

Thylakoid Membrane Biogenesis in *Chlamydomonas reinhardtii* 137⁺

II. Cell-cycle Variations in the Synthesis and Assembly of Pigment

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ABSTRACT Synthesis of the chlorophyll and the major carotenoid pigments and their assembly into thylakoid membrane have been studied throughout the 12-h light/12-h dark vegetative cell cycle of synchronous *Chlamydomonas reinhardtii* 137⁺ (wild-type). Pulse exposure of cells to radioactive acetate under conditions in which labeling accurately reflects lipogenesis, followed by cellular fractionation to purify thylakoid membrane, allowed direct analysis of the pigment synthesis and assembly attendant to thylakoid biogenesis. All pigments are synthesized and assembled into thylakoids continuously, but differentially, with respect to cell-cycle time. Highest synthesis and assembly rates are confined to the photoperiod (mid-to-late G₁) and support chlorophyll and carotenoid accretion before M-phase. The lower levels at which these processes take place during the dark period (S, M, and early-to-mid G₁) have been ascribed to pigment turnover. Within this general periodic pattern, pigment synthesis and assembly occur in a "multi-step" manner, i.e., by a temporally-ordered, stepwise integration of the various pigments into the thylakoid membrane matrix. The cell-cycle kinetics of pigment assembly at the subcellular level mirror the kinetics of pigment synthesis at the cellular level, indicating that pigment synthesis not only provides chlorophyll and carotenoid for thylakoid biogenesis but may also serve as a critical rate-determinant to pigment assembly.

The photosynthetic (thylakoid) membrane of the green-plant chloroplast is characterized by its content of colored neutral lipids, the green chlorophylls and the yellow-orange carotenoids (1). As "functional lipids" with direct and indirect roles in the capture and/or conversion of light energy, the pigments have distinctive distributions both spatially along the thylakoids and functionally among photosystems I and II and the light-harvesting complex (2). Although some carotenoids are present outside the thylakoids in osmiophilic plastoglobuli (3) and in the chloroplast envelope (4), the physiological importance of the pigments to photosynthesis is such that most of the carotenoid of green plants is localized in the thylakoids, and the chlorophylls are present exclusively in that membrane (1). At least some of the critical steps in pigment biosynthesis take place within the chloroplast itself (5).

The functioning of the pigments at the level of the thylakoid

membrane and their distinct intramembrane topologies which reflect this physiology make the biogenetic assembly of pigment into thylakoids important not only for membrane and organelle development but also for the continued photosynthetic metabolism of the plant as a whole. We have been investigating (6, 7) the relationships among lipogenesis, membrane biochemistry and biogenesis, and cell development by utilizing the Chlorophyte *Chlamydomonas reinhardtii* 137⁺ (wild-type). The ability to obtain highly-synchronous, axenic cultures of this green alga facilitates kinetic analysis of membrane biogenesis, and the high progeny yield every cell cycle (i.e., every 24-h) demands appreciable production of membrane constituents and their assembly into supramolecular structures (8). Further, the pigment complement of the alga has been shown by others (9, 10, 11) and was confirmed by us (12) to consist virtually exclusively (~98% of total pigment) of two chlorophylls (*a* [54.8%] and *b*

[24.2%]) and six major carotenoids: β -carotene (6.3%), lutein (4.4%), violaxanthin (3.1%), neoxanthin (2.8%), lorenzoanthin (1.9%), and luteoxanthin (0.7%). Here we detail the kinetics of pigment synthesis through the synchronous *Chlamydomonas* cell cycle and establish at the subcellular level the kinetics and mode of assembly of synthesized pigment into thylakoids during biogenesis of the photosynthetic membranes.

MATERIALS AND METHODS

Cell Culture

C. reinhardtii 137⁺ (wild-type) was grown phototrophically in minimal medium (13) in axenic, log-phase culture at $25 \pm 0.5^\circ\text{C}$ with continuous stirring and aeration (14). Cultures were synchronized by exposure to an automated program of alternating 12-h light (*L*)/12-h dark (*D*) periods for at least three complete 24-h cycles (6, 15). Therefore, the experimental cycle was the fourth *L/D* cycle or beyond. Under these culture conditions, the cells undergo a highly synchronous cycle every 24 h: cell number is constant through the photoperiod, and mitoses are restricted to between $\sim 2D^1$ and $\sim 5.5D$ with a fourfold increase in cell number every cycle (6). Patterns of protein (6, 16) and DNA (6, 8) accumulation are qualitatively similar to those reported by others, as are the cell-cycle phases (6, 8).

Pigment Purification and Chromatography

Lipids were quantitatively (>98%[6]) extracted and purified according to a modified Bligh-Dyer procedure (17). Purification of pigment to constant specific radioactivity from noncolored lipid and separation of the tissue pigments were achieved by thin-layer chromatography (TLC) on Merck Type-60 F-254 gel with fluorescence indicator (Merck and Co., Darmstadt, W. Germany) as described (12, 18). Noncolored lipid was visualized under ultraviolet light or by exposure to iodine vapor (19); colored lipid was visualized directly on the plate. The individual pigments were identified by a variety of chromatographic and chemical methods (12). When necessary, chlorophylls and carotenoids were quantitatively eluted into acetone and ethyl ether, respectively. Adsorbent containing radioactive lipid was counted by liquid scintillation spectrometry as detailed (12). Recovery of pigment and pigment-associated radioactivity applied to TLC plates was always $\sim 98\%$ of that chromatographed.

Pigment Quantification

Chlorophyll was quantitated spectrophotometrically in 80% acetone extracts using Arnon's coefficients (20). Carotenoid was quantified spectrophotometrically in anhydrous ethyl ether (3).

Assessment and Expression of Pigment Synthesis and Assembly

At various cell-cycle times, synchronous cultures were "pulsed" under growth conditions for 20 min with [³H]acetate (New England Nuclear, Boston, MA; sp act: 2.0 Ci/mmol) or [¹⁴C]acetate (sp act: 54.0 mCi/mmol) at, routinely, an 800- μM or a 1,600- μM final acetate concentration, with no difference in the results. These acetate concentrations are sufficient to saturate the (single) intracellular acetate precursor pool used for lipogenesis at all cell-cycle points (6). That the protocol allows reliable assessment of lipogenesis has been verified (6) by comparative, *in vivo* labelings with ³H₂O (sp act: 5 Ci/g).

Labeling was ended after 20 min by diluting the specific radioactivity of acetate in the culture 200-fold with nonlabeled acetate in ice-cold, sterile medium. The algae were immediately pelleted at 2°C and were washed three times with the ice-cold medium containing the excess of nonlabeled acetate. This treatment effectively quenches acetate incorporation into lipid and frees the cells from unassimilated precursor. The washed algae were subjected to lipid extraction, or, alternatively, thylakoid membranes were purified therefrom as described elsewhere (6, 21) and the lipids were immediately extracted from the isolated photosynthetic lamellae (17). The lipid extracts were used for the chromatographic purification of the various pigments as detailed (12). To avoid complication from biochemical parameters (such as pigment [Fig. 1] or protein [6, 16] masses) which are continually changing in the cell cycle, synthesis and assembly

¹ The number immediately preceding *L* or *D* denotes time in hours during the *L* or *D* period, respectively. Therefore, "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.

rates have been calculated as molar amounts of acetate incorporated into the particular pigment(s) assayed/ 10^6 mother cells/h. The calculation was possible knowing the amount of chlorophyll in the portion of lipid extract analyzed and the concentration of chlorophyll per 10^6 mother cells at any cycle time.

Miscellaneous Analyses

Cell number was determined by replicate serial-dilution hemacytometer counting of algae fixed in 0.2% glutaraldehyde (final concentration). Statistical evaluation of the significance of the difference between two means was made with a Student-type *t*-test; $P < 0.05$ was taken as indication of a significant difference (22). Area under a curve was determined with the aid of calibrated grids and by a "cut-and-weigh" technique (23).

RESULTS

Pigment Accretion during the Cell Cycle

In synchronous cultures of wild-type *Chlamydomonas*, the concentrations of both chlorophyll and carotenoid increase during the photoperiod (mid-to-late *G*₁ [6]) to reach values by 12*L*/0*D* which are some fourfold higher than at 0*L* (Fig. 1). Since the cell population subsequently quadruples at $\sim 2D$ (6), the pigment accretion observed is indicative of proportionate cell growth. Although an increase in carotenoid concentration is evident between 1.5*L* and 3*L*, significant chlorophyll accumulation begins only after a lag of ~ 3 h, as has been noted by others (8, 16). More than 90% of the total carotenoid and chlorophyll accumulation occurs by 10.5*L*. Both chlorophyll *a* and *b* accrue in parallel with patterns qualitatively similar to that of total chlorophyll accumulation, the chlorophyll *a*-to-*b* mass ratio 2.4 ± 0.2 (SD; $n = 6$) through the cycle. The concentrations of chlorophyll and carotenoid in the mother cells (or in the culture) are constant during the dark period (*S*, *M*, and early-to-mid *G*₁).

Determination and Expression of Assembly Kinetics

In a previous report (6), a pulse-labeling protocol with radioactive acetate as precursor was developed and was verified to reflect, accurately, lipogenesis through the *Chlamydomonas* vegetative cycle. Therefore, application of the protocol at the

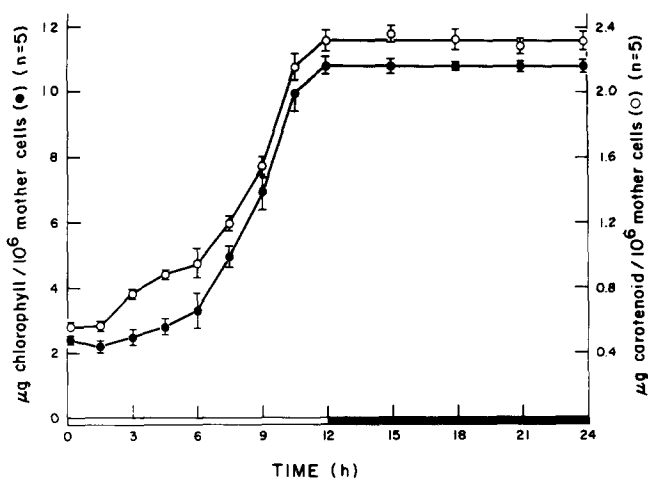


FIGURE 1 Pigment accretion in synchronous *C. reinhardtii* 137⁺. Samples taken from synchronous algal cultures at various times in the cell cycle were assayed spectrophotometrically for total chlorophyll (●) and total carotenoid (○) content. The pigment masses so obtained, normalized on a mother-cell basis, are plotted against cell-cycle time in hours. The data points are means \pm SD and are representative of five independent cultures.

cellular level followed by isolation of thylakoid membrane, quantitative purification of its pigment constituents, and measurement of the radioactivity incorporated into each pigment from labeled acetate during the pulse should allow direct and reliable assessment of the assembly of newly synthesized pigment into the photosynthetic lamellae. This conclusion, however, may be undermined by two additional factors: the degree to which the isolated thylakoids are representative of the membrane *in situ* and any alteration of thylakoid pigment during cellular fractionation.

The analytical nature of the thylakoid fraction, as isolated from asynchronous cells and from synchronous cells at various times during the cell cycle, has been described by others (21) and detailed by us (6, 24) using a variety of physical, fine-structural, and biochemical methods. To investigate possible alteration of thylakoid pigment during fractionation, we performed two types of "mixing experiments." In the first, a thylakoid fraction purified from cells exposed to [³H]acetate was added to a freshly prepared homogenate of unlabeled cells, from which thylakoid membrane was isolated; all the added radioactivity associated with thylakoid pigment was recovered in the final thylakoid preparation. A second mixing experiment combined an unlabeled thylakoid fraction with the pooled material discarded during the preparation of ³H-labeled thylakoid membrane; no pigment-associated radioactivity was found in the re-isolated thylakoids. The results of these "mixing experiments" substantiate the conclusion that no alteration of thylakoid pigment takes place during lamellar isolation. We were unable to detect by paper chromatography (25) or on TLC (12, 26) any substances in the total thylakoid-membrane lipid extract that would indicate pigment breakdown (e.g., phytol from the action of chlorophyllase, EC 3.1.1.14 [27]). Pigments extracted from the thylakoid fraction were well-resolved by TLC as discrete, defined bands with no streaking or smearing (12), further evidence that pigment does not degrade during thylakoid preparation.

On the basis of these data, the ability of our labeling protocol to yield reliable rates of pigment synthesis (6), and the quantitative nature of the pigment extraction and purification (12), we regard the amount of radioactive precursor (acetate) incorporated over pulse time into pigment(s) of isolated thylakoid membrane as the rate of biogenetic pigment assembly into that membrane. The 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 thylakoids (6). Since 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 also independent of thylakoid membrane recovery.

General Cyclic Variations in Pigment Synthesis and Assembly

The nature of the pigment dynamics attendant to thylakoid membrane biogenesis was defined by examining the synthesis of total cellular pigment and the assembly of total pigment into thylakoids through the *Chlamydomonas* cell cycle (Fig. 2). In both the cell and its photosynthetic membrane, total pigment is comprised virtually exclusively (~98% [9–12]) of two chlorophylls, *a* and *b*, and six major carotenoids: β -carotene, neoxanthin, luteoxanthin, violaxanthin, luteoxanthin, and lutein. Highest rates of pigment synthesis and assembly are confined to the photoperiod (mid-to-late G₁). Maxima are attained by

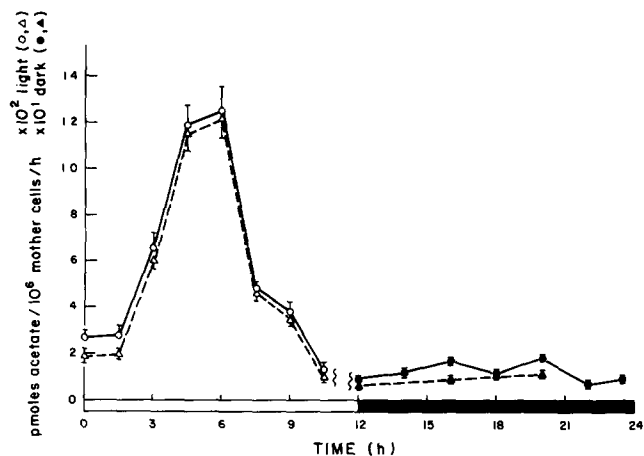


FIGURE 2 Pigment synthesis (\circ and \bullet) and assembly (Δ and \blacktriangle) during the cell cycle of *C. reinhardtii* 137⁺. Algae at various points in the synchronous cycle were pulsed for 20 min with radioactive acetate at saturating precursor concentration (800 μ M) and under culture and illumination conditions appropriate to the ongoing cycle. After termination of the pulse, the cells were processed either for lipid extraction or for thylakoid membrane purification. In the latter case, thylakoid lipids were extracted immediately upon recovery of the isolated membranes. The total-algal or thylakoid lipid extract was subjected to TLC to resolve the pigments from each other and from nonpigmented neutral and polar lipids (12, 18). All pigmented bands were recovered from the chromatogram and combined to constitute a pigment fraction consisting of the two chlorophylls *a* and *b* and the six carotenoids β -carotene, neoxanthin, luteoxanthin, violaxanthin, luteoxanthin, and lutein. The pigment was assayed by liquid scintillation spectrometry to quantify the radioactivity incorporated from acetate during the pulse labeling. Rates of synthesis are expressed as picomoles acetate incorporated into cellular pigment/ 10^6 mother cells/h \pm SD ($n \geq 4$), and rates of assembly are expressed as pmoles acetate incorporated into thylakoid pigment/ 10^6 mother cells/h \pm SD ($n \geq 4$). All rates are plotted at the times pulses were initiated. Note the difference in exponent of the y-axis between the light (open symbols) and the dark (closed symbols) periods. Identical kinetics were obtained at a final acetate concentration of 1,600 μ M or with ³H₂O (5 mCi/ml).

4.5L (i.e., in cells at 4.5 h in the cycle pulsed for 20 min with radioactive acetate) after four- to sixfold rate increases beginning by 1.5L. From the 4.5L–6L maxima, pigment synthesis and assembly decline and between 10.5L and 12L/0D drop by over an order of magnitude to establish relatively low levels in the dark (S, M, and early-to-mid G₁) at most 10% of those of 0L. With the beginning of the subsequent photoperiod and the dark-to-light transition, pigment synthesis and assembly increase sharply.

Synthesis and Assembly of the Chlorophylls

The kinetics of chlorophyll *a* and chlorophyll *b* synthesis and assembly into thylakoids are plotted in Fig. 3 over cell-cycle time. Qualitatively, both chlorophylls display similar kinetic patterns through the vegetative development of the alga. The chlorophylls are synthesized and are introduced into the thylakoid membrane mainly during the photoperiod, maximal rates for these processes attained by 6L. The chlorophyll *a* maxima represent approximately 22-fold increases over the synthesis and assembly rates at 0L, whereas these differences for chlorophyll *b* are approximately 17-fold. Chlorophyll synthesis and assembly decline through the last half of the light period and between 10.5L and 12L/0D drop by a factor of ~58 for chlorophyll *a* and ~17 for chlorophyll *b* to very low levels

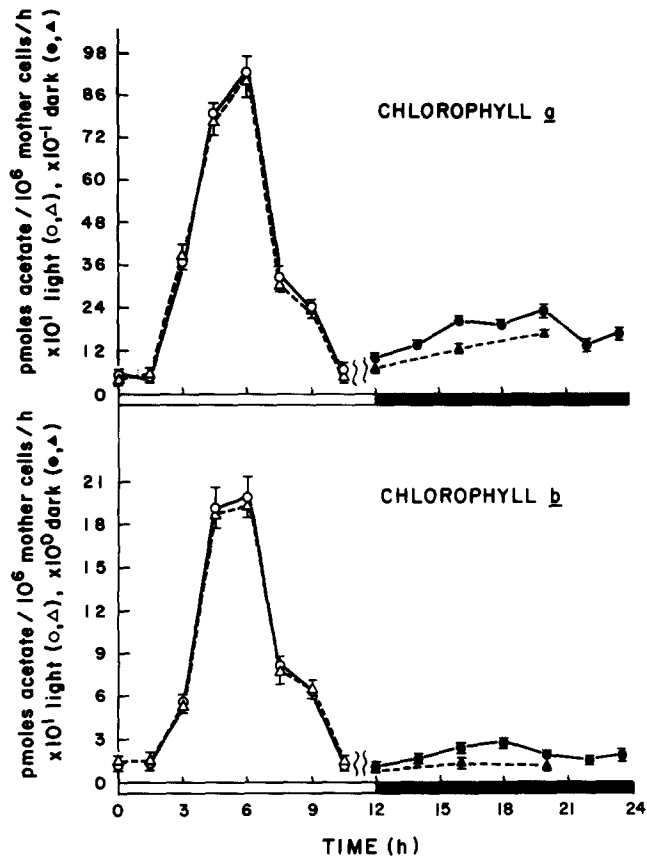


FIGURE 3 Synthesis (○ and ●) and assembly (△ and ▲) of the chlorophylls in synchronous *C. reinhardtii* 137⁺. Experimental procedures are those in Fig. 2, except that chlorophyll *a* and *b* were each recovered from the pigment chromatograms and assayed for radioactivity incorporated from acetate during the 20-min pulse labeling. Rates of synthesis are expressed as pmoles acetate incorporated into cellular chlorophyll *a* or chlorophyll *b*/10⁶ mother cells/h ± SD ($n \geq 4$), and rates of assembly are given as pmoles acetate incorporated into thylakoid-membrane chlorophyll *a* or chlorophyll *b*/10⁶ mother cells/h ± S.D. ($n \geq 4$). All rates are plotted at the cell-cycle times pulses began in both the light (open symbols) and the dark (closed symbols). Note the difference in exponents of the y-axes between light and dark.

in the dark which increase significantly only with the start of the next cell cycle.

Synthesis and Assembly of the Carotenoids

In Fig. 4, the rates of synthesis and assembly of the sole cellular carotene and the main cellular and thylakoid carotenoid, β -carotene, are plotted through the synchronous cycle. β -carotene is synthesized and is assembled into chloroplast lamellae most rapidly very early in the cell cycle. Highest rates are established virtually at the start of the photoperiod and are maintained until at least 4.5L, whereupon β -carotene synthesis and assembly decline, dropping over one order of magnitude by 12L/0D at the start of the dark period. Only with the beginning of the next photoperiod do β -carotene synthesis and assembly return to appreciable levels.

The synthesis and assembly of the five major algal xanthophylls (oxygenated carotenoids) also undergo periodic variation in cell cycle time (Fig. 5). The synthesis of each xanthophyll and its assembly into thylakoid membrane increase from 0L to achieve maxima by 3L in the case of violaxanthin and by 4.5L for the others. From the maxima, xanthophyll synthesis

and assembly decline in a generally discontinuous fashion to levels in the dark which are a significant (~20% on average) proportion of each respective 0L rate. There are marked quantitative differences, though, among the individual xanthophylls in the extent to which the synthesis and assembly rates vary during the cycle. The maximal rates of neoxanthin, loroxanthin, and violaxanthin synthesis and assembly represent approximately 4.5-fold increases over the respective 0L rates, whereas the maxima of lutein are approximately 5.4-fold increases from 0L, and those of luteoxanthin, approximately sevenfold increases. In the dark period, the rates of loroxanthin and luteoxanthin synthesis and assembly may reach 60–70% of the 0L values; the highest dark-period rate of each of the other xanthophylls, however, is always <50% of the respective rate at 0L.

DISCUSSION

Pigment dynamics related to membrane biogenesis have been investigated most extensively at the tissue level during the "greening" or dark-grown, etiolated plants and algae (28), including the *Chlamydomonas* yellow mutant *y-1* (3, 10, 29). Results from such studies support a general correlation among morphologically recognizable thylakoid formation, chemically measured accumulation of chlorophyll and carotenoid, and enhanced pigment labeling from radioactive precursor. The indirect nature of such greening studies, though, precludes assessment of the rates and mechanisms of pigment assembly into the thylakoids.

We studied the pigment synthesis and assembly attendant to membrane biogenesis in a direct manner by utilizing highly-synchronous, phototrophic cultures of the green alga *C. reinhardtii* (wild-type). Our analyses demonstrate that each thylakoid pigment is introduced into the membrane with distinct kinetics that undergo periodic variation through the cell cycle as part of a general pattern which restricts pigment synthesis largely to the photoperiod (mid-to-late G₁). Such a "multi-step" mode of pigment assembly implies that the pigment

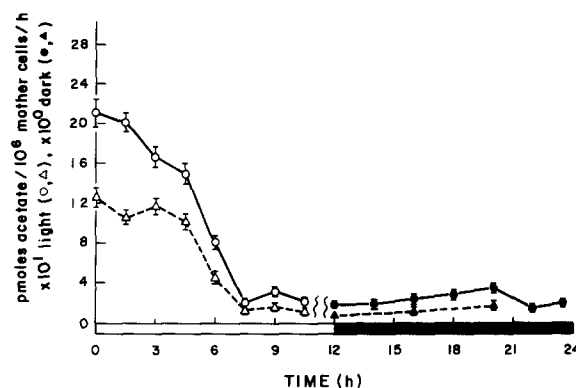


FIGURE 4 Synthesis (○ and ●) and assembly (△ and ▲) of β -carotene in synchronous *C. reinhardtii* 137⁺. Experimental procedures are those in Fig. 2, except that β -carotene was recovered from the pigment chromatograms and assayed for radioactivity incorporated from acetate during the 20-min pulse. Rates of synthesis are expressed as pmoles acetate incorporated into cellular β -carotene/10⁶ mother cells/h ± SD ($n \geq 4$), and rates of assembly are expressed as picomoles of acetate incorporated into thylakoid-membrane β -carotene/10⁶ mother cells/h ± SD ($n \geq 4$). Rates are given at the cell-cycle times that pulses were initiated during the light (open symbols) and the dark (closed symbols) periods. Note the change in exponent of the y-axis with the light/dark transitions.

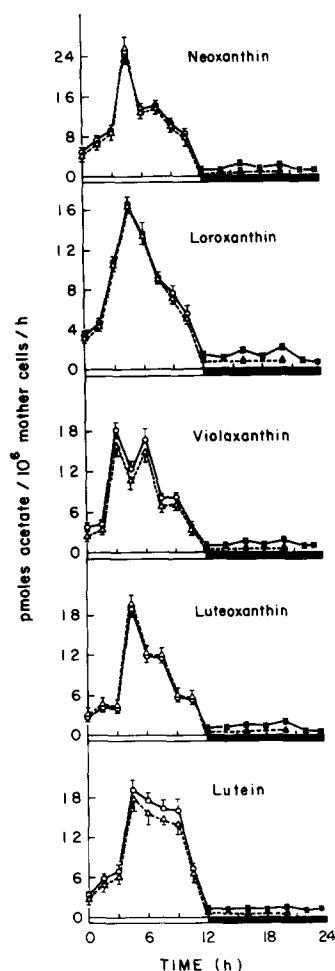


FIGURE 5 Xanthophyll synthesis (○ and ●) and assembly (△ and ▲) in the cell cycle of *C. reinhardtii* 137⁺. Experimental conditions are those in Fig. 2, except that each major xanthophyll was recovered from the pigment chromatogram and assayed for radioactivity incorporated during the 20-min exposure to labeled acetate. Rates of synthesis are given as picomoles acetate incorporated into each respective cellular xanthophyll/ 10^6 mother cells/h \pm SD ($n \geq 4$), and rates of assembly are given as picomoles acetate incorporated into each respective thylakoid xanthophyll/ 10^6 mother cells/h \pm SD ($n \geq 4$). Each rate is plotted at the cell-cycle time a pulse began in both the light (open symbols) and the dark (closed symbols).

biochemistry of the thylakoids changes with development, as is the case during greening of the *Chlamydomonas* y-1 mutant (3).

The masses of chlorophyll and carotenoid in the culture do not increase in the dark (Fig. 1). The relatively low levels of pigment synthesis and assembly during S, M, and early-to-mid G₁, therefore, are probably indicative of pigment turnover, although a small amount of net pigment production for cell division cannot rigorously be excluded. The rapid pigment synthesis in the light period is consistent with net pigment accumulation and with the elaboration of thylakoids prior to M-phase cell division at $\sim 2D$ (6, 30). Since the areas under the pigment accretion curves approximate the areas under the corresponding synthesis curves to within 5%, the contribution of turnover to the high rates of pigment synthesis and assembly is relatively slight over the photoperiod. The rapidity with which the rates of pigment synthesis change at the L/D transitions suggests that short-term photocontrols operate along with longer-term controls over the light and dark periods to modulate pigment production. The metabolic basis for the modulation of lipogenesis in the *Chlamydomonas* cell cycle will be the subject of future reports.

The distinctive pigment biochemistry of the two photosystems (1) and their ordered distribution in the thylakoids between stacked (grana) and unstacked (stroma) regions (2) suggest that the biogenetic incorporation of pigment into the thylakoids takes place with some specificity at the level of the photosynthetic unit and is thereby reflected in the chemistry

and functioning of, for example, the various chlorophyll-protein complexes (31). In these regards, Schor et al. (32) have demonstrated that the photoreductive activities of photosystems I and II are maximal between 3L and 6L of the *Chlamydomonas* vegetative cell cycle, an interval of rapid thylakoid pigment assembly (Fig. 2). Whether the ordered distribution of photosystems in the plane of the chloroplast lamellae would necessitate a strict topological order to thylakoid pigment assembly is intriguing in the face of suggestions (8, 32) that *Chlamydomonas* thylakoids unstack late in the dark and remain so through most of the subsequent photoperiod, inviting lateral intramembrane movement of the photosystems (33).

For brevity, we have termed the incorporation of synthesized pigment into thylakoid membrane "assembly." The assembly measured, though, is the result of at least three processes (34): synthesis of pigment, transit of pigment so produced to the sites of assembly at the thylakoids, and integration of the pigment into the membrane. During the photoperiod of the *Chlamydomonas* cell cycle, the rates of synthesis and the rates of assembly of the chlorophylls and most of the carotenoids are statistically equal. We presume that the differences between the rates of β -carotene and violaxanthin synthesis and their respective rates of assembly into thylakoids reflect pigment production for extra-thylakoid structures, since β -carotene is the major carotenoid in plastoglobuli (3, 35) and violaxanthin is the major chloroplast-envelope carotenoid (4). Such parallelism between cellular pigment synthesis and thylakoid pigment assembly strongly indicates that pigment transit and integration are so rapid as not to be limiting to thylakoid biogenesis, a conclusion equally applicable to the nonpigmented thylakoid lipids as well (6).

Rather, it appears that pigment synthesis itself may be the rate-determining, if not rate-limiting, step in the pigment assembly supporting the extensive photoperiod biogenesis of thylakoid membranes. That the tight coupling between pigment synthesis and assembly indicative of *Chlamydomonas* vegetative development may be less direct operatively, however, is suggested, for instance, by the pigments' abilities to act as feedback regulators of their own complex and rather poorly-delineated synthetic pathways (36). In light of the specific quantitative association between a portion of the thylakoid pigments and pigment-binding proteins (31, 37), synthesis of the binding proteins or their disposition within the photosynthetic lamellae may also exert a modulatory influence upon the assembly of pigment into the thylakoids. Therefore, it has become of interest to detail the physiological chemistry of lipogenesis during *Chlamydomonas* vegetative development in order to gain further understanding of the mechanisms underlying membrane biogenesis and organelle formation.

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