BIOGENESIS OF CHLOROPLAST MEMBRANES

IV. Lipid and Pigment Changes during Synthesis of Chloroplast Membranes in a Mutant of *Chlamydomonas reinhardi y-1*

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ABSTRACT

The glycolipid, phospholipid, pigment, and fatty acid content in whole *y-1* cells during the greening process have been investigated. The time course of their changes indicates that phosphatidyl glycerol and glycolipids are the main lipids synthesized specifically during illumination of dark-grown cells, concomitant with an increase in the polyunsaturated C18:2 and C18:3 fatty acids. The pigment complex of light-grown cells consists mainly of chlorophylls a and b, lutein, β-carotene, violaxanthin, and neoxanthin. During the greening process, chlorophylls a and b are synthesized in constant proportions (ratio a/b equals 2.6), β-carotene and violaxanthin do not change significantly, and lutein and neoxanthin increase. The molar ratios of the different lipids and pigment to total chlorophyll during greening has been calculated. It was found that during the initial phase of greening when chlorophyll is synthesized at increasing rates, the molar ratios of various lipids and pigments to chlorophyll decrease and tend to become constant when chlorophyll and membrane synthesis proceed at constant rates. The implication of these findings with respect to the concept of membrane assembly through a spontaneous single step process is discussed

INTRODUCTION

In a previous work (1) the process of light-induced synthesis of photosynthetic membranes in dark-grown *Chlamydomonas y-1* cells was investigated. On the basis of the kinetics of the increase in total chlorophyll, membrane content, and photosynthetic activity, it was suggested that the formation of the photosynthetic membranes from newly synthesized and preexisting building blocks might occur through a "single step assembly" mechanism. This would imply that the chemical composition of the newly formed membranes (in terms of lipid constituents, pigments, and proteins) would be constant throughout the process. In the present work the composition and content of lipids, fatty acids, and pigments during the greening process were analyzed. Since it has not yet been possible to obtain pure chloroplast preparations from *Chlamydomonas* cells, the work was carried out with whole cells. Following the onset of illumination the rate of photosynthetic membrane synthesis increases and becomes constant after about 2.5–3.5 hr. While changes in the molar ratio of lipid constituents are to be found during the initial phase of synthesis, a

marked tendency towards constant molar proportions between chlorophylls a and b, lutein, total glycolipids, and polyunsaturated fatty acids was found during the phase when membrane synthesis proceeded at a constant rate.

MATERIALS AND METHODS

Chlamydomonas reinhardi y-1 cells were grown in the dark as reported previously (2). The initial chlorophyll content in all greening experiments was below 2 $\mu g/10^7$ cells. For greening experiments, the cells were harvested by centrifugation, washed, and resuspended in fresh growth medium at a concentration ranging from 5×10^6 to 1.2×10^7 cells/ml and exposed to light as described (1).

Preparations of Extracts

For chromatographic analysis, the lipids were extracted with chloroform-methanol (2:1 v/v) at 4°C for 10 min by using portions of 1 ml solvent for 10^7 cells and repeating the extraction until the residue was colorless. Samples of cells from different times in a greening experiment were either extracted immediately or stored under nitrogen at −20°C overnight before extraction. The chloroform-methanol extract was evaporated under nitrogen, and the residue was dissolved in a small volume of chloroform-methanol and stored under nitrogen in the dark at 4°C until use.

Separation and Analysis of Pigments

Thin-layer chromatography (TLC) on glass plates (20 × 20 cm) was performed according to Hager and Bertenrath (3). Carotenoids were completely extracted from the absorbent with chloroform-methanol. The extract of each spot was dried under nitrogen and redissolved in hexane or petrol ether (bp 80–100°C), for spectrophotometric analysis, and in mixtures of aqueous methanol-hexane for identification according to their partition coefficients (4). For spectrophotometry a Cary Model 14 spectrophotometer was used (Cary Instruments, Monrovia, Calif.). Chlorophyll could not be extracted com-

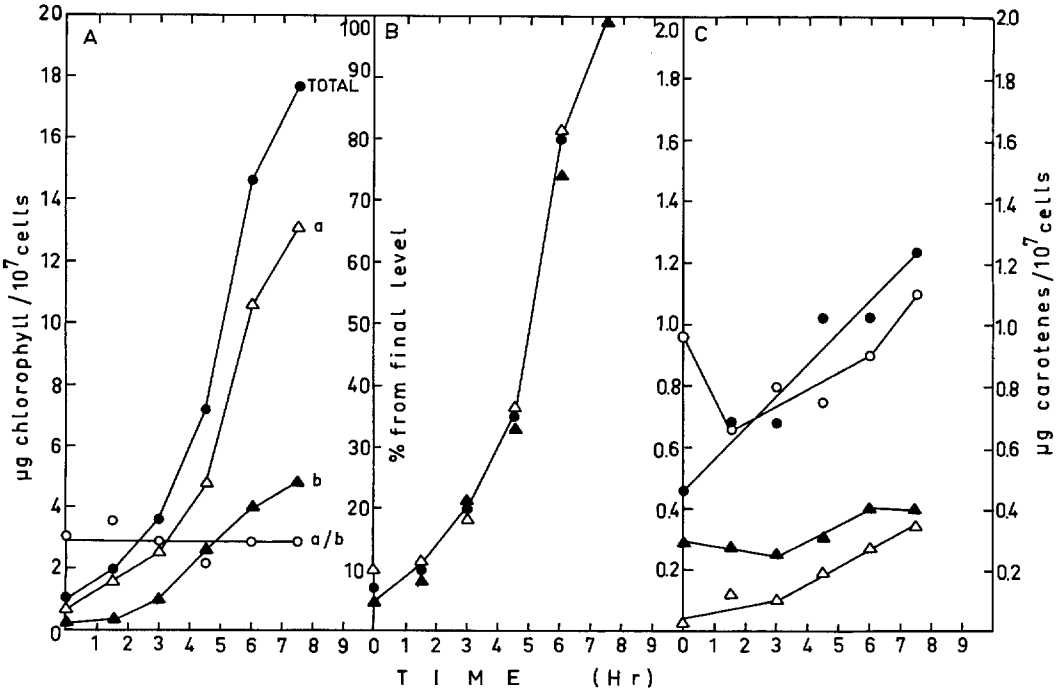

FIGURE 1 Changes in different pigments' content during the greening process of *y-1* cells. Dark-grown cells were washed and suspended in growth medium at a final concentration of 4×10^6 cells/ml and incubated at 25°C in light (700 ft-c). *A*. Increase in chlorophylls a, b, total chlorophyll, and the ratio of chlorophyll a/b as a function of time. *B*. Same as in Fig. 1 *A*. The average of 10 different experiments is plotted as % of change from the final level attained in each experiment. △——△, chlorophyll a; ▲——▲, chlorophyll b, ●——●, total chlorophyll. *C*. Increase in different carotenoids as a function of time. ●——●, lutein; ○——○, β carotene; ▲——▲, violaxanthin; △——△, neoxanthin.

pletely from the thin-layer absorbent used in this and other TLC methods tested (5). The recovery was between 40 and 70% in different experiments. Therefore, chlorophyll was quantitated in 80% acetone extracts of the whole cells by the absorbancy at 649, 652, and 665 mμ (6). When acetate-^{3}H was used for incorporation experiments, the spots were scraped from the glass plates, suspended in Bray phosphorus solution, and counted in a scintillation counter (Packard Tricarb Model 3003, Packard Instrument Co., Inc., Downers Grove, Ill.) with an efficiency of ≈15%. Quenching was measured in each sample by the addition of known internal standards.

Separation and Analysis of Lipids

For analysis of nondeacylated lipids the chloroform-methanol extracts (see above) were dried under nitrogen and redissolved in 80% acetone. The insoluble colorless precipitate was removed by centrifugation at 3,000 *g* for 5 min, the clear solution was reevaporated under nitrogen, and the residue was dissolved in chloroform-methanol. The dissolved lipids were washed free of nonlipid components by the procedure of Munro and Downie (7). Thin-layer chromatography of nondeacylated lipids was carried out on 20 × 20 cm silica gel G plates containing $CaSO_4$ as a binder, with chloroform-methanol-water mixture (65:25:4, by volume) and containing 0.05% butylated hydroxy toluene (w/v) as a solvent (8). Iodine vapors were used for spot detection. The radioactivity content of the whole lipid extracts or of the separated spots was estimated as described above. Galactose-containing lipids and sulfolipid were determined according to Rougham and Batt (9), while phosphate content was determined as described by Ames (10). Recovery of lipids from chromatograms was above 80%.

Analysis of Fatty Acids

The total fatty acid content of whole cell extracts was estimated following methylation by the hydroxamate method (11). Methylation was carried out at 100°C for 2 hr in sealed tubes with anhydrous methanol containing 5% hydrochloric acid (12). The methylation was complete as tested by thin-layer chromatography on silica gel G plates by using petrol ether (bp 60–80°C), diethylether and glacial acetic acid (90:10:1 v/v) as solvent. The methylated fatty acids were extracted from the methylation mixture by adding NaCl to a final concentration of 0.6% and extracting twice with petrol ether (bp 60–80°C). The solvent phase was dried over Na_2SO_4. Fatty acids were separated and analyzed by gas-liquid chromatography with the aid of a Packard GLC apparatus equipped with a hydrogen flame detector. Two different columns were used: (*a*) 19% ethylene glycol-succinate 100/120 mesh with gas chrom P (Applied Science Laboratories Inc., State College, Pa.) operated with argon 85 ml/min, air 700 ml/min and hydrogen 85 ml/min at a column temperature of 170°C and detector temperature of 179°C, running

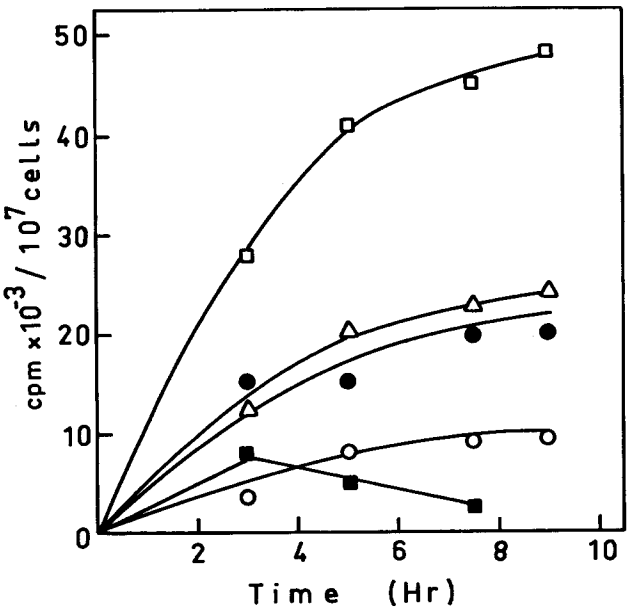

FIGURE 2 Incorporation of acetate-^{3}H into different pigments during the greening process of *y-1* cells. Dark-grown cells were washed and resuspended in growth medium at a final concentration of 10^7 cells/ml. Acetate-^{3}H was added (3 μCi/μmole) and the cells were incubated in the dark or light (700 ft-c). The initial chlorophyll content was 1.76 μg/10^7 cells and did not change during incubation in the dark. The final chlorophyll content in the cells incubated in the light was 21.1 μg/10^7 cells. The total radioactivity in the pooled pigments was 4.0 × 10^4 cpm/10^7 cells in the dark and 14.4 × 10^4 cpm/10^7 cells in the light. The results are expressed as the difference between light and dark values for each pigment. □, total chlorophyll; ●, lutein; △, neoxanthin; ○, violaxanthin; ■, β-carotene.

time, 1 hr; and (*b*) 10% Apiezon L on Chromosorb W 60–80 mesh (Packard) with argon 80 ml/min, air 800 ml/min, and hydrogen 100 ml/min at a column temperature of 197°C and detector temperature of 200°C, running time about 6 hr.

Fatty acids were identified by known markers (Applied Sciente Laboratories) and also by running samples after hydrogenation according to Burchfiled and Storrs (13).

The number of moles of each fatty acid was determined by measuring the relative area of each peak and dividing by the molecular weight.

Acetate-^{3}H, 2 cmmole was purchased from the Radiochemical Centre, Amersham, England. All reagents and solvents used in this work were of analytical grade.

RESULTS

Light-grown *y-1* cells contain chlorophylls a and b, lutein, β-carotene, and relatively small amounts of two additional carotenoids identified according to their chromatographic and spectrophotometric properties and their partition coefficients in different solvents as neoxanthin and violaxanthin (14). The weight ratio of chlorophyll to carotenoids in light-grown cells was about six. Dark-grown cells contain relatively high amounts of β-carotene, while the chlorophyll and other carotenoids content is reduced. The weight ratio of chorolphyll to carotenoids was about two.

During the greening process chlorophylls a and b were synthesized in constant proportions and lutein and neoxanthin content were increased in amount, whereas the content of β-carotene and violaxanthin varied widely around the initial level present at the start of the greening experiments. The kinetics of the synthesis of these pigments during the greening process is shown in Fig. 1. Changes in carotenoids, especially β-carotene, were small as compared with the initial background content. Quantitative analysis of chlorophylls a and b was done spectrophotometrically in total extracts due to difficulties in extracting the pigments from thin-layer chromatographic plates. As a control for the data obtained by this

TABLE I

The Incorporation of Acetate-^{3}H into Various Lipids and the Increase in Phospholipids and Glycolipids during the Greening of Dark-Grown y-1 Cells

Lipid	R_f value	Dark	Light	Dark	Light	Dark	Light
		cpm/10^7 cells		*μmole phosphate/10^9 cells*		*μmole sugar/10^9 cells*	
NL*	0.94	2,700	32,000	—	—	—	—
MG	0.79	2,400	60,100	0.05	0.05	2.72	4.05
PEA + X	0.70	4,950	10,000	0.05	0.05	0.05	0.05
DG	0.61	7,350	20,400	0.05	0.05	2.11	3.17
PG	0.54	1,300	6,300	0.64	1.49	0.05	0.05
PC	0.49	—	—	0.40	0.14	0.05	0.05
SL	0.32	388	2,040	0.09	0.11	0.24	0.51
PI	0.24	1,100	1,350	0.06	0.09	0.05	0.05
PS	0.15	490	530	0.08	0.08	0.05	0.05
Total		20,678	132,720	1.37	1.96	5.07	7.73

The data are derived from two different experiments. For phosphate and galactose measurements, dark-grown cells containing 0.56 μg chlorophyll/10^7 cells were washed and suspended in growth medium at a final concentration of 1.48 × 10^7 cells/ml. The final chlorophyll content in the light-incubated cells was 11.0 μg/10^7 cells.

Cells containing 1.76 μg chlorophyll/10^7 were used for radioactivity measurements. Acetate-^{3}H (3 μCi/μmole) was added to the cell suspension containing 10^7 cells/ml. The final chlorophyll content in the light-incubated cells was 21.1 μg/10^7 cells. Incubation was carried out at 25°C for 9 hr both in the dark and light (700 ft-c).

* The following abbreviations are used in this work: FA, fatty acids (the figure before the colon indicates the number of carbon atoms in the chain; the figure after the colon, the number of double bonds); MG, monogalactosyl diglyceride; DG, digalactosyl diglyceride; SL, sulfolipid; PS, phosphatidyl serine; PG, phosphatidyl glycerol; PI, phosphatidyl inositol; PC, phosphatidyl choline; PEA + X, phosphatidyl ethanolamine + unidentified lipid; NL, neutral lipids (including pigments).

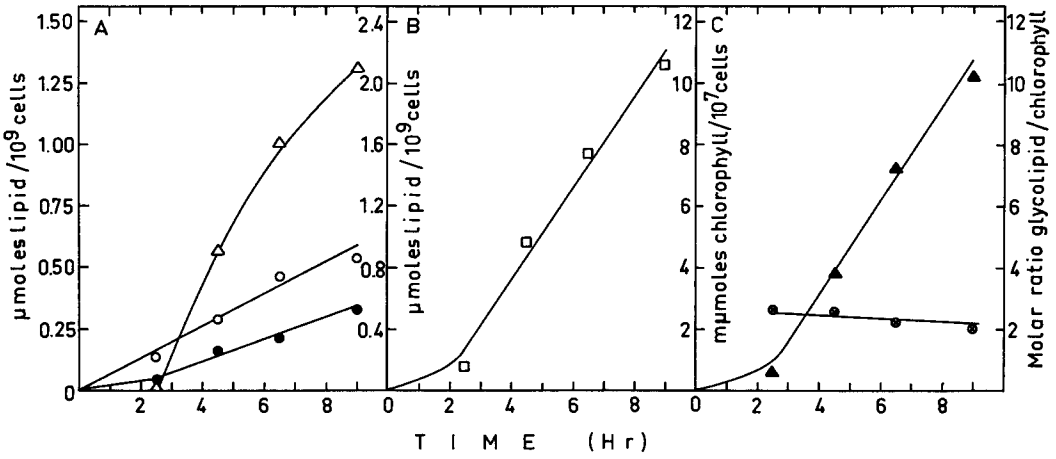

FIGURE 3 Kinetics of increase in glycolipid content and their ratio to chlorophyll during greening of dark-grown *y-1* cells. *A*. Increase in different glycolipids as a function of time. △——△, *MG*; ○——○, *DG*; ●——●, *SL*. *B*. Increase in total glycolipids as a function of time. *C*. Increase in chlorophyll (▲——▲) and ratio of total glycolipid to chlorophyll (⊗——⊗) as a function of time. Same experimental conditions as in Table I. All data are represented as the difference light-dark.

method, the incorporation of acetate-^{3}H into the lipids and pigments was also measured. The results show that little radioactivity is incorporated into β-carotene and violaxanthin as compared with lutein and neoxanthin (Fig. 2).

The incorporation of acetate-^{3}H and the measurement of changes in sugar and phosphate content of different lipids of *y-1* cells during the greening period show that, in addition to the neutral lipid fraction which includes the pigments, mainly four lipids are rapidly synthesized. These lipids are mono- and digalactosyl diglycerides, sulpholipid, and phosphatidyl glycerol (Table I).

The kinetics of glycolipid synthesis during the greening process is shown in Fig. 3. The results indicate that, whereas the different glycolipids are synthesized in different proportions, the ratio of total glycolipids to chlorophyll is almost constant.

The changes in fatty acid composition of dark-grown *y-1* cells occurring during incubation for 9 hr in the dark or in the light are shown in Table II. A total increase of about 80% was found in light-incubated cells as compared with the 30% increase in the dark control. Whereas saturated and monounsaturated acids showed only slight changes independent of illumination, a marked light-dependent increase was observed in the polyunsaturated fatty acids (18:2 and 18:3). The changes in fatty acid composition and the ratio

TABLE II

Changes in Fatty Acids Content During the Greening of Chlamydomonas reinhardi y-1 Cells

No. of carbon atoms in fatty acids	*hr* 0	9 (dark)	9 (light)
		µmoles ester/10⁹ cells	
14:0	2.75	2.07	1.45
16:0	6.70	9.95	11.10
16:1	1.06	1.61	2.11
16:2	0.50	0.50	0.50
16:3	1.88	2.92	3.55
18:0	1.32	1.92	2.32
18:1	3.07	4.65	5.00
18:2	3.75	5.35	10.65
18:3	4.55	6.10	9.75
Unidentified*	0.96	1.43	1.81
Total	26.04	36.00	47.74

Dark-grown cells were washed and incubated at 25°C in growth medium at a final concentration of 5.6 × 10^5 cells/ml in the dark or light (700 ft-c). Initial and final chlorophyll content in the light was 1.95 and 22.5 µg/10^7 cells, respectively.

For the procedure of lipid extraction, fatty acid methylation, separation and determination of esters, see Methods.

* Unidentified unsaturated acid with a retention value (16:0 = 1) of 4.7 on the ethylene glycol-succinate column.

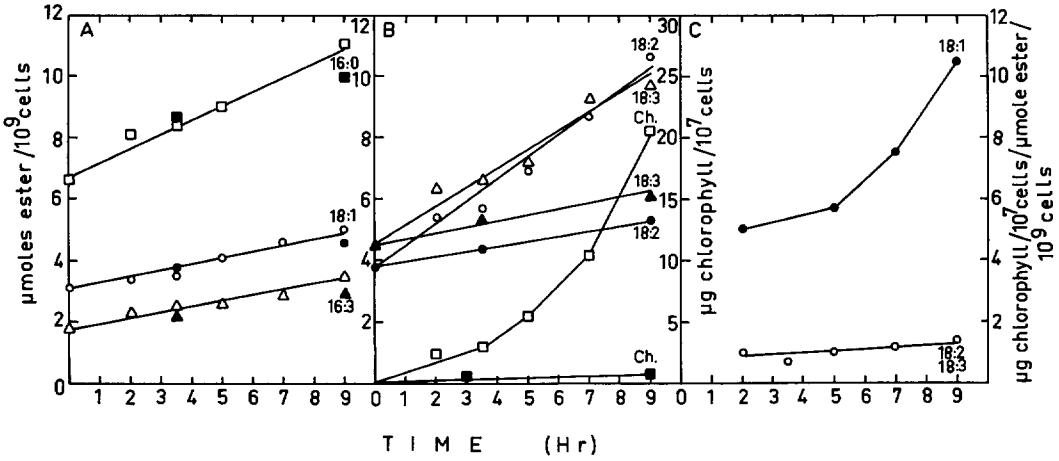

FIGURE 4 Changes in fatty acid composition and ratio of chlorophyll to polyunsaturated acids during the greening of *y-1* cells. *A*. Fatty acids whose changes were unaffected by light. *B*. Increase in chlorophyll and fatty acids affected by light. *C*. Ratio of chlorophyll to polyunsaturated fatty acids (18:2 + 18:3) as compared with saturated acid (18:1). Open figures, incubation in light; closed figures, incubation in dark. *Ch.* = chlorophyll. Same experimental conditions as in Table II.

TABLE III

Molar Ratio of Various Lipids and Pigments to Total Chlorophyll during Light-Induced Synthesis of Membranes in Chlamydomonas reinhardi y-1

Compound	Light												Data from literature (21)
	1.5	2	2.5	3	3.5	4.5	5	6	6.5	7	7.5	9	
						hr							
Chlorophyll a*	0.74			0.75		0.70		0.74			0.72		0.70
Chlorophyll b*	0.26			0.25		0.30		0.26			0.28		0.30
Lutein*	0.60			0.32		0.26					0.11		0.09
MG‡						1.35			1.25			1.15	1.50
DG‡			1.87			0.71			0.55			0.50	0.62
SL‡			0.45			0.33			0.24			0.27	0.20
Total glycolipids			2.24			2.26			1.90			1.82	2.30
FA C18:2§		6.70			5.75		4.05			3.50		2.28	—
FA C18:3§		4.15			4.65		3.45			2.70		1.82	—

The data are computed from three different experiments. The initial and final chlorophyll contents ($\mu g/10^7$ cells) were:
* Initial, 1.0, final, 18.0.
‡ Initial, 0.56, final, 11.0.
§ Initial, 1.9, final, 22.5.

of total unsaturated acids to chlorophyll during the greening process are shown in Fig. 4.

On the basis of all the above mentioned data, the molar ratios of the different components tested to total chlorophyll were calculated for different time points during the greening process (Table III). The results show that the ratio of the different lipid constituents to chlorophyll tend to decrease more rapidly during the first 2–4 hr of greening. When the rate of chlorophyll synthesis reaches its maximum, the ratio of the different components to chlorophyll approaches a constant value.

DISCUSSION

In the present work, the changes in lipid and pigment content of whole cells during the greening

of *y-1* cells were analyzed, and an attempt was made to correlate these results with changes occurring in only one of the many cellular compartments—the chloroplast. To do this, we have assumed that all changes in lipid composition occurring in the dark are not related to photosynthetic membrane synthesis and can be considered as a blank in the calculation of changes in lipid composition related to the formation of these membranes in the light. This assumption is supported by results obtained by radioautographic methods, which showed that during greening macromolecular synthesis is predominantly confined to the chloroplast[1].

The type of lipids synthesized more rapidly during illumination are specific to chloroplast membranes which were shown to be rich in glycolipids and polyunsaturated fatty acids (16). The changes in the molar ratios of the different glycolipids, fatty acids, and pigments to chlorophyll are most pronounced during the initial phase of greening. In part this might be the reflection of the redistribution of synthetic activities within the cell induced by the transition from heterotrophic growth on acetate in the dark to autotrophic metabolism in the light (17, 18). Later, when chlorophyll and membrane synthesis proceed at their maximal and constant rates, the ratios of total glycolipids including mono- and digalactosyl diglycerides and sulfolipid to chlorophyll are constant to within $\pm 10\%$. Within the pigment fraction, both chlorophylls a and b are synthesized in constant proportions, the a/b ratio being 2.6. This ratio is similar to that usually found in other systems (19–22). The same value was also reported by Hudock et al. (23) for a similar *Chlamydomonas* mutant. The changes in β-carotene content were not consistent with the increase in membrane content. β-carotene was recently found to be the major constituent of the eye-spot globules in this organism (14). In the dark-grown cells the globules are still present and contain the bulk of all β-carotene of the cells. Although β-carotene is a constituent of photosynthetic membranes (21), its relative concentration is small and therefore its increase over the background value during membrane synthesis is difficult to quantitate. A similar situation exists for the other carotenoids. An increase was observed only for lutein and to some extent for violaxanthin. Therefore only the molar ratio lutein/chlorophyll was calculated. It was found to decrease from 0.32 after 3 hr of greening to 0.11 after 7.5 hr of greening. The *y-1* mutant cells are able to synthesize all the glycolipids but not chlorophyll in the dark (2). Dark-grown cells are particularly rich in carotenoids. The initial molar ratio of total carotenoids to chlorophyll is about 1.1 as compared with about 0.2 in light-grown cells (0.23 in isolated spinach chloroplast lamellae [21]). Other carotenoids which were reported to be present in the wild type *Chlamydomonas reinhardi*, such as α-carotene, luteoxanthin, zeaxanthin, and trollein (24), were probably present in *y-1* cells in amounts too small to be detected on the chromatograms.

Dark-grown cells contain structural elements, remnants of the original photosynthetic membranes diluted through growth in the dark (2). It is possible that these structures in the form of irregular, distended vesicles are rich in carotenes and glycolipids as well as in cytochrome *f* (2, 25), and that during the initial phase of chlorophyll synthesis they are used as building blocks. Alternatively, chlorophyll might be added to them through a 'repair' mechanism before new membranes are synthesized. This would account for the observed rapid changes in the molar ratio of carotene to chlorophyll.

Analysis of the lipid composition in etiolated and green algae was carried out in other organisms including *Chlorella* (26, 27), *Euglena* (17, 18, 28) and higher plants (28–31). In general, findings were reported which bring out the close association of glycolipids and polyunsaturated fatty acids with the photosynthetic apparatus. This work presents the time-course changes in the relative composition of the aforementioned constituents.

It has been shown in a previous work that in the *y-1* mutant cells the specific activity per chlorophyll unit of photosystem II as measured by oxygen evolution or ferricyanide reduction was constant throughout the whole greening process.

[1] The relative ratio of radioautographic grains (grains/unit area) over the chloroplast (following incorporation of acetate-^{3}H) to that of the grains over the whole cell was found to be about 1:1 in the dark control, 2:1 during the first 2 hr of greening, 3.2:1 after 3.45 hr, and 4:1 after 6.15 hr of illumination. Radioautographic grains found outside the chloroplast were mainly located over mitochondria, Golgi apparatus, cell membrane, and nucleolus (1, cf. also 15).

For No. III of this series see ref. 25.

However, the specific activity of the photosynthetic proton pump and photooxidation of cytochrome f per chlorophyll unit were very low during the first hour of greening and increased rapidly up to a constant level during the phase of constant rate of membrane synthesis (25).

It seems that the changes in the molar ratios of the different lipids to chlorophyll during the initial phase of greening are associated with changes in electron transport system activity and the structural reorganization of the remnants of the photosynthetic lamella (2). The small changes observed in the molar ratio of total glycolipids, pigments, and polyunsaturated fatty acids of chlorophyll during the phase of constant rate synthesis of membranes might be the expression of their mutually controlled synthesis (2). It is possible that the control on the membrane lipid composition is effective only at the assembly step by a limiting factor such as chlorophyll. This possibility should be considered in view of results obtained with organisms deficient in chlorophyll synthesis in which the pattern of lipid synthesis is significantly altered (32).

It is conceivable that an assembly mechanism, such as to ensure constant proportions of constituents (and thus constant biochemical activity) in the newly synthesized membrane, might be operative when synthesis proceeds at the maximal constant rate under normal physiological conditions. Deviation from such a constancy when membrane synthesis occurs under unbalanced or unfavorable conditions, such as nutrient deficiency (32), inhibition of protein (33), or chlorophyll synthesis (32), might reveal the degree of control exerted by the system on both levels of synthesis and assembly of components into a functional membrane.

More data should be available in order to evaluate the types of mechanisms involved and their relative contribution to the formation of biological membranes.

The results presented in Fig. 1 were obtained with the cooperation of Mrs. E. Gan-Zvi, whom we wish to thank for careful and skilled work. This work is part of a Ph.D Thesis to be submitted to The Hebrew University by I. Goldberg.

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