

CHANGES IN CHEMICAL COMPOSITION OF
THYLAKOID MEMBRANES DURING GREENING
OF THE *y-1* MUTANT OF
CHLAMYDOMONAS REINHARDI

BENITA DE PETROCELLIS, PHILIP SIEKEVITZ, and
GEORGE E. PALADE

From the Department of Cell Biology, The Rockefeller University, New York, New York 10021.
Dr. De Petrocellis' present address is International Laboratory of Genetics and Biophysics,
Naples, Italy

ABSTRACT

Two fractions of thylakoid membranes (TMF) have been isolated from disrupted (French press) algal cells by using a discontinuous sucrose gradient. TMF-II consists mostly of thylakoid membranes still partially organized in grana; it contains also fragments of chloroplast envelope, pyrenoid tubules, and starch granules; thus it amounts to a fraction of chloroplast fragments which have lost practically all matrix components. TMF-I consists of smaller chloroplast fragments and is contaminated to a larger extent than TMF-II by other subcellular components, primarily mitochondria. TMF-II accounts for about 12% of the protein and 30% of the chlorophyll of the whole cell; it contains cytochrome 554 and carotenoids in the same ratio to chlorophyll as the latter, and shows photosystems I and II activities but lacks enzymatic activities characteristic of the dark reactions. During the greening of the *y-1* mutant of *Chlamydomonas*, TMF's have been isolated over a range of chlorophyll concentrations from 5 to 25 $\mu\text{g}/10^7$ cells. The results showed that during this period the ratios of chlorophyll to cytochrome 554 and of chlorophyll to carotenoids, and the relative concentrations of individual carotenoids were continuously changing. The findings support the view that during greening, thylakoid membranes are produced by multistep assembly.

INTRODUCTION

Previous work with the *y-1* mutant of *Chlamydomonas reinhardtii* has shown that dark-grown cells, extensively depleted of chlorophyll and chloroplast discs (thylakoids), are still capable of synthesizing many of the chloroplast enzymes involved in both light and dark photosynthetic reactions, including cytochrome 554 (cytochrome *f* or cytochrome 553 [cf. 1]) and ferredoxin (2, 3). When exposed to light, such cells begin chlorophyll synthesis, thyla-

koid formation, and photosynthesis in apparent synchrony. At first, it seemed that electron carriers already present in dark-grown cells were used together with newly synthesized chlorophyll and possibly other compounds to form photosynthetic membranes in a single-step operation (2, 3). However, more recent work (4) has shown that the assembly of various components into developing disc membranes can occur stepwise. To look

further into this problem, it became necessary to isolate thylakoid membranes at various stages of the greening process, and to examine their biochemical composition and photosynthetic properties.

METHODS

Culture

Chlamydomonas reinhardtii *y-1* was grown in the light in a semicontinuous culture apparatus, as previously described (2). For fractionation, the cells were harvested in late logarithmic phase, when their chlorophyll content ranged from 20 to 30 $\mu\text{g}/10^7$ cells. At all times, 7.5×10^8 to 12.5×10^9 cells were used in each experiment. For studying the photo-reductive activities of whole cells and cell fractions, it proved advantageous to use algae in a lag phase after culture dilution (cf. 2).

Enzyme Assays

Alkaline fructose diphosphatase (FDPase), NADPH-linked glyceraldehyde-3-phosphate dehydrogenase, ribulose diphosphate-carboxylase activities, photoreduction of NADP, and the Hill reaction were assayed as already described (2, 3), except that the inhibitor used in the Hill reaction was 3(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) (2×10^{-5} M) instead of O-phenanthroline. Cytochrome *c* photoreduction was measured according to Keister and San Pietro (5) in a system containing, in 3 ml total volume: 50 μmoles of Tris-HCl buffer, pH 7.5, 1 μmole of KCN, 1 μmole of MgCl_2 , 1.5 mg of cytochrome *c*, and either 0.1 mg of *Clostridium pasteurianum* ferredoxin (Sigma Chemical Co., St. Louis, Mo.) or 1 mg of a spinach protein preparation made as described by San Pietro and Lang (6). A dark control and a control with DCMU (1.2×10^{-5} M) were concurrently run. Light was shown at 30 sec intervals, and the change in OD at 550 nm was measured with a Zeiss spectrophotometer.

Chemical Determinations

Total chlorophyll was measured in 80% acetone extracts (7). Chlorophyll *a*: chlorophyll *b* ratios were determined by the Arnon (7) or Vernon (8) method. Proteins were measured by the procedure of Lowry et al. (9). Total lipids were extracted and the individual lipids identified as previously described (1) using two-dimensional paper chromatography and the Marinetti solvent system (10). Deacylations of the extracted lipids were performed as recommended by Wintermans (11). Quantitative measurements of the incorporation of radioactive precursors (acetate- ^3H and ^{14}C) into these lipids were performed as previously described (1).

RNA was measured by the orcinol procedure (12) and DNA by Burton's method (13) on hot 5% trichloroacetic acid (TCA) extracts (14) of samples previously precipitated with cold 10% TCA. These samples had previously been treated several times with 5% TCA in methanol and finally with ethanol-ethyl ether, 3:1 (v/v) in the cold. When the starch content of the samples was high, no accurate RNA determinations could be made.

Determination of Cytochromes

The pink residue, left after the extraction of chlorophyll by 80% cold acetone, was further extracted at $\sim 20^\circ\text{C}$ for 10 min with vigorous stirring with 1.5 ml of a 1% deoxycholate (DOC)-1% Triton X-100 solution in 0.1 M Tris-HCl buffer, pH 7.5. The operation was repeated four times. The first and second extract contained about 75% of the cytochromes and were combined. The third and the fourth were separately assayed to check for completeness of extraction. Difference spectra of the reduced and oxidized cytochromes were recorded at $\sim 20^\circ\text{C}$ with a Cary Model 14R spectrophotometer (Cary Instruments, Monrovia, Calif.). To determine cytochrome amounts and states by difference spectrophotometry, 0.1 M hypochlorite (instead of 0.1 M ferricyanide) was used as oxidant (15), and 0.1 M ascorbate neutralized to pH 6.0 immediately before use was chosen as reductant. (With a redox potential of +0.065 v, the ascorbate is expected to reduce cytochrome 554 but not cytochrome 559 [cytochrome b_6] [16].) 25 μliter hypochlorite or sodium ascorbate were added to 0.6 ml samples in the cuvettes, while the reference cuvette and the cuvette containing solid dithionite each received 25 μliter water. The following spectra (Fig. 1) were recorded for whole cell—and thylakoid fraction extracts: for oxidized cytochrome 554, the ascorbate reduced—minus the untreated (*A-U*) spectrum; for reduced cytochrome 554, the untreated—minus the hypochlorite oxidized (*U-H*) spectrum; for total cytochrome 554, the ascorbate reduced—minus the hypochlorite oxidized (*A-H*) spectrum (15, 17); for cytochrome 559 plus cytochrome 563, the dithionite reduced—minus the ascorbate reduced (*D-A*) spectrum (16, 17); for total cytochrome 554 plus cytochrome 559 and cytochrome 563, the dithionite reduced—minus the hypochlorite oxidized (*D-H*) spectrum (16, 17); and for oxidized cytochrome 554 plus cytochrome 559 and cytochrome 563, the dithionite reduced—minus the untreated (*D-U*) spectrum. The cytochrome 554 concentration was calculated from the (*A-H*) spectrum, using a difference molar extinction coefficient ($E_{554 \text{ nm}} - E_{575 \text{ nm}}$) of 2.5×10^4 (18). The cytochrome 559 plus cytochrome 563 amount was determined from the (*D-A*) spectrum, using a difference molar extinction coefficient ($E_{559 \text{ nm}} - E_{575 \text{ nm}}$) of 2.0×10^4 (18).

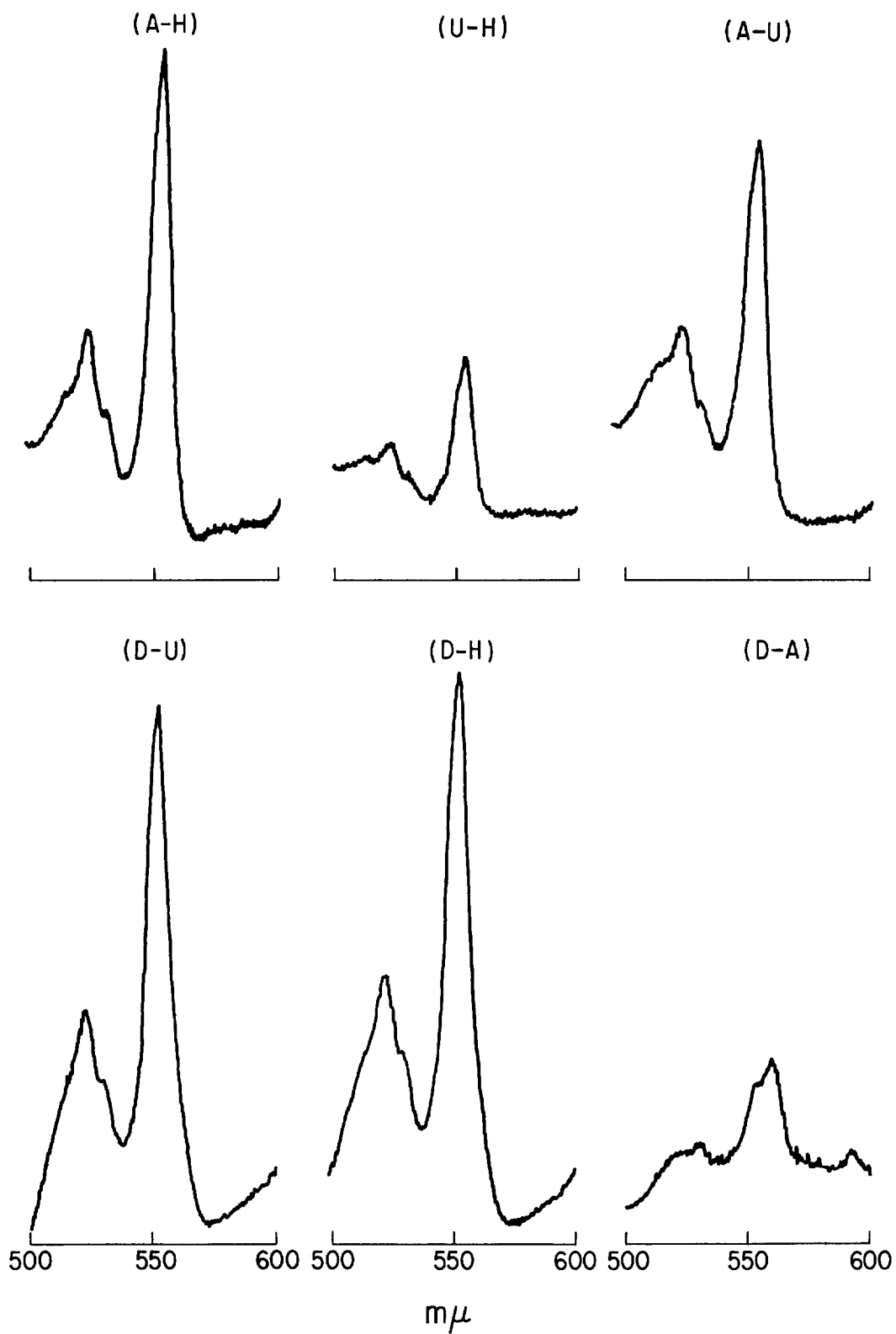


FIGURE 1 Difference spectra of the DOC-Triton X-100 extract from TMF-II. Methods given in the text. (A-H) = ascorbate minus hypochlorite; (U-H) = untreated minus hypochlorite; (A-U) = ascorbate minus untreated; (D-U) = dithionite minus untreated; (D-H) = dithionite minus hypochlorite; (D-A) = dithionite minus ascorbate.

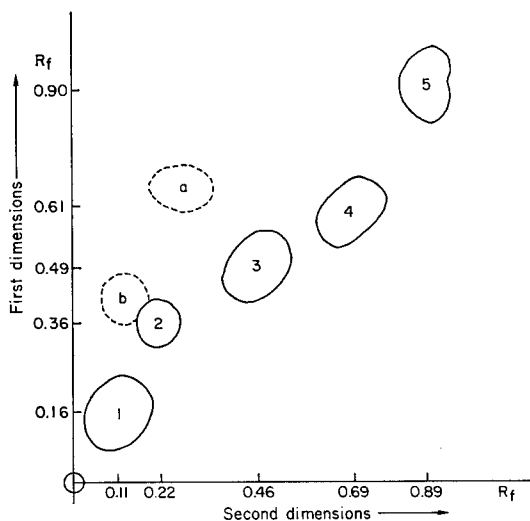


FIGURE 2 Paper chromatography of carotenoid extract from combined TMF-I plus II fraction. Methods are given in the text. Spots marked (1) and (2) are tentatively identified as trollein plus unknown, and neoxanthin respectively, while those marked (3), (4), and (5) are violaxanthin, lutein, and mixture of α - and β -carotenes, respectively. (a) and (b) are the positions of chlorophylls *a* and *b*, when present. The R_f 's in the two directions are: (1) = 0.16, 0.11; (2) = 0.36, 0.22; (3) = 0.49 and 0.46; (4) = 0.61, 0.69; (5) = 0.90, 0.89.

Determination of Carotenoids

Carotenoids were quantitatively transferred from the 80% acetone extract to ether by adding to the extract an equal volume of ether, mixing, separating, and then washing the ethereal layer with 1% NaCl solution, as described by Boardman and Anderson (16). The ethereal extract was saponified with methanolic KOH (1 volume of 60% aqueous KOH added to 10 ml methanol; 2 ml reagent; 10 ml extract) and washed several times with 4% NaCl. Total carotenoid concentrations ($\mu\text{g}/\text{ml}$) were calculated on the washed extracts by dividing adsorbancy at 442 nm by 0.24 (16).

After concentration in nitrogen, the extracts were subjected to two-dimensional ascending paper (Whatman 3 MM) chromatography at room temperature, in the dark, to separate the various carotenoids (19). The developing solvents were similar to those described by Jeffrey (19): 2% n-propanol (v/v) in light petroleum (bp 60–80°C) for the first dimension, and 30% chloroform (v/v) in light petroleum (bp 60–80°C) for the second dimension. The extract, containing about 20–30 μg of pigments, was applied

at the origin on paper which was cut into a square shape (23 cm side), clipped together to form a cylinder, and placed in a jar previously equilibrated with fresh solvent mixture. For quantitative measurements, the individual spots were cut out and eluted with diethyl ether. The pigments were partially identified by their color, by their positions on the paper (19) (Fig. 2), and by their absorption spectra (16, 19, 20) (Fig. 3). Concentrations of the individual carotenoids were determined by using an average specific absorption coefficient of 240 ($\text{mg}/\text{ml}/\text{cm}$) at 442 nm (20).

Preparation of Specimens for Electron

Microscopy

The chloroplast fractions were sedimented from the 0.5 M sucrose-Tris-HCl medium, resuspended in 0.5 M sucrose–0.15 M phosphate buffer (pH 7.4),¹ and fixed in suspension in either 2% glutaraldehyde, or 1% OsO_4 . In each case, the fixative was buffered to pH 7.4 with phosphate buffer, and in some cases the tonicity of the fixative solution was adjusted by adding sucrose to a concentration of 0.5 M.

After fixation, the suspension was centrifuged at 10,000 $\times g$ and the pellet processed for electron microscopy by dehydration in graded ethanols and embedding in Epon. In the case of the glutaraldehyde fixed material, this processing included postfixation in 1% OsO_4 , buffered as above. In some cases, OsO_4 or glutaraldehyde- OsO_4 -fixed pellets were stained in block in UO_2 -acetate before dehydration.

The pellets were cut and embedded in such a way as to allow systemic examination of their entire depth, from top to bottom. Thin sections were cut from the embedded pellets with a Sorvall microtome model MT2 (Ivan Sorvall Inc., Norwalk, Conn.) provided with diamond knife. They were mounted on Formvar carbon-coated grids, were stained in uranyl acetate and lead citrate, and were finally examined in a Siemens (Elmiskop I) electron microscope.

Preparation of Chloroplast Lamellae

Since repeated attempts to isolate whole intact chloroplasts were unsuccessful, we concentrated on the isolation of thylakoid membranes for which we worked out the following procedure carried out at $\sim 4^\circ\text{C}$ (cold room). Cells harvested from an ~ 5 liter culture ($4\text{--}5 \times 10^6$ cells/ml) and washed once with fresh medium by low speed centrifugation were finally

¹ Replacement of the Tris by phosphate buffer is necessary, because in the presence of Tris, fixation is unsatisfactory; it leads to extensive fragmentation of membranes.

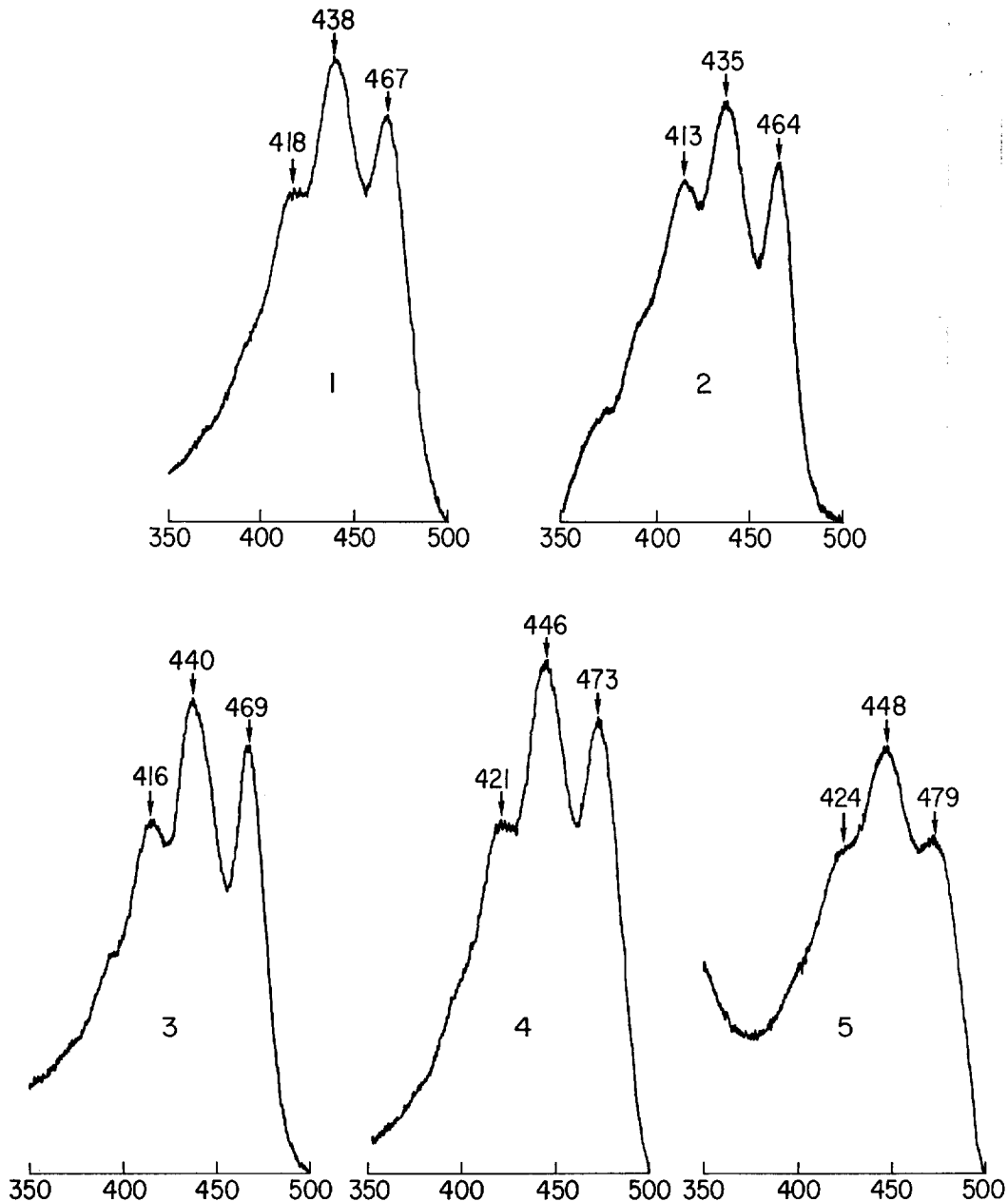


FIGURE 3 Absorption spectra in ether of carotenoids of combined TMF-I plus II. Methods are given in text. The number references are given in legend to Fig. 2.

resuspended and incubated for 15 min at $\sim 4^{\circ}\text{C}$ in 30 ml of 0.3 M sucrose, 0.05 M Tris-HCl buffer (pH 7.2). The thick cell suspension was then forced through a French press twice, at 2000 and 1000 psi, respectively. The incubation time in Tris-sucrose was critical for efficient cell disruption; without any incubation only a small percentage of cells were broken,

while with incubations longer than 15 min the cells began to swell.²

² Sucrose-Tris was preferred to sucrose-phosphate as a suspension medium, since in the latter the photo-reductive activity of the isolated thylakoid fractions was lost more rapidly than in the former.

The French press effluent was carefully layered onto a three-layer discontinuous density gradient, made up of 8 ml of 2 M, 10 ml of 1.5 M, and 8 ml of 1.0 M sucrose, all in 0.05 M Tris-HCl buffer, pH 7.2. The loaded tubes were spun in a Spinco SW 25.1 rotor (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) for 1 hr at 25,000 rpm. Thylakoid membranes separated as two green bands, one narrow at the 1:1.5 M interface and the other broader at the 1.5:2 M interface. Unbroken cells, cell walls, and starch granules sedimented to the bottom of the tube. A yellow band containing eyespots (stigmata) remained at the 0.3:1.0 M interface. Since the chloroplast membrane fractions were contaminated by mitochondria and other organelles, further purification was necessary.

The green broad bands were collected by syringe, diluted with 0.05 M Tris-HCl to bring the molarity of sucrose back to approximately 0.5 M, and centrifuged at $2900 \times g$ for 20 min. The resultant pellets were re-suspended by gentle swirling in 0.5 M sucrose-Tris buffer, and the suspensions layered on the same type of discontinuous gradient, and centrifuged as before. The broad band that formed at the 1.5:2.0 M interface was collected and the operation repeated once or twice under the same conditions, except that the third and fourth gradients were made up of only two layers, i.e., 10 ml of 2 M and 15 ml of 1.5 M sucrose in Tris-HCl buffer. The centrifugation times could be reduced to 40 min and 20 min for the third and fourth run, respectively.

The same general purification procedure was used for the narrow green band except that in that case the discontinuous gradients contained only 1 M and 1.5 M sucrose in Tris-HCl buffer, pH 7.2. After separate purification the fractions were combined in experiments in which a large amount of thylakoid membranes were needed. In the rest of the text, the purified fraction derived from the narrow band at the 1.0:1.5 M interface will be referred to as thylakoid membrane fraction I (TMF-I), and the corresponding preparation derived from the broad band at the 1.5:2.0 M interface as TMF-II.

In preliminary experiments, continuous sucrose gradients ranging from 1.5 to 2.0 M sucrose and from 1.0 to 1.5 M sucrose were also used to purify both fractions. The largest membrane fragments sedimented at 1.7–1.6 M sucrose, and the smallest fragments at 1.4–1.3 M sucrose. Since in this case more time was required to obtain a good separation of the bands, and since the results were the same, the discontinuous gradient method was the one routinely used.

The same fractionation procedure was applied to *y-I* cells at different time points during greening. As chlorophyll concentration decreases, separation of purified TM fractions becomes progressively more

difficult. Below 5 μg chlorophyll/ 10^7 cells the extent of contamination increased and identification of thylakoid membranes in these fractions became uncertain.

RESULTS

TMF-II consists of chloroplast fragments (Fig. 4) which have retained their thylakoids, but have lost their stroma components, either completely, in the case of the matrix and ribosomes, or extensively, in the case of osmiophilic (Fig. 5) and starch (Fig. 6) granules. A few thylakoids are disrupted; the rest, i.e., most of them, appear as intact, closed vesicles swollen to a varied extent, yet often still piled in stacks like the thylakoids of the grana of intact chloroplasts (Figs. 5, 7, 9). The dense fusion layers between granular thylakoids are, however, largely lost; they can still be recognized in a few places only (Fig. 7). The separated surfaces of the thylakoids are covered by an irregularly distributed, finely granular material which can be better seen in glutaraldehyde-OsO₄-fixed preparations (Fig. 7).

Many chloroplast fragments are still partially surrounded by one or both membranes of their envelope (Fig. 8). The ruptured envelope may show free membrane margins, but quite often it "heals" into closed, flattened vesicles (Figs. 5, 9), which can be easily recognized because the two membranes of the envelope appear connected by dense crossbars (Figs. 7, 8) in isolated chloroplast fragments. Partially disorganized stigmata and pyrenoids can be recognized in many chloroplast fragments (Fig. 9). In the pyrenoids, the tubules appear distended but otherwise well preserved; the starch plates are partly lost, and the fine particles which make the mass of the organ are completely extracted, with the exception of some fine granular or possibly filamentous material which covers the outside surface of the tubules (Fig. 9).

The fraction contains only occasional contaminants, mainly mitochondria either swollen or disrupted (Fig. 4), and very few incompletely disrupted cells.

TMF-I consists of smaller vesicular aggregates which retain less of the chloroplast architecture, and hence are less certainly identifiable as intact or fragmented thylakoids. The fraction contains, in addition, recognizable mitochondria, a few nuclear fragments, a few pieces of cell wall, and occasionally fragments of flagella.

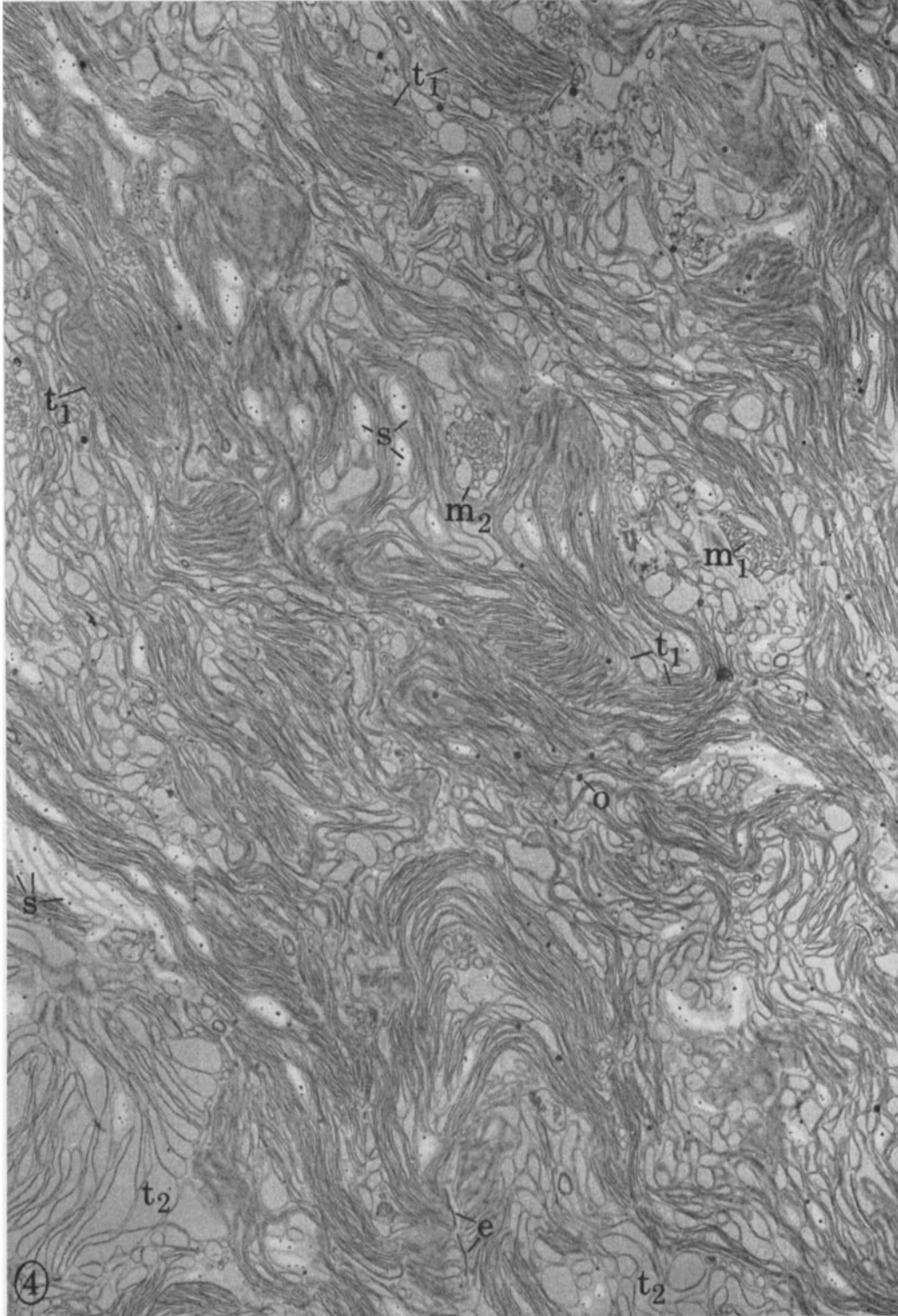


FIGURE 4 Thylakoid membrane fraction II. Representative field in the pelleted fraction which consists of packed chloroplast fragments. Their thylakoids (t_1) are slightly distended but still piled up as in the grana of chloroplasts *in situ*. More disorganized fragments with extensively swollen thylakoids appear at t_2 . Fragments of chloroplast envelope can be seen at e , osmiophilic granules at o , and starch plates or granules at s . The latter did not "stain", and hence appear negatively contrasted; their surface is contaminated by fine metal (stain) deposits. The few contaminants of the fraction visible in this field are partially (m_1) or extensively (m_2) disrupted mitochondria. Preparation fixed in suspension in 1% OsO_4 in 0.1 M phosphate buffer, pH 7.2, containing 0.5 M sucrose; stained in block in 0.5% uranyl acetate in acetate-Veronal buffer, pH 5.0; embedded in Epon. Section stained with uranyl acetate in 50% ethanol, and lead citrate in 20% ethanol. $\times 9000$.

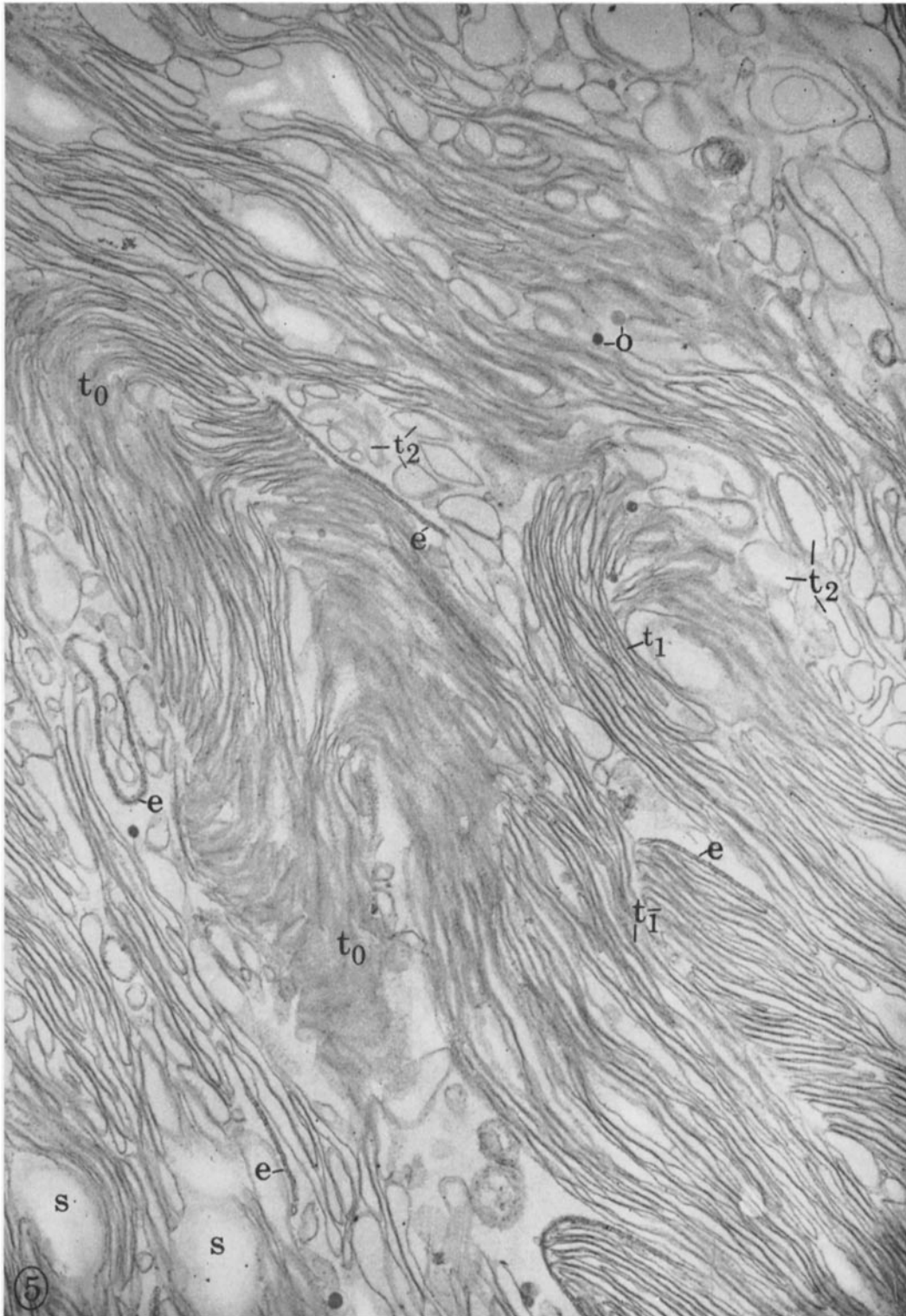


FIGURE 5 Thylakoid membrane fraction II. This higher magnification shows more clearly the following structural details in chloroplast fragments: pieces of chloroplast envelope at *e*; moderately swollen still piled up in partially disorganized grana at *t*₁ (normally cut) and at *t*₀ (obliquely sectioned); scattered and extensively swollen thylakoids at *t*₂; osmiophilic granules at *o*; and starch granules at *s*. Specimen prepared as for Fig. 4. $\times 35,000$.

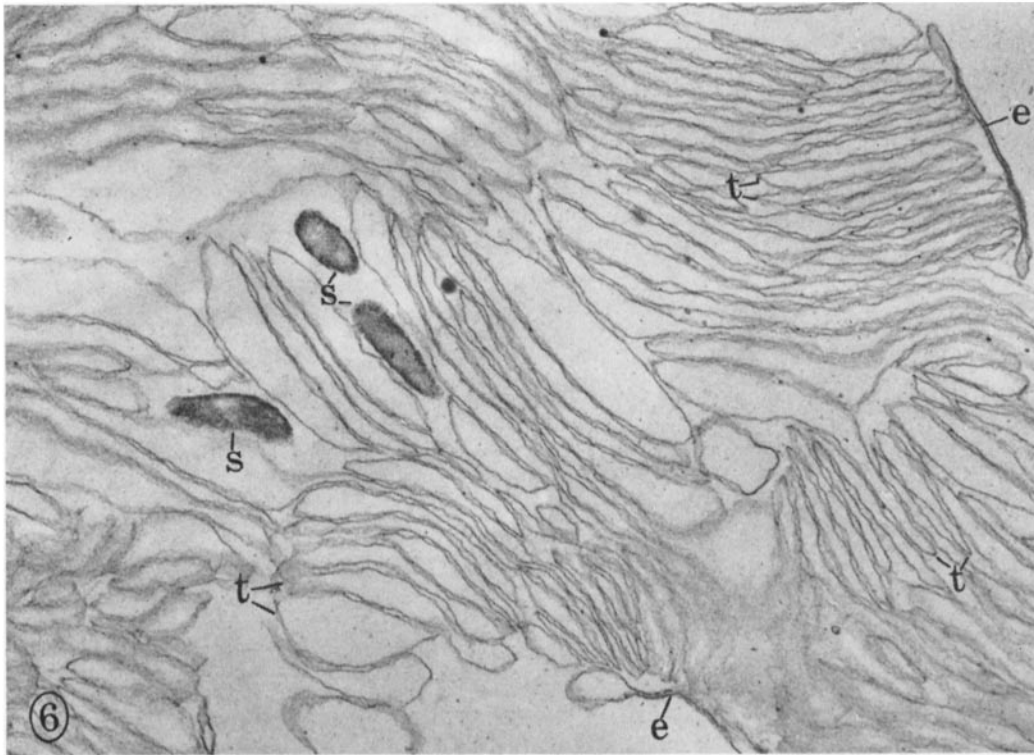


FIGURE 6 Thylakoid membrane fraction II. Part of a chloroplast fragment which shows positively stained starch granules (*s*), distended thylakoids (*t*) which retain their grana-like stacking, and pieces of chloroplast envelope (*e*) healed into closed, flattened vesicles. Specimen fixed in 1% OsO₄ in 0.1 M phosphate buffer, pH 7.4, for 16 hr at 0°; embedded in Epon. Sections stained with uranyl acetate in 50% ethanol and lead citrate in 20% ethanol. × 32,000.

Biochemistry

CHLOROPHYLL: The chemistry of these fractions is given in Tables I–III. TMF-II (Table I) accounts for ~12% of the protein and ~30% of the chlorophyll of the whole cell, and hence represents an ~2.5-fold purification of chlorophyll containing membranes. Based on chlorophyll recovery, TMF-II together with TMF-I account for about 40% of the thylakoid membranes of the cell. The ratio of chlorophyll *a*:chlorophyll *b* is the same in these fractions as in the whole cell.

CYTOCHROMES AND CAROTENOIDS: The concentrations and amounts of cytochrome 554 and carotenoids recovered in TMF-I and II parallel closely the data obtained for chlorophyll (Table II). Extrapolation to 100% chlorophyll recovery indicates that practically all of the cytochrome 554 and carotenoids of the cells are located in thylakoid membranes (Table II).

Cytochrome 554 had an α band peak at 553–555 nm, in agreement with the originally found (21) 555 nm peak. In all experiments, the total measured cytochrome 554 (*A-H* spectrum, Fig. 1) was found to be the sum of the separately determined oxidized cytochrome 554 (*A-U*, Fig. 1) plus reduced cytochrome 554 (*U-H*, Fig. 1). However, while Fig. 1 shows a preponderance of oxidized cytochrome 554, the red:ox ratios varied in different experiments, from 0.4 to 2.7; in plant chloroplast preparations James and Leech found a ratio of 1.6 (15).

Our results on cytochrome 559 and cytochrome 563 distribution were less satisfactory. It was difficult to detect and to separate these cytochromes in whole cells, even at low temperature, and the amount recovered in thylakoid membranes fractions gave a calculated cytochrome 559 plus 563/cytochrome 554 ratio of 0.5, which is much lower

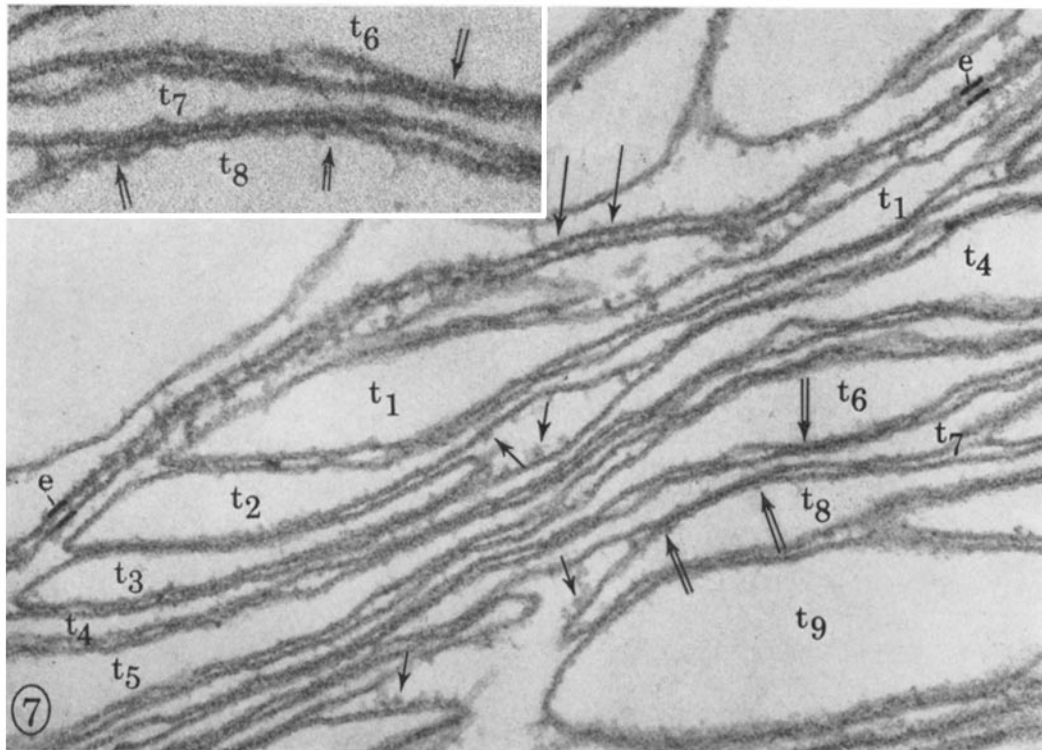


FIGURE 7 Thylakoid membrane fraction II. Periphery of a chloroplast fragment whose envelope is marked *e* and distended thylakoids t_1 – t_9 . Note the bars of dense material which stretch from one membrane of the envelope to the other (long arrows), the fine granules irregularly scattered on the stromal surface of the thylakoids (short arrows), and the few sites where dense fusion layers (double arrows) persist among thylakoids. The latter detail is shown at higher magnification in the *inset*. Specimen fixed in suspension in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, containing 0.5 M sucrose; washed overnight in buffered sucrose; postfixed in 1% OsO_4 in the same buffer; washed in acetate-Veronal buffer; stained in block in 0.5% uranyl acetate in acetate-Veronal buffer; embedded in Epon. Sections stained as for Fig. 4 $\times 135,000$; *inset*: $\times 275,000$.

than the values of cytochrome 559:cytochrome 554 recorded in the literature (1.3 to 4.0) for different plant cells, including a ratio of 4.0 for wild strain *Chlamydomonas* (22). This point should be re-investigated.

The chromatography of the carotenoids gives five distinct spots (Fig. 2) of which only three, namely neoxanthin, violaxanthin, and lutein, have been definitely identified. Carotenes α and β appear in a single unresolved fourth spot. The fifth spot, which is made up of two components—one yellow and the other orange—is tentatively identified as trollein on the basis of a comparison of its spectrum with that of a carotenoid fraction isolated from the wild strain of *C. reinhardtii* and from

a yellow mutant, γ -2 (23), similar to our γ -1. The epoxides neoxanthin and violaxanthin showed the characteristic hypsochromic shift of ~ 20 nm towards the blue when treated with dilute acid, whereas the nonepoxides, i.e. lutein and the carotenes, as well as the presumed trollein, did not. The latter result is in agreement with the findings of Krinsky and Levine (23) on their trollein fraction.

LIPIDS: Table III gives the specific radioactivity, normalized to chlorophyll, of the main membrane lipids after 7 hr *in vivo* labeling with acetate- $2\text{-}^{14}\text{C}$.

Since 7 hr represent one generation time, and since the cell population is asynchronous, one

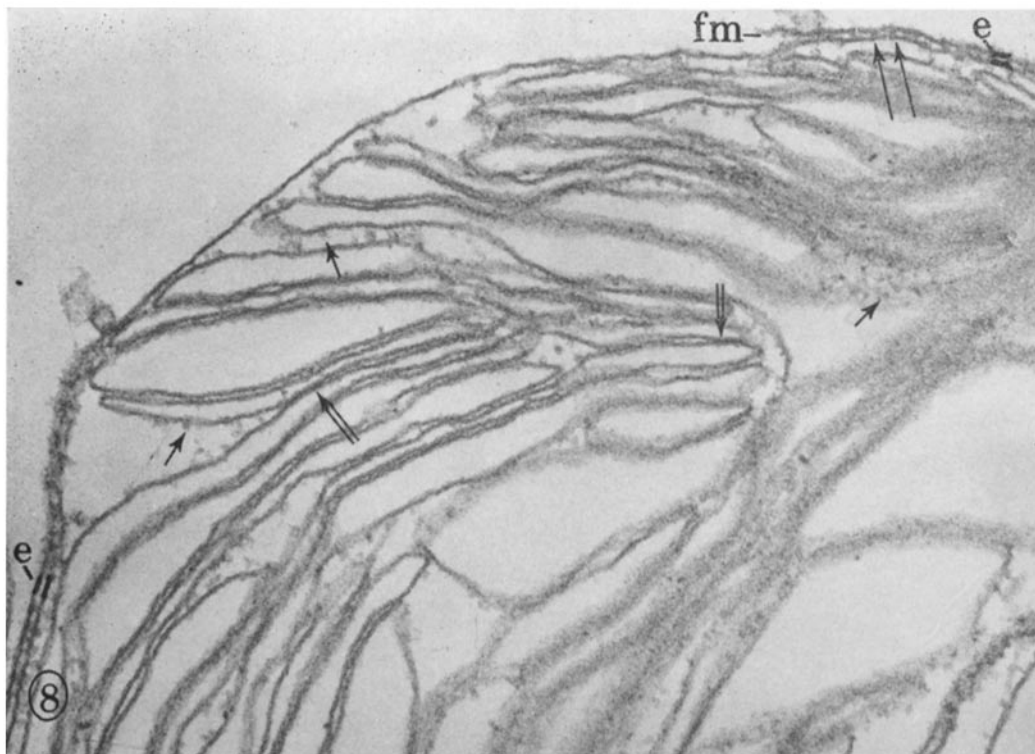


FIGURE 8 Thylakoid membrane fraction II. Chloroplast fragment which has lost part of the outer membrane of its envelope (*e*). The torn margin of this membrane is marked *fm*. As in Fig. 7, note the crossbanding of the envelope (long arrows), the fine particles (short arrows) distributed irregularly on the stromal surface of normally and obliquely sectioned thylakoids, and the persistence of a few sites of thylakoid membrane fusion (double arrows). Specimen prepared as for Fig. 7. $\times 92,000$.

would expect general and more or less uniform labeling of these lipids. Under such conditions, the radioactivity distribution should parallel the quantitative distribution of each lipid among different subcellular components. Based on these assumptions, normalization to chlorophyll should show how much of each lipid is associated with thylakoid membranes. The data suggest that only monogalactosyl glyceride is predominantly ($\sim 75\%$) associated with photosynthetic membranes, while the others, for instance the digalactosyl glyceride and the sulfolipid (6-sulfo- α -D-quinovopyranosyl-(1 \rightarrow 1')-2,3 diacyl-D-glycerol) are about evenly distributed between such membranes and the rest of the cell. In the case of the sulfolipid, this distribution pattern was confirmed by using $^{35}\text{SO}_4^{=}$, instead of acetate- ^{14}C , as precursor.

It has been assumed that the sulfolipid is a characteristic component of photosynthetic mem-

branes in bacteria, green algae, and higher plants (24). For instance, Wintermans found that in spinach leaves 75% of this lipid is in the chloroplast (11). Our results are, in part, in agreement with this view; they suggest that in *Chlamydomonas* $\sim \frac{1}{2}$ of the sulfolipid is in thylakoid membranes, with the remainder either in other chloroplast structures, such as the chloroplast envelope, or in other subcellular components. The same conclusion applies for digalactosyl glycerides.

NUCLEIC ACIDS: TMF-II contains about 10% of the RNA and DNA of the whole cell. The DNA is practically all chloroplastic (B. De Petrocellis, P. Siekevitz, and G. E. Palade, data to be submitted), and the fact that it separates with a relatively light membrane fraction suggests that it is anchored *in situ* to some thylakoid membranes (B. De Petrocellis, P. Siekevitz, and G. E. Palade, data to be submitted).

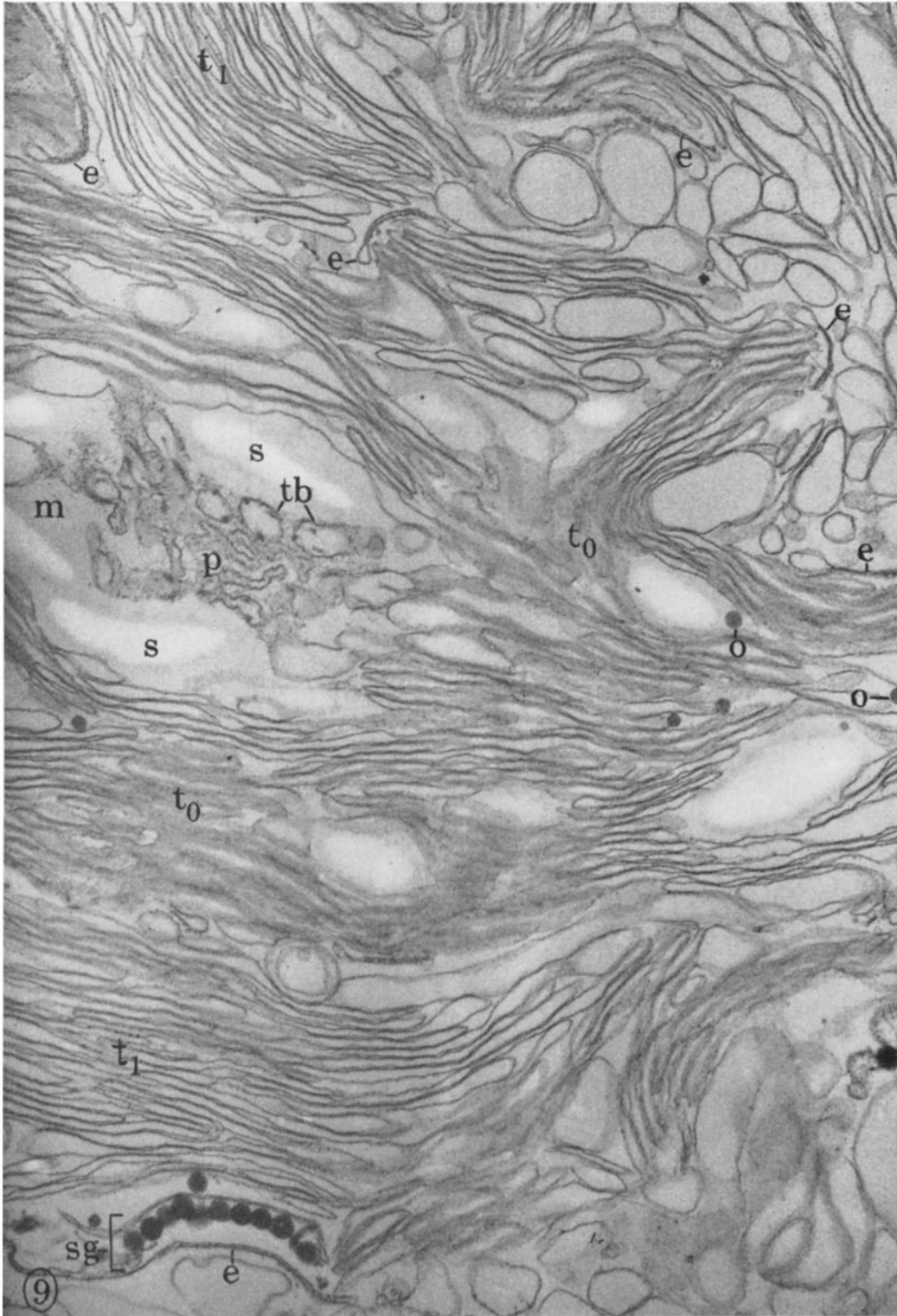


FIGURE 9 Thylakoid membrane fraction II. Packed chloroplast fragments in which a stigma (sg) and a pyrenoid (p) with distended tubules (tb), extracted matrix (m), and negatively contrasted starch plates (s) can be recognized. Thylakoids appear in normal section at t_1 and in oblique section at t_0 . Osmiophilic granules are marked o , and fragments of chloroplast envelope e . Many of the latter have formed closed, flattened vesicles. Specimen prepared as for Fig. 1. $\times 40,000$.

TABLE I
Recovery of Chlorophyll and Protein in TMF-I and II

Methods are given in experimental section

Exp.	Whole cells	TMF-I	TMF-II
	$\mu\text{g}/10^7$ cells	$\mu\text{g}/10^7$ cells	$\mu\text{g}/10^7$ cells
1 Chlorophyll	33.0	3.3	10.7
Protein	480.0	22.3	59.5
Chl/Prot	0.07	0.14	0.18
2 Chlorophyll	35.0	—	11.6
Protein	455.0	—	52.7
Chl/Prot	0.08	—	0.22
3 Chlorophyll	26.3	—	8.4
Protein	395.0	—	43.4
Chl/Prot	0.07	—	0.19

PHOTOREDUCTIVE ACTIVITIES: Data from a representative experiment given in Table IV show that isolated thylakoid membranes have photoreductive activities characteristic of photosystems I and II. Their specific activities could not be reliably determined, however, since they underwent considerable losses during cell fractionation. This loss is directly visible in the low ratios: activity:mg chlorophyll, and offsets the expected concentration effect in the ratios:activity:mg protein. For all activities tested, the loss ranges

from 50 to 70%. These activities are more sensitive to aging in thylakoid fractions than in homogenates; for preparations made in sucrose-Tris buffer, the inactivation amounts to 20% and 5%, respectively, in 3 hr.

SOLUBLE ENZYMES: Consistent with their appearance in electron micrographs, thylakoid membrane fractions contained none of the chloroplast soluble enzymic activities for which they were assayed, i.e., alkaline fructose-1,6-diphosphatase, ribulose diphosphate carboxylase, and NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase.

Changes Undergone during the Greening Process

CAROTENOIDS: During greening the pigment content per *y-1* cell increases two-fold for carotenoids and 20- to 40-fold for chlorophyll (2, 3). As Table V shows, the various carotenoids increase at different rates; hence, their relative amounts in the whole cell and the TM fractions change with greening. The yellow *y-1* cell has the same major carotenoids as the greened *y-1* (with the exception of an additional minor component in the carotene α and β fraction) but their proportions are different. A comparison of our data (Table V) with those of Krinsky and Levine (23) on *y-2* indicates that the increase in carotenoid contents follows rather different patterns during greening in the

TABLE II
Cytochrome 554 and Carotenoid Contents of Combined TMF-I Plus II as Compared to Whole Cells
Methods are given in experimental section

Exp.	Whole cells			TMF-I plus II*		
	$\mu\text{g}/10^7$ cells	$\mu\text{g}/\text{mg chl.}$	$\mu\text{g}/\text{mg prot.}$	$\mu\text{g}/10^7$ cells	$\mu\text{g}/\text{mg chl.}$	$\mu\text{g}/\text{mg prot.}$
1 Chlorophyll	8.	—	47.0	(8)*	—	155.0
Cyt. 554‡	0.016	1.78	0.084	0.015	1.76	0.273
Carotenoids	1.7	210.0	9.9	1.55	194.0	30.0
2 Chlorophyll	15.0	—	49.0	(15)*	—	158.0
Cyt. 554‡	0.023	1.52	0.075	0.022	1.42	0.225
Carotenoids	3.05	201.0	9.9	2.9	192.0	31.0
3 Chlorophyll	20.0	—	46.0	(20)*	—	174.0
Cyt. 554‡	0.037	1.87	0.086	0.028	1.4	0.244
Carotenoids	3.05	154.0	7.1	2.7	138.0	24.0

* The values for the chloroplast fractions are calculated for 100% recovery of chlorophyll and chloroplast membranes.

‡ The values for cyt. 554 are in μmoles .

two mutants. To begin with, the relative amounts are different; the carotenes, for instance, account for $\frac{1}{4}$ of the total carotenoids in *y-1* and for $\frac{1}{2}$ in *y-2*; and to end with, the rates of increase of indi-

TABLE III
Lipid Constituents of Isolated TMF-II as Compared to Whole Cells

Green cells ($17 \mu\text{g}$ chlorophyll/ 10^7 cells) were incubated in the light for 7 hr in the growth medium containing $1 \mu\text{mole}$ acetate/ml and $1 \mu\text{Ci}$ acetate- $2\text{-}^{14}\text{C}$ /ml. At the end of the incubation, the cells were harvested and washed free of radioactivity; a portion was used for the isolation of thylakoid membrane fraction II. The lipids were extracted from whole cells and from thylakoid membrane fraction II, chromatographed, eluted from the paper, and counted as described in the text. *MG* and *DG* are monogalactosyl and digalactosyl glycerides, *SL* is sulfolipid, and *PL* are various unidentified phospholipid components. The figures represent cpm/ μg chlorophyll.

	Whole cells	TMF-II
	cpm/ μg chlorophyll	
MG	164	120
DG	153	88
SL	46	24
PL ₁	44	25
PL ₂	51	66*
PL ₃	24	
Neutral	376	144

* The two *PL* spots could not be separated on paper and were counted as one.

vidual carotenoids vary as much as five times from one mutant to the other. Our data, together with these of Krinsky and Levine (23), indicate that there is little similarity in individual carotenoid content among the genotypes and phenotypes so far studied (i.e., wild strain and either yellow or greened *y-1* and *y-2*), although some of them are equally active in photosynthesis (e.g., wild type and fully greened *y-1*). Our findings support, therefore, the already expressed view (23, 25) that the totality of the carotenoids are not inextricably linked to the photosynthetic process, at least in *Chlamydomonas*.

A comparison of whole greening cells and thylakoid fractions (I and II) derived therefrom shows no difference in individual carotenoid concentrations (Table V) and reveals that for each time point investigated, chlorophyll:carotenoid ratio is equal in whole cells and TMF's (Fig. 10). These data suggest that, apart from $\sim 20\%$ of the total β -carotene found in the isolated eyespot fraction (B. De Petrocellis, P. Siekevitz, and G. E. Palade, data to be submitted. I. Ohad, personal communication), practically all the other carotenoids are associated with thylakoid membranes from the earliest point at which we could isolate such fractions (i.e., at a concentration of chlorophyll of $\sim 6 \mu\text{g}/10^7$ cells). Assuming that this association exists in extensively degreened cells and persists throughout the entire greening process, the carotenoid:chlorophyll ratio should be two times and ten times higher when the chlorophyll concentration drops from 25 to $5 \mu\text{g}$ and $1 \mu\text{g}/10^7$ cells, respectively. Since all these membranes appear to

TABLE IV
Photoreductive Activity of Homogenates and TMF-II

The enzymatic assays are described in the text. The values for each assay are representative of many experiments. The Hill oxidant was dichlorophenolindophenol. TMF-II were obtained from cells with a chlorophyll content of from $26\text{--}33 \mu\text{g}/10^7$ cells, and represented $31\text{--}33\%$ recoveries based on chlorophyll content. Both the homogenate, obtained from the French press, and TMFs-II were suspended in 0.5 M sucrose, 0.05 M Tris-HCl buffer, pH 7.2, for the assays. The values represent μmoles substrate metabolized per min.

Enzyme assay	Whole cell homogenate			TMF-II		
	/ 10^7 cells	/mg chloro.	/mg protein	/ 10^7 cells	/mg chloro.	/mg protein
Hill reaction	0.022	0.670	0.046	0.012	0.365	0.067
Photored. of NADP	0.007	0.216	0.015	0.004	0.108	0.017
Photored. of cyt. <i>c</i>	0.022	0.816	0.053	0.011	0.400	0.077

TABLE V
Changes during Greening of Carotenoid Components
in Whole Cells and in TMF-I Plus II

Methods are given in the experimental section. Data are given as % of total carotenoid content. The total carotenoid content ($\mu\text{g}/10^7$ cells) were: 1.9 for whole cells having 3.8 μg chl./ 10^7 cells; 3.9 for whole cells having 26 μg chl./ 10^7 cells.

μg chlorophyll/ 10^7 cells	3.8	6.4	11.9	19.8	26.0
Whole cells					
Trollein	19.1	—	25.6	23.7	37.2
Neoxanthin	1.9	—	2.5	2.4	3.1
Violaxanthin	17.0	—	17.8	18.7	18.9
Lutein	41.2	—	26.2	31.6	27.8
α - and β -carotenes	20.9	—	27.8	23.7	13.0
TMF-I plus II					
Trollein	—	17.0	31.0	29.5	39.9
Neoxanthin	—	1.8	2.4	1.7	2.0
Violaxanthin	—	25.7	18.7	17.6	18.5
Lutein	—	35.3	28.8	37.0	27.5
α - and β -carotenes	—	20.6	19.0	14.1	12.0

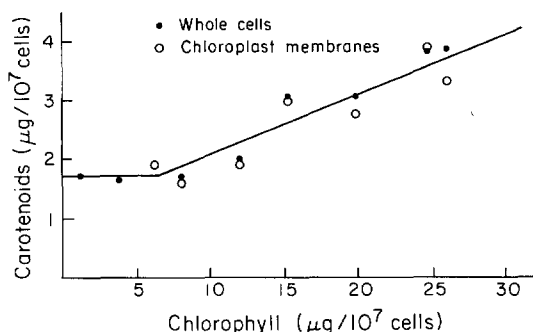


FIGURE 10 Changes in total carotenoid concentration of whole cells and combined TMF-I plus II fraction as a function of chlorophyll content of cells during greening. Methods are given in the text.

be about equally effective in photosynthesis on a chlorophyll basis (2, 3)³, it again follows that the absolute carotenoid concentration is not directly relevant for the photosynthetic activity of the thylakoid membrane.

CYTOCHROME 554: As in the case of carotenoids, the cytochrome 554 content of the γ -1 cells

³ It should be noted that during the late phases of regreening (cf. Fig. 25 of ref. 3), there is a decrease in photosynthetic efficiency.

increases two-fold during greening. Since the concomitant increase in chlorophyll is 20- to 30-fold, the ratio, chlorophyll:cytochrome 554 also increases continuously in whole cells as well as in their TMF's (Fig. 11). In the latter, for instance, the calculated molar ratio changes from 300:1 to 1200:1 as the chlorophyll content increases from 5 to 26 $\mu\text{g}/10^7$ cells. Since on a chlorophyll basis, these two membranes have equal photoreductive activities, it follows that at the beginning of the greening process, the thylakoid membranes must contain an excess of cytochrome 554. For each time point investigated during the first hours of the greening process the ratio, chlorophyll:cytochrome 554 was the same for whole cells and their TMF's (Fig. 11), indicating that all the cytochrome 554 of the cell was firmly bound to thylakoid membranes. Toward the end of the process, however, the situation changed; only 70% of the cytochrome 554 was recovered in these membranes. The rest was soluble or easily extractable, as it is known to become under certain growth conditions in *Chlamydomonas* (26).

LIPID: Table VI gives the protocol and the results of an experiment in which yellow cells are doubly labeled (acetate-¹⁴C, acetate-³H) in the dark, washed, and then greened in the light in the presence of one of the radioactive precursors (acetate-³H). The results indicate that all the lipid synthesized in the dark is retained in the greening cell, although only ~40% of it is recoverable in thylakoid membranes.

Preliminary experiments in which mono- and digalactosyl glycerides synthesized in the dark

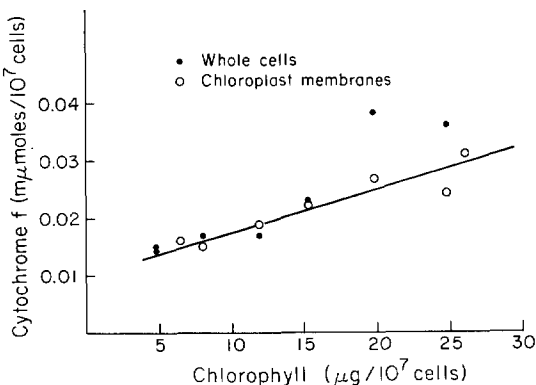


FIGURE 11 Changes in cytochrome 554 concentration of whole cells and of TMF-II fraction as a function of chlorophyll content of cells during greening. Methods are given in the text.

TABLE VI

Utilization during the Greening Process of Lipid Synthesized in the Dark

Yellow cells ($1.2 \mu\text{g chl./}10^7$ cells) were incubated in the presence of acetate- $2\text{-}^{14}\text{C}$ ($1 \mu\text{Ci/ml}$) and acetate- $\text{U-}^3\text{H}$ ($3.6 \mu\text{Ci/ml}$) for 17 hr in the dark. During this period, they divided once and their chlorophyll content was reduced accordingly to $\sim 0.5 \mu\text{g}/10^7$ cells. At this time, the cells were harvested, an aliquot was saved for lipid extraction (yellow cells), while another one was placed in the light in the presence of acetate- $\text{U-}^3\text{H}$ ($4.7 \mu\text{Ci/ml}$). After 10 hr in the light, when chlorophyll content had reached $13.4 \mu\text{g}/10^7$ cells, the cells were harvested and an aliquot was used for the preparation of TMF-II as given in Methods. The yellow cells, the green cells, and the TMF-II were all extracted for lipids, as given in the text, and the ^3H and ^{14}C radioactivity in the total lipid extract were determined.

Fraction	^3H	^{14}C	^3H	^{14}C	$^3\text{H}/^{14}\text{C}$
	<i>dpm/10⁷ cells</i>		<i>dpm/μg chlorophyll</i>		
(1) Yellow cells	58,400	118,000	118,000	238,000	0.5
(2) Green cells	275,000	117,500	20,400	8,650	2.4
(3) TMF-II	149,000*	48,000*	11,200	3,570	3.1
(4) (1)/(2)	0.21	1.00	5.8	27.5	
(5) (3)/(2)	0.54	0.41	0.55	0.41	

* Counts corrected for full thylakoid recovery based on chlorophyll recovery (22%) in TMF-II.

were isolated and deacylated indicated that the ^{14}C label was mostly retained in the fatty acids of these lipids during greening. This finding and the nearly complete retention of lipid radioactivity acquired before greening imply either extensive reutilization of the label, primarily by transacylation, or considerable reduction in the rate of lipid turnover during greening. Extensive lipid degradation followed by active metabolism of the degradation products seems unlikely. The experiment also suggests that the lipids synthesized in the dark as well as in the light are used in the formation of new membranes.

DISCUSSION

Our data show that it is possible to isolate from greened *y-1* cells of *Chlamydomonas reinhardi* a fraction which consists almost exclusively of structurally recognizable thylakoid membranes, which accounts for $\sim 30\%$ of the chlorophyll of the cell, and in which there is a three-fold increase in chlorophyll concentration over the cell homogenate. Morphologically and biochemically, this fraction appears to be a representative sample of thylakoid membranes freed completely or extensively of other chloroplast components (soluble enzymes, ribosomes, starch) with the exception of chloroplast DNA. On the reasonable assumption that chlorophyll is exclusively located in thylakoid membranes, the data indicate that the carotenoids (with the exception of a certain amount of β -caro-

tene present in the eyespot), the cytochrome 554, and certain lipids (e.g., monogalactosyl glyceride) of the alga, are also mainly or exclusively located in the same structures. The findings suggest that about half of the total digalactosyl glyceride and sulfolipid is present in other plastid or cell components.

The isolated thylakoid membranes have measurable but unstable photoreductive activity which appears to be partially inactivated during the isolation procedure and which is sensitive to aging.

During the greening process, the chemical composition of the thylakoid membranes changes. From the earliest stage at which they can be isolated, these membranes account for all the carotenoids (with the exception mentioned above) and cytochrome 554 of the cell, but the ratios, chlorophyll:carotenoids and chlorophyll:cytochrome 554 change progressively, since chlorophyll increases \sim five-fold during the period investigated while carotenoids and cytochrome 554 increase only two-fold. The relative concentration of different carotenoids also changes. Extrapolating to the beginning of the greening process, the residual (vestigial) thylakoids present in the chloroplast probably have unusually high concentrations of carotenoids and cytochrome 554.

Lipid synthesized in the dark appears to be effectively retained by the greening cells and $\sim 40\%$ of it appears in the thylakoid membranes. However, desaturation of the fatty acids of these

lipids may occur as greening progresses, since the concentration of linolenic and linoleic acid is known to increase (27) in the total cell lipids.

The findings indicate that there is no set chemical formulation for the thylakoid membrane, as has already been shown for other cellular membranes (cf. 28). During greening of *y-1*, its chemical composition varies from step to step; hence its assembly follows a multistep pattern. This conclusion, different from the one previously considered (2, 3), reinforces recent findings published by Hooper et al. (4). Changes in chemical compo-

sition and activities (e.g., photooxidation of cytochrome 554 and proton pumping) of the *y-1* chloroplast have also been recorded by Ohad et al. (29) during the first phase (~3 hr) of greening; they assume, however, that one-step assembly is still possible in the second phase of the process.

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