REGULATION OF THE *CHLAMYDOMONAS* CELL CYCLE BY LIGHT AND DARK

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ABSTRACT

By growing cells in alternating periods of light and darkness, we have found that the synchronization of phototrophically grown *Chlamydomonas* populations is regulated at two specific points in the cell cycle: the primary arrest (A) point, located in early G_1, and the transition (T) point, located in mid-G_1. At the A point, cell cycle progression becomes light dependent. At the T point, completion of the cycle becomes independent of light. Cells transferred from light to dark at cell cycle positions between the two regulatory points enter a reversible resting state in which they remain viable and metabolically active, but do not progress through their cycles.

The photosystem II inhibitor dichlorophenyldimethylurea (DCMU) mimics the A point block induced by darkness. This finding indicates that the A point block is mediated by a signal that operates through photosynthetic electron transport. Cells short of the T point will arrest in darkness although they contain considerable carbohydrate reserves. After the T point, a sharp increase occurs in starch degradation and in the endogenous respiration rate, indicating that some internal block to the availability of stored energy reserves has now been released, permitting cell cycle progression.

Synchronous growth and division of photosynthetic cells exposed to alternating periods of light and darkness is a widespread phenomenon in nature. The synchrony attained by such populations is of such a high degree that it was first observed in the relatively uncontrolled environment of the natural habitat. More than 70 yr ago, L. H. Gough (12) reported that in nature the dinoflagellate *Ceratium fusus* divides only between 1:00 a.m. and 3:00 a.m. Although laboratory-controlled exposure to light/dark cycles has been used as a technique to synchronize cell division in many phototrophically grown algal species, including *Chlamydomonas reinhardtii* (7, 15, 18, 30), the mechanism of the synchronization is not known. Two hypotheses have been advanced: (*a*) synchronization results from the cell cycle being driven by the light and dark periods, i.e., by metabolic steps that are either light dependent or dark dependent (4); (*b*) synchronization results from the entrainment by the alternating light/dark periods of a preexisting endogenous circadian oscillation (i.e., a biological clock, 6) that regulates the cell cycle (17, 19).

The purpose of the work reported here is to clarify the cell cycle regulatory mechanism responsible for light/dark synchrony of the unicellular eukaryotic alga *Chlamydomonas reinhardtii.*

J. CELL BIOLOGY © The Rockefeller University Press · 0021-9525/80/04/0136/10 $1.00
Volume 85 April 1980 136–145

Chlamydomonas is especially suited for studies involving cell cycle specificity because the cells are easily cultured in defined media and are readily synchronized under phototrophic conditions by alternating cycles of light and darkness. In addition, the cells are amenable to genetic and biochemical analysis (5, 27–29); and temperature-sensitive cell cycle mutants are available (16).

The data we present in this report demonstrate that light/dark synchrony results from a forced oscillation of the cell cycle (as in hypothesis *a* above). The synchronization is a consequence of the properties of the cells at two specific G_1 regulatory points: a primary arrest point at which cell cycle progression becomes light dependent, and a transition point at which cell cycle progression becomes independent of light (analogous to the "restriction point" in mammalian cells [24]). Cells at cell cycle positions between the two regulatory points enter a dark-induced resting state in which they remain viable and metabolically active, yet do not progress through their cycles. From our data we conclude that the synchronization is mediated through photosynthetic electron transport in phototrophically grown cells. Although this suggests a nutritional basis to light/dark synchrony, cells prior to the transition point will arrest in darkness even though they contain considerable carbohydrate reserves. After the transition point, a sharp increase occurs in dark-induced starch degradation and in the dark endogenous respiration rate.

MATERIALS AND METHODS

Strains and Culture Conditions

To obtain strains that produce highly synchronous cultures, single colony isolates of the *Chlamydomonas reinhardtii* strain 21gr were selected as follows: 20–30 individual clones, which produce relatively large colonies on minimal (M) medium (29) agar, were each inoculated into 35 ml M medium in 125 ml capped flasks and incubated at 24°C with shaking in continuous light (15,000–17,000 lx). After growth to stationary phase, each culture was diluted 1:100 into fresh M medium, grown for 10 h in light, darkened by wrapping with aluminum foil for 12 h, shifted back to continuous light for 18–20 h, and then examined microscopically. Those cultures containing multiple (i.e., unseparated) cells at the time of examination were discarded, and the culture containing cells of most uniform size was transferred to M medium agar plates and used in the procedures reported below. This selection process was repeated approximately every 3 mo to maintain cultures that yielded good synchronization and that showed complete separation of daughter cells when grown in continuous light.

Asynchronous cultures were obtained by inoculating M medium from a stationary-phase culture (previously grown from a plate inoculum) to $\sim 10^3$ cells/ml in bubbler-equipped flasks. Cultures were incubated with shaking in continuous light at 24°C and bubbled with 5% CO_2 in air. By diluting the culture 1:4 with fresh medium every 12 h, we maintained the cells in continuous logarithmic growth between 10^4 and 5×10^5 cells/ml for at least 6 d to ensure random distribution of the partial synchrony present in stationary-phase inocula.

Synchronous cultures were obtained by a modification of the procedure of Surzycki (32). A culture was inoculated to 10^4–10^5 cells/ml from a stationary-phase culture, incubated in continuous light for 10 h, placed in the dark for 12 h, and subjected to alternating 12-h periods of light and darkness.

3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU), recrystallized Divron, from DuPont Biochemicals Department, Wynnewood, Pa., was obtained from Dr. J. Ellenson, Harvard Biological Laboratories, Harvard University, Cambridge, Mass.

Cell Counts and Laser Light-scattering Measurements

I/KI (iodine/potassium iodide solution) in 95% ethanol (prepared by the procedure of Pucher et al. [26]) was added at 40 μg/ml of culture to kill cells for hemocytometer counting and for determining laser light-scattering histograms of the cell populations. After being kept at 4°C overnight, the cells were pelleted at 13,000 *g* for 10 min at 4°C and washed with fresh medium. Histograms were determined with a cytofluorograph model 4800A (Bio/Physics Systems, Inc., Mahopac, N. Y.) set in light-scattering mode. Each histogram consists of values from ≥50,000 cells.

The instrument measures the low angle (1°–19°) light scattering by single cells exposed to a 10 mW argon-ion laser beam. Output signals from the scattered-light sensors are accumulated in a multichannel pulse height analyzer. In normal operation, the resulting histogram is projected onto an oscilloscope screen across which the total multichannel output spans 12.7 cm. In this paper, 1 U of laser light scattering represents 2.5 cm across the oscilloscope screen, which defines the *Arbitrary Unit* on Figs. 1, 2, 4, and 6. Selected channels were expanded with a gain-setting control to obtain greater detail, and the results were plotted in our standard units as explained above for the unexpanded scale.

Particle size has been shown to correspond linearly to channel number by measurements of standard polystyrene microspheres of known diameters (37). Although with *Chlamydomonas* cells a strictly linear relationship does not hold, we do observe monotonically increasing light scattering with increased cell volume (see Figs. 1 and 2).

Using this light-scattering method, we found essentially no resolvable variability in the histograms of samples from the same culture. The reproducibility of the position of the histogram peak from the same culture was <2% of the range of scattering values shown in the figures of this report. However, the specific peak position for a given sample examined on different days fluctuated by as much as 20% (for example, 12-h dark-arrested populations in three separate experiments performed on different days showed peaks at 1.45, 1.40, and 1.20 of our light-scattering units). For this reason, any comparison of values obtained in different experiments would need to be based on light scattering relative to a standard, such as a dark-arrested population.

Volumes were determined from ×1,000 magnified micrometer measurements of long and short axes of individual cells from synchronous cultures. We measured >70 cells for each mean volume reported. The cells were assumed to be oblate spheroids for volume calculations. DNA content per cell was determined by the method of Sueoka et al. (31).

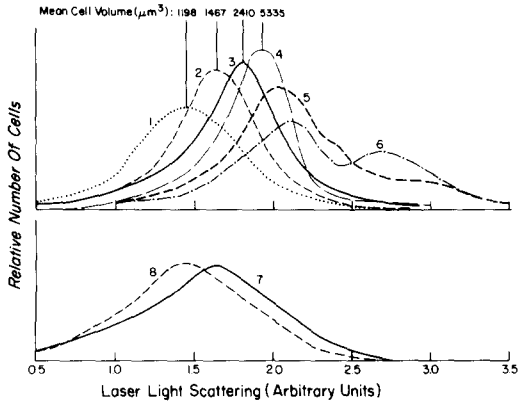

FIGURE 1 Light-scattering histograms of a synchronously cycling population. A culture of strain 21gr was synchronized with 12-h light/12-h dark cycles as described in Materials and Methods. During the second light/dark cycle, histograms and mean cell volumes were determined (as described in the text) on 50-ml aliquots taken at 0, 3, 6, 9, and 12 h in the light (histograms 1, 2, 3, 4, and 5 respectively) or at 1, 5, and 12 h in the dark (histograms 6, 7, and 8 respectively). For this strain, divided but unseparated cells began to appear between histograms *4* and *5* and cell separation occurred between histograms *6* and *7* (determined by microscopic examination and cell counts). Each histogram consists of values from 50,000 ± 1,000 cells.

Starch Measurement

Cellular content of starch was determined as follows: aliquots containing 5×10^6-2×10^7 cells were pelleted at 13,000 *g* for 10 min at 4°C. The pellet was resuspended in 20 ml of hot 80% ethanol and recentrifuged, and the ethanol extraction was repeated 3 ×. The cells were then washed with 20 ml of 2% Na_2CO_3, pelleted, resuspended in 5 ml of 2% Na_2CO_3, counted by hemocytometer, recentrifuged, and resuspended in 1 N H_2SO_4 at room temperature. The suspension was hydrolyzed for 10 min at 100°C, centrifuged at 13,000 *g* for 30 min at 4°C, and the sugar concentration determined in the supernate with the phenol-sulfuric acid reagent of Dubois as described by Ohad et al. (22).

RESULTS

Measurement of Cell Cycle Progression

We developed a rapid and precise method of monitoring cell cycle progression by the measurement of laser light scattering by individual cells. The results are expressed as light-scattering values in the form of histograms (Fig. 1). Synchronously cycling cells sampled at specific intervals show an increase in light scattering as the population proceeds through G_1 (histograms 1–4 in Fig. 1). The appearance of division forms, as determined by microscopic examination, correlates with formation of a high light-scattering peak (histogram 6 in Fig. 1). Cell separation results in a shift of the histogram back to low light-scattering values.

The light-scattering data were correlated with measurements of cell volume, which increases as cells progress through the cell cycle. We determined mean cell volumes from micrometer measurements of individual cells from populations with differing light-scattering peak positions. Fig. 2 shows that the relationship of mean cell volumes and histogram peak positions is not linear, but light scattering is a monotonically increasing function of cell volume. The histograms and cell volume data above the histograms in Fig. 1 show that both volume and light scattering increase with progression through G_1. After cell separation, cells decrease both in light-scattering values (cf. histograms 7 and 8 in Fig. 1) and in volume (data not shown) with continued incubation in the dark. This decrease after the first 5 h is gradual compared with the relatively large decrease resulting from cell separation, and does not interfere with the detection of cell separation in this method.

Additional cell cycle markers used below are daughter cell release (hatching), monitored microscopically by cell counts, and nuclear DNA synthesis, assayed by chemical determinations made at intervals over the cell cycle.

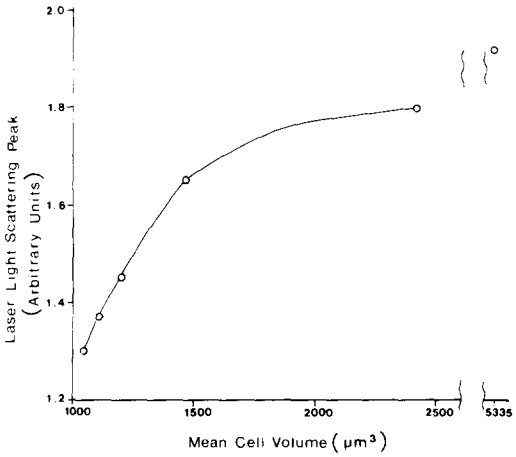

FIGURE 2 Correlation of mean cell volume and light-scattering peak. Laser light scattering and mean cell volumes were determined as described in Materials and Methods. The measured light-scattering histograms for each of the six samples were unimodal. The samples examined were from a light/dark synchronized culture exposed to (from lowest to highest volume): 36, 24, and 12 h of darkness, and 3, 6, and 9 h of light, respectively.

Darkness Restricts Cells to a Specific Portion of G_1

When a phototrophically growing asynchronous culture of wild-type *C. reinhardtii* is placed in the dark, the population undergoes growth and division as shown in Fig. 3. After about 12 h of darkness, no further change in cell number occurs for at least 60 h. By three lines of evidence we have shown that the population's residual growth in darkness ceases as its members reach a specific stage of the cell cycle. (*a*) The light-scattering histogram was restricted to low and fairly uniform values by the residual cycling in darkness (Fig. 4). Low light scattering is characteristic of cells at early cell cycle positions in synchronized populations (Fig. 1). The actual light-scattering values for cells after 12 h of darkness are lower than those of early G_1 cells because of a slow, continuous decrease in cell volume in the dark. (*b*) Dark-arrested populations showed a relatively synchronous release of daughter cells (Fig. 5) after exposure to light. (*c*) Entry of the population into S phase (nuclear DNA synthesis) after exposure to light was relatively synchronous, as judged from measurements of DNA content (Fig. 5).

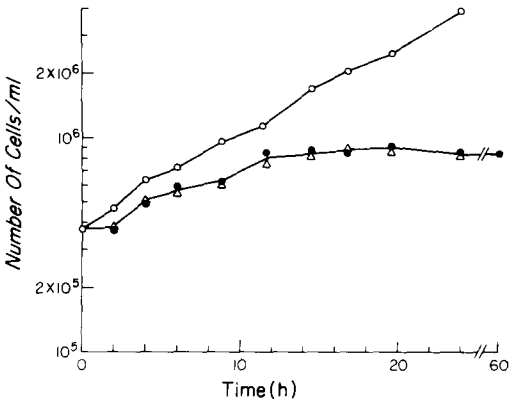

FIGURE 3 Residual cycling after dark or DCMU. An asynchronous culture of strain 21gr cells was grown in continuous light as described in Materials and Methods and divided into three 300-ml portions at time = 0 h. For the control culture, incubation of an asynchronous population in continuous light was continued (○). One culture (●), was darkened at time 0 as described in Materials and Methods, and to one (△) was added 0.6 ml of 5 mM DCMU in 95% ethanol. The DCMU culture was incubated in continuous light. Addition of 0.6 ml of ethanol alone has no effect on cell growth under these conditions. Cell number was determined by hemocytometer.

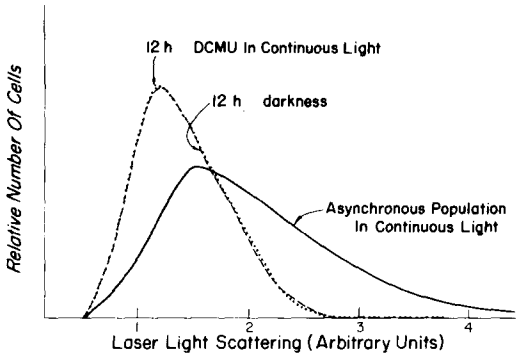

FIGURE 4 Effect of darkness and DCMU on light-scattering histograms of an asynchronous population. Histograms were determined as in Fig. 1 for the three cultures at 12 h in Fig. 3.

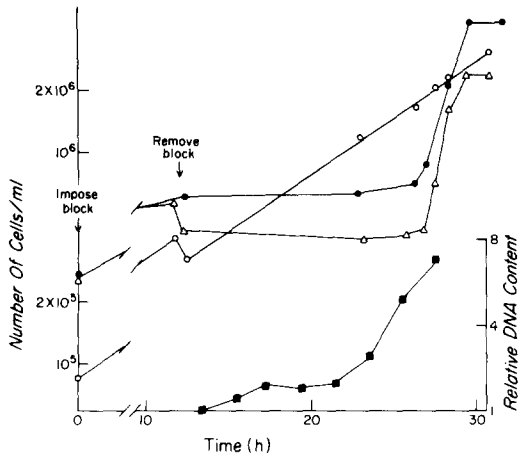

FIGURE 5 Synchronization of cell division by darkness and DCMU. At time 0, an asynchronous culture was divided into three portions and one was exposed to dark (●) and one to DCMU (△) as described in Fig. 3. The control (○) was diluted 1:3 with fresh medium to permit logarithmic growth over the duration of the experiment. The dark culture was reexposed to light at 12 h after time 0. The control and DCMU cultures were centrifuged at 6,000 *g* for 10 min, resuspended in fresh medium, recentrifuged, resuspended to the original volume, transferred to fresh flasks, and incubated in continuous light. Some loss occurred during the washing procedure. DNA content was determined on 250-ml aliquots from a 2-liter culture at 1.9×10^6 cells/ml after exposure to 12 h of darkness and subsequent transfer to the light (at time = 12 h).

Location of the Primary Arrest and Transition Points

From the results shown above, we can define a point in the cycle after cell separation (i.e., near

the beginning of G_1) that is the earliest cell cycle position at which a phototrophically grown cell requires light to progress further through its cycle. Because this is the earliest point of arrest, we call this cell cycle position the primary arrest (A) point.

Similarly, we can define the latest cell cycle position at which a cell still requires light for cell cycle progression. Beyond this point, a cell is committed to complete its cycle, whether in darkness or light. Using the terminology of Hamburger and Zeuthen (13), we call this point the transition (T) point.

We located the T point by shifting synchronous populations at various stages in G_1 from light to darkness and observing whether the cells were able to complete their cycles and arrive at the A point. If a cell is past the T point, it will complete its cycle in darkness and produce small daughter cells of low light-scattering values (Fig. 1). If a cell has not reached the T point, it will arrest in the dark, without completing its cycle, and will maintain the higher light-scattering values characteristic of the stage of G_1 it had attained.

Fig. 6 shows the results of such an experiment. Light-scattering histograms were determined with synchronously cycling populations shifted to darkness at various G_1 stages for a time sufficient for committed cells to complete their cycles. A population that received only 3 h of light (*L3*) was short of the T point, as the light-scattering peak did not migrate to the A point position in the dark. A population permitted to attain the middle of G_1 (*L6*) consisted of two subpopulations: one past the T point that completed the cycle to arrive at the A point, and a second subpopulation that remained arrested near the mid-G_1 position. Populations at later G_1 stages (*L9* and *L12*) showed essentially all cells completing their cycles in the dark and arriving at the A point. Therefore the T point occurs near the mid-G_1 stage attained by exposing a population arrested at A to 6 h of light.

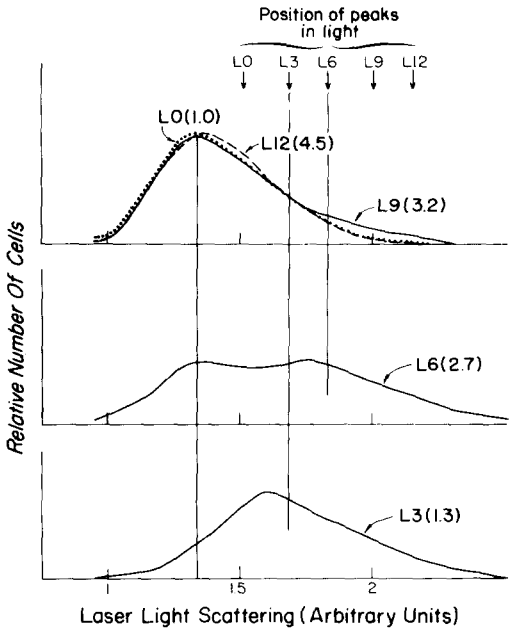

FIGURE 6 Effect of darkness at various times in the light period. A second-cycle synchronous culture was prepared and histograms were determined as described in Fig. 1. At the indicated number of hours in the light (*L*) aliquots were placed in the dark. After 24 h of dark incubation, histograms were determined and are shown in the figure. The numbers in parentheses are the ratios of the initial cell concentration in the light to the final concentration after 24 h of darkness.

TABLE I

Viability of Dark-arrested Cells

Hours in darkness	Colony-forming units light control ± SEM
	%
12	100 ± 4
36	98 ± 8
60	106 ± 9
84	53 ± 2
196	12 ± 3

Cells inoculated from stationary phase and exposed to 10 h of light were placed in the dark for the indicated number of hours. Colony-forming units were determined from triplicate agar platings as described in Fig. 7.

Characteristics of Dark-arrested Cells

In any system in which "quiescence" or "resting states" are observed, the question arises of whether the cells are truly arrested or are very slowly traversing the cycle. In the case of dark-arrested *C. reinhardtii*, this question can be directly tested because cells maintain complete viability for at least 60 h in the dark resting state (Table I). Table II shows that dark-arrested cells are not slowly traversing their cycles but rather are static in the sense that the amount of time the cells reside in the resting state does not alter the timing of cell cycle events (specifically cell separation) after removal of the block.

Our next concern was whether the cells are essentially inert in the dark-arrested condition or

TABLE II

Dark- or DCMU-arrested Cells are Static With Respect to Their Division Cycle

Blocking condition	Duration of block	Cell separation burst midpoint (hours after block removal)	Cell separation burst size
	h		
Dark	12	16–16.5	6.5
DCMU	12	15.5–16	5.9
DCMU	12	15–17	6.3
Dark	24	16–17	6.3
Dark	36	15.5–16.5	6.4
DCMU	36	16–18	7.5
Dark	48	15.5–16	5.7
Dark	48	15.5–16.5	7.4

Cells inoculated from stationary phase and exposed to 10 h of light were arrested by either 10^{-5} M DCMU or darkness and were released from the block after the indicated number of hours, as described in Fig. 5. Cell concentrations following removal of the block were monitored and, in each case, division synchrony was observed (>90% of the cells separated in a 2–4-h interval). The interval in which the cell separation burst reached 50% of its maximum value and the burst size (concentration of cells after the complete cell separation burst/concentration of cells at the time of removal of the block) were recorded.

metabolically active. Two types of measurements show that resting cells are metabolically active: (*a*) dark-arrested cells show a continuous volume (and light-scattering) decrease in the dark (data not shown) and (*b*) resting cells are dependent on mitochondrial oxidative phosphorylation to maintain viability (Fig. 7). Fig. 7 shows that dark-arrested cells lose viability upon exposure to dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, at concentrations of DNP that are better tolerated by cells growing either in the light, or in the dark with acetate as a carbon and energy source.

Thus, between points A and T, cells idle in the dark without further progressing through their cell cycle. Endogenous respiration is necessary for the maintenance of viability, and a slow shrinking of the cells is evident in the continuous volume and light-scattering decreases that occur in the dark-arrested condition.

DCMU Mimics Dark-induced Synchrony

The next concern of our study was the biochemical basis of the light requirement for cell cycle progression. It could be an energy requirement mediated through the chromophores of photosynthesis or through a nonphotosynthetic photoreceptor, perhaps in a biological clock mechanism (e.g., see reference 17). We have found that inhibition of photosynthetic electron transport with the photosystem II inhibitor DCMU mimics with precision the synchronization induced by darkness. Several lines of evidence support this conclusion.

(*a*) As shown in Fig. 3, the kinetics of residual growth after exposure of a culture to DCMU in continuous light are the same as those resulting from darkening the culture.

(*b*) A stricter test of mimicry is provided by comparison of the light-scattering histograms of cells from a culture arrested by darkness and from a culture treated with DCMU in continuous light. The histograms of DCMU- and dark-arrested cells are indistinguishable (Fig. 4). (Single colony isolates of *C. reinhardtii* are highly variable with respect to the degree of physical separation of cells

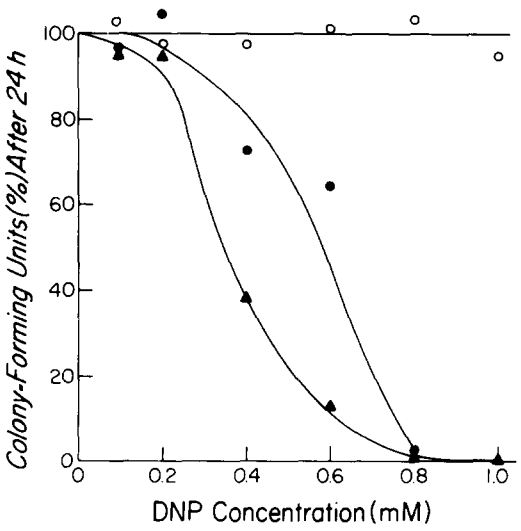

FIGURE 7 DNP sensitivity of arrested and cycling populations. 300-ml cultures of asynchronous phototrophically grown cells in continuous light (○), asynchronous heterotrophically grown cells in darkness and M medium + 0.1% sodium acetate (●), and a dark-arrested population obtained by placing an asynchronous phototrophically grown culture in the dark for 12 h (▲) were incubated with shaking. DNP (0.1 M in 95% ethanol) was added to the aliquots to give the final DNP concentrations noted on the abscissa, and incubation was continued. After 24 h, cells were counted, diluted into medium, and plated for determination of colony-forming units. Plating efficiencies determined for 0 DNP cultures were >90% in each of the three cases and were defined as 100% viability.

after division. For cultures showing significant numbers of unseparated cells in continuous light, darkness promotes separation, whereas DCMU does not [data not shown]. Therefore, light-scattering distributions of DCMU- and dark-arrested cells will not be identical for such cultures. All experiments reported here were performed with cells that separate completely even in continuous light.)

(*c*) The timing of the synchronous release of daughter cells after removal of the arresting condition is the same for DCMU- and dark-blocked cells (Fig. 5).

(*d*) DCMU-arrested cells, like dark-arrested cells, are static with respect to cell cycle progression, and cell separation burst sizes are similar for populations synchronized by DCMU treatment and by darkness (Table II).

Storage Reserve Catabolism Is Inhibited between the A and T Points

The main nutritional effect of blockage of photosystem II by either darkness or DCMU is the cessation of photosynthetic carbon fixation. Measurements with the same *C. reinhardtii* strain (21gr) used here show a complete inhibition of CO_2 incorporation at concentrations of DCMU below those used in this study (33); and we found a complete inhibition of O_2 evolution (which is required for CO_2 fixation) by 10^{-5} M DCMU (data not shown). Labeling studies (3) with *Chlorella pyrenoidosa* show that the concentrations of key metabolites (e.g., 3-phosphoglycerate and the adenylate energy charge) are maintained as high in the dark, in the absence of CO_2 fixation, as they are in the light. This homeostasis is accomplished by a shift from photosynthetic carbon fixation to glycolytic degradation of stored carbohydrate as the source for carbon and respiratory substrates.

C. reinhardtii storage carbohydrate is in the form of starch and possibly sucrose. Starch has been shown to be readily available for energy production and biosynthesis of proteins (20, 22), cell division (8), and cell wall or thecal formation (10, 11), and starch should be able to serve through established metabolic pathways as an ultimate substrate for the various anabolic reactions required for cell cycle progression.

The residual growth (Fig. 5) that permits cells past point T to reach A must occur at the expense of storage reserves, because in the dark such reserves are the only available source for new cellular material. Yet, although storage reserve degradation permits cells to progress in the region of the cycle after point T and before point A, it does not permit progression in the period between A and T. There are two possible explanations for this. (*a*) Storage reserves are exhausted in this stage of the cell cycle, as a result of either the failure to synthesize adequate reserves in this region in the light or of a relatively high rate of consumption of reserves in this region in the dark; or (*b*) storage reserves are available in the region between the A and T points, but they are not mobilized for biosynthetic reactions involved in cell cycle progression.

To distinguish between these two possibilities, we measured starch content, the rate of starch consumption, and the rate of respiration of endogenous reserves in the dark by cultures before and after the T point (Fig. 8). These measurements were made on a synchronous population cycling in continuous light, by shifting aliquots at various times to the dark.

Both the starch data and respiration rate data in

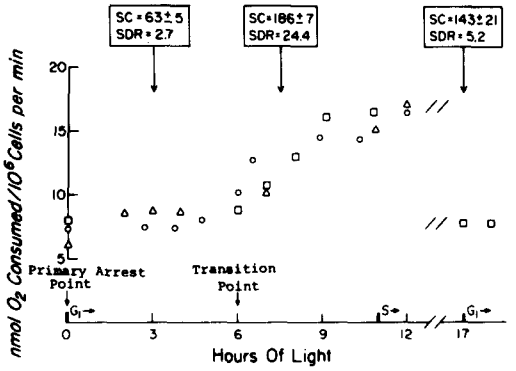

FIGURE 8 The three symbols indicate measurement from three independent dark-arrested cultures placed in the light at time 0. Each measurement was made by removing an aliquot from the culture in the light and measuring oxygen consumption in the dark with a polarigraph. The value on the ordinate is a linear rate of decrease in O_2 tension from its value in air-saturated medium measured for 10 min at 25°C with 10^6–3×10^6 cells in a 1.2-ml cuvette. Cells were delivered directly to the polarigraph cuvette from the culture. *SC*, starch content in nanomoles of hexose/10^6 cells ± SEM of four determinations, *SDR*, starch degradation rate in nanomoles of hexose/10^6 cells per hour, determined by the formula SDR = $(SC_i - SC_f)/3.5$, where SC_i = starch content in the light (at time indicated) and SC_f = starch content after 3.5 h of darkness. *S*, phase of nuclear DNA synthesis.

Fig. 8 rule out possibility (*a*). Starch content is appreciable before the commitment point and yet the cells do not show a high rate of starch consumption when placed in the dark (cf. 3 h of light with 7.5 h of light). After the populations reach point A in continuous light (17 h), the cellular starch content is nearly as high as in cells past point T in the previous cycle (7.5 h of light), yet such cells do not advance through their cycle and do not show the high starch-degradation rate of committed cells. A 17-h light culture, just as a dark-arrested culture, requires 5–7 h to reach its T point. Also, a 17-h light culture placed in the dark for 12 h requires the same number of hours of light to reach cell division as does a dark-arrested culture (data not shown).

Fig. 8 also shows that endogenous dark respiration rates are lower in the region between A and T than after T; and there is a sharp increase of the rate near the T point. Thus, the final reaction of carbohydrate degradation pathways (mitochondrial oxidation) is occurring at a minimum rate (rather than a maximum rate as in possibility *a*) in the region between the A and T points. A more gradual increase in endogenous respiration of *C. reinhardtii* with progression through the cycle was observed by Osafune et al. (23), and a stepwise increase similar to our finding was reported by Armstrong et al. (1).

Our conclusion is that storage reserve exhaustion is not responsible for the arrest in the region between the A and T points; rather, cells do not mobilize their available reserves in that region of the cell cycle.

DISCUSSION

By examining the effects of light and darkness on morphological and biochemical measures of cell cycle position, we have established that the synchronization of *Chlamydomonas* populations by alternating periods of light and darkness results from changes in cellular properties specifically at the A point, located at the beginning of G_1, and at the T point, located in the middle of G_1. At the A point, a cell acquires a dependence on light for further cell cycle progression. At the T point, cell cycle progression becomes independent of light and in the dark a committed cell will complete its cycle, divide, and arrest at the A point of the following cycle.

From the properties of the A and T points, it is clear in terms of cell cycle progression how alternating periods of light and dark synchronize populations. An asynchronous population consists of cells distributed throughout the cycle. Upon exposure to darkness, all cells in the population past point T complete their cycles and arrest at point A. Cells between A and T at the time of darkening do not progress through their cycles. Therefore, the resulting population is restricted to cell cycle positions between A and T. Exposing this partially synchronous population to a period of light sufficient to permit the cells at point A to progress past point T will enable the entire population to accumulate at point A after a second exposure to darkness. If the synchronization were initiated with a totally asynchronous (i.e., exponential) population, the second period of darkness would give a higher degree of synchrony than the first period, which has in fact been observed (4). Subsequent alternating light and dark periods prevent synchrony breakdown by successive arrests of the population in darkness at point A.

The hypothesis that light/dark synchronization derives from the existence of separate light-dependent and light-independent phases of the cell cycle was first proposed by Bernstein (4), who determined a 4-h light-dependent period for predivision processes in *Chlamydomonas moewusii.* Our determination of a mid-G_1 dark transition point is in accord with the *C. moewusii* data as well as with the determination by Howell and co-workers (15) of the *C. reinhardtii* transition point. For a number of different growth inhibitors, Howell and co-workers (15) estimated transition points from the amount of residual division after inhibitor addition. Although none of the inhibitors examined was shown to induce division synchrony, their method assumes that the inhibitors act at specific stages of the cell cycle. We demonstrate here that this assumption of specificity is valid for darkness and DCMU, because each of these agents induces cell cycle synchrony. With their method, Howell and co-workers (15) found that the positions of the DCMU and dark transition points differed. They place the dark transition point at 31% of the distance through the cell cycle (within the first third of the cycle) and the DCMU transition point at 48% of the distance through the cycle (midway through the cycle). By our criteria, we find that DCMU and darkness are indistinguishable in their induction of synchrony and their points of action in the cell cycle. Possibly the

disagreement is accounted for by variability in the residual division method applied by Howell and co-workers (15). In the same work, these investigators report a dark arrest point, which they designate the "accumulation stage." By using a theoretical relationship between cell cycle stage and relative cell volume, they calculate the accumulation stage to be at 29% of the distance through the cell cycle for cells with a transition point at 31% through the cycle. This implies that for phototrophically grown cells, cell growth is light dependent for only 2% of the cycle. In contrast, we find the light-dependent period (the period from the A point to the T point) to span the first half of G_1, constituting 35% of the cell cycle.

Resting states induced by nutrient deprivation are specific to G_1 in diverse eukaryotic cells (21, 24, 25, 34, 35; for review, see reference 2). In all these systems, cells past some critical point in G_1 complete their cycles and arrest at a block (restriction point [24]). Without this residual cycling by committed cells, the arrest could not be specific to a particular phase of the cell cycle. In *Chlamydomonas*, such committed cells are able to transit the cycle because the mobilization of internal reserves provides a substitute for energy from photosynthesis. Our results show that starch degradation is inhibited in early G_1 (i.e., in the arrest region). This part of the cycle may be the only one in which darkness induces an internal deprivation of metabolites. If this is the case, then the inactivation of enzymes of starch catabolism in early G_1 and their reactivation in mid-G_1 would ensure in a simple way that darkness arrests the cells specifically in early G_1. In view of this hypothesis, it is interesting that Wanka et al. (36) have determined that in the green alga *Chlorella*, the activity of starch phosphorylase, the principal enzyme of starch catabolism, is virtually absent in early G_1 and is produced in mid-G_1.

In this report, we have shown that the photosystem II inhibitor DCMU mimics with precision the cell cycle effects of darkness that are responsible for light/dark synchrony. We infer that the dependence on light between A and T is a dependence on photosynthetic electron transport. This may seem contradictory to the finding of Jarret and Edmunds (17) who reported that light/dark cycles will synchronize populations of a mutant of *Euglena* (grown on acetate) with severely impaired photosynthetic capacity, thus suggesting that the synchronization operates through nonphotosynthetic chromophores. The likely explanation of this apparent contradiction follows from the work of Epel and Butler (9) showing that wavelengths and intensities of light that synchronize the colorless (nonphotosynthetic) alga *Prototheca zopfii* also inhibit respiratory electron transport by photodestruction of mitochondrial cytochromes. A hypothesis that accommodates all of these results is that the light/dark synchrony of both photosynthetic and nonphotosynthetic algae results from the forced periodicity in the availability of high-energy metabolites from electron transport. For phototrophically grown species, energy is acquired through photosynthetic electron transport, and the inhibitory effect of darkness would synchronize the cells. For colorless algae or the *Euglena* photosynthesis mutant, energy is provided through respiratory electron transport, and the inhibitory effect of light would synchronize the cells. In either case, one might expect that synchronization results from regulatory points similar to the A and T points described here.

Another observation reported here clearly rules out any role of an endogenous circadian (i.e., ~24-h) oscillation in light/dark synchrony. We show that cells can be arrested in the dark in the region between A and T for up to 48 h without altering their cell cycle position, as determined by the timing of subsequent cell cycle events when the cells are placed in the light (Table II). This means that any period of darkness from a minimum equal to the amount of time for the cells to reach point A, to at least an additional 48 h, can be used to maintain a synchronously cycling population. Therefore, the total light/dark cycle length need not be circadian (i.e., 24 h) nor a multiple of 24 h.

Viable resting states at specific regions of the cell cycle have been observed after nutrient deprivation of diverse eukaryotic cells. For example, cultured mammalian cells arrest at a cell cycle position called the restriction point by Pardee (24), and yeast arrest at a position termed "start" by Hartwell (14). There are several advantages to investigating cell cycle regulation in *Chlamydomonas*. The difference between the permissive condition (light) and the restrictive condition (dark) is absolute, cells can be easily shifted from one condition to the other, and the metabolic effects of light/dark transitions in algae are relatively well studied. Furthermore, dark-induced activation of starch catabolism and a sharp increase in dark endogenous respiration rate provide easily monitored markers for the commitment to continuation around the cell cycle in *Chlamydomonas*.

We wish to acknowledge stimulating and informative discussions with Dr. A. B. Pardee.

This investigation was supported by grant GM 22874 from the National Institutes of General Medical Sciences and a grant from the Jane Coffin Childs Memorial Fund for Medical Research. J. L. Spudich is a Fellow of the Fund.

Received for publication 9 October 1979.

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