 1967. Nonidentical subunits of ribulose diphosphate carboxylase. *Biochem. Biophys. Res. Commun.* **28**:531–537.
14. KAWASHIMA, N., and S. G. WILDMAN. 1971. Studies of fraction I protein. II. Comparison of physical, chemical, immunological and enzymatic properties between spinach and tobacco fraction I proteins. *Biochim. Biophys. Acta*. **229**:749–760.
15. MOON, K. E., and E. O. P. THOMPSON. 1971. Separation and characteristics of subunits of ribulosediphosphate carboxylase. *Aust. J. Biol. Sci.* **24**:755–764.
16. CRIDDLE, R. S., B. DAU, G. E. KLEINKOPF, R. C. HUFFAKER. 1970. Differential synthesis of ribulosediphosphate carboxylase subunits. *Biochem. Biophys. Res. Commun.* **41**:621–627.
17. KAWASHIMA, N. 1970. Non-synchronous incorporation of $C^{14}O_2$ into amino acids of the two subunits of fraction I protein. *Biochem. Biophys. Res. Commun.* **38**:119–124.
18. GOODING, L. R., H. ROY, and A. T. JAGENDORF. 1973. Immunological identification of nascent subunits of wheat ribulose diphosphate carboxylase on

ribosomes of both chloroplast and cytoplasmic origin. *Arch. Biochem. Biophys.* **159:**324–335.

19. BLAIR, G. E., and R. J. ELLIS. 1973. Protein synthesis in chloroplasts. 1. Light driven synthesis of the large subunit of fraction I protein by isolated pea chloroplasts. *Biochim. Biophys. Acta.* **319:**223–234.

20. GRAY, J. C., and R. G. O. KEKWICK. 1974. The synthesis of the small subunit of ribulose 1,5-bisphosphate carboxylase in the French bean *Phaseolus vulgaris. Eur. J. Biochem.* **44:**491–500.

21. ARMSTRONG, J. J., S. J. SURZYCKI, B. MOLL, and R. P. LEVINE. 1971. Genetic transcription and translation specifying chloroplast components in *Chlamydomonas reinhardi. Biochemistry.* **10:**692–701.

22. HONEYCUTT, R. C., and M. M. MARGULIES. 1973. Protein synthesis in *Chlamydomonas reinhardi.* Evidence for synthesis of proteins of chloroplastic ribosomes on cytoplasmic ribosomes. *J. Biol. Chem.* **248:**6145–6153.

23. SAGER, R., and S. GRANICK. 1953. Nutritional studies with *Chlamydomonas reinhardi. Ann. N.Y. Acad. Sci.* **56:**831–838.

24. OHAD, I., P. SIEKEVITZ, and G. E. PALADE. 1967. Biogenesis of chloroplast membranes. I. Plastid dedifferentiation in dark-grown algal mutant (*Chlamydomonas reinhardi*). *J. Cell Biol.* **35:**521–552.

25. DAVIS, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121:**404–427.

26. LAEMMLI, N. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* **227:**680–685.

27. MANS, R. J., and G. D. NOVELLI. 1961. Measurement of the incorporation of radioactive amino acids into protein by filter paper disc method. *Arch. Biochem. Biophys.* **94:**48–53.

28. WARD, S., D. I. WILSON, and J. J. GILLIAM. 1970. Methods for fractionation and scintillation counting of radioisotope-labelled polyacrylamide gels. *Anal. Biochem.* **38:**90–97.

29. JONES, K., and J. G. HEATHCOTE. 1966. The rapid resolution of naturally occurring amino acids by thin-layer chromatography. *J. Chromatogr.* **24:**106–111.

30. LOWRY, O. H., J. H. ROSEBROUGH, A. C. FARR, and J. RANDALL. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193:**265–275.

31. ARNON, D. I. 1949. Copper enzymes in isolated chloroplast. Polyphenoloxidase in *Beta vulgaris. Plant. Physiol.* **24:**1–15.

32. JONES, R. F. 1970. Physiological and biochemical aspects of growth and gametogenesis in *Chlamodomonas reinhardtii. Ann. N.Y. Acad. Sci.* **175:**648–659.

33. KATES, J. R., and R. F. JONES. 1967. Periodic increases in enzyme activity in synchronized cultures

of *Chlamydomonas reinhardtii. Biochim. Biophys. Acta.* **145:**153–158.

34. SCHOR, S. L. 1971. Regular variations in chloroplast functional activity during the division cycle of *Chlamydomonas reinhardtii.* Ph.D. Thesis. The Rockefeller University, New York.

35. PESTKA, S. 1971. Inhibitors of ribosome functions. *Ann. Rev. Microbiol.* **25:**487–562.

36. STRIJKERT, P. Y., R. LOPPES, and J. S. SUSSENBACH. 1971. Arginine metabolism in *Chlamydomonas reinhardi.* Regulation of uptake and breakdown. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **14:**329–332.

37. SCHATZ, G., and T. L. MASON. 1974. The biosynthesis of mitochondrial proteins. *Annu. Rev. Biochem.* **43:**51–87.

38. BOULTER, D., R. J. ELLIS, and A. YARWOOD. 1972. Biochemistry of protein synthesis in plants. *Biol. Rev.* **47:**113–175.

39. TOGASAKI, R. K., and R. P. LEVINE. 1970. Chloroplast structure and function in ac-20, a mutant of *Chlamydomonas reinhardi.* I. CO_2 fixation and ribulose-1,5-diphosphate carboxylase synthesis. *J. Cell Biol.* **44:**531–539.

40. HUDOCK, G. A., and R. P. LEVINE. 1964. Regulation of photosynthesis in *Chlamydomonas reinhardi. Plant Physiol.* **39:**889–897.

41. OHAD, I., P. SIEKEVITZ, and G. E. PALADE. 1967. Biogenesis of chloroplast membranes. II. Plastid differentiation during greening of a dark-grown algal mutant (*Chlamydomonas reinhardi*). *J. Cell Biol.* **35:**553–584.

42. MOLLOY, G. R., and R. R. SCHMIDT. 1970. Studies on the regulation of ribulose-1,5-diphosphate carboxylase synthesis during the cell cycle of the eucaryote *Chlorella. Biochem. Biophys. Res. Commun.* **40:**1125–1133.

43. LIEN, T., and G. KNUTSEN. 1972. Synchronous cultures of *Chlamydomonas reinhardtii.* Synthesis of repressed and derepressed phosphatase during the life cycle. *Biochim. Biophys. Acta.* **287:**154–163.

44. PETERSEN, L. W., G. E. KLEINKOPF, and R. C. HUFFAKER. 1973. Evidence for lack of turnover of ribulose-1,5-diphosphate carboxylase in barley leaves. *Plant Physiol.* **51:**1042–1045.

45. SITZ, T. O., G. R. MOLLOY, and R. R. SCHMIDT. 1973. Evidence for turnover of ribulose-1,5-diphosphate carboxylase and continuous transcription of its structural gene throughout the cell cycle of the eucariote *Chlorella. Biochim. Biophys. Acta.* **319:**103–108.

46. HOOBER, J. K. 1972. A major polypeptide of chloroplast membranes of *Chlamydomonas reinhardi.* Evidence for synthesis in the cytoplasm of a soluble component. *J. Cell Biol.* **52:**84–96.

47. KAWASHIMA, N., and S. G. WILDMAN. 1972. Studies on fraction I protein. IV. Mode of inheritance of primary structure in relation to whether chloroplast or nuclear DNA contains the code for a chloroplast

protein. *Biochim. Biophys. Acta.* **262**:42–49.

48. CHAN, P. M., and S. G. WILDMAN. 1972. Chloroplast DNA codes for the primary structure of the large subunit of fraction I protein. *Biochim. Biophys. Acta.* **277**:677–680.

49. KATES, J. R., K. S. CHIANG, and R. F. JONES. 1968. Studies on DNA replication during synchronized vegetative growth and genetic differentiation in *Chlamydomonas reinhardtii. Exp. Cell Res.* **49**:121–135.