INCORPORATION OF POLYPEPTIDES INTO THYLAKOID MEMBRANES OF *CHLAMYDOMONAS REINHARDTII*

Cyclic Variations

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ABSTRACT

A purified fraction of unstacked thylakoid membranes (TMF_{1u}) has been obtained from homogenates of *Chlamydomonas reinhardtii* (wild type 137+) by using repeated centrifugations in sucrose density gradients and low salt treatment. The contaminants of the fraction are reduced to a few mitochondria ($\sim$3% of the total mitochondrial population), a few osmiophilic granules, and fragments of chloroplast envelopes. By SDS-polyacrylamide gel electrophoresis the polypeptide components of TMF_{1u} were resolved into at least 30 bands. To determine the relative rates of assembly of newly synthesized polypeptides into thylakoid membranes, synchronized algal cells were doubly labeled in vivo with L-[^{14}C] and L-[^{3}H]arginine—used for long- and short-term labeling, respectively. TMF_{1u}'s were isolated from the labeled cells at selected time points during the cycle and the distribution of radioactivity was assayed in the gel electrophoretograms of their solubilized polypeptides. Incorporation of newly synthesized polypeptides into the bands of the gels was found to occur continuously but differentially throughout the cycle. Maximal rates of incorporation for the majority of the polypeptides were detected shortly after cell division (6D–7D; equivalent to early G_1 phase). The rates of radioactive labeling decreased gradually to a low level at the end of the dark period and then rose slightly at the beginning of the next light period. The findings suggest that, in addition to the light/dark control postulated in the past, assembly of newly synthesized proteins into thylakoid membranes is activated by signals at work in the early G_1 phase.

The production of thylakoid membranes has been investigated extensively in the green alga *Chlamydomonas reinhardtii* by the use of either the yellow mutant y-1 (1–5) or the wild type (WT) strain (6–7). Most of the work has been carried out on the y-1 mutant during the greening process it undergoes upon exposure to light. In this case, chlorophyll is synthesized and thylakoid membranes are apparently assembled only while the cells are exposed to light; this condition makes the system attractive since it provides a simple means for regulating membrane production. The problem has been studied less extensively in the wild type strain, although in this case the results would apply

directly to the normal process. Cyclic changes in the activity of photosystem I (PSI)[1] and PSII and some of their component enzymes, as well as in the amounts of cytochromes 553, 559 and 563, have been recorded in synchronized cultures of the alga (6, 8). The components of PSI and PSII and the cytochromes can be considered either intrinsic or peripheral proteins of the thylakoid membranes (9). Cyclic variations were also found in the attachment of polysomes to thylakoid membranes, presumably in relation to the synthesis of certain membrane proteins (10, 11). More recently, the kinetics of assembly of a few proteins into thylakoid membranes have been investigated over part of the life cycle of *Chlamydomonas* (7). Since a comprehensive study covering all resolvable polypeptides of the thylakoid membranes over the entire cell cycle was still missing, we undertook the work reported in this paper.

METHODS AND RESULTS

Growth and Synchronization Conditions

C. reinhardtii (WT 137[+]; originally obtained from the collection of Indiana University) was used in all experiments. The cells were grown in 200-ml cultures at 25°C in the minimal medium prescribed by Sager and Granick (12) with continuous aeration and stirring as in (1). Cell synchronization was achieved by exposing the cultures to a program of alternating periods of 12 h light and 12 h dark for at least two complete cycles. During mitosis, each parental cell divided into either four or eight daughter cells; hence the cell number increased by six on the average for each cell cycle.

C. reinhardtii WT 137[+] cultures are known to be readily synchronized by a cyclic light (L)/dark (D) program (13), but the duration of the cycle and the degree of synchronization depend on culture conditions (e.g. light intensity, CO_2 concentration, and cell density). Therefore, it was necessary to establish the degree of synchronization and the kinetics of the cell cycle under our experimental conditions. Fig. 1 shows cells sampled at 10 differ-

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[1] *Abbreviations used in this paper*: D, dark; L light; PSI, photosystem I; PSII, photosystem II; SDS, sodium dodecyl sulfate; TKME buffer, 25 mM Tris-HCl (pH 7.6), 25 mM, KCl, 1 mM, $MgCl_2$, 3 mM EDTA; TMF, thylakoid membrane fraction; TMF_1, first (heavy) TMF; TMF_{1s}, firs TMF fraction, stacked; TMF_{1u}, first TMF fraction, unstacked; TMF_2, second (light) TMF; tricine, N-Tris(hydroxymethyl)methylglycine; WT, wild type.

ent time points during the third light/dark cycle of a representative culture. Judging by the sequence of cell appearances recorded, the relatively homogeneous size of the cells at each time point, and the occurrence of well-defined mitoses clearly restricted between 2.5D and 4.5D, it appears that the cells go through a full, satisfactorily synchronized cycle in 24 h. As shown in Fig. 2, ~85% of the DNA produced during a cycle is synthesized in ~2 h beginning shortly before the end of the light period (~11L). We assume that the DNA synthesized at this time is nuclear (S phase). The balance, i.e. ~15% of total DNA, is synthesized during the first 3 h in the light. This DNA fraction has been identified by Chiang and Sueoka (14) as chlorplast DNA. Since mitoses (M phase) have been observed between 2.5D and 4.5D, the G_1 phase of the cell cycle actually extends from ~5D up to the end of the next light period (~11L). The G_2 phase is short, apparently 1 h, but its exact duration cannot be determined because the degree of cell synchronization is less than 100%. Chlorophyll accumulation occurs in the light period and follows an S-shaped curve with an initial lag period of 2 h and a terminal leveling off at ~10L.

Isolation of Thylakoid Membranes

Procedures for the isolation of thylakoid membranes have been developed in the past primarily for the study of the greening process of the yellow mutant of *C. reinhardtii* (2, 4). These procedures were adapted as follows for the purpose of our work.

Synchronously grown *C. reinhardtii* cultures (WT 137[+]) were harvested (by cenrifugation at ~2,000 g_{av} for 5 min) after at least two full cycles, when the density generally reached 1.5–2.0 × 10^{6} cells/ml. The harvested cells were washed three times with cold TKME buffer, resuspended in cold 0.22 M sucrose in the same buffer, and disrupted by using a precooled French pressure cell operated at 5,000 lb/in_2. The resulting pressurate was layered on top of a discontinuous sucrose gradient consisting of three layers: 2.0 M, 1.5 M, and 1.0 M sucrose, respectively. Centrifugation of the gradient for 1 h at 100,000 g_{av} in an SW 27 rotor (Beckman centrifuge L3-50, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 4°C gave a dark green band (TMF_1) at the 1.0 M–1.5 M sucrose interface, and a light green band (TMF_2) at the 1.5 M–2.0 M sucrose interface. Each band was collected for further purification
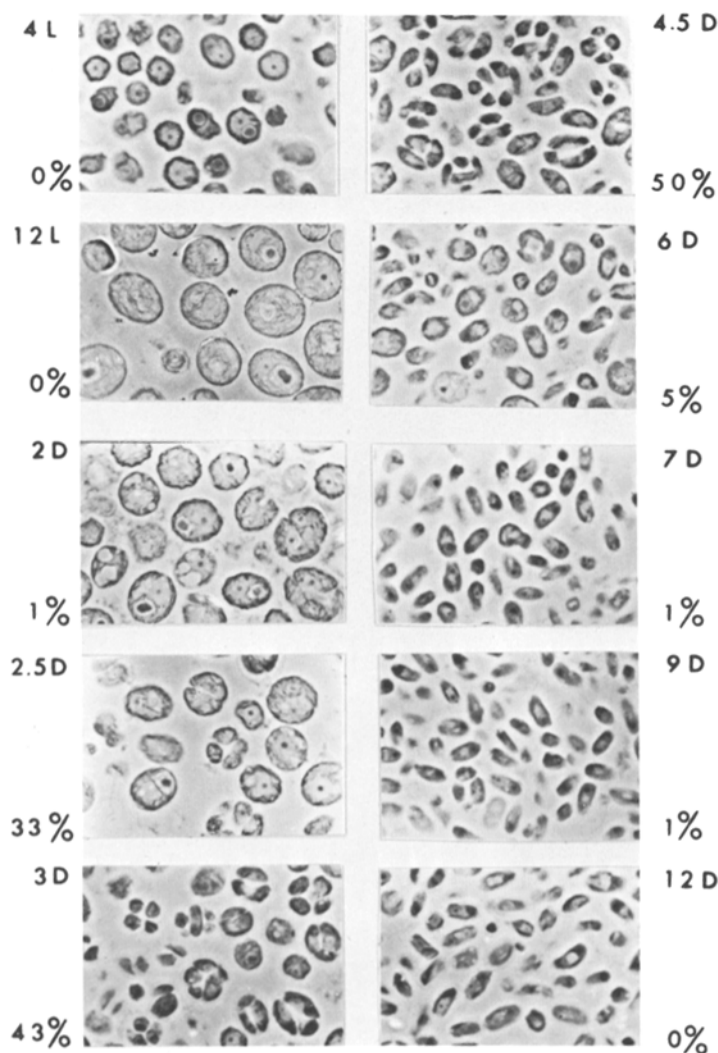
FIGURE 1   Morphology of *C. reinhardtii* (WT 137⁺) throughout the cell cycle. Culture samples, taken at various time points (indicated in the upper corners) throughout the cell cycle, were fixed for 1 h at 0°C with 0.25% glutaraldehyde in 10 mM $CaCl_2$ and 20 mM cacodylate buffer, pH 7.4, (final concentrations). The cells were subsequently pelleted, postfixed with 1% O_sO_4 in the same buffer, dehydrated, and embedded in Epon. Sections, 1–2 μm thick, were prepared, examined, and photographed under a phase-contrast microscope. In such sections, mitoses were recognized as clusters of two to eight (depending on the position of the plane of sectioning) daughter cells still kept together by the mother cell wall. The figures in the lower corners represent the percent of dividing cells in the whole cell population. (Divisions were easier to recognize in sectioned specimens than in fixed, whole-cell suspensions.) For each time point, 100–150 cells were counted at random throughout the sections which included in all cases the total thickness of the pellets. × 375.

and/or electron microscope examination. As indicated in Table I, ~90% of the loaded chlorophyll is recovered in TMF_1, while TMF_2 contains only ~4% of the pigment. Electron microscope examination shows that TMF_1 consists of chloroplast fragments containing thylakoids, stigmata, pyrenoids, chloroplast envelope fragments and starch grains, in addition to nonchloroplast contaminants, primarily mitochondria. TMF_2 contains the same components, but is very heavily contami-
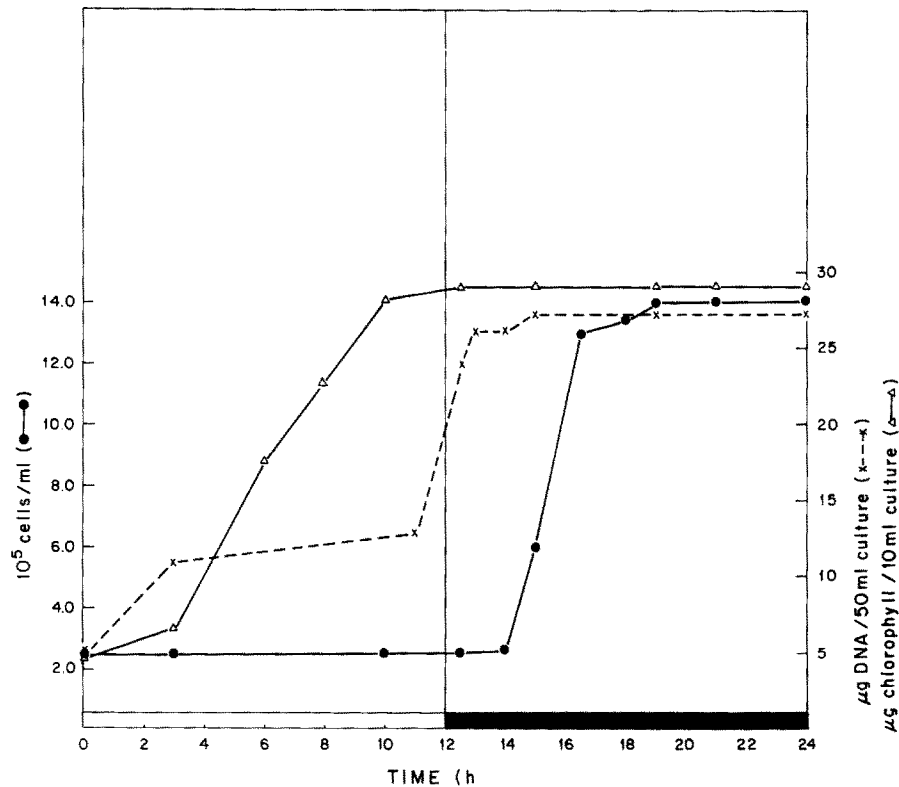
FIGURE 2   Variations in cell number, DNA and chlorophyll in synchronous cultures of *C. reinhardtii* (WT 137⁺). Culture samples were fixed with 0.25% glutaraldehyde (final concentration) and counted in a hemocytometer. The assays used were: Burton (22) for DNA and Arnon (23) for chlorophyll. The data are representative for three to eight experiments. (●——●), cells; (×----×), DNA; (△——△), chlorophyll.

TABLE I

Distribution of Chlorophyll in C. reinhardtii Cell Fractions

Fractions	Percent of total chlorophyll	
Homogenate	100	
0.22 M–1.0 M sucrose interface	4.0	
1.0 M–1.5 M sucrose interface (TMF₁)	90.7	100
1.5 M–2.0 M sucrose interface (TMF₂)	4.0	—
Pellet	1.2	—
TMF₁ₛ	81.1	90.8
TMF₁ᵤ	80.0	89.8

nated by broken cells and fragmented cell walls.

TMF₁ was selected for further purification on account of its morphology and its high chlorophyll content. The fraction was purified by flotation into a continuous sucrose gradient as follows: after adjusting the sucrose concentration to 1.5 M, the suspension was overlaid with a 1.5 M–1.0 M continuous sucrose gradient and centrifuged at

100,000 g_{av} in an SW 27 rotor for 18 h at 4°C. A green band formed in the 1.40 M sucrose region of the gradient (specific gravity (ρ) $\simeq$ 1.179 g/cm³). Elecron microscopy of the pelleted band showed that it consisted primarily of thylakoids swollen, but still largely fused into grana (Figs. 3 and 5). Other chloroplast components (starch grains, pyrenoids, envelope fragments, and stigmata) were lost or greatly reduced in number. This fraction can be described as stacked thylakoid membranes (TMF₁ₛ).

To minimize the possible trapping of stromal contaminants among stacked membranes, "unstacked" thylakoid membranes were prepared from TMF₁ₛ by a modification of the method of Goodenough and Staehelin (15). Unstacked thylakoids were obtained by dialyzing the TMF₁ₛ against 100 vol of 0.05 M Tricine-NaOH buffer (pH 7.3) for at least 10 h at room temperature. Subsequently the dialysate was sedimented through a 1.0 M –1.5 M continuous sucrose
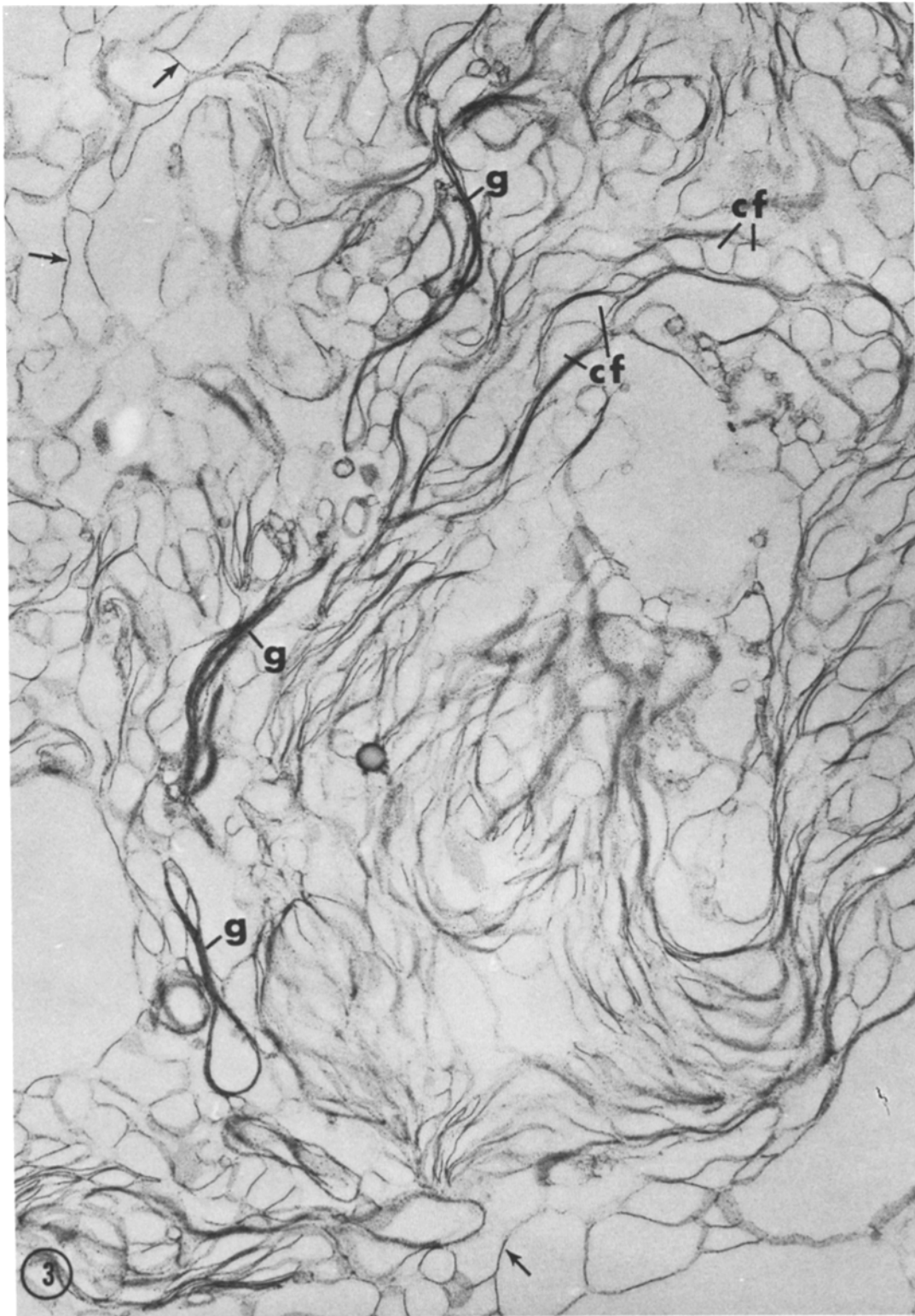
FIGURE 3 Representative field in a TMF$_{1s}$ pellet. The fraction consists of chloroplast fragments with swollen but still stacked thylakoids (cf) and swollen partially dispersed thylakoids; in the latter case areas of membrane fusion persist (arrows). Residual grana in the chloroplast fragments are marked g. × 16,000.
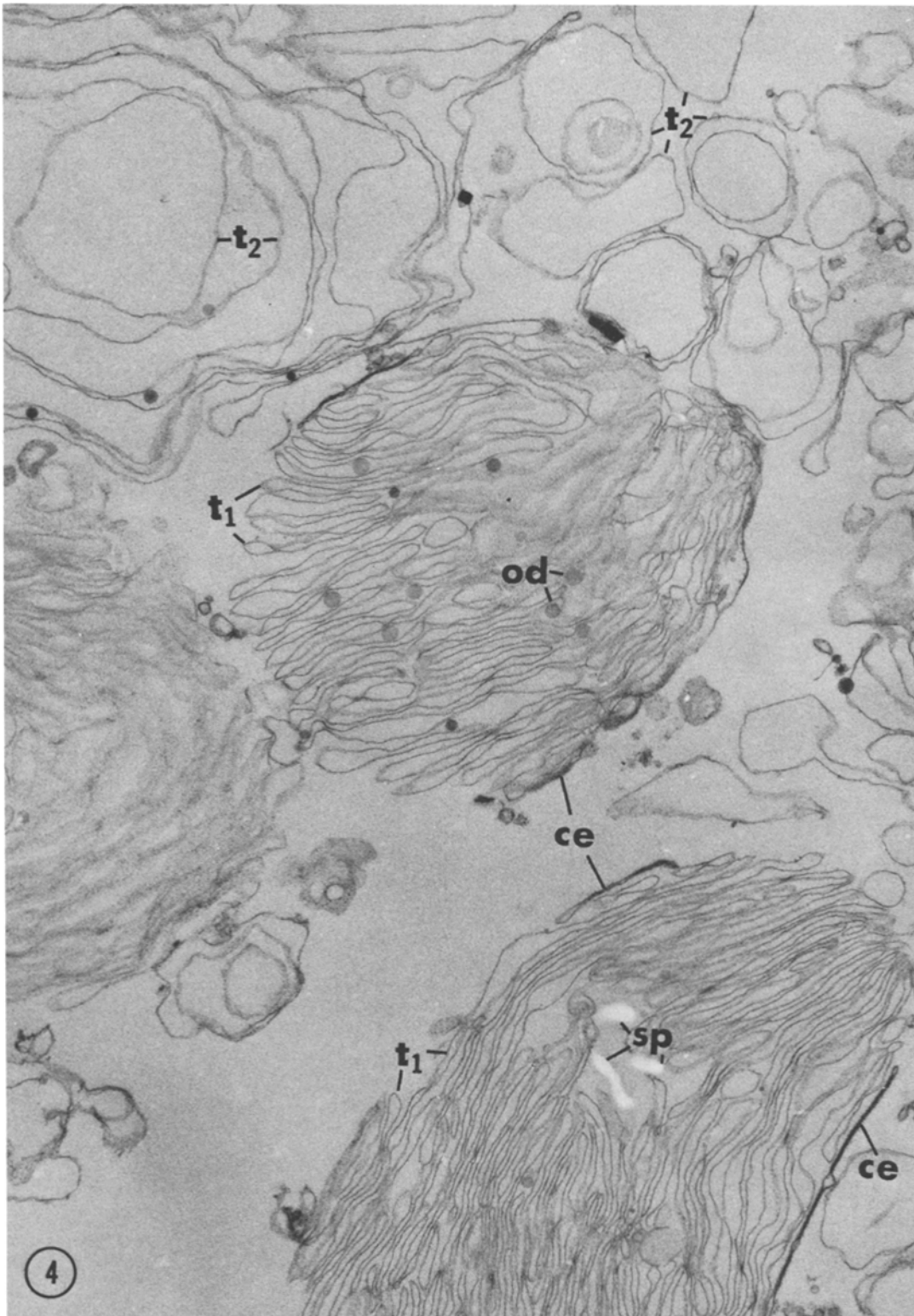
FIGURE 4   Representative field in the lower half of a TMF$_{1u}$ pellet. The fraction consists of recognizable chloroplast fragments whose thylakoids (t_1) are moderately swollen and fully unstacked. A number of isolated swollen thylakoids (t_2) surround the fragments. Chloroplast envelope fragments (ce) and osmiophilic droplets (od) can be recognized as chloroplastic contaminants of the fraction. Starch plates are seen (in negative images) at sp. × 18,000.

gradient in the same Tricine-NaOH buffer for 18 h at 100,000 g_{av} in an SW 27 rotor. The green band collected in the $\sim$ 1.40 M (ρ $\simeq$1.179 g/cm³) sucrose region consisted of a mixture of isolated thylakoids and piles of unstacked thylakoids; few envelope fragments were still present (Figs. 4 and 6). This preparation will be referred to as the unstacked thylakoid membrane fraction or TMF_{1u}. At equilibrium, the exact position of TMF_{1s} and TMF_{1u} in the gradient varied with the phase in the cell cycle reflecting cyclic variations in the protein to chlorophyll ratio of the membranes. At any time, however, the band could be easily located on account of its color. TMF_{1u} prepared as indicated accounted for a very large fraction (80%) of the chlorophyll of the homogenate (Table I); preliminary assays indicated that it retains PSI and PSII activities.

Contamination of TMF_{1u} by morphologically recognizable nonchloroplast components was reduced to a few mitochondria which accounted for less than 5% of the volume of the fraction. Assays for cytochrome c oxidase (Table II) indicated that most of the activity was satisfactorily recovered in the various cell fractions with only $\sim$6% and $\sim$3% of the original activity remaining in the TMF_{1s} and TMF_{1u}, respectively.

SDS-Polyacrylamide Gel Electrophoresis of Thylakoid Membrane Proteins

We have used sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and Neville's discontinuous buffer system (16) to analyze the polypeptide composition of thylakoid membranes in *C. reinhardtii*. A 0.1 × 30 × 20 cm slab gel consisting of separating and stacking gels was cast between two glass plates (35 × 24 cm). The separating gel was composed of a 3–20% continuous gradient of acrylamide—N,N'methylenebisacrylamide (wt/wt 30:0.8); the other components (maintained constant) were 0.4 M Tris-HCl (pH 9.18), 0.1% SDS, 0.03% (vol/vol) N,N,N', N'-tetramethylethylenediamine and 0.025% ammonium persulfate. The stacking gel consisted of 4.5% acrylamide—N,N-methylenebisacrylamide (wt/wt 30:0.8), 0.05 M Tris-H_2SO_4 (pH 6.1), 0.1% SDS, 0.1% N,N,N',N'-tetramethylethylenediamine and 0.1% ammonium persulfate. The buffers were 0.04 M Tris-boric acid, (pH 8.64) with 0.1% SDS, and 0.4 M Tris-HCl, (pH 9.18), for the upper and lower reservoirs, respectively.

Membrane samples were treated with 0.4 M dithiothreitol and then solubilized by adding SDS to a total volume of $\sim$0.1 ml for 4 mg protein (SDS to protein weight ratio was 8:1). The mixture was heated at 100°C for 1 min. 5- to 10-μl aliquots of solubilized membranes containing $\sim$200–400 μg protein were then layered on top of the gel. Under these conditions, the membranes were completely solubilized, and no aggregated polypeptides were left on top of either the stacking or separating gels. After acetone extraction, the solubilization of the membranes by the procedure described above was incomplete; some proteins were partially extracted, and some retention of proteins occurred above the stacking and separating gels.

Electrophoresis was carried out at a constant current of 30 mA for 12 h at room temperature. The gels were stained with 0.2% Coomassie brilliant blue in 30% methanol, 7% acetic acid for at least 6 h and then destained in 30% methanol, 7% acetic acid overnight.

The electrophoretic patterns were recorded by scanning the gels at 580 nm in a Beckman spectrophotometer (model Acta III) equipped with a linear transport gel scanner. Using this technique, we were able to resolve the thylakoid membrane polypeptides into at least 30 bands. For the convenience of description, we have classified these polypeptide bands into three major groups: large (mol wt 70,000-45,000), medium (mol wt 45,000-25,000), and small (mol wt 25,000-10,000) polypeptides. Within each group, we have arbitrarily labeled the major polypeptides L_1, L_2 . . . M_1, M_2 . . . S_1, S_2 . . . as shown in Figs. 7 and 8 which are electrophoretograms of TMF_{1u}.

A comparison of the electrophoretograms of TMF_1, TMF_{1s}, and TMF_{1u} reveals only minor differences in their band patterns: namely, the presence of additional minor bands (two in the L and two in the M group) in TMF_1. We have chosen to work primarily with TMF_{1u} which, a least on morphological grounds, is more homogeneous than TMF_1 and TMF_{1s}.

Cyclic Variations in the Rates of Protein Incorporation into Thylakoid Membranes

GENERAL APPROACH: To determine the rates of incorporation of new proteins into pre-existing bulk thylakoid membranes, we have used a double-labeling procedure which involves a long-term labeling of cell proteins with L-[¹⁴C]-
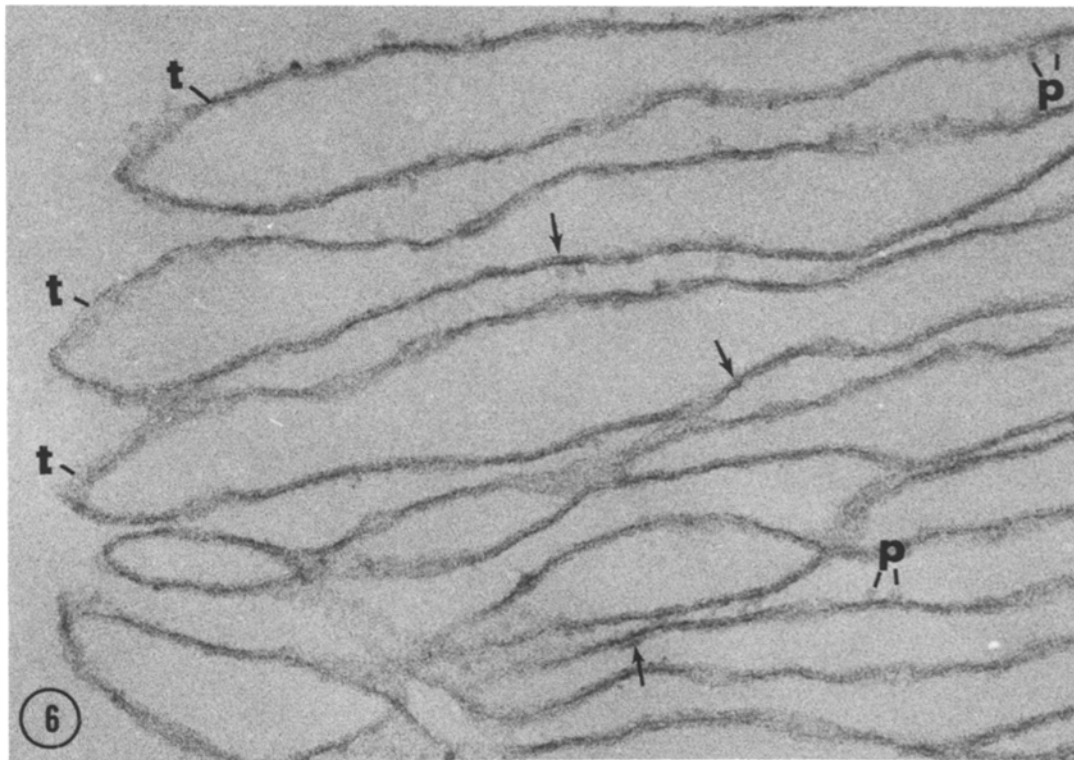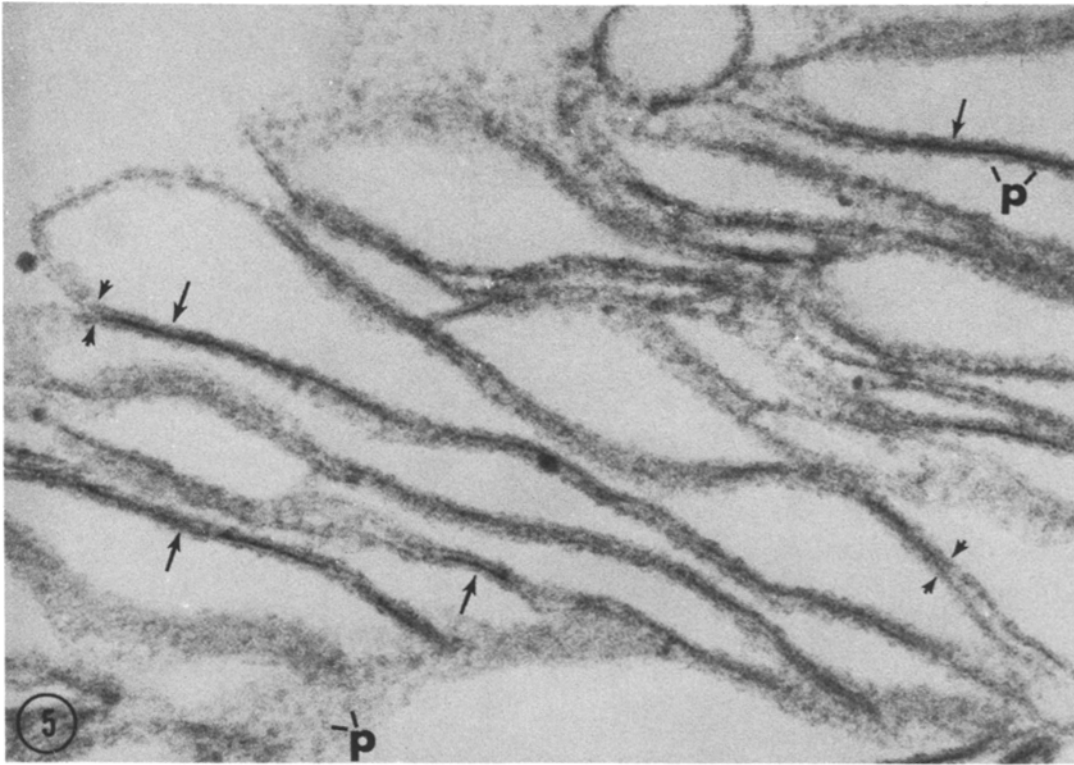
FIGURE 5   Representative field in a TMF_{1a} pellet. Pile of swollen thylakoids in a partially disorganized granum. The micrograph demonstrates the lines of fusion (arrows), points of separation of fused thylakoids (arrowheads) and arrays of particles (p) which probably correspond to the photosynthetic coupling factor identified (in sections) by Oleszko and Moudrianakis (26). × 146,000.

FIGURE 6   Small field in a TMF_{1u}. The micrograph demonstrates the complete unstacking of the otherwise intact thylakoids (t). The layered character of the membrane is visible in a few places (arrows). The outer surface of the thylakoids (involved in stacking) is marked by small, irregularly distributed, dense particles (p). × 146,000.

TABLE II

Distribution of Cytochrome c Oxidase Activity in C. reinhardtii Cell Fractions

Fractions	Percent of cytochrome *c* oxidase activity	
	(*a*)*	(*b*)‡
Homogenate	100	100
0.22 M–1.00 M sucrose interface	26	—
1.00 M–1.50 M sucrose interface (TMF₁)	32	32
1.50 M–2.00 M sucrose interface (TMF₂)	12	—
Pellet (a the bottom of the gradient)	30	
1.00 M–1.45 M sucrose region		1
1.45 M–1.50 M sucrose region		8
Pellet (at the bottom of the gradient)		15
TMF₁ₛ		6
TMF₁ᵤ		3

The cytochrome *c* oxidase was assayed by the procedure of Cooperstein and Lazarow (25).

* (*a*) Fractionation of the homogenate in a discontinuous sucrose gradient (1.0 M, 1.5 M, and 2.0 M sucrose in TKME buffer).

‡ (*b*) Fractionation of TMF₁ in a continuous sucrose gradient (1.0–1.5 M sucrose in TKME buffer).

arginine followed by a short-term ("pulse") labeling with L-[³H]arginine. The labeled cells were used for the isolation of thylakoid membrane fractions which were subsequently processed for SDS-polyacrylamide gel electrophoresis. Radioactivity distribution was determined in the resolved polypeptide bands. Variations in radioactivity distribution should indicate variations in the rate of incorporation into thylakoid membranes of proteins synthesized in any cell compartment during the pulse period. These rates are not expected to be identical to the rates of synthesis of the proteins under investigation (see Discussion for further considerations on this point).

QUANTITATION OF DATA—LONG-TERM LABELING: To be able to compare rates of protein assembly into thylakoid membranes at different time points in the cell cycle, we needed a procedure for estimating protein amounts in gel bands. We could not rely on the densitometry of Coomassie brilliant blue-stained gels since this

staining is preferential for basic amino acids and reaches saturation at high protein concentrations. Hence, we decided to attempt mass labeling or extensive labeling of membrane proteins with available radioactive precursors. Among the latter, radioactive bicarbonate requires special precautions, and radioactive acetate may change the growth condition of the algae from phototrophic to mixotrophic. Leucine and mixed amino acids were tried, but were taken up poorly; uptake of radioactive arginine was much more rapid; therefore, this amino acid was used for all subsequent experiments.

Synchronously grown cultures were first exposed to 0.05 μCi/ml L-[¹⁴C]arginine for exactly 48 h (two complete light/dark cycles) for each time point studied in the cell cycle. The patterns of radioacivity distribution after long-term arginine labeling showed only minor differences among TMF₁, TMF₁ₛ and TMF₁ᵤ electrophoretograms (at the level of resolution achieved in our experiments). Moreover, the radioactivity distribution pattern for TMF₁ᵤ was found to agree reasonably well with the Coomassie brilliant blue staining pattern of the same SDS-polyacrylamide electrophoretograms with the exception of few minor peaks (Fig. 7). Control experiments showed that with a systematically staggered 48-h period of exposure to L-[¹⁴C]arginine, the extent of labeling was very nearly identical for all the time points (i.e. 4L, 6L, 8L, 4D, 6D, 7D, 8D, 9D, and 11D) investigated in the cell cycle. The radioactivity distributions in SDS-gel electrophoretograms at four time points (4L, 8L, 6D and 9D) are presented as an example (Fig. 9).

Separate experiments were carried out to find out whether L-[¹⁴C]arginine was available or not to the cells over the entire 48-h period used for long-term labeling in our experimental protocol. The results showed (Table III) that about one-third of the initial radioactivity was still present in the culture medium after 48 h (the standard duration of our long-term labeling), and that the specific radioactivity of cellular proteins remained apparently constant past 24 h. Since, in our protocol, cells were exposed to L-[¹⁴C]arginine for two complete cell cycles (48 h) for each time point investigated irrespective of its position in the cycle, it follows that each individual membrane polypeptide was labeled to practically the same extent at all time points examined.

Although the long-term labeling procedure illustrated in Figs. 7 and 9 and in Table III appears to
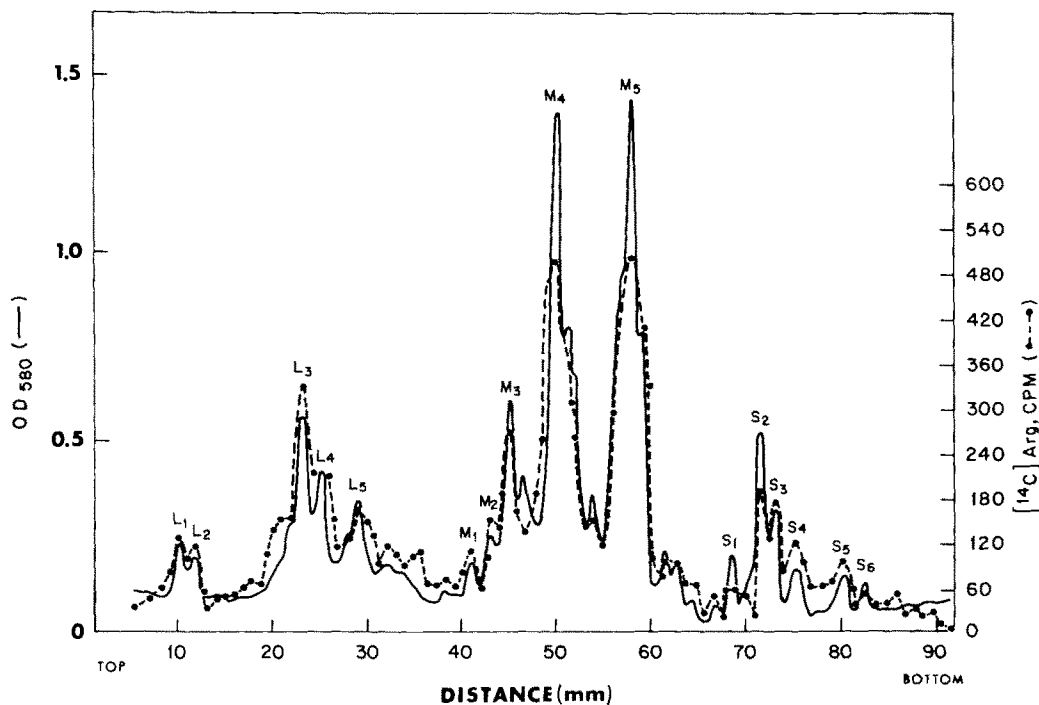
FIGURE 7   Comparison of the distribution of radioactivity and staining in an SDS-polyacrylamide gel electrophoretogram of thylakoid membranes (*C. reinhardtii*). A synchronously growing WT 137⁺ culture was labeled with 0.05 μCi/ml L-[^{14}C]arginine (spec act 22 mCi/mmol) for 48 h. A TMF$_{1u}$ was isolated from the labeled cells and its proteins were solubilized and analyzed on an SDS-polyacrylamide gel as given under Methods. The solid line profile (————) is the densitometer tracing (OD 580) of a gel stained with Coomassie brilliant blue. The dash line profile (●----●) shows the radioactivity distribution in the same gel determined in 1-mm slices, cut, processed and counted as given under Methods. The polypeptide bands resolved in this gel have been arbitrarily classified into L(L$_1$, L$_2$, L$_3$, L$_4$, L$_5$), M(M$_1$, M$_2$, M$_3$, M$_4$, M$_5$), and S(S$_1$, S$_2$, S$_3$, S$_4$, S$_5$, S$_6$) groups as described in the text.

be satisfactory for our purposes, it should be understood that it is dependent on the arginine content of the individual membrane proteins. Hence, comparisons of the same protein at different time points during the cycle are valid, but comparisons among different proteins should be made only with appropriate reservations.

SHORT-TERM LABELING: At various time points during the cell cycle, L-[^{3}H]arginine (10 μCi/ml) was added to the cultures (already labeled for 48 h with L-[^{14}C]arginine) for a standard period of 20 min to label newly synthesized proteins. An exposure of this duration, i.e. 1.4% of the entire cell cycle, is short enough to allow adequate time resolution of the process under study, yet long enough to permit the incorporation of sufficient radioactivity into thylakoid membrane proteins for reliable determinations. The 20-min "pulse" was ended by transferring the cultures to an ice bath and adding a 5,000-fold excess of nonradioactive

arginine. The cells were immediately harvested and washed three times with TKME buffer containing the same concentration of nonradioactive arginine. After the last wash, the pelleted cells were stored at $-70°$C for subsequent TMF$_{1u}$ isolation. The membrane fractions obtained were solubilized and processed for SDS-polyacrylamide gel electrophoresis as given previously. For radioactivity assays, the gels were cut (with a Rockefeller University gel slicer) into 1-mm slices. Each slice was dissolved by incubation in 0.5 ml of 30% H$_2$O$_2$ at 50°C for 17 h and subsequently counted in a Beckman liquid scintillation spectrometer using ^{14}C/^{3}H double-labeling settings. The scintillation fluid used contained 75% xylene, 25% Triton X-114, 0.3% 2.5-diphenyloxazole (PPO), and 0.2% p-phenylenebis-(5-phenyloxazole) (POPOP). The ratio ^{3}H:^{14}C was finally calculated for each major TMF$_{1u}$ band at each time point investigated during the cell cycle.

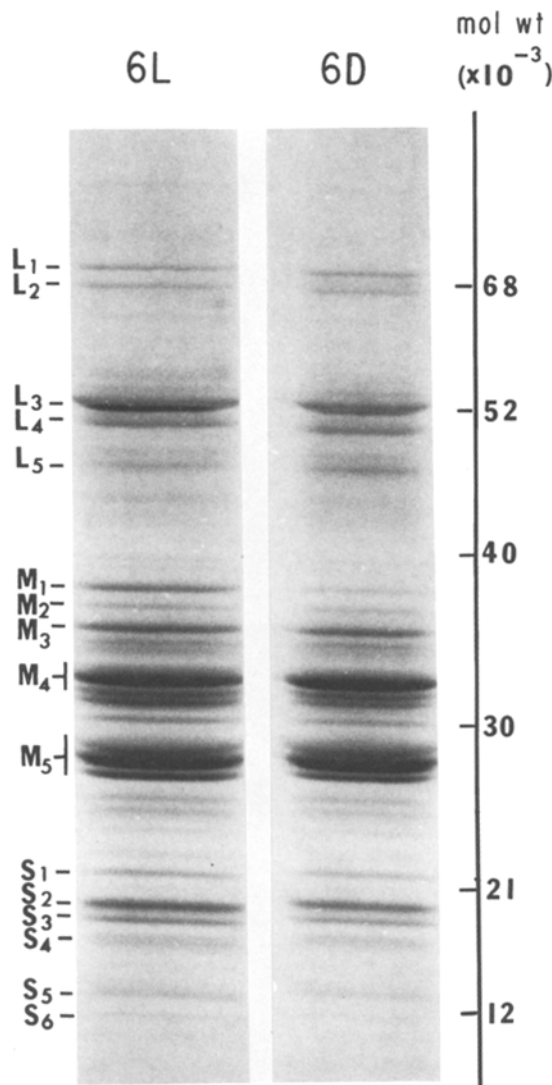RESULTS OBTAINED: Figs. 10 and 11 and

FIGURE 8   Comparison of the distribution of Coomassie brilliant blue staining patterns in an SDS-polyacrylamide gel electrophoretogram of thylakoid membranes (*C. reinhardtii*). Samples from a synchronously growing WT 137+ culture were harvested at 6L and 6D, respectively. In each case, a TMF$_{1u}$ was isolated from the cells and its proteins were solubilized and analyzed on a 3–20% polyacrylamide gel (in SDS) as given under Methods. With the exception of M$_1$, there were apparently few differences in Coomassie brilliant blue staining intensity between 6L and 6D. L$_3$ is a doublet, and M$_4$ and M$_5$ are partially resolved into triplets.

Table IV present in condensed form the results obtained in these experiments. They show that minimal values, which correspond to the least active incorporation of newly synthesized protein into thylakoid membranes, are found at the end of the dark period (11D) for all the polypeptide bands analyzed. The ratios appear to increase by a factor of 2 to 4 at the beginning of the light period (4L); they vary within narrow ranges during the rest of the light phase and the beginning of the dark period of the cycle (6L to 4D); and they rise to maximal values for all bands at 6D–7D, immediately after cell division, indicating that this is the period of the most active incorporation into pre-existing thylakoid membranes for all the polypeptide bands resolved in our gel system. However, the 6D–7D increase is not the same for all bands: it is large (> three times the 4L value) for most of the bands corresponding to large and small polypeptides (class L and S); and it is small (<2.5 times the 4L value) for the major bands that contain the polypeptides of the M class, especially for M$_5$. In the case of the bands designated M$_3$, M$_4$ and M$_5$, the ratios are relatively high during the entire light period, and for M$_4$ and M$_5$ there is even a weak suggestion of secondary peaks at 6L and 8L. The only other bands approaching this behavior (relatively high rates of incorporation during the light period, and small increases in the rates at 6D–7D) are L$_3$, L$_4$, and S$_3$.

From the data in Table IV it can be estimated that the average rate at which polypeptides L$_1$ and L$_2$ are introduced into the thylakoid membranes is four times higher at the beginning of G$_1$ (4D–8D) than during the second half of G$_1$ (4L–8L). The latter is the period characterized by rapid chlorophyll accumulation. For polypeptide(s) M$_4$, the increase in rate from (4L–8L) to (4D–8D) is considerably smaller (1.4); and for polypeptide(s) M$_5$, the same rate actually decreases slightly from the light to the dark period considered.

As already established (1, 3, 4) and indicated clearly by the data in Fig. 2, chlorophyll accumulates rapidly in the cells (more precisely in their thylakoid membranes) between 4L and 10L. Therefore, chlorophyll synthesis is out of phase by 12 h with the period of the most rapid accumulation of many proteins in the same membranes. Table V shows that the ratio of protein to chlorophyll varies cyclically in isolated thylakoid membranes from a minimum at 12L to a maximum at 7D. We do not have a satisfactory explanation for the large decrease of this ratio which apparently occurs between 7D (or later) in one cycle and 3L in the next cycle. It might be connected with the periodic unstacking of thylakoid membranes, which characterizes the beginning of the light
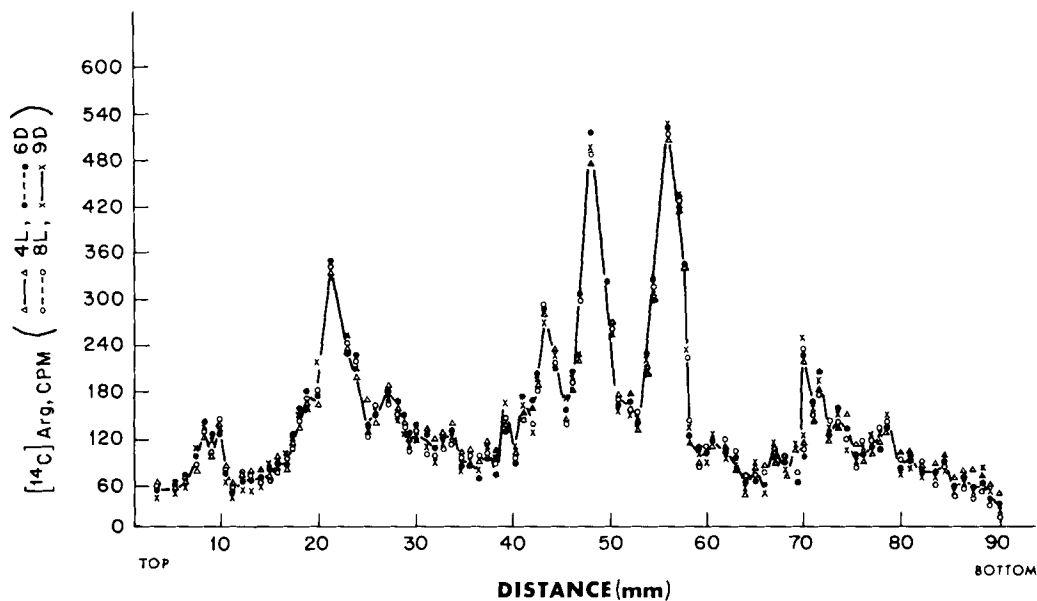
FIGURE 9 Comparison of radioactivity distribution in TMF₁ᵤ polypeptides labeled in vivo for a standard 48-h period beginning at four different time points in the cell cycle. L-[¹⁴C]arginine (spec act 22 mCi/mmol) was added at 0.05 μCi/ml to synchronously growing WT 137⁺ cultures either at 4L or 8L or 6D or 9D in the cell cycle. In each case, growth was continued in the presence of the labeled precursor for exactly 48 h. At the end of this period the cells were harvested. The corresponding TMF₁ᵤ were isolated and their proteins were processed through SDS-polyacrylamide gel electrophoresis. The gels were finally sliced and counted as described in the text. (△———△), 4L; (●———●), 6D; (○———○), 8L; (×———×), 9D.

phase of the cycle (unpublished observations), a possibility which remains to be explored by future work.

DISCUSSION

Definition of the System Used

ADVANTAGES: Wild type *C. reinhardtii* has two signal advantages as a material on which to study membrane biogenesis: its cultures can be satisfactorily synchronized and its progeny yield is high. Synchronization makes possible kinetic studies on morphogenesis in general and, in addition, it allows a reasonably accurate estimate of the duration and timing of the successive phases of the cell's cycle. Our observations show that G_1 lasts ~19 h (from 4.5D to 11L), S takes ~2 h (from 11L to 1D), G_2 is probably no longer han 1 h, and M is carried through in ~2 h (from 2.5D to 4.5D). The large progeny provides a substantial increase in membrane amount with a ratio of new to old membrane components that approaches 5 for each cell cycle. In principle, these advantages apply to

all types of cellular membranes in *Chlamydomonas*, but in the present state of development of cell fractionation procedures in this organism they can be put to good use only in the case of thylakoid membranes.

The fraction used for our observations, TMF₁ᵤ, is undoubtedly representative (in view of its high recovery figure for chlorophyll), but not truly homogeneous. It contains a small amount of morphologically recognizable particulate contaminants of nonchloroplastic origin—primarily mitochondria—which on morphological grounds represent < 5% of the volume of the fraction, and on the basis of biochemical assays account for ~3% of the total mitochondrial population of the cell. In addition, TMF₁ᵤ still comprises small amounts of structurally recognizable chloroplastic contaminants, primarily osmiophilic granules and fragments of chloroplast envelope and pyrenoid tubules. Although there is still room for improvement in homogeneity, we considered this fraction acceptable as a starting preparation since the amounts of individual "foreign" molecules provided by pariculate contaminants are expected to

TABLE III

Long-Term Labeling with L-[¹⁴C]Arginine of a Synchronous Culture of C. reinhardtii (WT 137⁺)

Exposure to L-[¹⁴C] arginine	Cell numbers/ml	TCA-soluble medium	TCA-insoluble cell pellet	Specific radioactivity (cell pellet)
h		*cpm*	*cpm*	*cpm/μg protein*
0	6×10^4	1,138,000 (100%)	1,260	17
24	3.6×10^5	1,017,300 (90%)	119,051	276
48	2×10^6	399,200 (35%)	668,820	278

L-[¹⁴C]arginine, (0.05 μCi/ml), was added to a 200 ml synchronous culture of *C. reinhardtii* (WT 137⁺) at 5L in the third cell cycle. Aliquots (10 ml) were collected immediately upon the addition of the label (0 h) and at a number of subsequent time points, including 24 h and 48 h, while the culture was maintained under synchronous growth. Each aliquot was separated into cells (cell pellet) and medium by centrifugation at 2,000 g_{av} for 5 min; and each ensuing fraction was treated with 10% TCA (final concentration) to yield TCA-soluble and TCA-insoluble subfractions. Radioactivity was determined in each subfraction. Results are tabulated only for cell pellet -(TCA-insoluble) and medium -(TCA-soluble). TCA-soluble radioactivity in the cell pellet leveled off at ~150 cpm/aliquot past 7 h, and TCA-insoluble radioactivity in the medium increased very slowly to reach ~1,800 cpm/aliquot by 48 h.
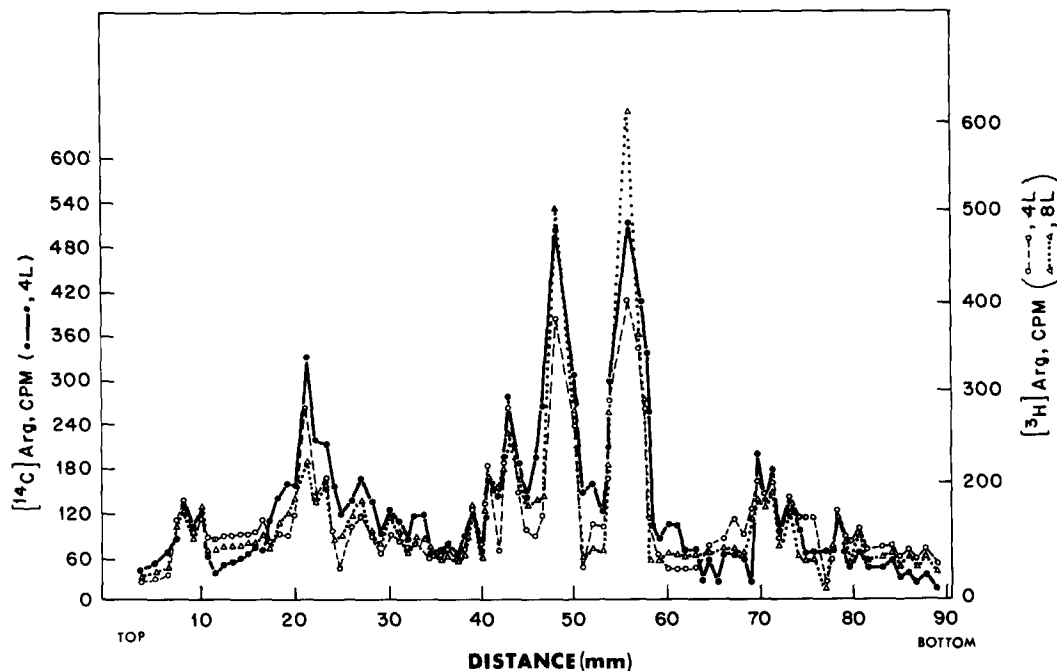

FIGURE 10 Radioactivity distribution in TMF₁ᵤ polypeptides after in vivo labeling for 48 h with L-[¹⁴C]arginine followed by a 20-min exposure to L-[³H]arginine. Comparison of two time points in the light period of the cell cycle. Two synchronously grown WT 137⁺ cultures were labeled with 0.05 μCi/ml L-[¹⁴C]arginine (spec act 22 mCi/mmol) for 48 h beginning at 4L and 8L, respectively. At the end of this period, 10 μCi/ml L-[³H]arginine (spec act 27.3 Ci/mmol) were added to each culture for 20 min. The procedures used to isolate TMF₁ᵤ, separate TMF₁ᵤ polypeptides on SDS-polyacrylamide gels, and determine ³H- and ¹⁴C-radioactivity in the gels are given under Methods. (O---O), L-[³H]arginine (cpm), 4L; (Δ···Δ), L-[³H]arginine (cpm), 8L; (●——●), L-[¹⁴C]arginine (cpm), 4L; L-[¹⁴C]arginine (cpm) at 8L was determined but not included in this figure. The two (4L and 8L) nearly coincidental ¹⁴C-radioactivity profiles of this experiment are shown in Fig. 9.
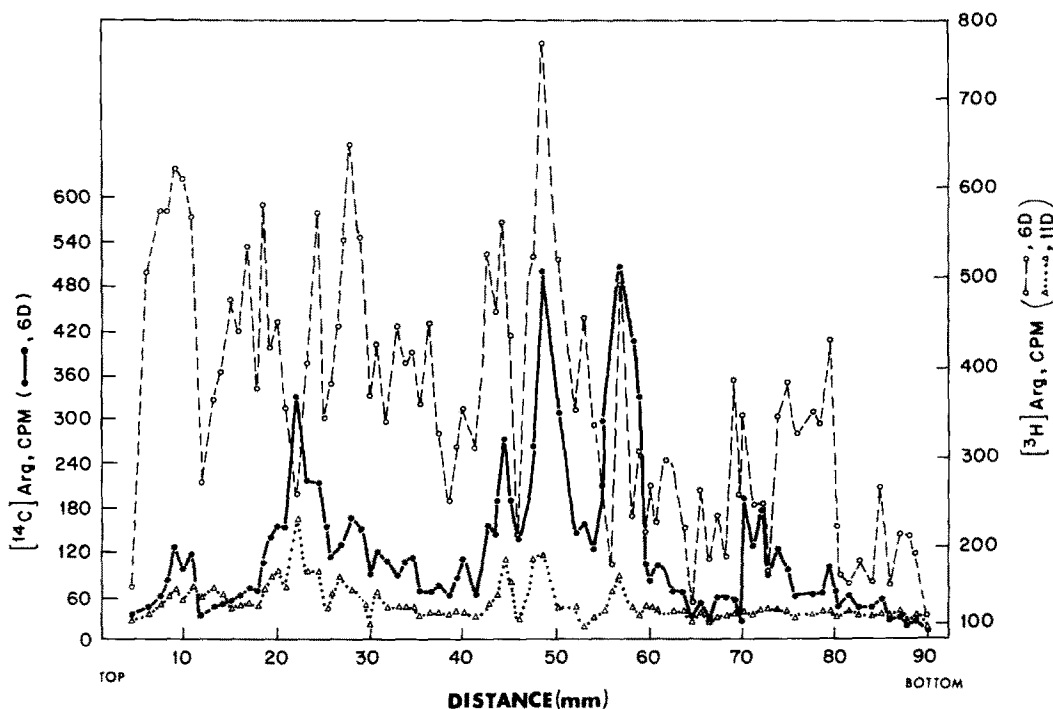
FIGURE 11 Radioactivity distribution in TMF₁ᵤ polypeptides after in vivo labeling for 48 h with L-[¹⁴C]arginine followed by a 20-min exposure to L-[³H]arginine. Comparison of two time points in the dark period of the cell cycle. Two synchronously grown WT 137⁺ cultures were labeled with 0.05 μCi/ml L-]¹⁴C]arginine (spec act 22 mCi/mmol) for 48 h beginning at 6D and 11D, respectively. At the end of this period, 10 μCi/ml L-[³H]arginine (spec act 27.3 Ci/mmol) were added to each culture for 20 min. See Methods for the procedures used to isolate TMF₁ᵤ, separate polypeptides on SDS-polyacrylamide gels, and determine ³H and ¹⁴C radioactivity distribution in the gels. (O- - - -O), L-[³H]arginine (cpm), 6D; (△· · · ·△), L-[³H]arginine (cpm), 11D; (●——●), L-[¹⁴C]arginine (cpm), 6D; (L-[¹⁴C]arginine at 11D was determined but not given; the two ¹⁴C radioactivity profiles were nearly coincidental.)

be below the limit of sensitivity of the next step in our analysis, i.e. SDS-gel electrophoresis of solubilized membrane proteins. As already mentioned, there are only minor differences in the electrophoretogram patterns of TMF₁, TMF₁ₛ and TMF₁ᵤ, and negligible variations in radioactivity distribution among the gels of the three fractions after long-term labeling.

With our solubilization procedure, all the polypeptides of TMF₁ᵤ were shown to enter the separating gels; and with the conditions used for SDS-polyacrylamide gel electrophoresis, the mixture of membrane polypeptides was reproducibly resolved into more than 30 reasonably well separated bands of characteristic distribution. The number of resolved bands varies with the type of polyacrylamide gradient in the gel; hence, the estimate given above should be considered as a minimal figure.

LIMITATIONS: The main limitations in our general approach are anticipated at the level of protein recovery in TMF₁ᵤ and polypeptide resolution in SDS-gels. At present, thylakoid membrane proteins are operationally defined as proteins present in TMF₁ᵤ. We do not know how many of the bona-fide membrane proteins are lost during the preparation procedure (due to their peripheral or extrinsic character); and we do not know either how many of the proteins present in the fraction are molecular contaminants (adsorbed from the soluble protein pool upon or after cell homogenization). Such points remain to be clarified by future work. The resolution obtained in the gels is not better than 500–1,000 daltons (depending on size); hence, any band in the gel may represent one polypeptide or two or more polypeptides of nearly the same (or even the same) electrophoretic mobility. Our results will be discussed while keeping in

Table IV

Variations in the Ratio of Radioactive Labeling of Thylakoid Membrane Polypeptides during the Cell Cycle of C. reinhardtii (WT 137⁺)

Polypeptides/time	4L	6L	8L	4D	6D	7D	8D	9D	11D
L₁	0.9	0.7	1.1	1.7	**5.0***	3.4	3.4	1.6	0.5
L₂	1.0	0.7	1.0	1.5	**4.3***	2.9	3.0	1.5	0.6
L₃	0.9‡	0.7	0.7	1.0	1.2	**1.5**	1.2	0.7	0.3
L₄	1.0	0.7	0.8	1.4	2.3	**2.5**	2.1	1.3	0.6
L₅	0.9	0.7	0.9	1.5	**3.9***	2.7	2.6	1.3	0.5
M₁	0.9	1.1	0.8	1.1	**3.7***	2.7	2.9	1.5	0.4
M₂	1.0	1.0	0.9	1.3	**3.7***	2.4	2.1	1.6	0.5
M₃	1.0	0.9	0.8	1.0	**2.5**	2.0	1.5	0.9	0.4
M₄	0.8‡	1.0	1.0	0.9	**1.8**	1.7	1.0	0.9	0.3
M₅	0.8‡	1.0	1.1	0.6	**1.2**	0.9	0.6	0.6	0.2
S₁	0.9	1.1	0.9	1.2	**3.5***	2.3	2.1	1.7	0.5
S₂	0.9	1.0	1.0	0.9	**2.7***	1.6	1.0	1.0	0.3
S₃	1.0‡	0.8	0.8	1.1	**2.1**	1.7	1.3	1.0	0.3
S₄	0.8	0.8	0.7	1.2	**3.0***	2.2	2.1	1.8	0.3
S₅	1.0	1.4	0.9	0.9	**4.0***	1.9	1.7	1.5	0.3
S₆	1.0	1.0	0.9	1.0	**3.4***	2.0	1.7	1.7	0.3

For each polypeptide band, the maximal value is in boldface, and the minimal value is underlined once.

The tabulated data come from four different experiments, each overlapping with another by at least one time point. The results are expressed as ratios of radioactivity incorporated during a pulse labeling (20 min; L-[³H]arginine) to radioactivity incorporated during continuous labeling (48 h; L-[¹⁴C]arginine). For 6L, 8L, 4D, 8D, 9D and 11D, the ratios were obtained in single experiments; for 7D, they are averages of results from two experiments; for 6D and 4L, they are representative figures for a series of three experiments.

* Polypeptide(s) with a ratio 6D:4L greater than 3.0.

‡ Polypeptide(s) with a ratio 6D:4L smaller than 2.5.

Cyclic Variations in the Rates of Incorporation of Thylakoid Membrane Proteins

We have selected 16 representative bands and have determined for each of them the ratio of short-term labeling (20-min pulse) with L-[³H]arginine to long-term labeling (48 h) with L-[¹⁴C]arginine. This ratio represents the relative rate at which the polypeptide (or polypeptides) of a given band synthesized during the "pulse" period appears (or appear) in thylakoid membranes. For each polypeptide, this figure is a function of its rate of synthesis,[2] its rate of transport from the site of

[2] For all polypeptides, the rate of labeling during the [³H]arginine "pulse" is expected to depend on the rate of arginine uptake by the cells and on the size of the intracellular arginine pool. Variations in the latter have

synthesis to its site of assembly, and finally its rate of assembly into the membrane. Each of these rates may be limiting. Notwithstanding the complexity of the whole process (which for convenience will be referred to as "incorporation of proteins into thylakoid membranes"), we recovered enough short-term label in all the bands investigated to suggest that transport as well as assembly occurs at a reasonably high rate. In view of the nature of the labeling, the comparison of the ratios for the same band at different times in the cycle is valid; but the comparison of one band to another depends on the relative arginine content of the corresponding polypeptides which is unknown at present. Our inter-

been recorded (17), but they appear to have little influence on our results since the pool is expandable (17), and since a comparison of the data of Iwanij et al. (17) with our results shows that there is no correlation in time between the size of the pool and the rates of synthesis of the various membrane polypeptides.

TABLE V

Cyclic Variations of the Protein*: Chlorophyll Ratio in TMF_{1u}

Time	3L	7L	12L	2D	4D	5D	6D	7D
Protein (mg)/chlorophyll (mg)	7.3	7.2	7.1	8.4	8.7	8.8	11.1	11.4

* Protein was assayed by the method of Lowry et al. (24).

pretations will rely primarily on cyclic variations of the ratio for each band considered separately.

Our results show that the polypeptides of all the bands investigated are incorporated into thylakoid membranes continuously throughout the cycle, albeit at rates that vary reproducibly from time point to time point within the cycle. For all of them the rate of incorporation reaches a minimal value at 11D and a maximal value at 6D–7D. The burst of activity which follows cell division and corresponds to the beginning of the G_1 phase is, however, different from one band to another: high for most large and small molecular weight polypeptides, and relatively low for the major polypeptides of intermediate size especially for those in the bands designated M_4 and M_5. The latter are major bands each resolved into a triplet in gels wih a different (less steep) polyacrylamide gradient.

Our results seem to be in contradiction with data already recorded in the literature. During the greening of the y-1 mutant of *C. reinhardtii*, membrane assembly appears to be tightly coupled to chlorophyll synthesis (1–5), whereas in the wild type a substantial fraction of the protein is incorporated almost 12 h after the bulk of the chlorophyll. It follows that in the wild type and under conditions comparable to those prevailing in nature, the two processes are dissociated (at least for a large number of specific polypeptides). For the moment, we do not have a satisfactory explanation for this difference.

In the wild type, cyclic variations in the activity of photosystems I and II and in the concentration of cytochromes 553, 559, and 563 have been recorded with maximal values at 4L to 6L (6). The position of these components in gel electrophoretograms is unknown; hence, for the moment, the extent of the disagreement (if any) cannot be assessed.

A population of polysomes (penta-to octamers) attached to thylakoid membranes has been detected both biochemically and morphologically during the light phase of the cycle (4L to 8L), but not during the dark phase (10). These polysomes are assumed to be involved in the synthesis and insertion of new proteins into thylakoid membranes, and are apparently detached and inactive during the dark phase.

Finally, Beck and Levine (7) showed that a series of major polypeptide bands, ranging in size from 40,000 to 27,000 daltons, starts being incorporated into thylakoid membranes (and reach maximal rates of incorporation) at different times during the 4L–12L part of the cycle, and are no longer added to the membranes at 1D. These bands probably correspond to M_3, M_4, and M_5 in our electrophoretograms. In our hands, M_4 and M_5 are composite bands which belong to a group characterized by small differences in their rates of incorporation into thylakoid membranes between 4L and 6D; moreover, their rates show suggestions of secondary peaks during the light period. Beck and Levine (7) did not include in their paper data on the rate of incorporation of new peptides into these thylakoid bands past 1D,[3] but showed that labeling of total cell proteins with exogenous radioactive acetate proceeded at a lower rate (one-third) in the dark than in the light. The reason for the apparent discrepancy beween their results and ours remains to be explained by further work. The exogenous radioactive acetate entering the inracellular pool during the night might be diluted by endogenous, not labeled (or less labeled) acetate derived from the starch reserves of the algae.

In part, at least, the discrepancies mentioned are probably due to incomplete separation of polypeptides in SDS-polyacrylamide gels. Bands like M_4, M_5 and L_3 are, or may be, composite bands comprising peptides with maximal rates of incorporation in the light [as postulated in (7)], in addi-

[3] J. Beck and R. P. Levine (personal communication) have investigated the labeling of membrane polypeptides with radioactive acetate during the dark phase of the cycle (past 1D) and have found that, under their experimental conditions, the labeling was not as extensive as it was during the light phase.

tion to polypeptides incorporated maximally at 6D-7D. Alternative explanations, which assume extensive involvement of chloroplast polysomes, are: (a) the inactivity of chloroplastic ribosomes during the dark phase is not complete, or concerns only a fraction of the whole chloroplastic ribosome population; and (b) some thylakoid membrane proteins are synthesized by free, rather than attached, chloroplastic polysomes. Low but persistent activity of chloroplastic ribosomes during the dark phase was detected recently by Iwanij et al. (17) in connection with the synthesis of ribulose-1,5-bisphosphate carboxylase (large subunit). In any case, our data definitely indicate that a large fraction of the proteins of the thylakoid membranes is incorporated therein during the dark.[4] These data also illustrate the advantages of our comprehensive approach which aims at investigating, throughout the entire cell cycle, most—if not all—of the resolvable polypeptides of the thylakoid membranes.

At present, very few of the gel bands can be identified with known membrane proteins. M_5, the major composite band of our medium molecular weight group, probably corresponds to the major membrane protein described as L protein by Eytan and Ohad (3, 5) and as fraction c polypeptide by Hoober (18). L_5 contains a protein tentatively identified as the "Q factor" by Chua and Bennoun (19), and L_2 is derived from the protein-chlorophyll complex of PSI, according to Chua, Karl and Bennoun (20).

Previous studies on thylakoid membrane biosynthesis have emphasized the asynchronous mode of assembly of these membranes during the greening of the y-1 mutant (2, 4, 5) as well as during synchronous growth in the wild type (6). In the latter case, the separation in time of the peaks of PSII and PSI activities (and cytochrome concentration) from the peak of chlorophyll accumulation was stressed, but the data for PS activities and cytochromes were obtained on cell homogenates rather than on isolated thylakoid membranes (6). Similar differences in time between enzyme activi-

ties and chlorophyll accumulation have been reported by Armstrong et al. (8). Our new findings concern directly components of isolated membranes and point to the existence of a large and different type of asynchrony than the one previously discussed. Most proteins are incorporated into thylakoid membranes at the beginning of G_1 in the dark, whereas chlorophyll is accumulated in the same structures in the light in the last third of G_1 just before the beginning of S. In addition, small scale asynchrony appears to exist from one polypeptide component to another, since the small variations in incorporation rates do not follow a common pattern as shown by the data in Table IV. Asynchrony of insertion of certain protein components at the beginning of the light period is not excluded by our results, but, if it does occur, it is expected to be dwarfed by the surge in protein incorporation at the beginning of the second half of the dark period. Thylakoid lipids appear to be incorporated into the membranes during the light (21).

The thylakoid membrane appears to be a highly dynamic structure whose chemical composition varies continuously throughout the cell cycle. The asynchrony of its assembly is confirmed by the results of our comprehensive survey, but, for the moment, at least, our data do not add strength to the hypothesis (7) of membrane assembly by successive waves of specific protein incorporation.

Previous studies have stressed the importance of light control in thylakoid membrnae biosynthesis (1-7). Our results point out the importance of the cell cycle, namely of G_1, in triggering a striking step-up in the rate of synthesis of a large number of new membrane proteins and in the rates of their incorporation into thylakoid membranes.

Finally, results obtained with specific inhibitors of protein synthesis on either chloroplastic or cytoplasmic ribosomes in *Chlamydomonas* (4) indicate that the majority of the thylakoid membrane proteins are synthesized in the cytoplasm on cytoplasmic polysomes. It is assumed that, upon termination, they (or their precursors) are transported across the chloroplast envelope to their sites of assembly in chloroplast membranes. Since our findings indicate that the rates of transport must be relatively high and that a large fraction of the operation is concentrated at the beginning of G_1, it becomes of interest to investigate structural modulations in the chloroplast envelope, which may appear in relation to this massive transport of macromolecules.

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[4] Iwanij et al. (17) have detected active incorporation of labeled arginine in total cell protein during most of the dark phase of the cycle. Since they found that the amount of protein in the culture does not increase during this phase, they concluded that the incorporation reflects active turnover of cell proteins. Our data suggest that at least part of this incorporation is connected with the synthesis of thylakoid membrane proteins.

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