Thylakoid Membrane Biogenesis in *Chlamydomonas reinhardtii* 137$^+$: Cell Cycle Variations in the Synthesis and Assembly of Polar Glycerolipid

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ABSTRACT The synthesis and assembly of thylakoid membrane polar glycerolipid (glycolipid, phospholipid, and ether lipid) have been monitored in synchronous cultures of the green alga *Chlamydomonas reinhardtii* 137$^+$. A "pulse" protocol using radioactive acetate as the lipogenic precursor was devised to allow assessment of both processes during the 24-h (12-h light/12-h dark) vegetative cell cycle. Under these conditions, acetate incorporation into each chromatographically resolved lipid at the cellular level reliably reflects lipid synthesis, and the appearance of radiolabeled lipid in purified photosynthetic membrane is indicative of the lipid assembly attendant to thylakoid biogenesis. Our results demonstrate that polar glycerolipid is synthesized by the alga and is assembled into its thylakoid membrane continuously, but differentially, with respect to cell cycle time. Synthesis and assembly are most rapid during the photoperiod (mid-to-late G_1), reach maximum rates at mid-photoperiod, and are comparatively negligible in the dark (S, M, and early-to-mid G_1). The extent to which synthesis and assembly vary within this general kinetic pattern, though, is characteristic of each thylakoid lipid, suggesting that the processes take place in a multistep manner with some temporal coordination among the different lipid types. Parallelism between the cyclic patterns of polar lipid synthesis at the cellular level and of polar lipid assembly into photosynthetic membrane at the subcellular level indicates that lipid production is not only essential to continuing thylakoid biogenesis but is also the critical determinant of the kinetics of thylakoid lipid assembly.

Production of biological membranes can be resolved into at least three steps: the synthesis of constituent molecules, their transport from sites of synthesis to sites of assembly, and, finally, their integration into the membrane matrix (1). The intracellular topologic and metabolic complexities of these steps hinder the in vivo, kinetic dissection of membrane biogenesis in most experimental systems. Grown in synchronous culture, the green alga *Chlamydomonas reinhardtii* 137$^+$ (wild-type) has proven to be an excellent organism for the study of membrane biogenesis. The high level of synchrony that the chlorophyte displays during a cyclic light (L)/dark (D)[1] program that closely mimics the conditions encountered by the alga in nature (2) facilitates obtaining samples representative of discrete cell cycle stages and allows kinetic studies on biosynthesis. Further, the quadrupling of the algal population every cycle (i.e., every 24 h [2]) demands substantial synthesis of cellular macromolecules and the production of massive amounts of rather easily purifiable photosynthetic membrane (thylakoid) before mitosis (3). Although measurement of rates of synthesis for individual polypeptides has not been carried out, most attention has been directed to the incorporation of polypeptides into thylakoids during the cell cycle (3–7). Notwithstanding the rich lipid biochemistry characteristic of green-plant photosynthetic lamellae (8), only one study (9) has attempted even partial characterization of the assembly of some polar glycerolipids into *Chlamydomonas* thylakoids through a portion of the synchronous cycle.

[1] The number immediately preceeding L or D denotes time in hours during the light or dark period, respectively. Thus, "2D" represents the point in the cell cycle 2 h into the dark period, the equivalent of hour 14 of the 24-h cell cycle.

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Our previous work (10, 11, and Janero and Barrnett, submitted for publication) on the lipid biochemistry of *Chlamydomonas* has demonstrated that the wild-type alga contains virtually exclusively (~95% of total) five polar glycerolipids: the glycolipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SL); the phospholipid phosphatidylglycerol (PG); and the ether lipid diacylglyceryl-trimethylhomoserine (DGTS). Although these five lipids are also the sole thylakoid polar glycerolipids, each lipid in the thylakoid membrane is characterized by a markedly distinctive fatty acid profile in comparison to its extrathylakoid counterpart. Questions arise, therefore, as to how the alga temporally coordinates production of these various lipids during its cell cycle and how, in kinetic terms, the lipids so produced are assembled into the thylakoid membrane of the chloroplast, the major cellular membrane (12). We define here parameters that allow accurate experimental assessment of lipogenic rate and establish the pattern of synthesis of each thylakoid polar lipid throughout the *Chlamydomonas* vegetative cycle. Subcellularly, the assembly kinetics of synthesized lipid into photosynthetic membrane during thylakoid biogenesis are detailed within the context of the cyclic variations in cellular lipogenesis.

MATERIALS AND METHODS

Cell Culture

C. reinhardtii 137$^+$ (wild-type) was grown in axenic, log-phase culture at 25 ± 0.5°C in minimal medium (13) with continuous stirring and aeration (14). Cultures were synchronized by exposure to an automated program of alternating 12-h L/12-h D periods for at least three complete 24-h cycles (2). Therefore, the experimental cycle was the fourth L/D cycle or beyond. Light intensity was 7,600 lux as supplied by fluorescent lamps.

Lipid Purification and Chromatography

Lipids were extracted and purified from algal tissue according to a modified Bligh-Dyer method (15). All solvents were of analytical grade as purchased. The final chloroform phase containing the purified lipid represents quantitative recovery of cell lipid; >98% of the radioactivity associated with the organic fraction obtained by direct alkaline saponification (16) of an aliquot of algal culture exposed to radioactive acetate (as below) is recovered in the saponified fraction from the cellular lipid extracted out of an identical portion of the same culture. Thin-layer chromatography (TLC) was carried out on plates coated with Merck type-60 F-254 adsorbent (Merck and Co., Inc., Darmstadt, Germany) in the solvent system of Allen and Good (17). Portions of the cellular chloroform lipid extract were resolved into fractions containing the total cellular polar glycerolipid and total neutral lipid in the first dimension only, whereas resolution of individual polar lipids required two-dimensional TLC. The sum of the total lipid-associated radioactivity recovered from the TLC plates was always >98% of that applied at the origin as quantitated radiochemically (below). Lipids were identified by their chemical, chromatographic, and staining properties (cf. 17). Quantitative elution of lipid from adsorbent was achieved by extracting the gel (15).

Radioactivity Measurements

Radioactivity was quantitated by liquid scintillation spectrometry using Biofluor (New England Nuclear, Boston, Mass.) as scintillant. Samples that were colored were bleached with stabilized sodium peroxide before the introduction of scintillant. For single-isotope counting, efficiencies, measured by an internal standard technique (18), were >55% for ^{3}H and >80% for ^{14}C. In double-isotope ^{3}H/^{14}C counting, efficiencies, assessed by a channels'-ratio method (19), were >25% for ^{3}H and >65% for ^{14}C. Sufficient raw counts were always accumulated to make for a counting error of <1.0%. Radioactivity was calculated as molar amounts of labeled precursor incorporated into the lipid assayed. Radiochemical purity of precursor used was >99% as verified in the TLC systems specified by the manufacturer (New England Nuclear).

Chemical Microquantitation

Chlorophyll (20), protein (21), and DNA (22) were quantitated by spectrophotometric assays. Glycerolipid mass was estimated as hydroxamic acid ester (23).

Miscellaneous Analyses

Cell number and estimations of division profiles in the cycle were determined by replicate hemacytometer counting of fixed cells (3). Statistical evaluation of the significance of the difference between two means was made by a Student *t* test (24). The area under a curve was estimated both by a "cut-and-weigh" technique and by calculation with the aid of calibrated grids (25).

Swamping of Intracellular Lipid Acetate Precursor Pool

Cultures at various times in the synchronous cycle were exposed for 20 min, under growth conditions and illumination appropriate to the time of the cycle to [^{3}H]acetate, sodium salt (2.0 Ci/mmol sp. act.; New England Nuclear) at acetate concentrations of 25, 50, 100, 200, 400, 800, 1,600, and 3,200 μM. To end a pulse, sufficient nonlabeled acetate in sterile, ice-cold medium was added to dilute the acetate specific radioactivity 200-fold and to simultaneously chill the cells. The algae were immediately pelleted at 2°C and were washed three times with the ice-cold medium containing the 200-fold excess of nonradioactive acetate. Lipids were quantitatively extracted and purified from the resulting cell pellets, and portions of the final chloroform extracts were assayed for chlorophyll and incorporation of radioactivity into total cellular lipid as described. Another portion of each cellular lipid extract was separated by one-dimensional TLC into polar lipid and neutral lipid fractions, which were also assayed for radioactivity. To avoid complication from biochemical parameters (e.g., chlorophyll or DNA masses [Fig. 1]) that continually change during the cell cycle, rates of lipid radiolabeling were calculated as molar amounts of acetate incorporated by algae at a given cell cycle time into total-cell, neutral, or polar lipid/10^6 mother cells per hour for each precursor concentration. The calculation was possible knowing the amount of chlorophyll in the portion of lipid extract analyzed and the concentration of chlorophyll/10^6 mother cells at any cell cycle time. The reciprocals of the incorporation rates were plotted against the reciprocals of the appropriate acetate concentrations. From the resulting double-reciprocal plots, the acetate concentration at which swamping of the cellular lipid acetate pool was achieved could be calculated (26, 27), at each cycle time sampled, for total algal lipid, and the neutral lipid and polar lipid subfractions therefrom.

Assessment of Lipogenic Rates

At various cell-cycle points, synchronous cultures were exposed for 20 min under growth conditions to [^{3}H]acetate, sodium salt (2.0 Ci/mmol sp act) or to [^{14}C]acetate, sodium salt (54.0 mCi/mmol sp act) at an 800 μM final acetate concentration. All experiments were also carried out at an acetate concentration of 1,600 μM with no difference in the results. The pulse was ended and the cellular lipid was extracted and purified from the washed algae as above. Portions of the chloroform extract were assayed for chlorophyll and for radioactivity incorporated into total cellular lipid as described previously. Other portions of the extract were fractionated into their individual polar lipid constituents by TLC (17), and adsorbent containing each resolved glycerolipid of interest was recovered and assayed for radioactivity. In some experiments, cultures were pulsed for 20 min with 5 mCi/ml ^3H_2O (5 Ci/g sp act) alone or with 5 mCi/ml ^3H_2O and 800 μM [^{14}C]acetate together, instead of radioactive acetate alone. For these experiments only, labeling was ended by chilling the cultures and immediately centrifuging them. Radioactivity was routinely calculated as molar amounts of acetate or H_2O so incorporated. Lipogenic rates are expressed as picomoles of incorporated precursor per 10^6 mother cells per hour for reasons already described and are each given at the time a pulse treatment was initiated.

Thylakoid Membrane Isolation

An analytical fraction of thylakoid membranes was purified from *Chlamydomonas* French-cell homogenates according to Chua and Bennoun (28). In brief (Janero and Barrnett, submitted for publication), the thylakoid fraction represents >75% of the 95% of chlorophyll recovered in the procedure, and is purified ~2.7-fold over the starting homogenate. Chlorophyll *a* to chlorophyll *b* ratios for the cells as a whole and the isolated membrane are identical, 2.1 ± 0.1 (SD; n = 5). Analysis of the thylakoid fraction by isopycnic sucrose density gradient centrifugation and electron microscopy supports results from marker enzyme assays[2] that the fraction is virtually homogeneous, the only detectable

[2] Janero and Barrnett submitted for publication. The enzymes assayed include: 5′-nucleotidase (EC 3.1.3.5), antimycin A-sensitive and antimycin A-insensitive NADH cytochrome *c* reductases (EC 1.6.2.2), nucleoside (inosine-5′) diphosphatase (EC 3.6.1.6), and cytochrome *c* oxidase (EC 1.9.3.1). Recovery of all activities through the fractionation with respect to starting homogenate was >98%, and the only activity detected in the thylakoid fraction was ~3% of the total homogenate cytochrome *c* oxidase activity.

contamination being ~3–4% of the total cellular mitochondrial population. These properties are in full agreement with the description of the fraction by Chua and Bennoun (28).

RESULTS

Properties of the Cell Cycle

Although wild-type *Chlamydomonas* is readily synchronized by alternating periods of light and darkness, the progression and timing of the cell cycle and the degree of synchrony vary with culture parameters such as CO_2 concentration, cell density, and temperature (2, 3). Therefore, it is necessary to establish the kinetics of the cycle for our cultures. The concentrations of cells, chlorophyll, and DNA in replicate cultures sampled hourly during the fourth L/D cycle are plotted in Fig. 1. Cell number remains constant throughout the light period and into the dark, the quadrupling of the cell population between ~2D and ~5.5D delimiting and M phase of some 3.5 h. DNA accumulates in a biphasic manner; most (~83%) is produced from ~11L to ~2.5D and probably represents nuclear DNA replication (29) to define an S phase lasting ~3.5 h and overlapping the beginning of the M phase by some 0.5 h. Approximately 15% of the total DNA produced in the cycle accumulates during the first 3.5 h of the photoperiod; this DNA has been identified as chloroplast DNA (30). Overall, a fourfold increase in cellular DNA is achieved before ~2.5D. Because mitoses are restricted to between 2D and 5.5D, and nuclear DNA accumulates from llL to 2.5D, the G_1 phase of the cell cycle lasts some 17.5 h, beginning at ~5.5D and extending to ~11L of the subsequent photoperiod. A discrete G_2 period is not long enough to be detected because of the slight overlap of S and M phases indicative of logarithmically growing algae (31).

The chlorophyll content of the culture begins to increase at ~3.5L, levels off by 10L at a concentration fourfold the 0L value, and is invariant throughout the dark period. Protein concentration in the culture increases in a virtually linear fashion during the light, but remains constant in the dark after a fourfold increase over the amount at 0L is attained near the end of the photoperiod (not graphed). Light microscope examination of fixed cells throughout the cycle indicates that division profiles are largely (>99%) restricted to between 2D and 5.5D; <1.0% of the entire cell population throughout the other 20.5 h of the cycle is in mitosis. From the microscopic appearance of cells in the cycle, the occurrence of well-defined mitoses in a discrete M phase, and the proportionate quadrupling of both the algal population and the culture concentrations of DNA, protein, and chlorophyll, it appears that the cells go through a highly synchronized cycle every 24 h, with patterns of DNA (3, 31), chlorophyll (3, 31), and protein (3, 31, 32) accretion qualitatively similar to those reported by others.

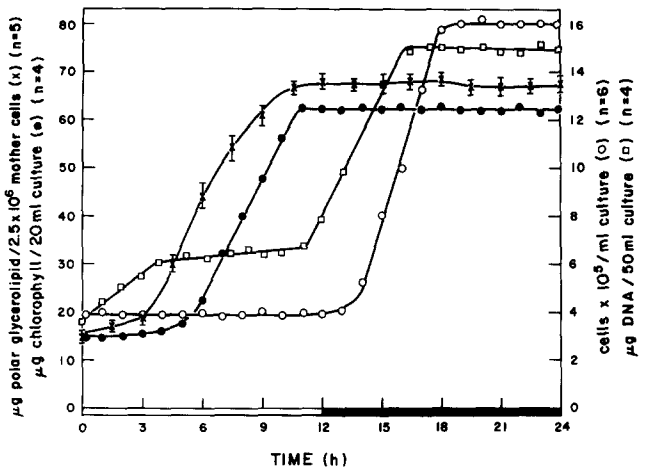

FIGURE 1 Variations in cell number, chlorophyll, DNA, and polar glycerolipid in synchronous cultures of wild-type *C. reinhardtii*. Cell number (○) was determined by replicate hemacytometer counting of algae fixed with 0.25% glutaraldehyde (final concentration). Chlorophyll (●) was quantitated spectrophotometrically in 80% acetone extracts using Arnon's coefficients (20); DNA (□) was assayed by a diphenylamine procedure (22). Polar glycerolipid (×) was purified by TLC and quantitated (±SD) as lipid ester (23). Values are plotted against cell cycle time and are representative of four to six independent cultures.

Polar Lipid Accretion during the Cell Cycle

The mass of polar glycerolipid in synchronous *Chlamydomonas* cultures was determined by hydroxamate ester analysis at 1.5-h intervals throughout the cell cycle (Fig. 1). Accretion of polar lipid occurs only during the light period, from middle to late G_1. Although accretion is detectable from the start of the photoperiod, >83% of the total lipid accumulated in the cycle is produced between 3L and 9L. The increase in the cellular concentration of polar lipid levels off between 10.5L and 12L/0D upon reaching a value fourfold higher than that at the start of the cycle (i.e., at 0L). No further increase in polar lipid concentration was detected in the 12-h dark period. Proportionate cell growth is again indicated by the correspondence between the fourfold increase in lipid mass during the light period and the quadrupling of the cell population beginning at ~2D.

Measurement of Cellular Lipogenesis

In preliminary experiments, we determined that acetate, even at low micromolar exogenous concentrations, is readily assimilated by the alga and incorporated into lipid throughout the cell cycle (33; also see reference 4). The assimilation and lipid labeling begin without detectable lags and remain linear for well over 20 min. However, the lipid labeling is markedly dependent upon the extracellular acetate concentration when the precursor is supplied in low micromolar concentrations (Fig. 2).

These characteristics of cellular lipid labeling from radioactive acetate at low precursor concentration can most readily be explained by rapid entry of the exogenously supplied radioactive acetate into the cellular lipid acetate precursor pool accompanied by dilution of the specific radioactivity of the pool by nonlabeled acetate probably derived from mobilized starch reserves (2). To obviate complication from fluxes in the cellular lipid acetate pool, conditions of treatment were established so that, irrespective of cell cycle time, the pool would be swamped with labeled precursor. Under these conditions, the intracellular acetate-specific radioactivity approaches that of the exogenous label (26). Because the intracellular acetate pool need not be the same for the two major lipid classes of the alga (cf. 27), it was necessary to assess the kinetics of acetate incorporation not only into algal lipid as a whole, but also into polar and neutral lipid resolved by TLC. We have restricted our labeling treatment to a pulse of 20 min, 1.4% of total cell cycle time. This interval is short enough to allow satisfactory kinetic resolution and minimize possible interference from lipid turnover, precursor catabolism, and long-term pool fluctuations

while permitting sufficient label to be incorporated into lipid for accurate radiochemical quantitation.

To define the exogenous concentration of acetate needed to swamp the intracellular lipid acetate pool throughout the cell cycle, aliquots removed from synchronous cultures were each pulsed for 20 min under growth conditions with a different concentration of radioactive acetate (from 25 to 3,200 μM), after which time the rates of lipid labeling during the pulse were assessed. When the labeling rates of total cellular lipid, and of the polar and neutral subfractions therefrom, are graphed against total acetate concentration in the medium, kinetic plots showing definite saturation are obtained (Fig. 2). However, the exogenous acetate concentration at which saturation is reached varies with cell cycle time. At any cycle point, the saturating concentration is the same for total algal lipid and for the polar and neutral subclasses therefrom, suggesting that the lipid acetate pool is a single, noncompartmentalized one. These results indicate that the rates of lipid labeling become independent of external acetate concentration only when the concentration is sufficient to swamp the intracellular lipid acetate pool in the face of cyclic variations in the pool's size. Expression of the data for the various cycle times in double-reciprocal plots allowed calculation that the highest external acetate concentration required to effectively swamp the pool for a 20-min labeling is <800 μM. Therefore, an appropriate labeling protocol for the reliable assessment of cyclic variations in lipogenic rate irrespective of the developmental state of the cells would be a 20-min pulse treatment at an acetate concentration of 800 μM under lighting that corresponds to the phase of the cell cycle.

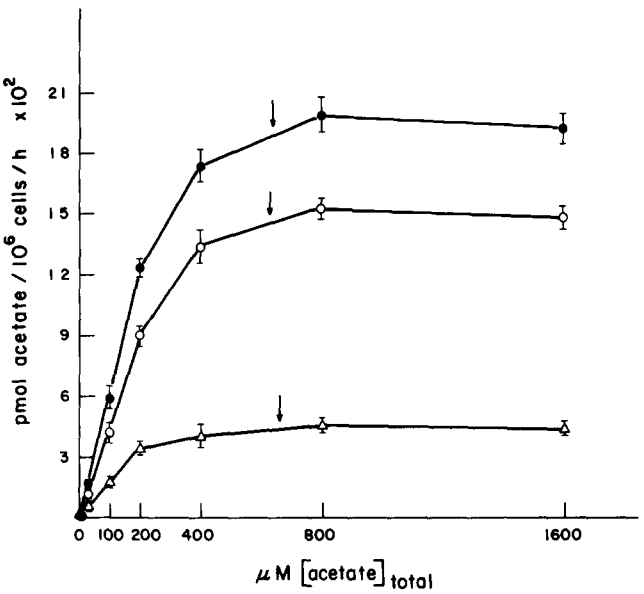

FIGURE 2 Influence of increasing precursor concentration on the rate of lipid labeling from acetate in synchronous *C. reinhardtii*. Aliquots of cells from replicate cultures at 9 *L* in the synchronous cycle were each "pulsed" for 20 min with [^{3}H]acetate at different concentrations, from 25 to 1,600 μM total acetate. The cells were washed free of unassimilated radioactivity, and their total lipid was extracted. A portion of this lipid was analyzed by scintillation spectrometry to determine the radioactivity associated with total algal lipid (●). Another portion was separated by TLC into total polar glycerolipid (○) and total nuetral lipid (△) fractions, each of which was similarly assayed for radioactivity incorporated from [^{3}H] acetate during the pulse. The rate of radiolabeling of each lipid class (pmol acetate/10^6 cells/h ±SD; $n = 5$) has been plotted against the appropriate total acetate concentration with which the algae were treated. Arrows indicate the concentrations at which the maximum rate (i.e., saturation) is attained, as calculated from double-reciprocal plots of the data plotted here.

Two independent checks were made on the ability of this protocol to yield reliable lipogenic rates. If saturation were achieved at all cycle time points by 800 μM acetate concentration, then treatment of cells at higher concentration should produce a labeling rate identical to that at 800 μM. The 800 and 1,600 μM data points in Fig. 2 for cells at 9L, as well as those in the similar plots for other cell cycle times, demonstrate this is indeed the case. A second check is based on the incorporation of tritium from ^3H_2O into lipid. As a lipogenic precursor, ^3H_2O has been proven to be independent of intracellular pools, free of permeability restrictions, and of high quantitative reliability in the measurement of fat synthesis (34). Exposure of algae at 0L, 6L, and 12L/0D to ^3H_2O and [^{14}C]acetate, either alone or together and with an acetate concentration of either 800 or 1,600 μM, yields the same patterns of lipogenesis for total cellular, polar, and neutral lipid (Table I). Therefore, the labeling protocol outlined in Materials and Methods allows

TABLE I

Measurement of Lipogenesis in Chlamydomonas with ^3H_2O and [^{14}C]Acetate

Precursor: cycle time of cell sample pulsed	Rate of precursor incorporation into: Total algal lipid*	Polar lipid*	Neutral lipid*
	%	%	%
^3H_2O, pmol ^3H_2O/10^6 cells/h			
0L	1,964 ± 107 (100)	1,671 ± 75 (100)	293 ± 21 (100)
6L	4,581 ± 389 (233)	3,519 ± 249 (211)	1,061 ± 63 (362)
12L/0D	40 ± 2 (2)	31 ± 2 (2)	9 ± 1 (3)
[^{14}C]acetate, pmol acetate/10^6 cells/h			
0L	2,768 ± 380 (100)	2,357 ± 361 (100)	411 ± 13 (100)
6L	6,851 ± 514 (247)	5,223 ± 554 (222)	1,628 ± 161 (396)
12L/0D	57 ± 6 (2)	44 ± 4 (2)	13 ± 1 (3)

Cultures of synchronous *C. reinhardtii* 137$^+$ were sampled at *0L, 6L,* and *12L/0D,* and aliquots at each time were pulsed under culture conditions for 20 min with ^3H_2O (5 mCi/ml) and [^{14}C]acetate (800 μM final concentration). After the pulse, cellular lipid was extracted, and a portion of the extract was analyzed by liquid scintillation spectrometry to determine the rate of incorporation of radioactivity from the precursors into total algal lipid. Another portion was separated by TLC into total polar glycerolipid and total neutral lipid subfractions, each of which was similarly counted to determine the rate of precursor incorporation into the lipid subclasses at each cycle time sampled. These rates are tabulated here ±SD ($n = 5$) for both ^3H_2O and [^{14}C]acetate, as is the percentage of the corresponding *0L* rate which the rates at *6L* and *12L/OD* represent. Results identical to these were obtained by pulsing cells with ^3H_2O and [^{14}C]acetate separately, at a final acetate concentration of either 800 or 1,600 μM.

* Percentage of precursor incorporation compared to 0L.

reliable determination of lipogenic rate through the cell cycle for algal lipid and, specifically, for polar lipid, which is the focus of this study. Further, because differences in lipogenic rate among 0L, 6L, and 12L/0D cells as determined by a ^3H_2O pulse are unaffected by the absence or presence of acetate (either 800 or 1,600 μM [Table I]), the precursor itself does not influence cellular lipid metabolism within our conditions of treatment.

Determination and Expression of Assembly Kinetics

In view of the suitability of our pulse protocol for determining cellular lipogenesis, algal labeling followed by isolation of thylakoid membrane, quantitative purification of its lipid constituents, and measurement of radioactivity incorporated into thylakoid lipid from acetate should allow reliable assessment of the assembly of lipid synthesized during the pulse into the photosynthetic membrane. This conclusion, however, could be significantly influenced by three additional factors: the homogeneity of the thylakoid fraction and the extent to which it is representative of the membrane *in situ*; the degradation of thylakoid lipid after cell homogenization; and the redistribution of lipid between thylakoid and other lipid-containing structures during cell fractionation.

The analytic nature of the thylakoid fraction used has been established by us (Janero and Barrnett, submitted for publication) and others (28) as isolated from asynchronous cells. In that nongreen organelles are elaborated during the cell cycle (35), we subjected thylakoid fractions isolated from algae at 6L, 12L/0D, and 8D to the complement of physical (sucrose density-gradient banding), ultrastructural (electron microscope examination), and biochemical (chlorophyll recovery; assay of mitochondrial, endoplasmic reticulum-nuclear envelope, Golgi, and plasma membrane marker enzyme[2]) analyses used to characterize the fraction from asynchronous cells and detailed elsewhere (Janero and Barrnett, submitted for publication). All thylakoid fractions share the same high degree of homogeneity, their contamination limited to ~3–4% of the total mitochondrial population. At all cell cycle times the isolated thylakoids represent >75% of the >95% of chlorophyll recovered in the fractionations and have the same chlorophyll a to chlorophyll b ratios as the whole algae. Regardless of the developmental stage of the cells from which they were isolated, then, the thylakoid fractions are of analytic quality and appear representative of the membrane *in situ*.

In other experiments, we were unable to detect on TLC (17) the presence of free fatty acid, lysolipids, phosphatidic acid, or pigment degradation products (36) in the thylakoid-membrane lipid extracts; nor could we detect chemically (37) any free fatty acid in these extracts. To further investigate possible alteration of thylakoid lipid during the fractionation, we added a thylakoid fraction prepared from cells treated in vivo with [^{3}H]acetate to a freshly prepared homogenate of unlabeled cells and then isolated thylakoids from the mixture; all the added radioactivity associated with thylakoid lipid was recovered in the final thylakoid fraction. We also combined an unlabeled thylakoid fraction with the pooled material discarded during the preparation of labeled thylakoids and reisolated the green lamellae; no lipid-associated radioactivity was found in the final thylakoid preparation. These "mixing experiments" provide additional evidence that there is no alteration of thylakoid lipid during lamellar isolation, a conclusion consistent with the separation of soluble cellular protein [presumably including any lipid-exchange proteins (38) or lipolytic enzymes (36)] from membrane within minutes of cell homogenization during an initial centrifugation.

Based upon these findings, the ability of our labeling protocol to reliably assess lipogenesis, and the quantitative recoveries obtained during lipid extraction and chromatography, we regard the amount of radioprecursor incorporated over pulse time into the lipid of isolated thylakoid membrane as indicative of the rate of lipid assembly into that membrane. All assembly rates have been normalized on a mother-cell basis to make them independent of cyclic changes in the concentrations of various substances in either the cell or the thylakoid. Furthermore, because the number of mother cells represented by a portion of a thylakoid membrane lipid extract is calculated from the amount of chlorophyll in the aliquot and the concentration of chlorophyll per mother cell, the assembly rates are thus independent of thylakoid recovery as well.

Kinetics of Thylakoid Polar Glycerolipid Synthesis and Assembly

The kinetics of synthesis and assembly of the five thylakoid lipids (MGDG, DGDG, SL, PG, and DGTS) as a whole during the *Chlamydomonas* synchronous cycle are presented in Fig. 3. Rates of synthesis and assembly are highest in the

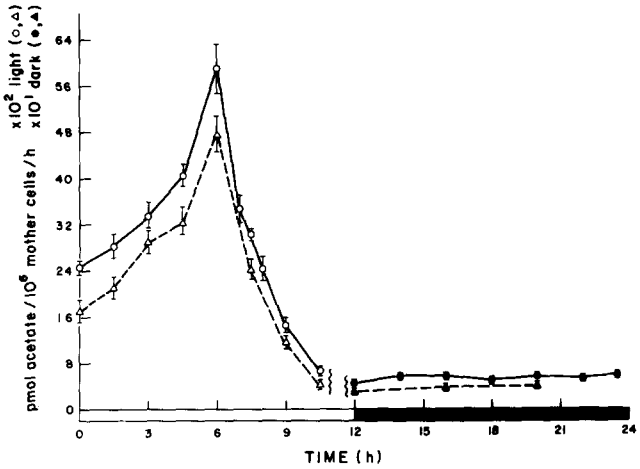

FIGURE 3 Synthesis (○, ●) and assembly (△, ▲) of polar glycerolipid during the vegetative cell cycle of *C. reinhardtii* 137$^+$. Synchronous cells were pulse labeled under growth conditions for 20 min with [^{3}H]acetate, sodium salt (final concentration, 800 or 1,600 μM with the same results) beginning at the times plotted during the light (open symbols) and dark (filled-in symbols) periods. The pulse was terminated, the cells were washed free of unassimilated radioactivity, and the algae were subjected either to lipid extraction or to thylakoid membrane isolation. In the latter case, thylakoid lipid was extracted subsequent to membrane purification. Cellular and thylakoid-membrane lipid were each chromatographed on TLC, and the major (~95% of total) cellular polar glycerolipids and the total thylakoid polar glycerolipids (i.e., MGDG, DGDG, SL, PG, and DGTS) were collectively recovered from the respective chromatograms and assayed for radioactivity incorporated during the pulse. The rate of lipid synthesis is expressed as picomoles acetate incorporated into the major cellular polar glycerolipids per 10^6 mother cells per hour ± SD ($n \geq 4$), and the rate of lipid assembly is expressed as picomoles acetate incorporated into total thylakoid polar glycerolipid per 10^6 mother cells per hour ± SD ($n \geq 4$). Each rate is plotted at the time a pulse labeling began; thus, the point at 0 *L* represents cells at 0 *L* in the synchronous cycle pulsed for 20 min. Note the difference in the exponent of the *y*-axis between light and dark.

photoperiod (mid-to-late G_1) and are negligible in the dark (S, M, and early-to-mid G_1). During the first half of the light period, both processes steadily increase to reach maxima at 6L (i.e., in cells at 6L pulsed 20 min with radioprecursor). The processes are then approximately threefold greater than their respective rates at 0L, the start of the cell cycle. From the maxima, synthesis and assembly decline, the sharp drop of well over one order of magnitude between 10.5L and 12L/0D leading to low, fairly constant rates in the dark which are <1% the respective 6L maxima. At the beginning of the subsequent cycle, with the end of the dark period, there is a marked step-up in synthesis and assembly. The cyclic variations of glycerolipid assembly into thylakoid are qualitatively similar in pattern to the lipogenic variations at the cellular level; quantitatively, the assembly rates vary from ~66% (at 0L) to ~80% (at 12L/0D) of the respective synthetic rates.

Synthesis and Assembly of the Individual Thylakoid Polar Glycerolipids

The kinetics in Fig. 3 were examined in greater detail through determination of the cyclic variations in the synthesis and assembly of each thylakoid polar lipid as resolved by TLC. The cyclic variations for the glycolipids are given in Fig. 4. High rates of galactolipid (MGDG and DGDG) and SL synthesis and assembly are exclusive to the photoperiod with maximum rates established at 6L. The processes take place at low levels in the dark which are negligible compared to light-period metabolism. MGDG and DGDG exhibit a striking parallelism in their synthesis and assembly kinetics through the cell cycle, and the synthesis and assembly of each glycolipid display similar qualitative variations in cycle time. Nonetheless, qualitative and quantitative kinetic differences between the galactolipids and the sulfated glycolipid are apparent; e.g., the maximum 6L rates of MGDG and DGDG synthesis and assembly are approximately three times higher than the respective 0L rates, whereas the 6L maxima for SL represent increases of approximately six times over the 0L values.

Similar to the glycolipids, the sole thylakoid phospholipid, PG, is synthesized and is assembled into the photosynthetic lamellae largely during the photoperiod (Fig. 5). The fairly constant, high rates of PG synthesis and assembly from 0L to 4.5L increase approximately twofold to 6L maxima.

The synthesis and assembly of ether lipid (DGTS) are also virtually restricted to the light period (Fig. 5). Increasing rates from 0L reach 6L maxima that are over three times the respective values at the start of the cell cycle. As is characteristic of the thylakoid glycolipid and phospholipid kinetics, synthesis and assembly of DGTS vary in a qualitatively coordinate manner during algal vegetative development.

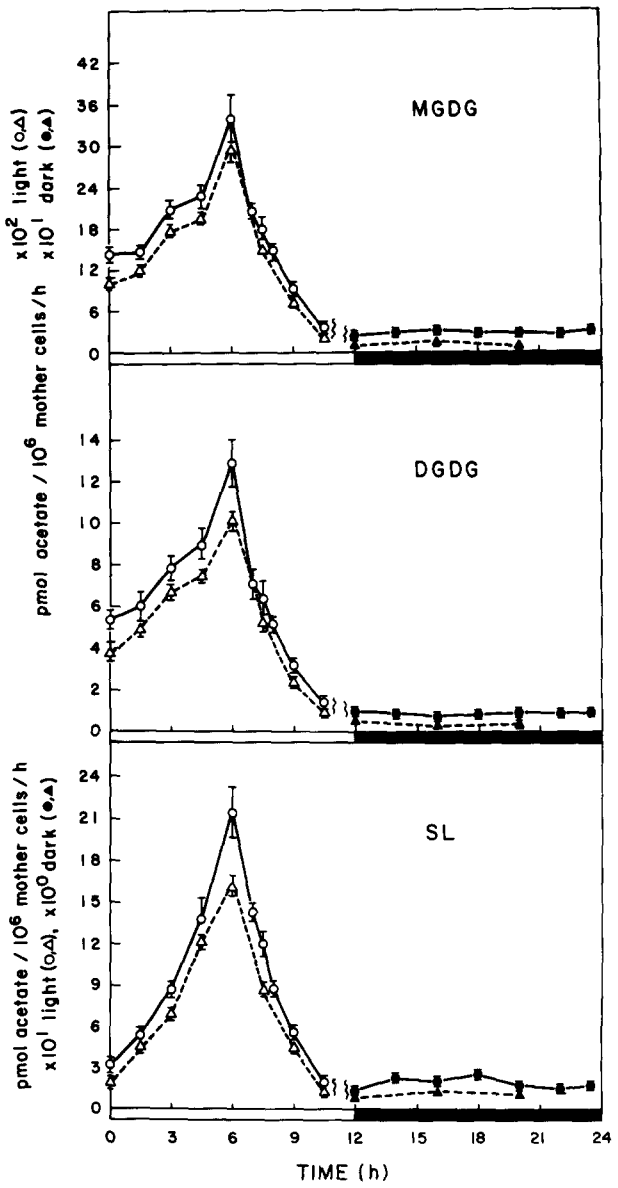

FIGURE 4 Synthesis (○, ●) and assembly (△, ▲) of glycolipids in synchronous *C. reinhardtii* 137⁺. Experimental conditions are identical to those in Fig. 3, except that each glycolipid (*MGDG, DGDG,* and *SL*) was recovered from the TLC chromatograms and assayed for radioactivity incorporated from lipogenic radioprecursor, acetate. The rates of synthesis are expressed as picomoles acetate incorporated into each respective cellular glycolipid per 10^6 mother cells per hour ± SD ($n \geq 4$), and the rates of glycolipid assembly into thylakoid are expressed as picomoles acetate incorporated into each thylakoid glycolipid per 10^6 mother cells per hour ± SD ($n \geq 4$). Rates are plotted at the start of a pulse treatment. The exponents associated with the *y*-axis are different for the light (open symbols) and the dark (filled-in symbols) periods.

DISCUSSION

Detailed kinetic dissection of biogenetic lipid assembly into the green-plant thylakoid membrane is complicated by the limited technology of plant cell fractionation (39) and by the fragmentary knowledge concerning the pathways and intracellular sites of plant lipid synthesis (40). As a result, the most widely used approach to study the relationship between thylakoid formation and lipid synthesis has been an indirect one at the intact tissue level and has used etiolated green-plant or algal mutants that apparently synthesize chlorophyll and assemble thylakoids only while exposed to light (41). The lipid biochemistry and radiolabeling patterns of tissue "greened" in the light are compared with those of the etiolated tissue left in the dark, and any differences are assumed to reflect thylakoid membrane production.

We have chosen to study the biogenesis of thylakoid membrane by using synchronous cultures of the unicellular green alga *Chlamydomonas reinhardtii* 137⁺ (wild-type). In contrast to etiolated cells, this phytoflagellate contains a fully functional and fully formed chloroplast lamellar system that undergoes elaboration in the course of the cell cycle (42), permitting isolation of thylakoid fractions throughout algal development.

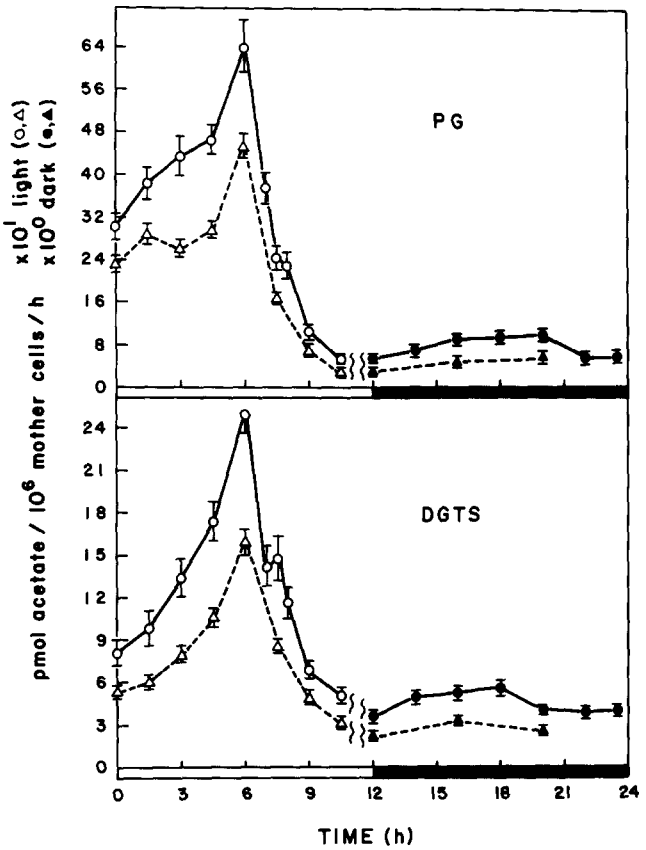

FIGURE 5 Phospholipid (*PG*) and ether lipid (*DGTS*) synthesis (○, ●) and assembly (△, ▲) in synchronous *C. reinhardtii* 137⁺. Experimental conditions are the same as in Fig. 3, except that incorporation of radioactive acetate into *PG* and *DGTS* are quantitated. The rates of synthesis are expressed as picomoles acetate incorporated into cellular PG or DGTS per 10^6 mother cells per h ± SD ($n \geq 4$), and the rates of PG and DGTS assembly into thylakoid membrane are given as picomoles acetate incorporated into thylakoid *PG* or thylakoid DGTS per 10^6 mother cells per hour ± SD ($n \geq 4$). Rates are plotted at the start of a 20-min pulse. Note the difference in exponent of the *y*-axis between light (open symbols) and dark (filled-in symbols) periods.

We have used short-term pulse labeling with radioactive acetate to determine variations in polar glycerolipid synthesis and assembly into thylakoids during the vegetative cell cycle of this chlorophyte. Experimental definition of the true extents of any differences in polar lipid synthesis among algae at distinct developmental stages depends critically on reliable assessment of lipogenic rate in the face of cyclic changes in the size of the intracellular lipid acetate pool brought about by the entry of nonlabeled acetate into this metabolite compartment. Similar cyclic variations have been reported (4) in the utilization of exogenously supplied acetate for protein synthesis and in the dependency of the rates of protein labeling on acetate concentrations below 100-μm in algae at 2L and 10L. Whether lipogenesis and protein synthesis use the same metabolite pools remains open to question. Nonetheless, the high affinity of the alga for a given metabolic radioprecursor, and its ready penetration into intracellular pools, cannot per se be taken to mean that molecular labeling obtained in vivo is directly indicative of synthetic rate (cf. 9).

An attempt has been made to overcome the problems attendant to cyclic fluctuations of the intracellular lipid acetate precursor pool by establishing a concentration at which the pool would be swamped throughout the cycle for a 20-min pulse period. Labeling patterns obtained at an acetate concentration (1,600 μM) double that needed for swamping and with ^3H_2O as precursor verify that our labeling protocol can be used to assess, accurately, lipogenic rate throughout the cell cycle. Correspondence between the cyclic rate patterns obtained with labeled acetate and ^3H_2O further indicates that the presence of acetate during the pulse alters neither the phototrophic metabolism nor the lipid metabolism of the cells.

Although pulse labelings shorter than 20 min could theoretically be carried out, the corresponding increases in the amounts of radioprecursor needed to obtain sufficient isotope incorporation into lipid for reliable radiochemical quantitation become a hindrance in practice. With a pulse period of 20 min, we obtain sufficient radiolabel in lipid at all cycle points so that the counting error is <1.0% and we simultaneously achieve kinetic resolution to an interval that is 1.4% of the cell cycle. Evidence from other actively cycling cells (43) and the proportionality between the fourfold increase in polar lipid mass during the light period and the subsequent mitotic quadrupling that synchronous, log-phase *Chlamydomonas* display suggest that this level of temporal resolution is sufficient to minimize possible effects of long-term intracellular pool changes and of precursor catabolism on the biosynthetic rate measurements.

Although the thylakoids of *Chlamydomonas* contain widely differing polar glycerolipid types, our results demonstrate that the synthesis and assembly of their five polar glycerolipids are largely confined to the light period of the synchronous cycle (mid-to-late G_1). During the first half of the photoperiod (0L–6L), synthesis and assembly rates for each polar lipid increase to reach maximal values at 6L and fall over one order of magnitude with the onset of the dark period, between 10.5 L and 12L/0D. A low level of thylakoid polar glycerolipid assembly is characteristic of the dark period, from which a striking step-up in assembly takes place with the start of the next light/dark cycle.

In view of the nature of labeling with a general lipogenic precursor, quantitative comparison among synthesis or assembly rates for any one lipid at different cell cycle times is valid; but comparison of absolute rates from one lipid to another at any given cycle time is complicated by factors other than synthesis (molecular structure, cellular prominance, etc.) that determine the amounts of radiolabeled moiety that can be incorporated into the various lipids from acetate (i.e., acetyl-CoA). Nonetheless, the magnitude of the changes in synthesis and assembly that the polar glycerolipids undergo through the cycle may be compared among the lipids. Such comparison reveals significant kinetic differences among the galactolipids, SL, PG, and DGTS. For example, within the first 6 h of the photoperiod, the increases in the synthesis and assembly rates of DGTS and, especially, SL are greater than the increases displayed by galactolipid and PG. The differences among thylakoid polar glycerolipids in the extents to which their synthesis and assembly rates vary through the cell cycle imply that thylakoid lipids are assembled in a multistep mode. The lipids are not introduced into the membrane in discrete, sequential "waves," as has been proposed for the photoperiod assembly of thylakoid protein (4). Rather, the multistep nature of polar lipid assembly is reflected in the varying degrees to which each lipid's assembly rate fluctuates during cell cycle time within a common pattern that restricts lipogenesis largely to the photoperiod.

The rapidity with which the *Chlamydomonas* lipogenic rate

drops as the photoperiod ends and with which it rises early in the light period suggests the operation of short-term lipogenic controls triggered by the light/dark illumination pattern. The short-term photocontrols, in turn, appear to be part of a longer term modulation of polar lipid synthesis and assembly over periods of several hours within both the light and the dark. Whether the short-term mechanisms involve regulation of enzymatic activity amid long-term fluctuations in the absolute amounts of key lipogenic enzymes, as in the hepatocyte (44), is under investigation. It has been reported (45) that acyl-carrier-protein-dependent fatty acid synthetase activity increases some threefold during the photoperiod as a result of *de novo* production. The temporal coincidence of lipid and protein accretion during the photoperiod would seem to provide an attractive situation if such long-term modulation were to depend upon enzyme production.

Details of the biosynthetic pathways for the polar lipids are not worked out in *Chlamydomonas* and cannot yet be deduced from evidence obtained in other plant systems (8, 40). The physiological bases for the similarities and distinctions among the cyclic synthetic patterns of each polar glycerolipid are, therefore, left largely to conjecture. For instance, the striking parallelism in the cyclic variations of MGDG and DGDG synthesis is consistent with hypotheses (46) that MGDG serves as the immediate precursor to DGDG, but the hypotheses do not yet provide a conclusive rationale. More generally, because there is no net increase in polar lipid concentration from ~10.5L to ~12D/0L in synchronous *Chlamydomonas*, the low lipogenic rates during the dark appear to support lipid turnover. Similar reasoning has been invoked (32) to explain the low levels of protein radiolabeling in the dark, when no protein accumulates. The slight, transient increases that span the M phase in the synthesis of some polar lipids (e.g., DGTS) may be indicative of a small amount of lipid production attendant to cytokinesis (47). The high levels of photoperiod lipid synthesis are most likely responsible for the fourfold increase in polar lipid mass that takes place in the light (mainly between 3L and 9L) before M phase at ~2D, and the rapidity of lipid assembly during the light period is consistent with the accumulation of thylakoids before cell division (1, 42). The close temporal coincidence between polar lipid synthesis and accretion and the equivalence of the areas under the respective kinetic plots suggest that the contribution of turnover to the measured rates of polar lipid synthesis is negligible during the photoperiod (43). Thus, lipid required by the daughter cells is produced some 2 h before mitoses begin.

Comparison of our assembly data at the subcellular level with lipid changes that occur in greening plant tissue is difficult, mainly because of the indirect nature of the latter studies. Greening of the y-1 mutant of *C. reinhardtii* is accompanied by the accretion and enhanced radiolabeling of MGDG, DGDG, SL, PG, and carotenoid in close temporal coincidence with chlorophyll accumulation (48, 49), but the data offer no detailed synthetic rates upon which even assumptions regarding subcellular assembly of lipid into thylakoid membrane can be reasonably made.

Beck and Levine (9) studied the appearance of label from $^{32}PO_4^{3-}$ into PG, from $H^{14}CO_3^-$ into MGDG and DGDG, and from $^{35}SO_4^{2-}$ into SL associated with a green membrane pellet centrifuged out of *Chlamydomonas* homogenates prepared by sonicating synchronous, wild-type cells labeled at different times during the cell cycle. Although the limited results of their study are consistent with our comprehensive survey in showing that high rates of lipid assembly into thylakoids characterize the photoperiod, the kinetics advanced do not agree with ours. Whereas it is not clear whether the labeling protocols of Beck and Levine accurately measure lipogenesis or whether their membrane fraction is of analytic quality, their data may have been influenced by factors such as differential cyclic fluxes in intracellular pools. The greater temporal resolution afforded us by a 20-min pulse (as opposed to a 1-h exposure) may also bear on the differences.

For convenience, we have termed the appearance of synthesized glycerolipid in purified thylakoid membrane "assembly." The assembly rates we measure, though, result from at least three coordinated processes (1): biosynthesis of glycerolipid in general; transit of some lipids so produced to the thylakoids; and integration of the lipid into the supramolecular structure. The subcellular lipid assembly kinetics can be placed in the context of cellular lipogenesis by comparing the cyclic variations in the rate of thylakoid glycerolipid assembly with the variations in lipid synthesis rate. The absolute rates of synthesis and assembly at any particular cell cycle time are not the same; because no polar glycerolipid is exclusive to the thylakoid, and because the molecular species of thylakoid and extrathylakoid glycerolipid are distinctive (10, 11; Janero and Barrnett, submitted for publication), we presume the differences reflect lipid production for the elaboration of extrathylakoid structures before the M phase. However, the cyclic variations of polar glycerolipid assembly into thylakoids strikingly mirror the cyclic variations in synthesis, a parallelism that also occurs at the level of each individual thylakoid polar lipid. The coordination between cellular glycerolipid production and the assembly into thylakoids of a portion of the lipids produced with specific molecular characteristics suggests that discrimination of polar acyl lipid takes place subsequent to synthesis, but before the introduction of the lipid into the membrane, which itself shows little (if any) capacity for polar glycerolipid synthesis (40).

Whatever the topology of lipid transit or even of lipid integration into the membrane matrix, the close temporal coincidence between the cyclic variations in polar glycerolipid synthesis and assembly in synchronous *Chlamydomonas* and the rapidity with which thylakoid lipids are radiolabeled in vivo suggest that the transit and integration steps are so rapid as to be nonlimiting to membrane biogenesis. The virtually instantaneous appearance of lipid synthesized by isolated spinach leaves in their chloroplast thylakoids is further support for the conclusion (50), and the rapid in vivo labeling of *Chlamydomonas* thylakoid polypeptides from exogenous radioprecursor (3) may warrant extention of the conclusion to thylakoid protein. Rather, such close temporal coincidence between lipogenesis and thylakoid lipid assembly through cell cycle time strongly indicates that lipid synthesis itself is the rate-determining, if not rate-limiting, step in the lipid assembly supporting thylakoid biogenesis. A similar relationship between lipid synthesis and membrane production has been indirectly deduced (51) from the requirement that the cycling of various transformed and untransformed animal cell lines display for membrane lipid (sterol and phospholipid). It is known that the control of membrane phospholipid production in *Escherichia coli* lies at the level of the synthesis of both phospholipid and its fatty acids, although the precise in vivo regulators are equivocal (52).

For synchronous *Chlamydomonas*, such reasoning would mean that diminution in the assembly of thylakoid membrane

lipid results from the lack of availability of the requisite polar glycerolipids during periods of decreasing lipogenesis. After synthesis of the various cellular glycerolipids, those species to be added to thylakoid membrane are discriminatorily and rapidly routed to the membrane and are assembled into it. Whether these mechanistic principles are valid during the greening of yellow *Chlamydomonas* mutants, whether the suggestive evidence for them in other eucaryotic cells will be augmented with additional supporting data, and whether they apply generally to all thylakoid lipids demands further work to ascertain. To this intent, the mechanisms regulating the synthesis and assembly of glycerolipid and other lipid types in *Chlamydomonas* are under study.

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