BIOGENESIS OF CHLOROPLAST MEMBRANES

V. A Radioautographic Study of Membrane Growth in a Mutant of *Chlamydomonas reinhardi y-1*

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ABSTRACT

The development of photosynthetic lamellae during greening of dark-grown *Chlamydomonas y-1* cells was investigated by radioautography. Acetate-^{3}H was used as a marker for membrane lipids. In short pulse-labeling experiments, about 50–60% of the radioactivity incorporated was found in the lipid fraction and about 25–50% in starch granules present in the chloroplast of these algae. The relative specificity of acetate-^{3}H used as a marker for membranes was artificially increased through quantitative removal of the starch granules from fixed cells by amylase treatment. Analysis of turnover coefficients of different membrane constituents and of the contribution of turnover and net synthesis to the total label incorporated in pulse experiments indicated that the incorporation of acetate into specific lipids was mainly due to net synthesis. The distribution of radioactivity in the different lipid constituents at the end of a short pulse and after 30- and 60-min chases indicated that transacylation is minimal and may be disregarded as a possible cause of randomization of the label. Statistical analysis of radioautographic grain distribution and measurements of different structural parameters indicate that (*a*) the chloroplast volume and surface remain constant during the process, whereas the growth of the photosynthetic lamellae parallels the increase in chlorophyll; (*b*) the lamellae do not develop from the chloroplast envelope or from the tubular system of the pyrenoid; (*c*) all the lamellae grow by incorporation of new material within preexisting structures; (*d*) different types of lamellae grow at different rates. The pyrenoid tubular system develops faster than the thylakoids, and single thylakoids develop about twice as fast as those which are paired or fused to grana. It is concluded that growth of the membranes occurs by a mechanism of random intussusception of molecular complexes within different types of preexisting membranes.

INTRODUCTION

The study of the degreening process in the *y-1* mutant of *Chlamydomonas reinhardi* has revealed that this organism, when grown in the dark for several generations, possesses a differentiated plastid containing only trace amounts of the photosynthetic lamellar system (1). When dark-grown cells are illuminated, most of their biochemical activity is shifted toward the synthesis of membrane components and their assembly into functional photosynthetic membranes (2, 3). Because of these properties the organism was considered to be a suitable model for the investigation of the mechanism of biological membrane formation.

Many problems concerning the biogenesis of

membranes are unanswered as yet. Thus, we do not know what is the mechanism controlling the synchronized synthesis of the membrane constituents, such as proteins, lipids, and pigments (4, 5). It is not yet clear whether the assembly of these building blocks into functional membranes is a spontaneous single step or a sequential process. Evidence has been presented in support of the single-step assembly hypothesis for the chloroplast membranes (2) and of a multistep assembly mechanism during the development of the endoplasmic reticulum in newborn rats (6–9). At the ultrastructural level it is not known whether membranes are formed *de novo* or grow by intussusception of new material into preexisting membranes. If membranes do grow, it is not known whether all the membranes at any stage of organization can accept new material at random, or whether growth is a vectorial process occurring in specialized centers or loci within the membranes.

With the aid of radioautographic techniques, experiments were designed to determine whether the growth of chloroplast photosynthetic membranes is a random or a vectorial process.

In order to obtain meaningful radioautographic results, the marker used should be incorporated into components highly specific for membranes with a low metabolic turnover and constituting a stable structural part of the membrane. This is necessary to prevent nonspecific migration of the marker between different membranes present in the chloroplast or cell.

These requirements were met when acetate-^{3}H was used as a marker for lipids. The relative specificity of acetate for membrane lipids was increased by quantitative removal of the starch granules containing the major fraction of nonlipid radioactivity incorporated in pulse-labeling experiments.

The results indicate, basically, that growth might occur by random incorporation of precursors into preexisting membranes.

MATERIALS AND METHODS

Chlamydomonas reinhardi y-1 cells were grown in the dark on a mineral medium with acetate as a carbon source (1). Greening experiments, extraction, and analysis of lipids and pigments were carried out as described before (1, 4).

The incorporation of acetate-^{3}H in pulse-chase experiments (2) was carried out in duplicate; one set was used for chemical and radioactivity analyses (specific activity of acetate-^{3}H, 2 μCi/μmole). and the other set (with a specific activity of acetate-^{3}H of 750 μCi/μmole) for radioautography. The high specific activity used in the latter case was necessary to ensure a final incorporation of about 1 dpm/cell in a 10 min pulse. This, in turn, would induce the appearance of about 15–30 grains/section after about 1–2 wk of exposure.

Starch content distribution of radioactivity in the lipid, starch and residue fractions were estimated as described before (1).

In order to increase the relative amount of acetate-^{3}H incorporated into the lipid fraction, amylase treatment was used to remove the starch granules which contained between 20 and 50% of the incorporated radioactivity, depending on the time point at which the pulse was given. To facilitate the quantitative chemical and radioactivity analyses of starch and lipids during amylase treatment, a procedure was developed to remove the starch from whole fixed cells. Several fixation procedures were tried, including different concentrations of glutaraldehyde and different buffers and fixation times. The best results were obtained with the following procedure. Samples containing $\backsim 3 \times 10^7$ cells were centrifuged in conical glass test tubes (12 ml) at 2000 *g* for 3 min to obtain a pellet whose thickness was about 1 mm. The pellet was fixed for 6 min in ice in 2% OsO_4 in sodium phosphate buffer, 0.074 M, pH 7.4 (1). The fixative was decanted, and the cells were resuspended by gentle pipetting in 10 ml of the same buffer in the cold, washed by centrifugation as above three times, and then pelleted again and fixed overnight in neutralized glutaraldehyde (acid content < 0.2%—Fluka AG, Basel, Switzerland), 0.6% in sodium phosphate buffer, 0.02 M, pH 7.0 at 4°C. The fixed cells were washed free of glutaraldehyde in 10 changes of sodium phosphate buffer 0.074 M, pH 7.4, containing 0.02 M sodium acetate (for complete dilution of traces of acetate-^{3}H), and then treated with α-amylase (see below), washed again four times with the same buffer, and finally fixed overnight at 4°C in 2% OsO_4 in phosphate buffer, as above. This last treatment was carried out to ensure complete fixation of the lipids and to prevent their extraction during dehydration (10, 11). Dehydration and embedding in Epon were done as described by Luft (12). All fixatives, alcohol, and propylene oxide supernatants from the dehydrated pellets were pooled for each sample and analyzed for radioactive content in order to account for loss of lipids during the processing.

Starch granules were removed from cells fixed as described above by digestion with a rat parotid gland extract. The glands were removed from six white Wistar rats ($\approx$ 250 g) starved overnight and were homogenized in sodium phosphate buffer, 0.02 M, pH 6.9, containing 7 mM NaCl. The homogenization was done in an ice cooled glass-teflon Potter-Elvjeheim homogenizer for 5 min. The homogenate was centri-

fuged at 60,000 *g* for 30 min. The clear supernatant (which will be further referred to as 'amylase') contained about 4,000 amylase units/ml (13). For starch digestion, a cell suspension containing 3×10^7 cells and 8000 amylase units in 3 ml of the same buffer as above was incubated at 37°C for 4 hr. Samples were taken at different times, the cells were removed by centrifugation, and the supernatant was analyzed for radioactivity, sugar, and lipid content as described above.

For radioautography, white to pale gold sections were cut on a Sorvall MT2 Porter-Blum-type microtome (Ivan Sorvall Inc., Norwalk, Conn.) with glass or diamond knives (Sugg. Rondikin Co., Honolulu, Hawaii). Sections were picked up on copper grids (400 mesh). The grids were mounted on glass slides and covered with Ilford L4 emulsion (Ilford, Essex, England) by the loop method, as described by Caro and van Tubergen (14). In order to reduce the thickness of the emulsion layer, the film formed on the 4 cm diameter loop was transferred onto a second loop (3.9 cm diameter). This was done by passing the second loop slowly through the first one before the gelation of the film. Electron microscopical examination of these films showed even distribution of the silver halide grains. The emulsion-coated sections were stored in the dark at 4°C in a desiccator, developed in Microdol X (Kodak) (14), and stained with uranyl acetate and lead citrate, as described by Reynolds (15). Electron micrographs were obtained with an RCA EMU 3G microscope at an electron magnification of × 7000. For statistical analysis, micrographs were enlarged to × 20,000 and the grain distribution on the different cell compartments or organelles was estimated according to the method described by Loud (16). A transparent grid with lines at 4 mm intervals (equal to 0.2 μ at a magnification of × 20,000) was used. A grain was considered to coincide with a certain structural element when it was included in a square superimposed on the structure (Tables IV, VI, VIII). In order to minimize the error due to the variation in the size of the developed grains, in certain cases a grain was considered to coincide with a certain structural element when the center of the minimal circle circumscribing a grain was superimposed on or fell within a distance of 0.5 grid unit of the structure (Tables V, VII). For each radiogram, the center of the grain, defined as above, was punctured with a needle and the statistical analysis of grain distribution was carried out on the resulting dots (17). The length (μ) of profiles of membrane sections was measured with a cartographic mile counter.

As a control for the randomness of grain distribution, a similar transparent grid punctured at random (400 dots) was superimposed on each radioautogram and the distribution of the randomly punctured "grains" was analysed and compared with that of the authentic grains.

The data are expressed as mean values ± confidence interval (SEM × *t*). The calculations were corrected by the *t* test, using, unless otherwise specified, a confidence interval of 95% and degrees of freedom between 7 and 14. Since the thickness of the sections used and the exposure time for grain development were not uniform for all preparations the data were standardized as follows. The total area of a cell analyzed for grain distribution was measured and taken as 100%. The total number of grains present over the cell was counted and taken as 100%. To calculate the specific activity of a subcellular structural element relative to the whole cell, the area of the structure was measured and expressed as % of the total cell area (*a*), and the grains present over that structure were counted and expressed as % of the total number of grains over the cell (*b*). The specific activity (grains/unit area) was expressed as the ratio b/a.

Electron microscopical examination of amylase-treated cells revealed that in almost 90% of the cell sections the starch granules were completely removed, leaving empty spaces filled with the plastic used for embedding. The remainder of the sections contained various amounts of starch granules at different stages of digestion. Only sections of cells which were completely devoid of starch granules were used for analysis of radioautographic grain distribution.

All the materials used in this work were of analytical grade.

RESULTS

Experiments were designed to find the proper conditions for pulse incorporation of acetate-^{3}H into the lipid fraction in order to determine the appropriate time of pulse and chase and to test the stability of the label. The results of a preliminary experiment showed that the incorporation rate increased with time and was higher in the cells incubated in the light than in the dark control. The radioactivity of acetate-^{3}H incorporated into the lipid fraction in a 10 min pulse was found to be rather stable; only a small amount was exchanged when chased with cold acetate for 30 and 60 min, respectively (Fig. 1). This exchange indicated that turnover of lipids might occur.

The exact determination of the contribution of turnover to the labeling of lipids is essential, since only radioautographic label due to net synthesis should be considered as indicative of membrane growth.

The fraction of label contributed by turnover of membranes during a pulse experiment would de-

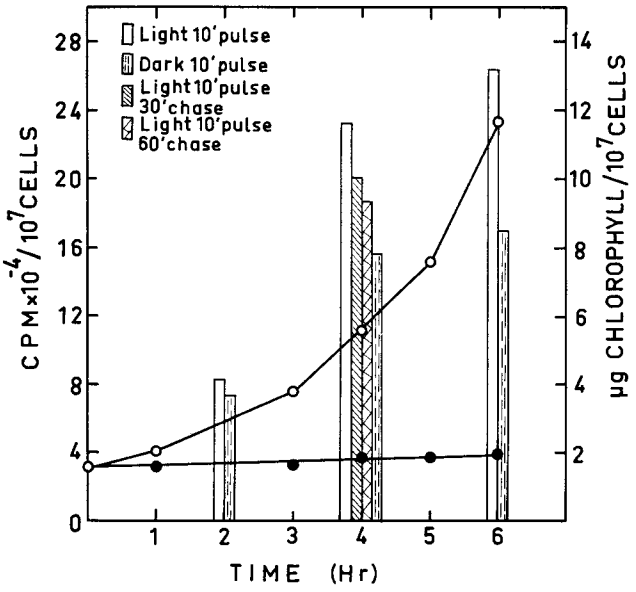


FIGURE 1 Pulse labeling of lipids with acetate-^{3}H during the greening of *y-1* cells. Dark-grown cells (3.5×10^6 cells/ml) were suspended in growth medium containing 7.5×10^{-3} M acetate and incubated either in the dark or light (700 ft-c) at 25°C. Acetate-^{3}H (30 μCi/ml) was added to samples of cells at different times and incubation was continued for 10 min. Incorporation was stopped by dilution with cold acetate, the cells were washed and lipids were extracted either immediately or after further incubation in growth medium as above for 30 or 60 min. Chlorophyll content in light (○——○); and dark (●——●).

pend on the total amount of membrane material (V_0) present in the cell at the time of the pulse (t), the rate of turnover per unit time (α), the total net increase in membrane material per unit time ($\bar{V}$), and the duration of the pulse (Δt).

The total amount of label (V_i) incorporated during the pulse can be expressed as:

$$\Delta V_i = \bar{V}\Delta t + (V_0 + \bar{V}t)\alpha\Delta t. \quad (1)$$

Assuming that the internal pool of precursors in these cells is infinitely small as compared with the external supply, that degradation products due to turnover are diluted within the pool, and that the net increase in membrane material during the pulse is linear with time ($\bar{V}/t$ = constant), we can write:

$$dV_i = \bar{V}dt + (V_0 + \bar{V}t)\alpha\, dt \quad (2)$$

$$V_i = \int_t^{\Delta t} (\bar{V} + V_0\alpha + \bar{V}\Delta t)\, dt \quad (3)$$

$$V_i = (\bar{V} + V_0\alpha)t + \frac{\bar{V}\alpha t^2}{2}. \quad (4)$$

The internal pool of acetate and precursors in *y-1* cells is small and in equilibrium with the external supply of acetate, as indicated by the immediate cessation of incorporation when radioactive acetate is diluted with cold acetate (2).

If the pulse is given during the linear phase of greening, then $\bar{V}/t$ = constant, so that we can use equation (4) for the calculation of label due to turnover ($V_0\,\alpha t$) and net synthesis ($\bar{V}$).

The coefficient α can be determined from chase experiments by analysis of decay of the radioactive label of different membrane components during the linear phase of membrane synthesis. The fraction V_0 for each component can be determined quantitatively at different times of the greening experiment, and from the shape of the curve V_0/t it is possible to calculate the value of $\bar{V}$ for the duration of the pulse for each compound.

A chase experiment was carried out with greening cells pulse labeled with acetate-^{3}H for 15 min at two different times during the linear phase of greening. The chase was continued for 2 hr while greening still proceeded at a constant rate. The turnover coefficient (α) was calculated for the total lipid and protein fraction, as well as for chlorophylls a and b, glycolipids, neutral lipids, and phospholipids (Fig. 2).

On the basis of measured or calculated values for V_i, $\bar{V}$, V_0, and α from the same experiment the ratio $\bar{V}/V_i$ and the relative contribution of net synthesis to the total radioactivity incorporated into different fractions were calculated and are given in Table I. The results show that most of the radioactivity in the glycolipid and chlorophyll fractions is due to net synthesis. About 43–46% of the label in the phospholipid fraction is due to turnover. The total radioactivity of the phos-

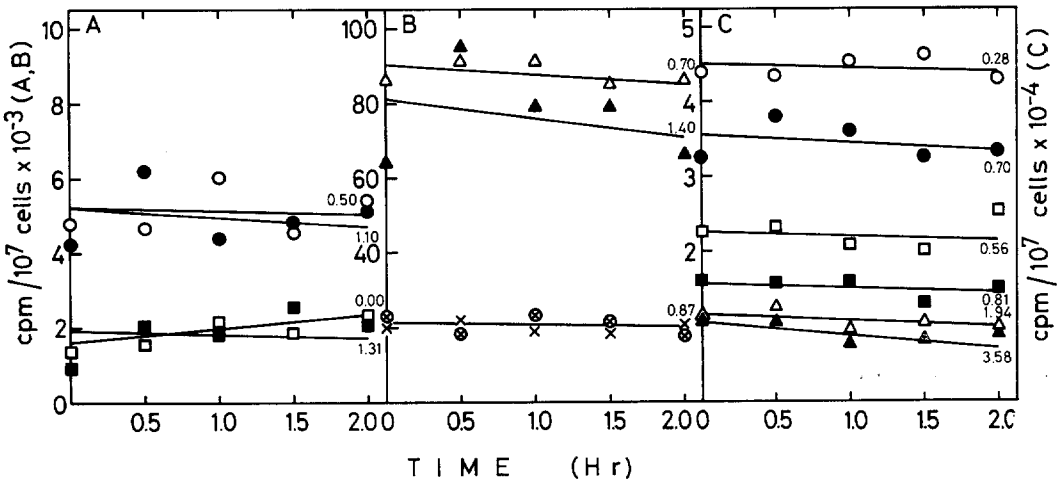


FIGURE 2 Changes in radioactivity of pulse-labeled lipids, pigments, and total protein during a chase experiment. Dark-grown cells were incubated in the light (700 ft-c) at a final concentration of 8×10^6 cells/ml in growth medium containing 2 mM KH_2PO_4. At different times, cell samples were pulsed with acetate-^{3}H (8 μCi/μmole, 0.5 mM final concentration) for 15 min in the light. The cells were washed three times by centrifugation at 2,000 *g* for 3 min in a medium containing 15 mM cold acetate, resuspended in the same medium, and further incubated in the light for 2 hr. Samples were taken for analysis of total radioactivity (V_i) and of net amount (V_0) of different components every 30 min. The cells were pulse labeled and processed as above at 4.5 and 5.5 hr of greening. From measurements of the chlorophyll content at one hour intervals throughout the whole greening process, it was found that chlorophyll synthesis proceeded at a constant rate (1.75 μg/10^7 cells/hr) from 4 hr after onset of illumination. The α coefficients were calculated from the slope of the curves and are given as % radioactivity lost in 15 min. The decay curves after pulse label at 4.5 and 5.5 hr are referred to as *P-1* and *P-2*, respectively. Analytical procedures were as described in Methods.

FIGURE 2 *A* Chlorophyll a ●——●, *P-1*; ○——○, *P-2*. Chlorophyll b ■——■, *P-1*; □——□, *P-2*.

FIGURE 2 *B* Total lipid ▲——▲, *P-1*;△——△, *P-2*; Protein ×——×, *P-1*; ⊗——⊗, *P-2*.

FIGURE 2 *C* Neutral lipid ●——●, *P-1*; ○——○, *P-2*. Glycolipids ■——■, *P-1*; □——□, *P-2*. Phospholipids ▲——▲, *P-1*; △——△, *P-2*.

pholipids amounts to only 12–15% of the total radioactivity in the lipids, so that the final contribution of phospholipid turnover to the radioactivity of the lipids is less than 8% of the total label. The radioactivity of the protein fraction was less than 10% of the total radioactivity incorporated into the cells during the pulse; about 20% of the total protein radioactivity was due to turnover (see Table III).

The possibility that reactions of transacylation of fatty acids between different lipids might cause randomization of the label was also considered. Cells were pulse labeled with acetate-^{3}H for 10 min at different greening times, and the distribution of radioactivity between the different lipid components at the end of the pulse and after 30 and 60 min chase with cold acetate was analyzed. We found that the relative radioactivity (%) of different lipids at the end of the pulse varied between 2 and 50% and was preserved as such, without extensive redistribution or randomization, at the end of a chase period of 30 or 60 min (Table II).

Not all the radioactivity incorporated in a pulse experiment was found in the lipid fraction. As shown in Table III, between 25 and 52% was found in starch granules and up to 17% was found in the residue fraction (cell walls, proteins, nucleic acids).

Since starch granules are located around the pyrenoid and within the chloroplast in close proximity to the developing membranes (1, 2), it is practically impossible to distinguish between

TABLE I

Turnover and Net Synthesis of Lipids and Proteins during Synthesis of Chloroplast Membranes

Component	V_0	$\bar{V}$*	V_1			$\frac{V}{V_1}$	$\frac{\bar{V}}{V_1} \cdot b$‡
			a	cpm/10^9 cells × 10^{-3}	b = radioactivity in lipids		
	μg/10⁹ cells	*μg/10⁹ cells*	*μg/10⁹ cells*		%	*μg/μg*	
I *A*							
Total lipids	—	—	—	6430	100.00	—	—
Phospholipids	135	6.56	11.6	1020	15.90	0.57	9.0
Glycolipids	1270	170.00	180.9	1620	25.20	0.94	23.8
Neutral lipids§	—	—	—	3260	50.30	—	—
Chlorophyll a	250	31.00	33.9	415	6.45	0.91	5.9
Chlorophyll b	75	6.00	7.0	94	1.47	0.85	1.3
Proteins	14700	500.00	631.2	2000	—	0.79	—
I *B*							
Total lipids	—	—	—	8600	100.00	—	—
Phospholipids	141	6.56	10.0	1080	12.50	0.66	8.25
Glycolipids	1440	170.00	178.0	2250	26.20	0.95	24.80
Neutral lipids§	—	—	—	4400	51.00	—	—
Chlorophyll a	375	31.00	32.9	475	5.50	0.94	5.20
Chlorophyll b	99	6.00	6.0	135	1.57	1.00	1.57
Proteins	16700	500.00	648.2	2300	—	0.77	—

a is calculated according to formula (4), using the values of αV_0 and V from this table.

I *A* Cells pulse labeled at 4.5 hr greening (*P-1*).

I *B* Cells pulse labeled at 5.5 hr greening (*P-2*).

Same experimental conditions as in Figs. 2 and 3.

* Calculated values from the slope of changes in net amount as a function of time during the greening phase of synthesis at constant rate.

‡ Relative contribution (%) of net synthesis to total radioactivity incorporated in lipids.

§ The neutral lipid fraction includes also the chlorophylls.

radiographic grains as originating from a starch granule or from an adjacent membrane. It was therefore necessary to remove the starch granules from the fixed cells prior to radioautography. It was found that incubation of the fixed cells with amylase for 3 hr is sufficient to remove 80% of the total sugar and radioactivity of the starch fraction (Fig. 3; Table III). Sugar analysis of the supernatant of the amylase digest showed, in different experiments, that it contained between 60 and 90% of the initial starch content of the cells. Thin-layer chromatography of the digest indicated that less than 5% of the radioactivity of the lipid fraction of the cells was released during the amylase treatment. The use of the parotid extract as a source of amylase was suitable since it is practically free of lipolytic activity, as shown also by the insignificant loss of lipid during starch digestion. The extract also contained RNase activity (18), and it is very likely that some RNA was digested. This could explain the lack of definition of the ribosomes in micrographs obtained from sections of the treated tissue. Since only a small fraction of the acetate-^{3}H incorporated in the cells was found in the RNA fraction (total radioactivity of the residue fraction, including protein, cell-wall carbohydrate, and nucleic acid, was less than 8%), RNA digestion was not quantitated.

The amount of radioactivity lost due to the processing of samples for electron microscopy (during fixation and dehydration) was less than 10% of the total radioactivity initially present in the sample.[1] Thus if we account for the radio-

[1] Part of this loss might be accounted for by fragmentation of a small amount of the fixed cells which are rendered brittle by fixation. During the repeated washings, some of the fragments might have been lost through decantation.

TABLE II

Distribution of Radioactivity (%) Incorporated into Various Lipids by Pulse Labeling of y-1 Cells during the Greening Process

Lipid	Experiment 1			Experiment 2		
	10′ pulse	30′ chase	60′ chase	10′ pulse	30′ chase	60′chase
Phosphatidyl serine	2.74	3.88	3.24	1.75	2.04	0.99
Phosphatidyl inositol	7.20	8.05	7.40	2.95	3.76	2.75
Sulfolipid	8.24	7.54	8.42	10.40	7.30	4.40
Phosphatidyl choline	3.34	3.01	4.05	5.12	9.20	9.35
Phosphatidyl glycerol	5.55	5.70	5.55	4.00	7.10	4.00
Digalactosyl diglyceride	24.00	26.40	26.00	29.20	20.00	19.60
Phosphatidyl ethanolamine*	12.40	10.10	8.12	7.93	4.25	3.40
Monogalactosyl diglyceride	14.40	11.10	10.60	16.40	9.78	13.50
Neutral lipid	23.70	24.30	26.80	49.00	36.50	42.50
% Recovery‡	88	92	100	70	94	87

For details of analytical procedures see Methods.

* This spot includes an unidentified lipid.

‡ Calculated as % of the total radioactivity placed at the chromatogram origin.

TABLE III

Radioactivity Distribution (%) Incorporated into Various Cellular Components by Pulse Labeling and its Removal by Treatment of Fixed Cells with Amylase

Component	Treatment			
	5 hr dark 10′ pulse	3.5 hr dark 10′ pulse	6 hr light 10′ pulse	6 hr light 10′ pulse 1 hr chase
Lipids	36 ± 2	70 ± 8	56 ± 6	54 ± 5
Starch	52 ± 3	24 ± 8	38 ± 3	42 ± 2
Residue*	17 ± 1	6 ± 2	6 ± 2	4 ± 2
Amylase digest	49 ± 19	16 ± 8	29 ± 9	26 ± 5

Same experimental conditions as in Fig. 3.

The data are represented as the average of three different experiments.

* The residue includes proteins, nucleic acids, and cell walls.

activity of the starch removed by amylase digestion and that lost during processing of samples for electron microscopy, about 64% of the total radioactivity incorporated into the cells at the end of a 10 min pulse labeling is left in the cell pellet at the time of sectioning. Of this radioactivity, 78% is lipid, about 14% is starch, and 7.8% is residue. When examined in the electron microscope, only about 10% of the cells still showed the presence of starch granules. Since only cells free of starch were used for analysis of radioautograms, we may consider that the specificity of the label in the lipid fraction of those cells was at least 90%.

Examples of radioautograms used for analysis of grain distribution are shown in Figs. 4–8. Despite the extensive manipulation of the cells in the course of preparation for electron microscopy, their preservation was relatively good, and the same morphological elements were shown as previously described (1).

The plasma membrane, cell wall, nucleus and nucleolus, dictyosome, mitochondria, vacuoles,

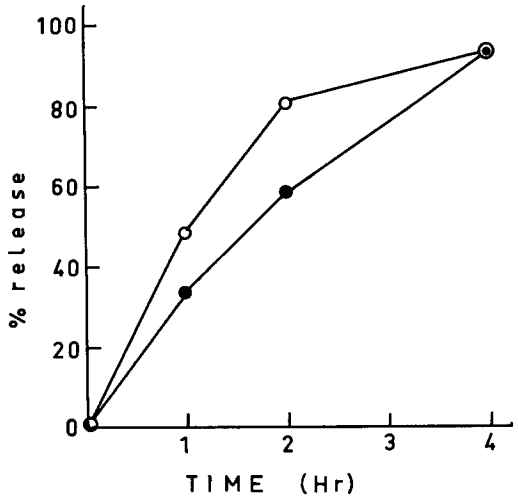


FIGURE 3 Release of sugar and radioactivity from fixed cells by amylase treatment. Dark-grown cells were washed and suspended in growth medium at a final concentration of 4.2×10^6 cells/ml. The cell suspension was incubated in light (700 ft-c) for 5.5 hr; acetate-^{3}H (2 μCi/μmole) was added and incubation was continued for 10 min. The cells were then diluted with cold acetate and samples were taken for fixation (4.7×10^7 cells), determination of total radioactivity incorporated, the distribution of radioactivity in different fractions, and total starch content. The chlorophyll content at 5.5 hr of illumination was 5.8 μg/10^7 cells. The starch content was 108 μg/10^7 cells and its radioactivity was 1220 cpm. These values represent the 100% level in the figure. Fixation and amylase treatment were done as described in Methods. O——O μg sugar; ●——● radioactivity.

chloroplast outer and inner membranes,[2] pyrenoid, and eyespot were clearly seen and exhibited no major structural alterations. In some cases there was a slight amount of plasmolysis, the plasma membrane being retracted from the cell wall and folded (Figs. 4, 5). Also, the membranes of the nuclear envelope was eventually vesiculated (Fig. 6). The cytoplasm and chloroplast matrix appeared granular in texture, rendering the identification of ribosomes difficult.

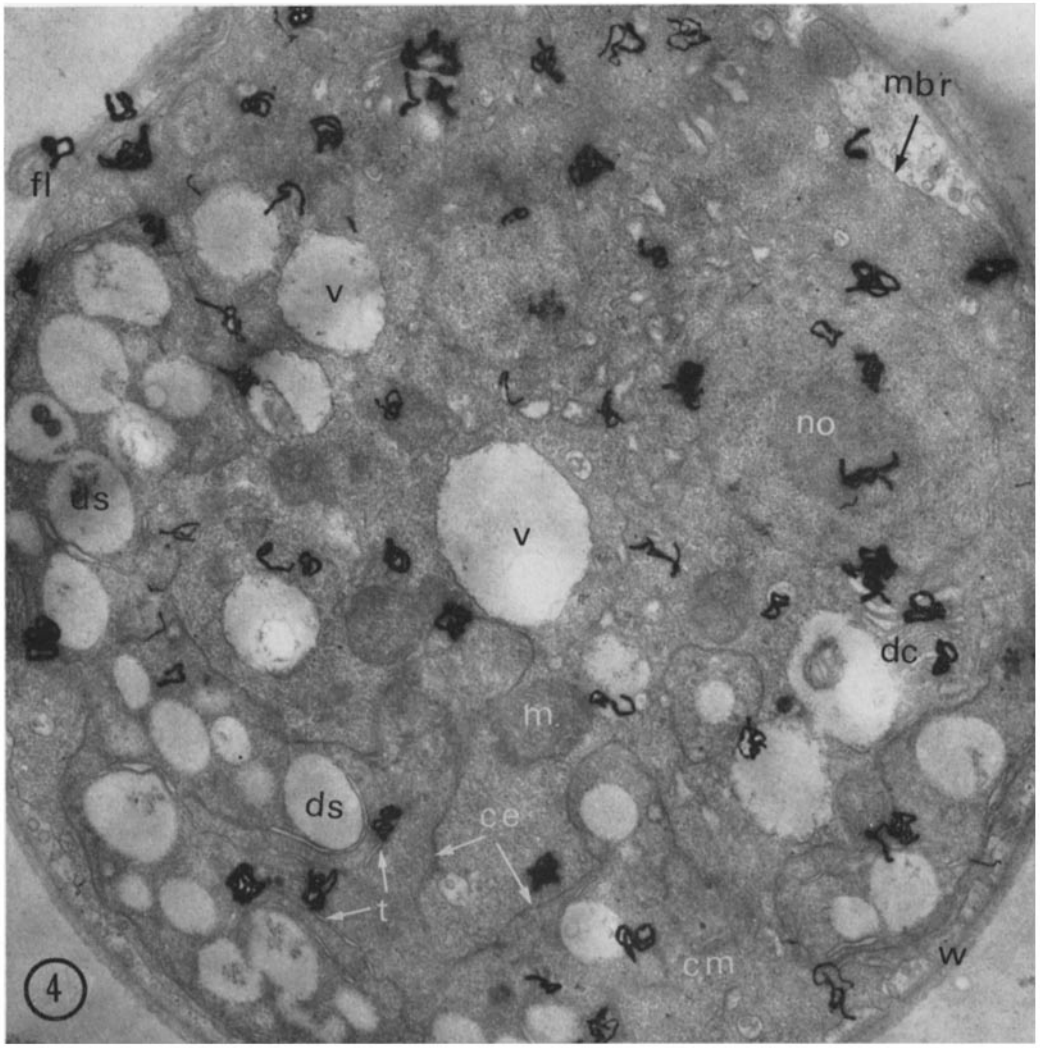
The distribution of radioautographic grains between chloroplast (*a*) and the protoplasm (*b*)[3] is shown in Table IV. The specific radioactivity of the chloroplast in the cells incubated in the dark is almost equal to that of the protoplasm and increases in the cells exposed to light as a function of greening. The ratio for *a*/*b* was between 3:1 and 4:1 after 6 hr illumination, as compared to 1.06 obtained from analysis of the distribution of randomly punctured grains. During the greening, the increase in the total amount of chloroplast photosynthetic membranes parallels the increase in chlorophyll (Fig. 9). The chloroplast volume, relative to the cell volume, can be estimated from the relative chloroplast and cell surfaces on micrographs of cell sections. It was found that during the greening the chloroplast surface is constant (Table IV). Within the chloroplast the radioautographic grains were located mostly on membranes, the distribution being significantly different from that of the randomly punctured grains (Figs. 5–7, Table V). The relative distribution of grains on the outer chloroplast envelope, photosynthetic lamellae (inner membranes), and membranes located within the pyrenoid is shown in Table VI. Although the method of analysis of grain distribution on different types of membranes used in Table VI differs from that used in Table V, the ratio of the specific activity of single to that of paired thylakoid is about the same ($\approx$ 2:1).

In a previous work it was found that the periphery of the thylakoid membranes presents a specific feature, described as a darkened outer rim (2), and which consists of discontinuous osmiophilic thickenings of the unit membrane. These thickenings appear in cross-sections as dark blebs located at the ends of discs, and in surface or tangential sections they appear as protruding globules (Fig. 7). These structures were not well preserved by the fixation method used in this work. The distribution of radioautographic grains on the outer rims or ends of thylakoids, as compared to the rest of the thylakoid cores, is shown in Table VII. It appears that the specific activity of the rims is significantly lower than that of the cores. No such difference was found when a similar analysis was carried out for randomly punctured grains (Table VII).

Within the protoplasm the specific activity of the mitochondira and nucleus is higher than that of the rest of the protoplasm, the mitochondria having the highest specific activity of all the cellular organelles (Table VIII). The specific activity of these organelles relative to that of the protoplasm increases slightly during the greening, while the

[2] Throughout this work, the term "inner membrane" refers to all the photosynthetic membranes within the chloroplast, including both single thylakoids and paired thylakoids forming grana.

[3] The term protoplasm is used to define the whole cell content without the chloroplast.



FIGURES 4–8 Radioautograms of sections from cells pulse labeled with acetate-^{3}H at different stages of the greening process. For experimental conditions see Methods. General abbreviations are the following: *ce*, chloroplast envelope; *cm*, chloroplast matrix; *dc*, dictyosome; *ds*, digested starch granule; *es*, eyespot; *fl*, flagella; *m*, mitochondria; *mb*, cell membrane; *mbr*, cell membrane retracted from the cell wall due to slight plasmolysis; *n*, nucleus; *ne*, nuclear envelope; *no*, nucleolus; *pt*, paired thylakoids; *py*, pyrenoid; *pyt*, pyrenoid tubules; *sg*, partially digested starch granules; *t*, single thylakoid; *v*, vacuole; *w*, cell wall.

FIGURE 4 Cells pulse labeled after 5 hr incubation in the dark. Notice the even distribution of the radioautographic grains over all cellular compartments. × 16,500.

specific activity of the protoplasm relative to that of the chloroplast decreases.

DISCUSSION

The aim of this work was to determine whether the growth of photosynthetic membranes is a random or a vectorial process.

This can be done by radioautographic methods if certain experimental conditions are fulfilled: specificity of the marker for membranes; net synthesis of membrane material with low turnover background; stability of the marker against transfer via biochemical exchange reactions or migration between similar structures; preservation of the

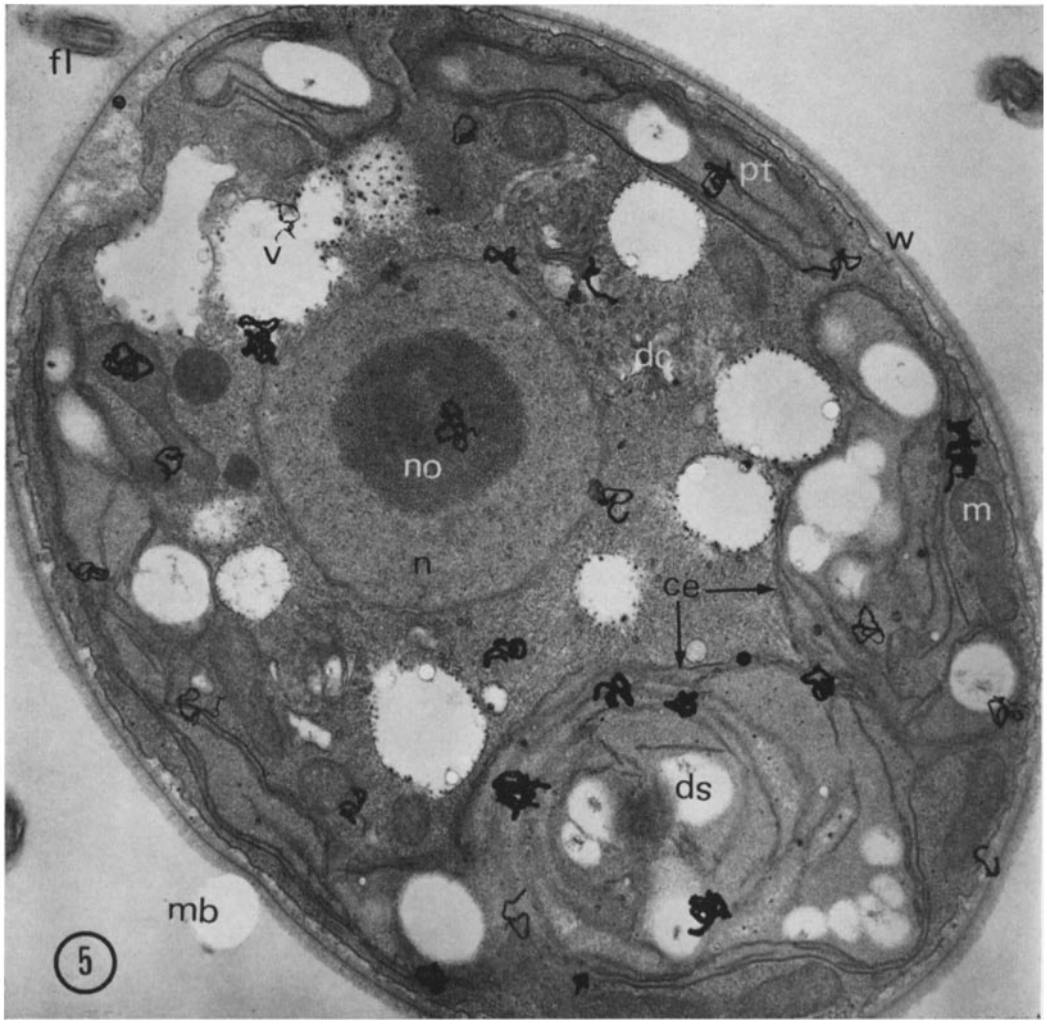


FIGURE 5 Cells pulse labeled after 3.5 hr incubation in the light. Many of the radioautographic grains are still found over the protoplasm, especially over the mitochondria. The dictyosome is also labeled. × 16,500.

structure with its marker during preparation of the sample for observation. The last two requirements are of special importance, since the building blocks forming the membrane are held together not by covalent bonds but through hydrophobic and associative interactions. Migration of lipid or lipoprotein micelles between different membranes or parts of a membrane would bring about randomization of the marker, thus invalidating any interpretation of the results. Such an occurrence should be considered possible in view of results indicating transfer of proteins from microsomes to mitochondria (19). In the following discussion we shall consider to what degree the above requirements were met.

Acetate-^3H as a Marker for Chloroplast Membrane Lipids

The photosynthetic membranes contain about 40% lipids by weight, their major components being galactosyl glycerides and sulfolipid. The glycolipids represent about 40% of the total lipid fraction, an additional 20% being represented by chlorophyll and carotenoids. The fatty acid moiety of these lipids consists mainly of polyunsaturated acids C18:2 and C18:3 (20–24).

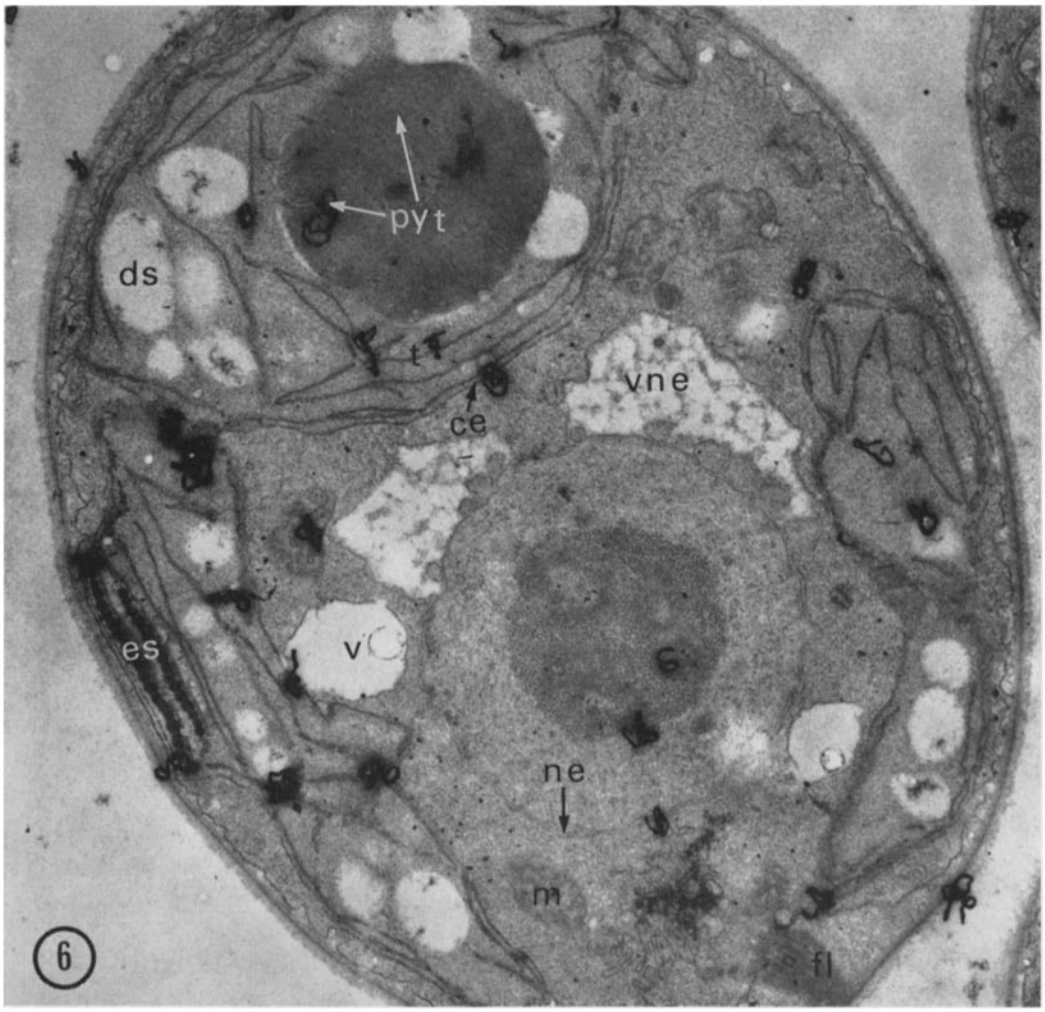


FIGURE 6 Cells pulse labeled after 6 hr incubation in the light and fixed after 1-hr chase with cold acetate. Most of the radioautographic grains are located over the chloroplast. A similar picture was found when cells were fixed immediately after the pulse. *vne*, vesiculated nuclear envelope. × 16,500.

It was previously shown that during the light-induced membrane formation in the *Chlamydomonas y-1* mutant, all these lipid constituents are preferentially synthesized (2, 4). Synthesis of chlorophyll was demonstrated to be an appropriate marker for membrane formation (2). In this work, too, a good correlation was observed between increase in the membrane content of the cells and increase in their chlorophyll content. During the linear phase of the greening, glycolipids are synthesized almost in constant proportions to chlorophyll, and acetate-^{3}H is incorporated rather specifically into these lipids, as well as into the neutral lipid fraction[4] including chlorophyll and carotenoids (4). Thus it can be concluded that acetate-^{3}H can be used as a specific marker for the synthesis of the chloroplast membranes in the *Chlamydomonas* mutant *y-1*.

Lipid synthesis during greening cannot be considered *a priori* as confined only to the chloroplast.

[4] The neutral lipid fraction is defined as the mixture of lipids including chlorophylls, carotenes, quinones, triglycerides, free fatty acids, and unidentified lipids moving with the solvent front on thin-layer chromatograms used in this work.

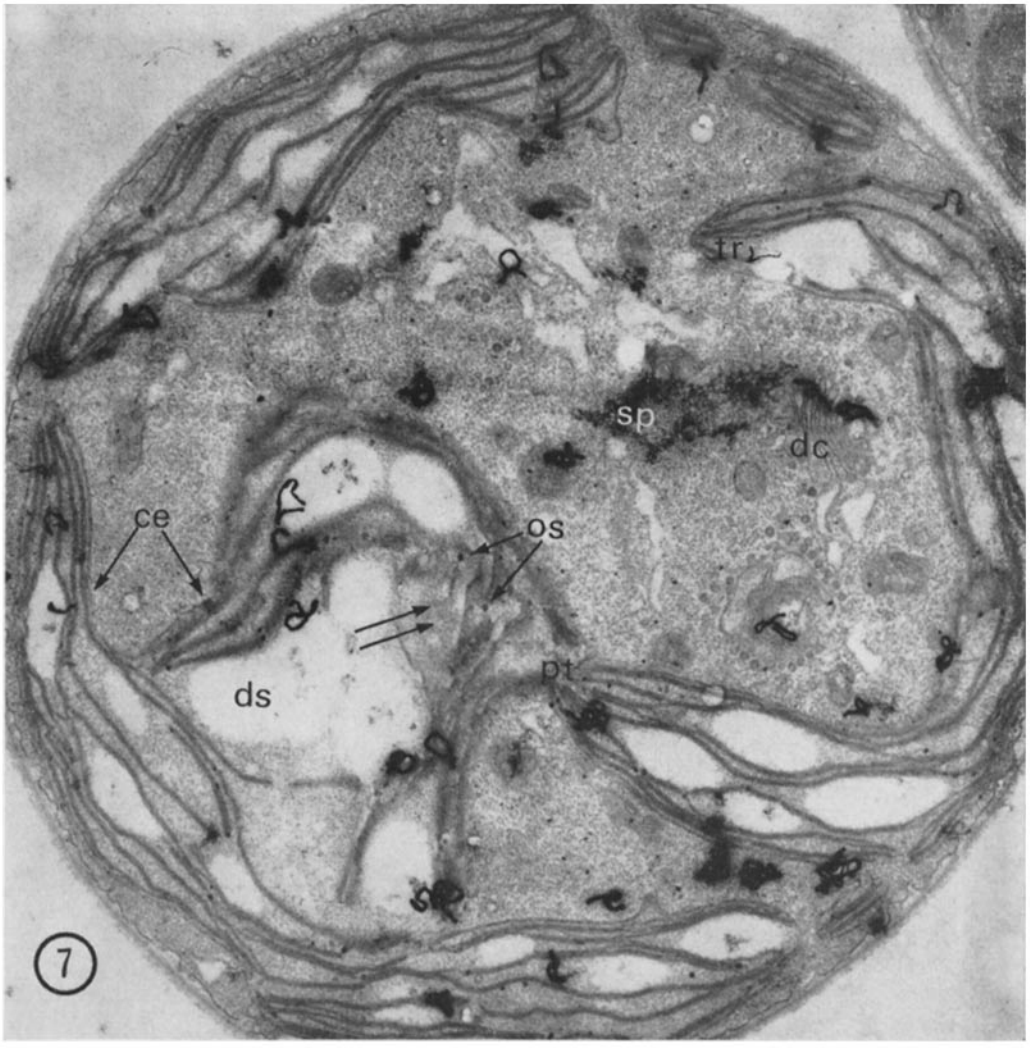


FIGURE 7 Same as Fig. 6. A section through the basal part of the cell. Notice the high concentration of radioautographic grains on the chloroplast. *os*, osmiophilic globules located at the ends of the thylakoids, better observed when membranes are sectioned tangentially (double arrow); *tr*, thylakoid outer rims; *sp*, stain precipitate, × 19,000.

It is conceivable that synthesis might also occur outside the chloroplast, the product being transferred afterwards to the chloroplast membranes. If this were the case, a short pulse labeling would indicate the site of synthesis rather than that of membrane growth. The final location of the product would be detected only after an appropriate chase period. The specific activity of the chloroplast (grains/unit area) during the greening was higher than that of the protoplasm (cf. also 2). The distribution of the radioautographic grains within the chloroplast was highly specific for membranes and was changed little during a chase of 1 hr. Thus, we can conclude that, under the experimental conditions used, the grains indicate the final location of the synthesized lipid when incorporated into a growing membrane.

Stability of the Label

The metabolic and structural stability of the lipids within a membrane determines to what extent the labeling of membranes by lipid precursors

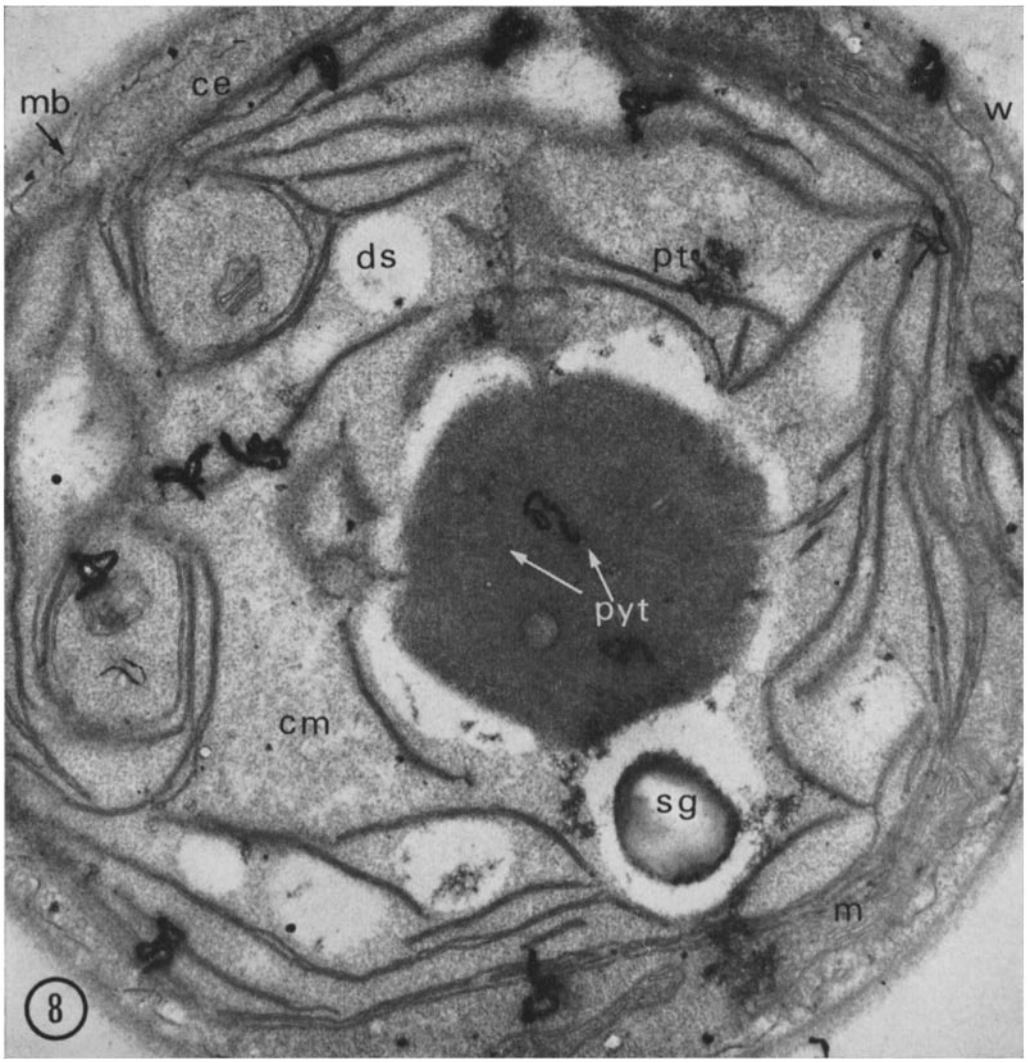


FIGURE 8 Same as Fig. 6. A section through the basal part of the cell at the level of the pyrenoid. A partially digested starch plate (*sg*) is seen near the pyrenoid. Notice that most of the radioautographic grains are located on membranes. × 24,000.

can be considered to be an indication of membrane growth. Different turnover rates for different lipid components, or for the same component in different cellular compartments, would cause variable patterns of radioautographic grain distribution. A similar situation would exist if transacylation reactions were to transfer or remove labeled fatty acids from different lipid molecules located within the same or different membranes. Even when the turnover coefficient is low, when pulse labeling is done at a stage when the total amount of membranes (V_0) is high, an important fraction of the resulting label might represent turnover ($V_0\alpha t$).

The results presented in this work indicate that the labeling due to turnover of chlorophylls and glycolipids is sufficiently small to be disregarded, $\overline{V}/V_i$ being close to unity. It was not possible to estimate the value of $\overline{V}$ or V_0 for neutral lipids, and thus we could not calculate the $\overline{V}/V_i$ ratio for this fraction. The value of α for this fraction is very low (0.6% in 15 min) and the amount of radioactivity incorporated varies in different experiments from

TABLE IV

Distribution of Radioautographic Grains over Chloroplast and Protoplasm of Cells Pulse Labeled with Acetate-^3H during the Greening Process

Treatment	Chlorophyll	Total radioactivity	Total grains counted	Grains in chloroplast of total grains/cell	Area of chloroplast of total cell area	Chloroplast specific activity* (*a*)	Protoplasm specific activity* (*b*)	*a/b*
	μg/10⁷ cells	*cpm × 10⁻⁶/10⁷ cells*		%	%			
5 hr dark 10′ pulse	1.46	9.80	332	34.2 ± 8.7	31.3 ± 6.8	1.1 ± 0.2	1.0 ± 0.1	1.2 ± 0.3
3.5 hr light 10′ pulse	2.56	18.10	356	55.5 ± 7.4	39.5 ± 4.2	1.4 ± 0.2	0.7 ± 0.1	2.0 ± 0.2
6 hr light 10′ pulse	6.10	17.50	320	67.0 ± 10.0	40.5 ± 9.0	1.7 ± 0.2	0.5 ± 0.1	4.0 ± 1.4
6 hr light 10′ pulse 1 hr chase	7.30	15.50	502	62.5 ± 4.7	40.5 ± 5.5	1.6 ± 0.2	0.6 ± 0.1	2.9 ± 1.0

Dark-grown cells with an initial chlorophyll content of 1.2 $\mu g/10^7$ cells were washed and suspended in growth medium at a final concentration of 4.2×10^6 cells/ml. The cells were incubated in dark or light (700 ft-c) at 25°C. Labeling was carried out with acetate^3H (750 $\mu Ci/\mu mole$) at times as shown in the table. Fixation, starch digestion with amylase, and radioautography were done as described in Methods.

* Specific activity is defined as % grains of total number of grains on the cell area, divided by % area of the total cell area.

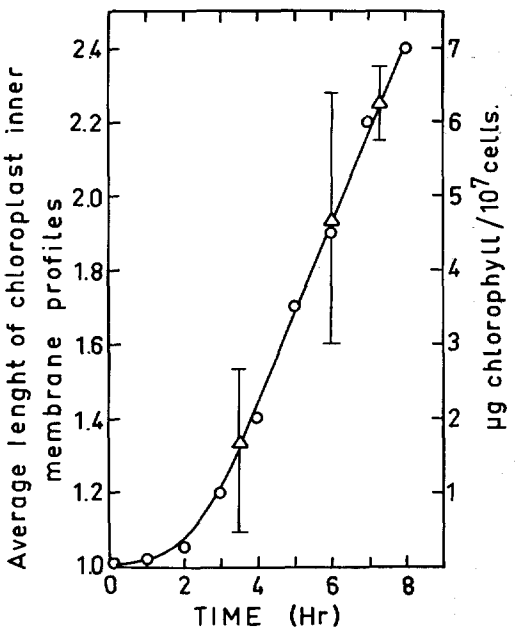


FIGURE 9 Increase in chlorophyll and chloroplast membranes during greening of dark-grown $y - 1$ cells. Membrane content is expressed as the average length of membrane profiles (μ). Since different micrographs represent different planes of cell sections including different amounts of the chloroplast area, standardization was obtained for all sections analyzed by dividing the length of the measured profiles for a given cell by the profile length of the envelope of the chloroplast area analyzed. The chloroplast envelope was used as an internal standard since it was found to have a constant length during the greening process (see Table VI). Same experimental conditions as in Table IV. ○——○, chlorophyll; △——△, length of membrane profiles.

20 to 40% of the total radioactivity incorporated during the pulse. Such a high rate of labeling can be achieved if (*a*) V_0 is very large; in this case most of the labeling would represent turnover; (*b*) the specific activity of the precursor incorporated into this fraction is higher than that of the rest of the pool; (*c*) a large net increase occurs in this fraction during greening.

Part of the radioactivity of this fraction is accounted for by the net synthesis of chlorophylls and carotenoids. In order to account for the rest as turnover, the V_0 of this fraction would have to be over twice that of all the glycolipids combined, which is rather improbable in view of the known composition of the lipids of these cells (20). If we compare the specific activities ($\overline{V}$ cpm/$\overline{V}$ μg) of different lipids we find a 1.5- to 2-fold variation between chlorophyll a, b, or glycolipids. It is possible that a higher specific activity is found in the neutral lipid fraction. This is not improbable since the radioactive acetate pool of these cells is small and can be diluted to different degrees, in different cellular compartments, with unlabeled acetate derived from the degradation of starch, which is known to occur during the greening process of *y-1* cells (1, 2). Even if some labeling due to turnover occurs in this fraction, it should not necessarily be located only in the chloroplast, since this fraction also contains lipid molecules from other cellular compartments. Thus, the possibility that most of the radioactivity in this fraction is due to net synthesis seems most probable.

Analysis of the distribution of radioactivity within different lipid fractions indicated that the majority of the lipids retained their radioactivity at the end of 30- and 60-min chases. Considering the experimental error (the recovery of radioactivity from the chromatograms was 70 to 100%), a loss of radioactivity between 0 and 30% for most of the lipids is in agreement with the loss of radioactivity from the total lipid fraction. Since the relative radioactivity of the different lipids varied between about 2 and 50% (Table II) and no major changes were observed in these values at the end of the chase period, we can consider that transacylation between different lipid species is minimal, although the possibility of such transfer within the same species of molecules cannot be excluded. Also, transfer or migration of lipids between different structures can be considered as minimal, since the specific activity of various types of membranes or cellular compartments was found to differ within a wide range and to remain constant for each one after chase. If migration or transfer occurred, we would expect a randomization of the label between different types of membranes or cellular compartments. This was not observed. However, incorporation is expected to be at random in the dark control, as clearly demonstrated by the distribution of the radioautographic grains.

Distribution of Radioautographic Grain on Cellular Components

The mitochondria, which represent in sections 8–10% of the protoplastic area, had a specific activity about twice that of the nucleus and five times that of the remaining protoplasm. Since no loss of radioactivity was observed during chase, it

TABLE V

Distribution of Authentic and Randomly Punctured Grains on Chloroplast Lamellae

Type of grains	Total number of grains counted	Total number of grid squares on chloroplast	Single membranes: Grid squares with single membrane	Single membranes: Grains on grid squares with single membrane	Single membranes: Specific activity*	Paired membranes: Grid squares with paired membrane	Paired membranes: Grains on grid squares with paired membrane	Paired membranes: Specific activity*	a: Grains on membranes relative to total grain on chloroplast	a/%: Squares containing membranes relative to number of squares on chloroplast
									%	
Radioautographic grains	40	1378	336	21	2.15	411	11	0.92	80	1.60
Randomly punctured grains	263	1378	336	42	0.66	411	55	0.70	37	0.72

Radioautograms were from sections of cells pulse labeled after 6 hr greening and fixed after 1-hr chase (see Table IV). For details of analysis see Methods.

* The total number of grains over the chloroplast and the total number of grid squares occupied by the chloroplast are taken as 100%. The specific activity is defined as the percentage of grains found over a specific structure within the chloroplast divided by the percentage of squares occupied by that structure.

appears that the incorporation represents net synthesis also for components of these organelles.

The area of the chloroplast, relative to the total cell area, and the length of its envelope profile can be considered proportional to the chloroplast volume and surface (25, 26). The measurements of these parameters showed that the chloroplast volume and surface are constant during the greening process, the volume representing about 40% of the cell volume. The specific activity of the chloroplast, as compared with that of the remaining protoplasm, increased as the greening proceeded up to a ratio of 4:1, indicating that most of the incorporated radioactivity is located inside the chloroplast (cf. 1). Incorporation of radioactivity into the outer chloroplast envelope[5] in the absence of growth might be partially due to turnover. The specific activity of the chloroplast envelope and that of the photosynthetic lamellae did not differ significantly, and no change was detected during chase for both types of membranes. This would indicate that, in agreement with previous findings (2), no precursor-product relationship exists between these two types of membranes in the *Chlamydomonas y-1* mutant. On the basis of electron microscopical observations, it was proposed that the chloroplast lamellae in algae such as *Euglena* develop from invagination of the inner layer of the chloroplast envelope (27). It was suggested in the past that the different ways of membrane growth are correlated with the state of differentiation of the plastid (2). However, in a recent study in which improved fixation techniques were used, it was shown that the proplastid in *Euglena* contains a prolamellar body, and that the chloroplast membranes develop in connection with it and out of the ground substance within the proplastid.[6]

The pyrenoid located in the chloroplast of the *Chlamydomonas* cells is traversed by a set of radially disposed tubules connected with the lamellar photosynthetic apparatus (1, 2, 28). The specific activity of this organelle as a whole is lower than that of the chloroplast. The total radioactivity incorporated into the pyrenoid represents about 12–19% of the total radioactivity of the chloroplast. The pyrenoid tubules also grow during the greening, and most of the pyrenoid grains coincide with these structures. If we were to attribute all the

[5] The chloroplast envelope consists of two layers of unit membranes: an external membrane similar in appearance to the outer plasma membrane, and an interior envelope similar to the photosynthetic lamellae. Both layers are very close to each other and fused in places (1, 2). Because of insufficient resolution, radioautographic grains located on the chloroplast envelope cannot be ascribed to one of these two layers and, for the purpose of this discussion, they are considered as a unit.

[6] J. A. Schiff, personal communication.

TABLE VI

Radioautographic Grain Distribution in Different Types of Membranes in the Chloroplast of Cells Labeled with Acetate-^3H during the Greening Process

Treatment	Chloroplast outer membranes		Chloroplast inner membranes			Pyrenoid membranes	
				Specific activity of the thylakoids			
	Length	Specific activity*	Total length	single	paired	Length	Specific activity
	μ/cell		*μ/cell*			*μ/cell*	
3.5 hr light 10′ pulse	34.00 ± 6.70	0.80 ± 0.17	43.50 ± 6.10	1.19 ± 0.15	0.71 ± 0.25	3.75 ± 1.70	3.13 ± 1.34
6.0 hr light 10′ pulse	32.50 ± 3.27	0.73 ± 0.14	59.00 ± 16.50	1.30 ± 0.22	0.72 ± 0.35	5.20 ± 1.37	3.05 ± 0.94
6.0 hr light 10′ pulse 1.0 hr chase	33.00 ± 2.17	0.90 ± 0.12	75.00 ± 16.80	1.25 ± 0.25	0.78 ± 0.30	6.95 ± 1.71	3.30 ± 2.40

* Specific activity was calculated for each type of membrane as % of grains on the membrane profiles of total grains in the chloroplast, divided by the length of profiles (μ) of total length of all chloroplast membrane profiles.

TABLE VII

Distribution of Radioautographic Grains between Cores and Periphery of Thylakoids

Treatment	l Total length of chloroplast inner membranes	n Total number of membrane 'ends'	Total grains		Specific activity of thylakoid cores		Specific activity of thylakoid periphery			
			Radioautography	Randomly punctured	*a*	*a′*	*b*	*b′*	*a/b*	*a′/b′*
	μ									
3.5 hr light 10′ pulse	456	972	96	257	1.28 ± 0.16	0.87 ± 0.1	0.76 ± 0.21	1.24 ± 0.20	2.64 ± 1.20	0.81 ± 0.21
6.0 hr light 10′ pulse	580	1254	78	144	1.26 ± 0.16	0.91 ± 0.10	0.83 ± 0.24	1.24 ± 0.24	2.52 ± 0.98	1.10 ± 0.23
6.0 hr light 10′ pulse 1 hr chase	830	1802	143	497	1.32 ± 0.17	1.05 ± 0.12	0.84 ± 0.23	1.22 ± 0.37	2.52 ± 0.94	1.15 ± 0.28

Calculation of grain distribution on the periphery of thylakoids was as follows: the fraction length (%) of membrane ends of total membrane length was calculated as $(0.2)^2 nl^{-1}$ when 0.2 represents the width of a square grid of 0.2 μ (4 mm at × 20,000); *l* is the total measured length of membrane profiles in μ and *n* the total number of membrane ends. The fraction (%) of grains on membrane ends of total number of grains on membranes was calculated as the number of membrane ends of 0.2 μ length containing a grain (the center of a minimal circle circumscribing a grain).

Specific activity is defined as the ratio % grains to % length.

a and *b*—authentic radioautographic grains; *a′* and *b′*—randomly punctured grains. The confidence interval (SEM × *t*) was 80% ($t = 0.9$).

TABLE VIII

Distribution of Radioautographic Grains over the Nucleus, Mitochondria, and Pyrenoid of Cells Pulse Labeled with Acetate-^3H during the Greening Process

Treatment	Nucleus			Mitochondria			Pyrenoid		
	Protoplasm grains	Protoplasm area	Specific activity*	Protoplasm grains	Protoplasm area	Specific activity	Chloroplast grains	Chloroplast area	Specific activity
	%	%		%	%		%	%	
5 hr dark 10′ pulse	18.8 ± 11.6	13.4 ± 3.1	1.3 ± 0.6	16.8 ± 6.1	8.0 ± 1.8	2.1 ± 0.7	—	—	—
3.5 hr light 10′ pulse	35.3 ± 7.3	20.0 ± 4.0	1.8 ± 0.2	22.0 ± 13.0	9.1 ± 3.5	2.4 ± 1.2	11.6 ± 4.1	10.8 ± 2.8	1.1 ± 0.2
6 hr light 10′ pulse	40.0 ± 14.0	20.0 ± 3.8	2.0 ± 1.1	24.0 ± 9.1	7.2 ± 3.2	3.4 ± 1.2	16.0 ± 6.6	15.1 ± 3.5	1.1 ± 0.4
6 hr light 10′ pulse 1 hr chase	31.9 ± 7.3	19.1 ± 3.6	1.8 ± 0.2	28.6 ± 6.2	8.3 ± 1.5	3.6 ± 0.9	19.6 ± 13.0	13.8 ± 3.7	1.4 ± 0.4

Same experimental conditions as in Table IV.

* Specific activity is defined as % grains:% area.

TABLE IX

Average Length of Single and Paired Thylakoids during the Greening Process

Treatment	Total length of thylakoids	Average length of thylakoids*	Total length of single thylakoids	Total length of paired thylakoids
	μ/cell	*μ*	*μ/cell*	*μ/cell*
3.5 hr light 10′ pulse	54.6 ± 6.2	0.83 ± 0.29	37.8 ± 3.2	16.7 ± 4.6
6.0 hr light 10′ pulse	64.5 ± 10.4	0.91 ± 0.31	36.8 ± 6.6	27.7 ± 10.7
6.0 hr light 10′ pulse	76.0 ± 12.7	0.96 ± 0.35	42.0 ± 6.7	34.0 ± 7.4

* Defined as the total length of inner chloroplast membranes divided by ½ the number of total membrane ends. The data are expressed as mean value ± SEM × t when t = 0.9 (the confidence interval is 80%).

radioactivity of the pyrenoid to this system, its calculated relative specific activity would be higher than that of all other chloroplast membranes. In this case, since no loss of radioactivity was observed during chase, we should conclude that the pyrenoid tubules do grow within the organelle but are not precursors or products of the extrapyrenoidal lamellae.

Growth of the Chloroplast Lamellar System

In the experiments reported in this work, pulses were given at two time points during the linear phase of membrane synthesis. In both cases most of the lamellae present in the chloroplast were single thylakoids, and only a few grana or paired thylakoids were found even after 6 hr of light. This is in agreement with the level of chlorophyll synthesized (∽ 7 $\mu g/10^7$ cells), since it was reported that, in this system, pairing and grana formation predominate above 6 μg chlorophyll/10^7 cells (2). Analysis of grain distribution disclosed that single thylakoids grow faster than those which are paired or form grana. The average length of single thylakoids was rather constant from 3 to 6 hr of greening. During this time the total length of membranes increased and resulted in the pairing of the newly formed lamellae (Fig. 9, Table IX). This would imply that the specific activity of paired membranes increases during the chase, if we consider the single thylakoids of a higher specific activity to be the precursor of the paired membranes. The results obtained in this work indicate that during chase of 1 hr there is no change in the specific activity of either single or paired thylakoids. Since the total increase in pairing was very small, the expected change in specific activity is below the limit of resolution of the method used. It is possible that the lower rate of labeling of paired membranes is due to the fact that less area of their surface is directly available for contact with the chloroplast matrix.

The rate of labeling of the thylakoid cores seems to be significantly different from that of the thylakoid outer rim or ends; the rate of labeling of the thylakoid cores is almost twice as high. This difference is also emphasized by the results of analysis of the distribution of randomly punctured grains. In a previous work it was reported that the edges or rims of the thylakoids terminate in special structures, differing from those of the rest of the disc (2). It was thought that these irregularities might be correlated with points of growth. In the method used for preparation of cells for radioautography in this work, these osmiophilic blebs were not well preserved. It is possible that labeled material from this region was specifically lost or that differences in the properties of this membrane region somewhat hindered the addition of new material. The present data do not allow us to draw a conclusion on this point.

The principal conclusions that may be drawn from this work are that chloroplast membranes are formed by random incorporation of new material within preexisting types of membranes, and that different types of membranes grow at different rates. It is attractive to think that this might be the case for other types of biological membranes.

The intimate structure of biological membranes is still a matter of controversy as to whether membranes are built up as a bimolecular leaflet of lipid and protein (29) or as a continuum of homogeneous lipoprotein particles (30, 31). The fact that the aggregation of membrane constituents is due to hydrophobic interactions rather than to covalent bonding allows us to ascribe to membranes a great deal of structural and chemical flexibility; this flexibility forms the basis of many cellular physiological activities, and the growth of

such structures by intussusception of new units can be easily envisaged. Different types of membranes exhibit varying degrees of metabolic and structural dynamics from the 'inert' myelin sheet to the pinocytotic and phagocytic vacuoles, zymogen granules of exocrine glands, etc., in which membrane-membrane interaction or fusion is a fundamental prerequisite of their function (32, 33). The photosynthetic lamellae represent a homogeneous system of a certain chemical and structural rigidity. The fact that the mechanism of growth of these membranes is of the random-intussusception type makes it even more probable that this mechanism might be operative also for other types of biological membranes.

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