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Interaction of the Circadian Cycle with the Cell Cycle in *Pyrocystis fusiformis*[1,2]

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BEATRICE M. SWEENEY
Department of Biological Sciences, University of California, Santa Barbara, California 93106

ABSTRACT

Dividing pairs or single cells of the large dinoflagellate, *Pyrocystis fusiformis* Murray, were isolated in capillary tubes and their morphology was observed over a number of days, either in a light-dark cycle or in constant darkness. Morphological stages were correlated with the first growth stage, G_1, DNA synthesis, S, the second growth stage, G_2, mitosis, M, and cytokinesis, C, segments of the cell division cycle. The S phase was identified by measuring the nuclear DNA content of cells of different morphologies by the fluorescence of 4′, 6-diamidino-2-phenylindole dichloride.

Cells changed from one morphological stage to the next only during the night phase of the circadian cycle, both under light-dark conditions and in continuous darkness. Cells in all segments of the cell division cycle displayed a circadian rhythm in bioluminescence. These findings are incompatible with a mechanism for circadian oscillations that invokes cycling in G_q, an hypothesized side loop from G_1. All morphological stages, not only division, appear to be phased by the circadian clock.

It has been quite clear for many years that there is an interaction between the cell division and the circadian cycles. The division of a number of different kinds of cells occurs only at a certain phase of the circadian cycle. This phenomenon has been documented in *Euglena* (6, 7), *Chlamydomonas* (1, 2), *Gonyaulax* (11, 22) and a number of marine phytoplankton organisms (4, 25, 26). The nature of the relationship, however, has never been clear. It has been suggested (3, 7) that the two cycles share the same timing mechanism. This seemed unlikely because circadian rhythms have often been observed in nondividing cultures and the cell division cycle is not temperature-compensated as are circadian rhythms. In *Gonyaulax*, for example, the small temperature dependence observed in the circadian period is opposite to that of the cell division cycle, the circadian period becoming longer as the temperature is increased while the cell division cycle shortens under these conditions. It has been suggested (22) that some phase of the cell division cycle is gated by the circadian oscillator so that only cells that have reached a certain stage of the cell division cycle at a given circadian time can progress to division, all others being required to await the next opening of the gate. This interpretation fits the data from *Gonyaulax* since the generation time of individual cells was found to be an even multiple of 24 h. There remains the possibility suggested for diatoms by Chisholm and Brand (3) that cells cycle in G_q, a time-loop annexed to G_1 (14), and can proceed to S only on completing one or more cycles in this loop. Such a situation would result in a quantized generation time as observed in *Gonyaulax* if the time to traverse this loop were 24 h. Temperature dependence in the average generation time of a cell population could arise from the effect of temperature on the number of transits that a cell would make in G_q.

The question then is: "Is there a G_q part of the cell cycle which corresponds to the circadian oscillator?" In order to answer this and related questions about the interrelationship between the circadian and the cell cycles, an experimental organism with a long generation time, longer than 1 d, is required to be able to distinguish the CDC[3] from the CR. Recognizable morphological states corresponding to the parts of the cell cycle would be an advantage. It goes without saying that the experimental cell should show a clear circadian rhythmicity in some physiological function other than cell division. Such an organism is the dinoflagellate, *Pyrocystis fusiformis*. Under the culture conditions used, this common marine dinoflagellate has a generation time of a minimum of 5 to 6 d. It is large, about 900 μm long, and can be easily seen under a dissecting microscope. It traverses five recognizable morphological stages between one cell division and the next. Populations are brilliantly bioluminescent (20), emitting about 1,000 times as many photons per cell as does *Gonyaulax*. Bioluminescence displays a distinct circadian rhythm, both in constant light and continuous darkness (19). Cell division occurs only during the night phase. In addition, chloroplasts migrate to the center of the cell during the night phase and disperse again to the cell periphery during the day phase in DD (18, 19). *P. fusiformis* then seemed an ideal experimental organism for the investigation of the interaction between the cell cycle and the circadian clock. In this paper, we report the results of such a study.

MATERIALS AND METHODS

Pyrocystis fusiformis Murray was isolated by the author from a net sample of surface plankton taken in the Halmehara Sea in S.E. Asia during the Bioluminescence Expedition of the R.V. Alpha Helix in 1975. Cells were maintained in culture in f/2 medium (9), with the omission of silicate and the addition of soil extract (1%), at 20°C and an irradiance of 1 mw cm^{-2} from cool-white fluorescent lamps during the light portion of a 12-h light, 12-h dark cycle. Irradiance was measured with a United Detector Technology model UDT-40x photometer.

For the determination of morphological state, individual *Pyrocystis* cells were picked up with a micropipette while observing them with a dissecting microsope and inserted into an autoclaved capillary tube containing a small amount of medium. Five such capillaries were held on a glass I-shaped bar in a Petri plate

[1] This paper is dedicated to the memory of my friend, Bill Hillman, with whom I shared many good discussions on circadian rhythms.

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[3] Abbreviations: CDC, cell division cycle; CR, circadian cycle; LD, environmental light-dark cycle; DD, constant darkness; CT, circadian time; 4′,6-diamidino-2-phenylindole diHCl.

containing medium to maintain a moist atmosphere within. Petri plates were placed in either LD or DD at 20°C. The cells within the capillaries were examined at frequent intervals with a dissecting scope and their morphological stage was recorded. This operation was done as quickly as possible so that cells were exposed to the microscope light for at most 0.5 min at each reading. Series of observations of change in stage were usually begun by isolating dividing pairs. The two resulting daughter cells remained in synchrony at least through one cell cycle, usually longer.

Bioluminescence was measured using the photomultiplier photometer described previously (21), standardized against scintillation solvent containing ^{14}C (12). Single cells or samples of a population were placed in shell vials in 2 ml medium during the light part of the LD cycle. Bioluminescence was stimulated by the addition of 0.5 ml of 50 mM acetic acid. Light emission was measured for 1 min. A small amount of light continues to be emitted for more than 30 min after acid stimulation. For this reason, the values given here for photons per cell are less than the total light of which a cell is capable.

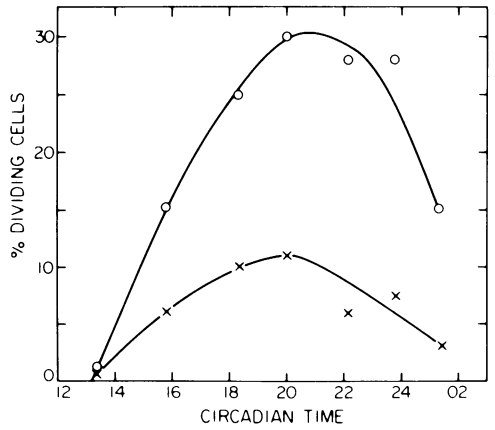

FIG. 1. The number of dividing cells in a culture of *P. fusiformis* as a function of time (CT) during a night in LD. Number of dividing cells, (○); cells forming two daughter cells, (×). Note that cells may go through all the stages of the cell cycle, but form only a single new cell. No dividing cells were found between 02 and 12 CT.

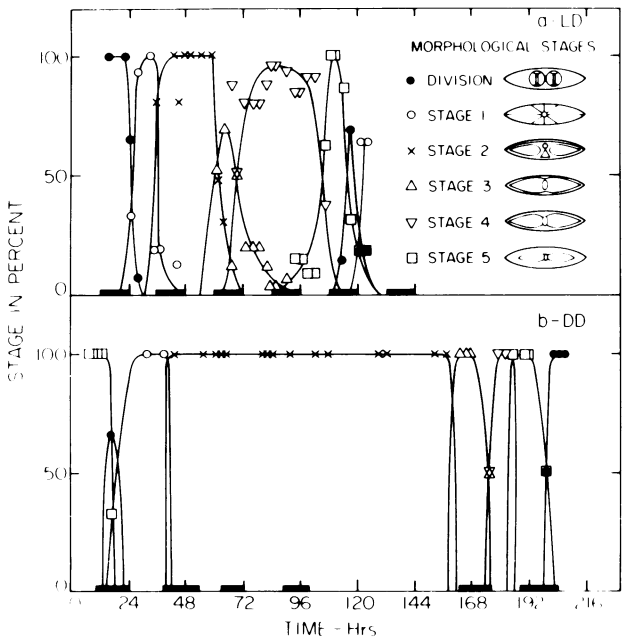

FIG. 2. The morphological stages of *P. fusiformis* (see insert for diagrammatic description and symbols) as a function of time in LD (a) and in DD (b). Zero time on the abscissa is the beginning of a environmental light period. The dark bars on the abscissa represent dark periods (a) or night phase as determined by the position of the chloroplasts near the nucleus (b). Gaps represent the absence of observations during the night. The points where lines cross the abscissa were omitted for clarity.

DNA per cell was measured as the fluorescence at 450 nm of nuclei stained with DAPI. Cells of known morphological stage were fixed in alcohol:glacial acetic acid, 3:1. They were dried on gelatin-coated slides (13), stained for 30 min in the dark in aqueous DAPI at 0.01 $\mu g\ ml^{-1}$, washed with H_2O, and mounted in glycerol (5, 16). After several days in darkness in the cold room, cells were examined with a Leitz fluorescence Dialux microscope and Nanometrics Nano Spec/100 microspectrophotometer which permitted the measurement of the fluorescence from only the nucleus. Cells of different stages on the same slide were compared. Filters were UG1 ultraviolet transmitting filter and a 430 nm cut-in filter. DAPI was obtained from Boehringer Mannhiem.

RESULTS

Morphological Stages of *Pyrocystis*. *P. fusiformis* is such a large organism (average length = 0.98 ± 0.1 mm) that cells can be isolated and observed easily. Their behavior in capillaries is the same as in populations in culture for at least 2 weeks, and generation times are the same in LD, usually either 5 or 6 d. As in many other dinoflagellates, cell division in *P. fusiformis* is strongly phased by the circadian oscillator to take place only during the night phase (Fig. 1).

Other distinct morphological stages have been recognized in *P. fusiformis* (23, 24, 28), and are diagrammed in sequence in Figure 2 as I shall refer to them. Recently divided cells are the most characteristically dinoflagellate in appearance of any of the cell stages. They are approximately spherical and have a girdle. We have once observed such cells to possess both a transverse and a trailing flagellum and to be motile for a brief time. When *P. fusiformis* divides, occasionally only a single daughter cell is formed. Within 3 to 5 h of cell division, the new cell enlarges rapidly to the fusiform, nonmotile form in which the cytoplasm is dispersed in strands across a large vacuole and the nucleus is central. These stage-1 cells later rearrange so that the cytoplasm forms a thick band on one side of the cell and a much thinner band on the opposite side, joined in the center of the cell by two distinct transverse cytoplasmic strands. In accord with the nomenclature of Swift and Durbin (23), this is a stage-2 cell. We have distinguished an intermediate stage, stage 3, between stage 2 and the third stage of Swift and Durbin, where the cytoplasm is withdrawn from the wall only in the center of the cell. The cytoplasm then contracts still further from the cell wall to form stage 4. This stage is followed by further contraction of the cytoplasm into a thin strip in the center of the vacuole (stage 5). This stage immediately precedes the rounding up of the cytoplasm in cell division. These stages follow one another in sequence even when only a single progeny cell is formed.

At night, the chloroplasts of *Pyrocystis* aggregate in the center of the cell around the nucleus. Plastids from the tips of the cell must migrate as much as 400 to 450 μm twice each 24 h. The alteration in the position of the chloroplasts changes the appearance of the cells, so that each day stage has a corresponding night stage if it lasts more than 12 h.

Timing of Morphological Stages. It was of interest to ask whether the other morphological stages are phased by the circadian clock as is division. In order to investigate this question, I have determined the times of day when one stage ends and the next begins by isolating single pairs in a capillary tube and observing them at frequent intervals for 10 d with a dissecting microscope. The capillaries were placed under culture conditions, either LD or DD. At least two cycles of cell division could be

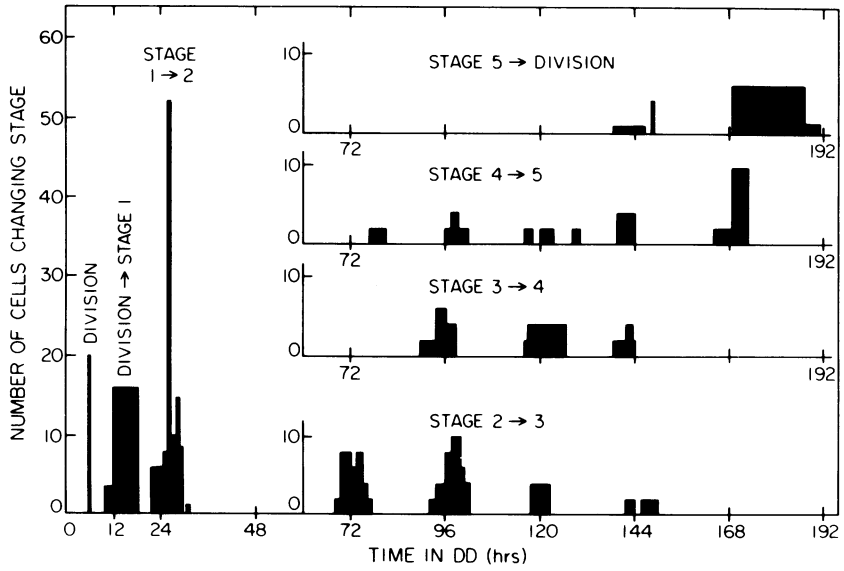

FIG. 3. The timing of transitions between morphological stages in *P. fusiformis* in DD, from data like tht in Figure 2. Only transitions where the change in stage could be resolved within several hours are plotted. Zero on the abscissa is the beginning of a night. Note that all changes are distributed discontinously in time, at intervals of about 24 h, hence are quantized to $n \times 24$ h.

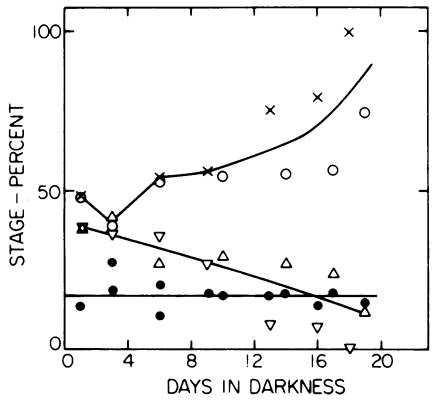

FIG. 4. The distribution of morphological stages in two cultures of *P. fusiformis* during 20 d in DD. Stage 2, culture 1, (○); stage 2, culture 2, (×); stages 3 + 4 + 5, culture 1, (△); stages 3 + 4 + 5, culture 2, (▽); dividing cells + stage 1, both cultures, (●).

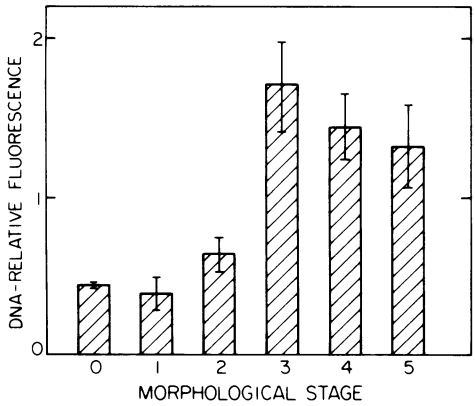

FIG. 5. The fluorescence relative to that of stage 3 nuclei stained with DAPI and measured at 450 nm. The bars at the top of each segment represent ± 1 SE.

observed without disturbing the capillaries. The cells in any given capillary remained in tight synchrony with respect to stage for at least one cycle.

The transition from a recently divided cell to stage 1 took place at the end of the night when division occurred or the beginning of the following day and lasted no longer than 12 h (Fig. 2a). During the early part of the next night, the two transverse bands of cytoplasm characteristic of cells in stage 2 began to be distinguishable. The transition was complete before the beginning of the next day. The duration of stages 2 and 4 were variable. Stages 3 and 5 each persisted for 1 d. At the end of 5 to 6 d, cell division stages appeared, most frequently during the latter part of the night.

During the progression through the cell cycle in LD, not only cell division but all morphological stages first appeared at certain characteristic times of day (Fig. 2b). In order to detect whether or not this behavior was under circadian control, pairs of dividing cells were isolated in capillary tubes and transferred to DD at the beginning of a normal dark period to allow the CR to express its free-running period. Cells were examined as frequently as possible and the stage of the cells was recorded.

P. fusiformis can survive and transit the cell cycle normally for at least two cycles, 12 d, in total darkness, probably because it contains a large amount of stored starch, as seen in electron micrographs (18, 19). The fact that a second cycle of cell division can be completed in darkness shows that these cells do not require light at any stage of the cell cycle, unlike *Chlamydomonas* (17).

In DD, the period of the rhythm in chloroplast position (19) could be observed in isolated cells and served to allow the determination of the night phase. The period was clearly a little longer than 24 h at 21°C. As in LD, stage-1 cells appeared at the end of the night in which the division had taken place and lasted about 12 h (Fig. 2b). At the beginning of the second night phase in DD, the cells passed through the transition to stage 2, as they did in a light-dark cycle. In DD, stage 2 was prolonged beyond 24 h, for five cycles in the example illustrated in Figure 2b. However, cells began stage 3 only during the early night phase whatever the duration of stage 2. This is evidence that the time of transition from stage 1 to 2 and stage 2 to 3 are controlled by a circadian clock, which continued to run in DD.

To investigate further the timing of transitions from one morphological stage to another, more than 100 dividing pairs were

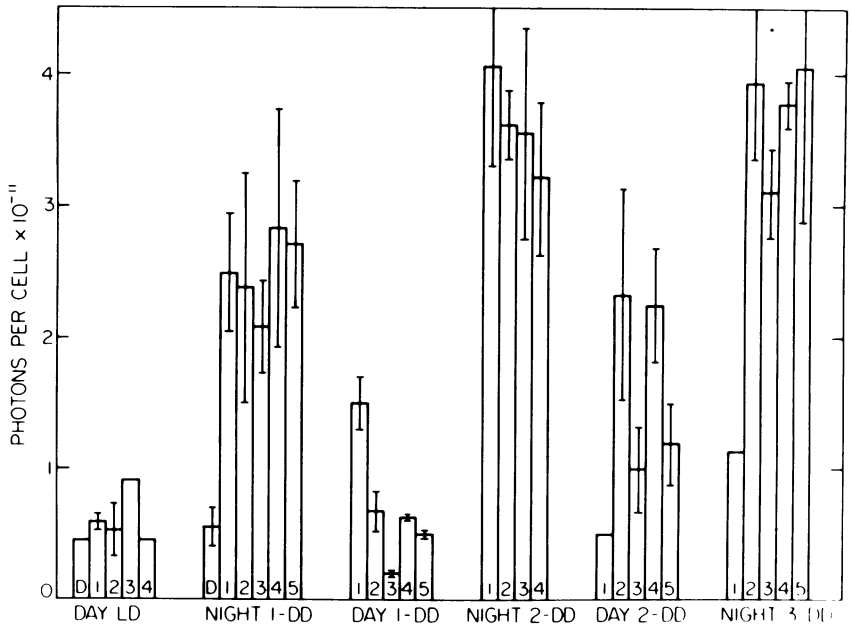

FIG. 6. The bioluminescence of single isolated *Pyrocystis* cells of different morphological stages as indicated at the base of each segment. Measurements were made for 1 d in LD and then in DD. The bars at the top of each segment represent ± 1 SE.

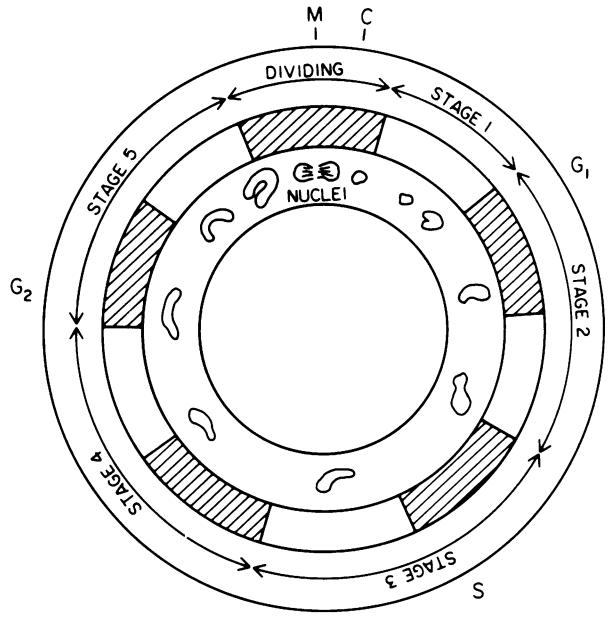

FIG. 7. A diagrammatic reprsentation of the relationship between the CDC and the CR. On the outermost circle are represented the segments of the CDC. The day phase and the night phase of each CR are shown as light and dark bars on the middle circular segment. The shape of the nucleus as seen with DAPI fluorescence is shown on the innermost circle. Note that the transitions from one morphological stage to the next (double arrowhead) occur at specific times with reference to the CR.

isolated in capillaries and placed in DD at the beginning of a dark period. Particular attention was focused on the time when cells changed from one stage to the next. The transitions that were observed with an accuracy of at least ± several hours were then plotted as a function of time (Fig. 3). From this representation of the data, it is evident that the changes in stage occur under circadian control. This is particularly clear with respect to the change from stage 2 to stage 3, but can also be detected from stage 3 to stage 4 and from stage 4 to stage 5. The distribution of transitions in time is discontinuous and occurs at intervals of approximately 24 h. Thus, cell division is not the only stage of the cell cycle which is under the control of the circadian oscillator.

When populations of *Pyrocystis* were kept in darkness for extended times, cells accumulated in stage 2 (Fig. 4). The numbers of cells in stages 3, 4, and 5 steadily declined. However, the number of dividing cells remained low but quite constant. Under these conditions of extended darkness, only a single daughter cell was produced, although the stages of the CDC appeared to be normal.

The Morphological Stage Corresponding to the S Phase of the Cell Cycle. Distinct and recognizable morphological stages are associated with each day and night of the cell cycle. To correlate these morphological stages with the corresponding stages of the cell cycle, G_1, S, G_2, M, and C, it was necessary to locate the S phase and mitosis. Radioactively labeled thymidine cannot be used for this purpose since thymidine is very poorly taken up by *Gonyaulax* and presumably other marine autotrophic dinoflagellates. Thus, cells were stained with the fluorescent DNA stain, DAPI, and the relative fluorescence of the nuclei of cells in different stages was measured (Fig. 5). This is a fairly crude method but was adequate to allow the localization of the synthesis of DNA at the end of stage 2 and the beginning of stage 3, probably during the night phase. Stages 1 and 2 are equivalent to G_1, stage 3 to S, and Stages 4 and 5 to G_2. DAPI fluorescence also outlined clearly the dividing nuclei and showed that nuclear division immediately precedes cell division in *Pyrocystis*. No evidence for a DNA content higher than twice that of newly divided cells was found.

Rhythmicity in Bioluminescence of Single Cells of All Morphological Stages. The bioluminescence emitted by one cell of *P. fusiformis* is very bright, more than 10^{11} photons. Thus, the light emitted from a single cell could easily be detected. To determine whether all morphological stages of the cell cycle displayed circadian rhythmicity, single cells were isolated and chemically stimulated to emit light. The morphological stage was determined immediately following the recording of bioluminescence. While the light emitted by single cells showed a fairly large variability, it was clear that all stages displayed circadian rhythmicity in bioluminescence (Fig. 6). Rhythmicity was observed both in LD and DD. Day-phase bioluminescence was lower in a light envi-

ronment than when the cells were in darkness, an indication of light inhibition as in *P. lunula* (10).

DISCUSSION

The results of these studies with isolated cells of *Pyrocystis* make it clear that cycling in a G_q (3, 14) cannot account for the phased cell division in this species. Although there is evidence that, when deprived of light for long periods of time, *Pyrocystis* cells accumulate in G_1 and the duration of this stage appears to be quantized, consistent with the Klevetz-Chisholm model, other parts of the cell division cycle also appear quantized, for example the transitions from stages 3 to 4, 4 to 5, and 5 to division (Fig. 3). Stages 3, 4, and 5 may each last more than 24 h, but the transitions from one stage to the next occur only at a certain CT. Furthermore, a rhythm in bioluminescence can be demonstrated in isolated cells of all stages of the cell division cycle. Were this circadian behavior the result of the time when a cell exited from a holding pattern in G_q, cells which had remained in stage 3, 4, or 5 for more than 24 h would not show rhythmicity. Thus, the circadian oscillator is running in all cells, although some have already left G_1 and presumably also G_q.

The evidence from experiments reported here supports the existence of multiple control of restriction points (7, 15) where the circadian clock interacts with the cell cycle. These all appear to be in the night phase of the CR, usually in the early night phase. The findings from this study may be best summarized in the form of a diagram (Fig. 7), which shows the correspondence between the CDC, the CR, and the nuclear shape during a cell cycle. In both *Euglena* (7) and *Chlamydomonas* (2), the CDC can be dissociated from the CR under certain environmental conditions. In *Pyrocystis*, however, there is no evidence that such uncoupling ever occurs.

Cell division in *Pyrocystis* is not so exactly timed as it is in *Gonyaulax* and some other species. Some dividing pairs can be found throughout the night phase. Edmunds (7) has remarked on this variability as observed in other species with phased cell division and considered it to be evidence for the insertion of variable time loops in the CDC.

It is interesting to find that *Pyrocystis* can initiate new rounds of cell division in continuous darkness. Thus, *Pyrocystis* does not require light for any part of its cell cycle, unlike *Chlamydomonas*, which requires a minimum of 4 h light per cell cycle (17).

A requirement for attaining a genetically determined cell size for progression from G_1 to S has been postulated to explain the experimental results obtained with synchronous cultures of the yeast, *Schizosaccharomyces pombe* (8). *Pyrocystis* attains full size early in morphological stage 1, clearly before the restriction point in G_1. The cell volume of *Pyrocystis* may vary by as much as a factor of 2 (27, 28), but there is no apparent correlation between the duration of G_1 and cell volume. Therefore, it seems unlikely that the CDC in *Pyrocystis* is under cell size control.

In conclusion, the study of isolated cells of *P. fusiformis* clearly shows that cells in all parts of the CDC display circadian rhythmicity. Furthermore, the transitions from one morphological stage to another, and presumably also from one CDC segment to another, are all under circadian control, 'phased' in the same sense as cell division.

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