INHIBITOR EFFECTS DURING THE CELL CYCLE IN *CHLAMYDOMONAS REINHARDTII*

Determination of Transition Points in Asynchronous Cultures

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ABSTRACT

A wide variety of inhibitors (drugs, antibiotics, and antimetabolites) will block cell division within an ongoing cell cycle in autotrophic cultures of *Chlamydomonas reinhardtii*. To determine when during the cell cycle a given inhibitor is effective in preventing cell division, a technique is described which does not rely on the use of synchronous cultures. The technique permits the measurement of *transition points*, the cell cycle stage at which the subsequent cell division becomes insensitive to the effects of an inhibitor. A map of transition points in the cell cycle reveals that they are grouped into two broad periods, the second and fourth quarters. In general, inhibitors which block organellar DNA, RNA, and protein synthesis have second-quarter transition points, while those which inhibit nuclear cytoplasmic macromolecular synthesis have fourth-quarter transition points. The specific grouping of these transition points into two periods suggests that the synthesis of organellar components is completed midway through the cell cycle and that the synthesis of nonorganellar components required for cell division is not completed until late in the cell cycle.

In synchronized cultures of *Chlamydomonas reinhardtii* a wide variety of cytological and biochemical activities have been shown not to be continuous processes, but discontinuous cell cycle events. Cell cycle events which have been identified include nuclear and chloroplast DNA synthesis (5), synthesis of pigment, electron transport components (1, 17), and membrane polypeptides (2) of the photosynthetic apparatus, expression of several autoregulated enzymes (13), and a period of gametogenic competency (16).

The regulation of cell cycle events in *C. reinhardtii* has been studied using conditional mutants which are defective in the cell cycle. Howell and Naliboff (11) described temperature-sensitive mutants, called cycle-blocked (*cb*) mutants, which, at restrictive temperature, are defective in their progress through the cell cycle. *Cb* mutants have discrete periods of temperature sensitivity during the cell cycle. The end point of the temperature sensitive period in a *cb* mutant is called the *block point* and is considered to be the cell cycle stage when the gene product required for cell division normally completes its function. A composite cell cycle map of block points for *cb* mutants has provided information about the timing of gene-controlled functions which regulate the cell cycle (10).

Another approach which has been used to investigate the programming of cell cycle events involves the use of various antibiotics and antimetabolites which have stage-specific effects during

the cell cycle. Hamburger and Zeuthen in their early studies of the cell cycle in heat-shocked synchronous *Tetrahymena* cultures first introduced the concept of the *transition point* to describe the effects of an inhibitor at various times during the cell cycle (9; for review, see reference 14). The transition point is defined as the stage beyond which an inhibitor, in the case cited here, 2,4-dinitrophenol, would not affect division in the ongoing cell cycle but would affect division in the subsequent cycle. In later studies, transition points for a number of different inhibitors in various synchronous cell systems have been described (20, 15, 7), and these studies have provided considerable information about scheduling during the cell cycle of general metabolic processes which are required for cell division.

The determination of transition points and other cell cycle parameters has usually required synchronous cultures. Ordinarily, transition points are measured by adding an inhibitor to samples from a synchronous culture at different cell cycle stages and monitoring the subsequent progress of the cells in each sample. In this paper, we describe a technique for determining transition points in asynchronous cultures. We have applied this technique to determine transition points for a number of different inhibitors in *C. reinhardtii*.

MATERIALS AND METHODS

C. reinhardtii strain 137C mating type$^+$, kindly supplied by W. T. Ebersold, was used throughout these experiments. The cells were grown autotrophically on 3/10 HSM at 23°C and bubbled with 3% CO_2 in air. Cultures were illuminated with cool white fluorescent lighting with a light intensity measured at the culture surface of 4,000 lx.

Cells in exponential growth were diluted to ca. 5×10^4/ml and subcultured in 20–250-ml cultures. Cells were grown to about $1-2 \times 10^6$/ml, and a small volume of a stock solution of inhibitor was added to each culture. Stock solutions of inhibitors dissolved or suspended in growth medium were as follows: acridine orange (Calbiochem, San Diego, Calif.) 200 μg/ml, actinomycin D (Calbiochem) 250 μg/ml, 5-bromouracil (Calbiochem) 10 mg/ml, colchicine (Sigma Chemical Co., St. Louis, Mo.) 100 mM, cycloheximide (Calbiochem) 100 μg/ml, 3(3,4-dichlorophenyl) 1,1 dimethylurea (Pfaltz & Bauer, Stamford, Conn.) 2.0 mM, 2,4-dinitrophenol (J. T. Baker Chemical Co., Phillipsburg, N. J.) 20 mM, ethidium bromide (Calbiochem) 200 μg/ml, hydroxyurea, (Calbiochem) 200 μg/ml, mycostatin (Calbiochem) 200 μg/ml, *p*-chloromercuribenzoate (Calbiochem) 1 mg/ml, potassium cyanide (Malinckrodt Chemical Works, St. Louis, Mo.) 50 mM, Rifampin (Calbiochem) 10 mg/ml, sodium azide (Matheson Co., Inc., East Rutherford, N. J.) 50 mM, spectinomycin (Upjohn Co., Kalamazoo, Mich.) 625 μg/ml. Chloroamphenicol (Sigma) 5 mg/ml was prepared in 0.1% ethanol in growth medium and 3-indoleacetic acid (Calbiochem) in 0.5% ethanol solution. All inhibitor stocks were prepared in sterile solution or were sterilized by Millipore filtration. Approximately 0.1-ml aliquots of culture were removed at periodic intervals after addition of inhibitors for cell density determination. Cell densities were measured with a Celloscope electronic cell counter and/or hemacytometer, and sizing distributions were determined with the cell counter. Signal peak height is proportional to cell envelope volume on the counter.

RESULTS

The technique used here for measuring transition points[1] is similar to that described by Howell and Naliboff (11) for determining block points in asynchronous cultures of cycle-blocked (*cb*) mutants. Transition points for various inhibitors in asynchronous cultures are determined by measuring the amount of cell division in a culture after the addition of an inhibitor. The amount of division (called residual division) is related to the proportion of cells in the asynchronous population which have already passed the transition point. By measuring the amount of residual division one can determine this proportion of the cell population and, hence, the transition point, provided the distribution of cells in the cell cycle is known.

In exponential asynchronous cultures, cells are distributed through the cell cycle according to an age distribution described by Cook and James (6). That age distribution can be represented by Eq. 2 from Howell and Naliboff (11):

$$y = \frac{\ln A}{A - 1} A^{(1-x)}, \qquad (1)$$

where y is the relative number of cells at cell cycle stage x (varying from 0 to 1), A is the number of daughter cells produced by a single parent cell at each cell generation, and $\ln A/A - 1$ is a normalization constant such that $\int_0^1 y\,dx = 1$. The portion (I_2) of the total cell population which lies between the transition point (x_{TP}) and the subse-

[1] The term transition point, denoted x_{TP}, defines the last cell cycle stage at which the addition of an inhibitor (drug, antibiotic, antimetabolite, etc.) will block cell division or any other cell cycle event. In this study, we will consider only transition points which relate to the blockage of cell division, where cell division is used in the general sense to denote the process of cell multiplication.

quent cell division (which is 1 on a cell cycle scale from 0 to 1) is therefore

$$I_2 = \frac{\ln A}{A - 1} \int_{x_1}^{1} A^{(1-x)} dx. \quad (2)$$

This portion, I_2, has escaped the effects of the inhibitor in the ongoing cell cycle and will undergo cell division. Therefore, the amount of residual cell division (N/N_0, where N = cell density at the termination of growth and N_0 = cell density at the time of inhibitor addition) is

$$N/N_0 = I_2(A - 1) + 1. \quad (3)$$

Evaluating the integral in Eq. 2, substituting in Eq. 3 and solving for the transition point (x_{TP}) gives:

$$x_{TP} = 1 - \frac{\ln (N/N_0)}{\ln A}. \quad (4)$$

Eq. 4 allows one to determine the transition point in an asynchronous culture by simply measuring the amount of residual cell division (N/N_o) after addition of an inhibitor. The preconditions for the application of Eq. 4 are the following: (*a*) the cell culture must be uniform and in exponential growth at the time of inhibitor addition (or in any *growth* phase where the age distribution of the cell population can be accurately described); (*b*) after addition of the inhibitor, the average number of daughter cells, A, produced by subpopulation I_2 must be constant and/or known; (*c*) eventually cell density increase must stop completely after addition of the inhibitor; and (*d*) the amount of residual cell division, N/N_o, must be less than the number of daughter cells, A, produced per dividing cell after addition of the inhibitor.

Cell Division after Inhibitor Addition

In attempting to find transition points for various inhibitors, it is important to consider what concentration of inhibitor to use when measuring the extent of residual cell division. One might want to use inhibitor concentrations which have been employed in other studies to inhibit specifically a single metabolic or macromolecular synthesis process. However, in many cases such documentation may not be available for the inhibitor in the cell system under study. Therefore, in this analysis the rule which we have applied is to use the lowest inhibitor concentration over a range where the amount of residual cell division is constant.

An example of this rule is shown in Fig. 1 A where chloramphenicol (CAP) is added to an asynchronous culture. In the control sample (no inhibitor added), the cells are in exponential growth during the time-course of the experiment, the cell doubling time is 8 h, and the cell generation time is 16 h since the number of daughter cells produced at each division is four.

At the lowest CAP concentration (0.2 mg/ml), CAP reduces the growth rate but does not block cell division altogether within 44 h. At higher concentrations (0.5 mg/ml), CAP completely blocks residual cell division by 24 h and cell density remains constant thereafter. At even higher CAP concentrations (1.0 mg/ml), the same cell density is reached within 24 h as with 0.5 mg/ml CAP, but the cell density begins to decline at about 30 h. Cytological observations showed that after addition of CAP, the cells which did divide produced four daughter cells, and at higher CAP concentrations the decline in cell density (as determined by electronic cell counter) is due to some cell lysis.

The lowest CAP concentration (0.5 mg/ml) over a range in which residual cell division is constant and which eventually results in the complete cessation of cell division was chosen to determine the transition point. The extent of residual division (N/N_0) determined from an average of the plateau data points was 2.03 (Table I) and the calculated transition point was 0.48 (fractional units of the cell cycle from 0 → 1).

The residual division kinetics in the presence of CAP present an example of an inhibitor with an

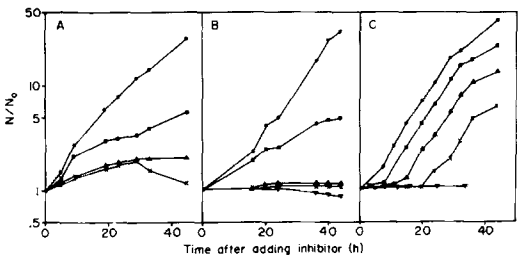

FIGURE 1 The effect of inhibitors on the increase in cell density of asynchronous cultures. Inhibitors were added at zero time and subsequent cell density changes expressed as N/No (see eq. 3) were monitored by an electronic cell counter. (A) Chloramphenicol (CAP). No inhibitor control, ●——●; 0.2 mg/ml, ■——■; 0.5 mg/ml, ▲——▲; 1.0 mg/ml, ×——×. (B) 2,4-dinitrophenol (DNP). No inhibitor control, ●——●; 0.5 mM ■——■; 0.75 mM, ▲——▲; 1.0 mM, ×——×; 2.0 mM, ▼——▼. (C) Cycloheximide (CHI). No inhibitor control, ●——●; 0.1 μg/ml, ■——■; 0.5 μg/ml, ▲——▲; 1 μg/ml, ×---×; 3 μg/ml, ▼——▼.

early-to-mid-cycle transition point, where, with the addition of higher inhibitor concentrations, residual cell division is significant (nearly a cell density doubling) but reaches a limit. In contrast to CAP, 2,4-dinitrophenol (DNP) has a late transition point. As illustrated in Fig. 1 B, 0.5 mM DNP slows the division rate, but does not completely block cell division within 44 h. At higher DNP concentrations (0.75–1.0 mM), residual division reaches a limit where almost no division is observed upon the addition of DNP. At even higher DNP concentrations (2.0 mM), cell densities begin to decline, a phenomenon which, again, appears to result from cell lysis. N/N_o was determined from an average of the data points on the plateau of the 1.0 mM DNP curve, and the transition point was calculated to be 0.98 (Table I).

The effect of cycloheximide (CHI) is an exception to the typical residual division inhibition curves described for CAP and DNP. As shown in Fig. 1 C, after the addition of 0.1–3.0 μg/ml CHI, cell division ceased rapidly as is characteristic for an inhibitor with a late transition point. However, cells recovered from the inhibitory effects of CHI, and the time for recovery was dependent on the initial concentration of CHI. It appeared as though the cells had metabolized or detoxified the inhibitor, since, when the CHO-containing medium in which full recovery had occurred was added to fresh cells, it no longer inhibited their growth. The transition point was therefore calculated from the lowest concentration curve which gave a significant plateau (1 μg/ml), and later recovery effects were disregarded. The transition point for CHI was 0.90 (Table I).

All transition points were determined for cells grown under constant conditions in autotrophic culture. It was necessary to protect certain photosensitive inhibitors from the effects of light while yet permitting photosynthesis to continue. Such photo-protection was provided by the use of colored celluloid filters which permitted light passage above 570 nm. These filters increased the cell generation time from 16 h to about 24 h, but otherwise had no other effect on the growth of the cells. Light filters were used in determining the transition points for 5-bromouracil (BU), ethidium bromide (EB), and actinomycin D(ACT-D). Acridine orange (AO) and Rifampin (RIF) colorized the growth media, but the cell division inhibition caused by these inhibitors was not due to light absorption since increasing light intensities from 4,000 to 10,000 lx gave similar transition points.

Accumulation Stage for Various Inhibitors

The transition point is not necessarily the cell cycle stage where inhibitor-treated cells accumulate; instead, they terminate their progress at an

TABLE I

Summary of Transition Points for Various Inhibitors

Inhibitor	Concentration	Residual division (N/N_0)	Transition point x_{TP}
Rifampin (RIF)	250 μg/ml	2.80	0.25
5-Bromouracil (BU)	1 μg/ml	2.75	0.26
Dark	—	2.59	0.31
Spectinomycin (SPEC)	30 μg/ml	2.44	0.35
$-PO_4$	—	2.33	0.41
Ethidium bromide (EB)	10 μg/ml	2.24	0.42
Dichlorophenyldimethylurea (DCMU)	5 μg/ml	2.13	0.45
Chloramphenicol (CAP)	0.5 mg/ml	2.03	0.48
p-Chloromercuribenzoate (pCMB)	10 μg/ml	1.85	0.56
Actinomycin D (ACT-D)	20 μg/ml	1.42	0.74
Colchicine (COLCH)	5 mM	1.37	0.77
3-Indoleacetic acid (IAA)	10 μg/ml	1.36	0.78
Acridine orange (AO)	10 μg/ml	1.29	0.82
Hydroxyurea (HU)	10 μg/ml	1.15	0.90
Cycloheximide (CHI)	1 μg/ml	1.15	0.90
Potassium cyanide (CN)	1 mM	1.14	0.91
Sodium azide (N_3)	0.5 mM	1.04	0.97
2,4-Dinitrophenol (DNP)	1 mM	1.03	0.98
Mycostatin (MYCO)	1 μg/ml	1.02	0.98

average stage termed the *accumulation stage*, x_{AS}. Determination of an accumulation stage is usually uncertain because it is often tied to the expression of a biochemical process or morphological change which may be out of step with other cell cycle events in inhibitor-treated cells. Thus, in attempting to describe accumulation stages for inhibitor-treated cells, we have expressed these stages not in absolute cell cycle time, but *with respect* to the progress of an ongoing process in the cell cycle such as cell growth or increase in cell volume. When relating cell cycle stage to cell volume, we have recognized that determination of the accumulation stage is a population problem. Inhibitor-treated cells may accumulate in nearly a normal distribution around a mean cell volume or they may stop immediately in their growth and be distributed in a skewed volume distribution similar to that observed for an untreated asynchronous culture.

To determine the accumulation stages with respect to cell volume in asynchronous cultures, it was first necessary to describe the growth kinetics of individual cells during the cell cycle in untreated cultures. The measurement of individual cell growth kinetics was approached as a cell population problem and analyzed by measuring with an electronic cell counter the relative cell volume distribution of cells in an untreated asynchronous culture. The relationship between the relative number of cells, y, at the relative cell volume, v, in such a culture can be described from the observed size distribution by the least squares fitted line represented by the equation:

$$y = 0.0025(7.5\text{-}v)^{3.05} \quad (5)$$

Eq. 5 has been normalized such that within a quadrupling of cell volume (from $v = 1$ to $v = 4$), which occurs in one cell generation, the total relative number of cells is one or $\int_1^4 y dv = 1$.

We assume that, during the cycle in asynchronous cells, cell volume continuously increases in some fashion. Therefore, from the age distribution (eq. 1) and the cell volume distribution (Eq. 5), we correlated the cell volume and cell cycle stage with increasing fractional cell population increments (Fig. 2). From this correlation, the curve representing relative cell volume (v) as a function of cell cycle stage, (x), was fit by the quadratic equation

$$v = 1 + 2.6x + 0.4x^2. \quad (6)$$

and solving for x

$$x = \frac{-2.6 + \sqrt{(2.6)^2 - 1.6(1 - v)}}{0.8}. \quad (7)$$

From Eq. 7, one can determine, in the specific case, the cell cycle accumulation stage, x_{AS}, for any mean cell volume distribution ($\bar{v}$) in an inhibitor-treated culture. Thus, the technique we have used to determine accumulation stage is to add the inhibitor to an asynchronous culture (at the same concentration used to measure the transition point) and perform a cell volume-sizing analysis after the cells have terminated growth. For the control cells (no inhibitor added), the mean relative cell volume ($\bar{v}$) is 2.15 and the population is significantly

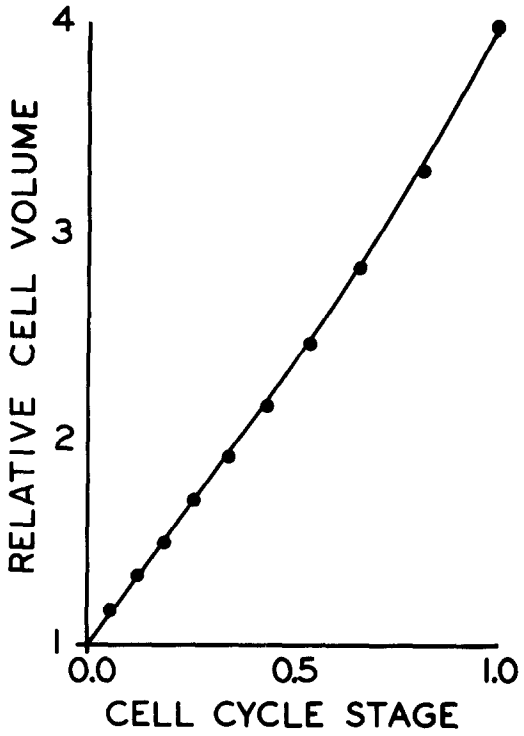

FIGURE 2 Relative cell volume (v) for asynchronous cells as a function of cell cycle stage (x). Relative cell volume was determined for discrete cell population increments from the cell size distribution (Eq. 5) obtained by an electronic cell counter. Average cell cycle stage for the same population increments was calculated from the theoretical age distribution (Eq. 1). Relative cell volume and cell cycle stage for 10% increments of the cell population are represented by points (●). Line is the best fit curve (Eq. 6).

skewed toward smaller cells. A summary of accumulation stages is shown in Table II.

With two inhibitors, colchicine (COLCH) and hydroxyurea (HU), cells accumulate at a cell cycle stage which is significantly beyond the transition point. For example, HU-treated cells accumulate with a mean relative cell volume ($\overline{v}$) of 4.43 which is equivalent to an accumulation stage of $x_{AS} = 1.1$ (Values of $x_{AS} > 1.0$ result from $\overline{v} > 4$ which untreated cells never reach during the cell cycle. Therefore, $x_{AS} > 1.0$ refers to stages in the subsequent cell cycle in the undivided cell.) The cell volume distribution is not significantly skewed but, instead, cell volumes are distributed rather evenly about $\overline{v}$. The accumulation stage is beyond the HU transition point, and so cells which fail to divide after the addition of the inhibitor continue to increase in volume, and those which do divide in the presence of the inhibitor also increase in volume to about $\overline{v} = 4.4$. The same holds for the effects of COLCH.

The effects of CHI are different from those of HU or COLCH. The accumulation stage for CHI (0.77) is relatively close, but before the transition point (0.90). Thus, cells before the CHI transition point continue to grow in size for a short while in the presence of CHI, but their final mean volume does not exceed that at the transition point. The final mean volume distribution for CHI-treated cells is less skewed than that for untreated cells, indicating that cells are being collected somewhat uniformly at the accumulation stage rather than being spread out throughout the cell cycle.

Another contrasting effect is that of EB. The accumulation stage, x_{AS}, for EB (0.30) is before the transition point, x_{TP}, (0.42). Therefore, cells which have escaped the effect of EB in the ongoing cell cycle undergo division and accumulate as smaller cells (with a mean relative cell volume less than that of untreated cells). The cells which have undergone division apparently do not continue to grow since the cell volume distribution is significantly skewed toward smaller cells.

DNP with a late transition point (0.98) stops the growth of all cells at any cell cycle stage, since the accumulation stage is early and the size distribution is significantly skewed. Since nearly all of the DNP-treated cells cannot divide, the fact that $\overline{v}$ has declined to a value less than that of the control cells indicates that most cells have slightly decreased in volume with 24 h of 1 mM DNP treatment.

DISCUSSION

Within an ongoing cell cycle, cell division in autotrophically grown *C. reinhardtii* cells is sensitive to a wide variety of inhibitors. Transition points for inhibitors are not spread throughout the cell cycle but are generally grouped into two broad periods in the second and fourth quarters (Fig. 3). Inhibitors with second-quarter transition points have distinctly different metabolic effects than

TABLE II

Summary of Accumulation Stages for Various Inhibitors

Inhibitor	Transition point x_{TP}	Time at sizing measurement	Mean relative cell volume	Volume distribution skewness*	Skewness *P* values	Accumulation stage‡ x_{AS}
		h				
RIF	0.25	28	1.84	−1.98	<0.005	0.30
Dark	0.31	41	1.79	−2.59	<0.005	0.29
SPEC	0.35	22	2.36	−1.61	0.005–0.010	0.49
EB	0.42	24	1.84	−2.03	<0.001	0.30
ACT-D	0.74	27	2.54	−2.04	<0.001	0.55
COLCH	0.77	22	5.82	−0.28	>0.50	1.5
HU	0.90	22	4.43	−0.44	0.400–0.500	1.1
CHI	0.90	21	3.24	−1.33	0.010–0.025	0.77
DNP	0.98	24	1.72	−2.90	<0.005	0.26
Control			2.15	−1.76	<0.005	

* g_1 values of Snedecor and Cochran (18). For a normal distribution in the absence of skewness $g_1 = 0$. Negative values represent left skew.

‡ Calculated from Eq 7.

those with fourth-quarter transition points. With respect to the inhibitors studied, those which block exclusively organellar macromolecular synthesis have second-quarter transition points, while those which effect macromolecular synthetic processes that are not exclusively (or not at all) organellar have fourth-quarter transition points. For example, organellar protein synthesis inhibitors, spectinomycin (SPEC) and chloramphenicol (CAP) (1), have second-quarter transition points, while CHI which blocks at these concentrations cytoplasmic protein synthesis in *C. reinhardtii* (1) has a fourth-quarter transition point. Organellar RNA and DNA synthesis inhibitors, RIF (19) and EB (8), have second-quarter transition points, while ACT-D (19), AO, and HU which may block both nuclear and organellar RNA and DNA synthesis have fourth-quarter transition points.

The general distribution of organellar- and nonorganellar-related transition points shows an interesting correlation with the schedule of cell cycle events in synchronous cells (Fig. 3). Events which involve the synthesis of chloroplast components are limited to the first half of the cell cycle in synchronous cells. These events include the synthesis of chloroplast DNA and chloroplast membrane components. By the end of the second-quarter, the chloroplast appears to have finished the reduplication of its components and is prepared to divide; however, chloroplast division itself does not occur until later in the cell cycle (12). Thus, the general grouping of transition points in the second quarter is consistent with the notion that by that cell cycle stage the *C. reinhardtii* cell has completed the synthesis of chloroplastic components, and that beyond that point the introduction of organellar DNA, RNA, and protein synthesis inhibitors has no effect on the subsequent cell division. The fine

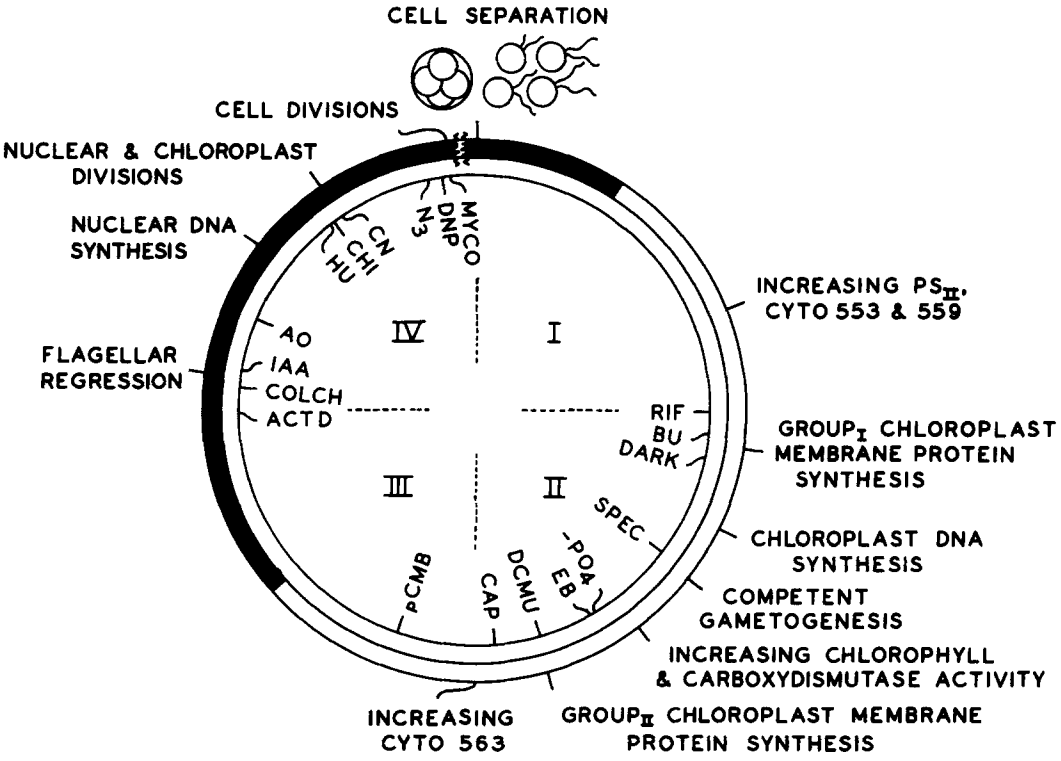

FIGURE 3 Cell cycle map for *C. reinhardtii*. Inner circle shows transition points for various inhibitors as determined from asynchronous cultures in continuous light. For comparison, outer circle represents cell cycle events in 12-h light–12-h dark synchronized cultures (1–6, 17, 19). Open bar, light phase; closed bar, dark phase. A 2-h time period has been deleted in the synchronous cell cycle (indicated by the broken lines in the dark phase) between cell division and cell separation. This period, as well as the entire cycle, is lengthened by about 2 h in synchronous cultures as compared to asynchronous cultures grown under the conditions as described in Materials and Methods.

positioning of transition points within the second or fourth quarter is more difficult to interpret, particularly the difference in transition points for inhibitors which supposedly have similar effects, such as SPEC and CAP. The difference in transition points could be due to slower permeability or rate of action of SPEC as compared to CAP. On the other hand, the inhibitors may not have exactly the same effects at the concentrations used since these concentrations were based not on the inhibition of a single biochemical process such as protein synthesis but were decided on using the criteria stated in Results.

In contrast to organellar macromolecular synthesis, nonorganellar DNA, RNA, and protein synthesis processes necessary for cell division are not completed until later in the cell cycle, in the fourth quarter. The ACT-D transition point, using ACT-D at concentrations which completely inhibit the synthesis of chloroplast and nuclear ribosomal RNA and ~80% of the rapidly labeled RNA (Howell, unpublished observation), suggests that the cell no longer requires RNA synthesis for division after the beginning of the fourth quarter. However, cytoplasmic protein synthesis, as judged by the CHI transition point, is needed until near the end, coincident with the point at which the cell becomes insensitive to HU.

Two other groupings of transition points appear significant, those involving uncouplers of electron transport inhibitors and those involving nutrient deprivation. Uncouplers and electron transport inhibitors, cyanide (CN), sodium azide (N_3), and DNP, have quite late transition points in the fourth quarter. This suggests that cells have an energy requirement almost until the time of cell division.

Transition points which result from sudden nutrient deprivation are located in the second quarter. In this study, all cells are grown under autotrophic (photosynthetic) conditions, and so, placing cultures in the dark or introducing dichlorophenyldimethylurea (DCMU), a Hill reaction inhibitor, is equivalent to nutrient deprivation. Thus, both the dark and DCMU have second-quarter transition points. Phosphate deprivation ($-PO_4$) will block a subsequent cell division ony if cells early in the cell cycle are deprived of phosphate. Deprivation of CO_2 will not block the cells within one cell cycle; apparently, autotrophic cells have adequate carbon skeleton stores to continue growing for an entire cell cycle in the absence of CO_2. The effects of ammonium deprivation during the cell cycle are complex and not analyzed here since NH_4^+ withdrawal at specific cell cycle stages triggers gametic differentiation and permits an extra round of cell division (16).

Inhibitor-treated cells accumulate at a mean cell cycle stage (accumulation stage) in either a relatively narrow or broad skewed distribution depending on the effects of the inhibitor. In Table II, it can be seen for inhibitors with fourth-quarter transition points that the accumulation stage and the distribution of cells around that stage can differ markedly. In general, inhibitors with late transition points, early accumulation stages and skewed distribution (such as DNP) have stopped the progress of all cells in an asynchronous culture regardless of their cell cycle stage. Inhibitors with similar late transition points, later accumulation stages, and unskewed distributions (such as with COLCH and HU) permit cells at nearly all cell cycle stages to continue on in many growth processes but prevent division. With the inhibitors studied with second-quarter transition points, the cells accumulate close to the transition point. This means that cells which can divide after the addition of the inhibitor discontinue their growth after division, and that cells which cannot divide are also blocked in further growth. In general, most inhibitors tested have one of three basic effects, they prevent the growth of most cells before the transition point, they allow cells to accumulate at the transition point, or they permit the growth of cells beyond the transition point. A determination of the accumulation stage permits one to distinguish among these effects.

The assumption was not made in this study that the inhibitors directly block cell division, since most of the inhibitors affect cell cycle events which take place before cell division. Obviously, those inhibitors with early accumulation stages must not affect cell division directly since these inhibitors prevent cells from progressing to the end of the cell cycle. We suggest that these findings serve as a cautionary note to situations where an inhibitor blocks a cell cycle event, and the interpretation is made that the inhibitor acts directly on that event and not on previous ones within a chain of prerequisite events. A case in point is the recent report by Blamire et al. (3) that inhibitors of chloroplast protein synthesis block nuclear DNA synthesis. They suggested that the inhibitors blocked the synthesis of a chloroplast protein product which was directly involved in the regulation of nuclear DNA synthesis. The alternative

interpretation suggested by this study is that inhibition of chloroplast protein synthesis prevents the cell from progressing to further cell cycle events which precede and are, perhaps, indirectly required for nuclear DNA synthesis.

The use of asynchronous cultures does not circumvent the argument that the general positioning of transition points only (or to a great extent) relates to the permeability of specific inhibitors at various times in the cell cycle. Methods which use either synchronous or asynchronous culture techniques for determining transition points are faced with this issue to the same degree. The stage-specific permeability argument is, however, countered by the finding that a variety of different inhibitors of organellar macromolecular synthesis (presumably all with different permeabilities) all have transition points concentrated during the second quarter and not spread throughout the cell cycle. Furthermore, biochemical effects of some of the inhibitors with early transition points (larger amounts of residual cell division) can be observed soon after their addition to a growing culture (1), indicating that they are probably not excluded for a longer period of time than those with later transition points.

The techniques described in this study are a convenient way for determining inhibitor transition points and accumulation stages in asynchronous cultures of *C. reinhardtii*. The general nature of the technique described in this paper for *Chlamydomonas* may permit its application to a wide variety of cell systems where the age distribution of the population is known.

This research was supported by grants GB-30237 and GB-37224x from the National Science Foundation and grant GM21579 from the National Institutes of Health.

Received for publication 19 February 1975, and in revised form 19 May 1975.

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