Circadian Rhythm of Cell Division in Euglena: Effects of a Random Illumination Regimen

Abstract. *A persisting, "free-running," circadian rhythm of cell division in autotrophically grown* Euglena gracilis *is obtained upon placing either an exponentially increasing population or a culture that has been synchronized by a 10:14 light-dark cycle in a random illumination regimen that affords a total of 8 hours of light each 24 hours. These results are interpreted as implicating an endogenous biological clock which "gates" the specific event of cell division in the cell developmental cycle.*

Cell division in cultures of *Euglena,* as well as numerous other microorganisms, can be synchronized by appropriately chosen 24-hour cycles of light and darkness (*1, 2*) and of temperature (*3*), so that the population approximately doubles every 24 hours. The mechanism by which cell division is synchronized, however, still remains obscure.

Synchrony is not necessarily due simply to the alternating environmental regime because rhythmic cell division persists under constant conditions of illumination and temperature where there are no shifts in growth conditions to be invoked (*4*). Under this particular set of conditions, division bursts in the population may occur for as long as 10 days (perhaps even longer in continuous culture) at intervals which approximate, but rarely exactly equal, 24 hours. Ordinarily one might expect the synchrony to quickly decay after removal of the synchronizing agent (*Zeitgeber*) because of the intrinsic variation in generation times of individual cells arising from karyotypic heterogeneity and stochastic processes inherent in the cell cycle (*5*). Indeed, the length of cell cycles in individual *Euglena* may vary from 9.5 to 23.7 hours under certain nutrient conditions (*6*).

As a working hypothesis, we have assumed that an endogenous, circadian, biological clock—similar in many respects to the coupled oscillator model proposed by Pittendrigh and Bruce (*7*)—underlies this persistent rhythm, although other mechanisms are almost certainly involved. Division of individual cells in a population would thus be entrained by appropriate *Zeitgeber* and under free-running conditions would be "gated" by an endogenous oscillation operating through molecular and biochemical events that control the cell cycle.

The use of so-called constant conditions, however, presents technical problems inherent in the culture of autotrophic organisms (*4, 8*). Another method for studying circadian rhythms which has yielded interesting results for hamsters (*9*) and rats (*10*), and which we now report for *Euglena,* involves the use of randomized illumination regimes in which periods of light and darkness follow each other at irregular intervals. Regimes of this type obviously can provide no direct timing clues or signals to the organism for synchronization of its internal rhythms with environmental time.

Cultures of *Euglena gracilis* Klebs (strain Z) (*11*) were grown axenically and autotrophically (*2*) in 9-liter serum bottles at 25°C (±0.5°C) on an inorganic salt medium to which vitamins B_1 and B_{12} were added. The cultures were aerated with 575 to 625 ml of air per minute and were magnetically stirred at a rate sufficient to counteract phototactic and other motility responses, thereby insuring a homogenous cell distribution. The serum bottles were maintained in Hotpack environmental chambers whose doors were furnished with banks of six fluorescent bulbs (Westinghouse 40-watt cool-white) which provided an incident illumination of about 8000 lux (as measured with a Weston illumination meter, model 756, quartz filter). The light regime was controlled by either a Flexopulse clock timer (40-hour dial) or an Intermatic 24-hour timer having 96 trippers. The cell number was automatically monitored every 2 hours with a Brewer pipetting machine, a miniaturized fraction collector, and a Coulter electronic particle counter (*2*).

The experimental protocol (*12*) for generating "random" light-dark (LD) cycles incorporated the following practical and arbitrary procedures: (i) the total duration of illumination during any given 24-hour time span was exactly 8 hours, a value which represented a compromise between very large and very small resultant division bursts in the population; (ii) the total duration of darkness during a given 24-hour time span was exactly 16 hours; (iii) the lengths of the light periods ranged between 0.25 and 1.0 hour; (iv) the lengths of the dark periods ranged between 0.5 and 1.5 hours; and (v) the values of the light and of the dark periods for a particular 24-hour time span were written on slips of paper and drawn alternately in random fashion from separate containers.

This method of alternate selection of light and dark periods ensured that no light "signal" would exceed 1.0 hour as a result of the chance drawing of a series of successive light periods had all the slips been mixed together; long light periods quickly give rise to exponential growth unless appropriate dark periods are intercalated (*2*). Obviously, as many "random" 24-hour regimes could be generated as desired by repeated drawings. For all intents and purposes, the cells were exposed to an unpredictable illumination regimen of indefinite length and devoid of any time clues.

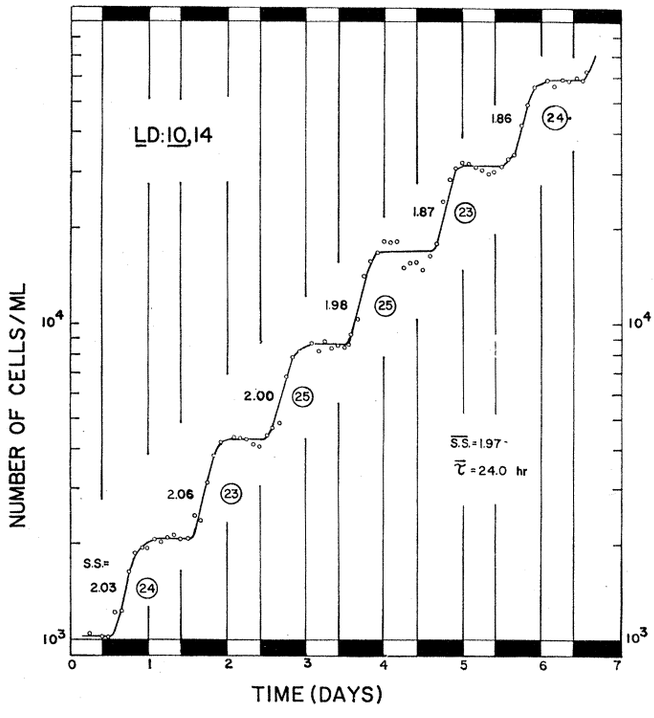
Typical light-induced synchrony of cell division in cultures of *Euglena* is shown in Fig. 1; a *10*:14 cycle was employed. This particular regime was chosen empirically: it phased cell division primarily to the dark periods with an overall doubling or generation time of 24 hours; each individual cell, on the average, divides once, and only once, during a 24-hour *Zeitgeber* period (*T*). The period of the division rhythm itself (τ) is taken as the time between successive onsets of division (cytokinesis) in the population; here it averages 24.0 hours, thereby matching the period of the imposed, entraining LD cycle. The spread of ±1 hour largely reflects the limitations of our system of monitoring cell number, which samples the culture at 2-hour intervals.

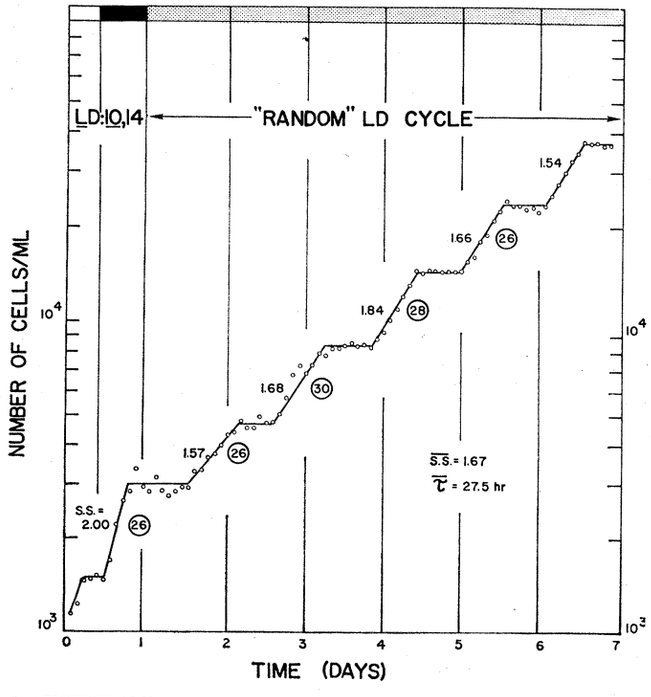
Over the range of cell concentrations used, the step-size, defined as the ratio of the cell concentration just after the division "burst" in the population to that just prior to the onset of the burst, remains almost constant (Fig. 1) with an average value of 1.97 (where 2.00 indicates a theoretically perfect doubling). One is justified, therefore, in considering the synchronized population as a model to the first approximation of an individual cell cycle; this assumption is supported by studies of the patterns of biosynthesis during a synchronized cycle (*1, 2*). The synchrony, however, is by no means perfect, since the total duration of the fission burst in the population is somewhat longer than the average fission time for the individual cell under these conditions (*2, 8*). These results are com-

parable to those reported (*2*) for *L*D: *14*,10 in a slightly different culture system.

As the light period is progressively decreased, the population still manifests rhythmic cell division, although the average step-size gradually decreases toward a limiting value of 1.0 (Fig. 2) for *L*D: *8*,16 (this cycle serves also as a control for the random regime). In this particular regime, the average step-size was 1.68. Nevertheless, the system is still synchronized with respect to the onset of cell division in the population, since τ equals T which equals 24 hours. These observations indicate that only a portion of the cells divide during any given 24-hour *Zeitgeber* cycle, but those that do, do so synchronously. There is no evidence that these cycles select for a smaller, functional subpopulation. We use the term "synchrony," therefore, to refer primarily to the event of cell fission itself within the population and not to a one-to-one mapping of individual cell cycles in those cases where average step-size does not equal 2.00.

Let us consider the effect of a "random" illumination regime upon a culture previously synchronized by a *L*D: *10*,14 cycle to a 24-hour period (Fig. 3). Synchronous fission bursts continued to take place but with average step-size equal to 1.67 for the five cycles shown. The onsets of the bursts, however, no longer occurred at intervals of 24.0 hours but rather at intervals of about 27.5 hours (average $\tau =$





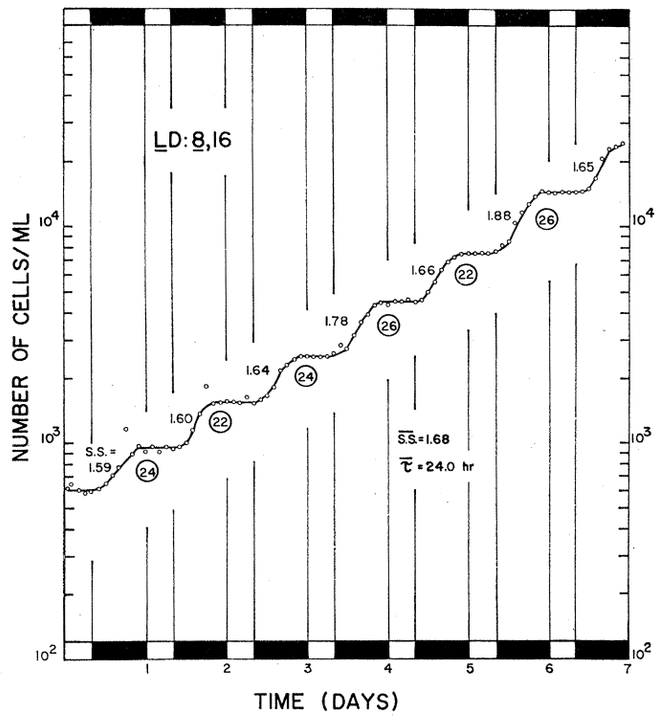


Fig. 1 (top left). Entrainment of the cell division rhythm in a population of *Euglena* grown autotrophically at 25°C in *L*D: *10*,14. Ordinate: cell concentration (cells per milliliter); abscissa: elapsed time (days). Step-sizes (ratio of number of cells per milliliter following a division burst to that just before the onset of divisions) are indicated for the successive division bursts. The period of the rhythm is also given in hours (encircled just to the right of each burst). The average period ($\bar{\tau}$) of the rhythm in the culture is essentially identical to that of the synchronizing cycle, and a doubling of cell number usually occurs every 24 hours. Fig. 2 (top right). Entrainment of the cell division rhythm in a population of *Euglena* grown autotrophically at 25°C in *L*D: *8*,16. Other labels as for Fig. 1. Although the average period of the rhythm is precisely that of the entraining light-dark cycle, the average step-size of the successive fission bursts is substantially less than 2.0, indicating that not all cells divide during any one cycle. This cycle serves as a control for the random regime (Fig. 3). Fig. 3 (bottom left). Persisting, "free-running," circadian rhythmicity in the cell division rhythm of *Euglena* cultures exposed to a "random" light-dark cycle after synchronization by *L*D: *10*,14. The generation of the "random" cycle is illustrated in reference (*12*); other labels as for Fig. 1. The average "free-running" period ($\bar{\tau}$) of the population rhythm is 27.5 hours. The average step-size ($\bar{s}.\bar{s}. = 1.67$) is almost identical to that found in *L*D: *8*,16 (Fig. 2), which affords the same total duration of illumination in a given 24-hour time span. Similar results are obtained upon exposure of an exponentially growing culture (no prior synchronization) to the random regime.

27.5 hours). The size of the individual division bursts was almost identical to that observed in *LD*: *8*,16 (Fig. 2) where the total duration of light afforded in a 24-hour time span was equivalent to that received in the random-illumination regime. These results indicate that the system was unable to entrain to the aperiodic regime and suggest that the putative underlying circadian oscillation was free running with an innate period (τ) that only approximated 24 hours.

Another series of experiments was performed to determine whether prior synchronization was necessary in order for a subsequent random illumination regime to elicit a circadian rhythm of cell division in the population. Cultures of *Euglena* which had been growing exponentially (a developmentally asynchronous population) in continuous bright light (8000 lux) with a generation time of about 13 to 15 hours were then exposed to an aperiodic regime. Synchronous division bursts immediately occurred with average τ and average step-size values almost identical to those observed in the preceding experiments.

It appears, therefore, that a free-running circadian rhythm can be induced almost immediately upon exposure of a developmentally asynchronous population to an appropriate aperiodic regime. It is not clear, however, whether (i) the hypothesized endogenous oscillations in the individual cells are themselves asynchronous but running in continuous light and subsequently "reset" and synchronized by the first dark period (or following light signal) of the imposed random illumination regime; or, alternatively, (ii) the circadian oscillations are either absent or damped out in continuous bright light (as in many higher systems) and subsequently initiated or released when the random regime is imposed.

These results compare favorably with those few studies on the effects of random illumination in higher systems. For example, it has been reported that the activity rhythms of hamsters (*9*) and of rats (*10*), previously synchronized to a 24-hour period by a light-dark cycle, revert to a circadian rhythm upon exposure to a random lighting regimen. The individual animals do not become aperiodic in their running-wheel behavior, although group desynchronization may eventually occur (*10*), owing to interindividual differences in the free-running period.

Our findings, taken with the previous demonstration for *Euglena* that (i) rhythmic cell division in the population will persist with a circadian periodicity for many days under constant conditions of illumination and temperature (*4*); that (ii) "skeleton" photoperiods—such as a *LD*: *3*,6, *3*:12 cycle which comprises the framework of a normal *LD*: *12*,12 cycle—will entrain the population rhythm to a precise 24.0-hour period, indicating that the continuous action of light is not required for synchrony (*8*); and that (iii) high-frequency LD cycles, such as a *LD*: *1*,2 regime ($T = 3$ hours) or a *LD*: *¼*,½ cycle ($T = 0.75$ hour) which elicit free-running circadian rhythms ($\tau = 26$ to 27 hours) (*8*), support the concept of an endogenous, circadian oscillation which underlies and "gates" at least the ultimate, terminal act of cell division similar to that envisaged for the *Drosophila* eclosion system (*7*, *13*).

This "gating" oscillation would be essentially independent of the rate and stage of the cell developmental cycle; that is, the act of division itself—not just differential growth rates before division—appears to be clock-controlled and thereby restricted to a specific phase of the underlying circadian oscillation. In making this conceptual distinction, which stresses the separability of the circadian oscillation in *Euglena* and the events that constitute the developmental sequence comprising the cell cycle, however, we do not wish to rule out the possibility that the very presence of the circadian oscillation may also contribute in some way to the direct control of prior developmental steps (*13*, *14*). But this does not imply in any way that the observed division rhythm is merely a trivial "population effect" resulting from the summation of the circadian rhythms of the preceding developmental stages.

From the empirical observation that division bursts occur in a population exposed to a random illumination regime at intervals of τ, and by making the likely assumption that not all cells divide during any given burst, it can be inferred that division of the *individual* cells occurs at times given by the expression $n\tau$, where n is an integer that is not necessarily constant (*8*).

Furthermore, the value of τ of the putative circadian oscillation in the individual cell must be quite precise; also, there must be little interindividual variation in τ. Otherwise, the synchrony in cell division observed in the population would soon decay due to the differences in the lengths of individual cell cycles stemming from karyotypic heterogeneity and stochastic processes (*5*, *6*). This does not seem implausible in view of our assumption that the oscillation is probably not gating the entire cell cycle but only those more immediate events controlling cell division and cytokinesis. An alternative hypothesis that division is synchronized by either known or unknown subtle geophysical variables such as fluctuating magnetic fields or cosmic rays seems unlikely for our system, although this possibility cannot, of course, be ruled out with absolute certainty. Also, it does not seem probable (*8*) that the observed division synchrony is merely the result of interaction among the cellular oscillators via repressor molecules or other chemical signals (*15*).

Finally, our interpretation of these results for *Euglena* are consistent with a growing body of literature reporting persisting circadian rhythms of cell division (or of cell "hatching") under "constant conditions" in populations of *Gonyaulax* (*16*), *Chlorella* (*17*), *Paramecium* (*18*), *Tetrahymena* (*19*), and *Chlamydomonas* (*20*). Collectively, these observations suggest that a cellular circadian clock, capable of being entrained by LD and temperature cycles, may be more deeply involved in the control of the cell cycle than heretofore anticipated (*21*).

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11. Culture supplied by Dr. V. G. Bruce, Princeton University; also available as culture 12716, American Type Culture Collection, Rockville, Md.
12. The generation of a typical segment of a random illumination regime is illustrated below. There were a total of 18 slips in each container. The various light periods oc-

curred with the following frequencies: eight 0.25-hour exposures (= 2 hours); eight 0.50-hour (= 4 hours); and two 1.00-hour (= 2 hours), yielding a total of 8 hours of illumination. The dark periods were represented as follows: three 0.50-hour exposures (1.50 hours); five 0.75-hour (= 3.75 hours); eight 1.00-hour (= 8.00 hours); one 1.25-hour (= 1.25 hours); and one 1.50-hour (= 1.50 hours), affording a total of 16 hours of darkness.

No.	Duration	
	Light period (hr)	Dark period (hr)
1	0.25	1.00
2	0.25	1.00
3	0.50	0.50
4	0.25	0.75
5	1.00	0.50
6	0.25	0.75
7	0.25	1.00
8	0.25	1.00
9	0.50	0.75
10	0.50	1.00
11	0.50	1.00
12	0.25	1.00
13	0.50	1.50
14	0.25	1.25
15	0.50	0.75
16	0.50	0.50
17	1.00	1.00
18	0.50	0.75

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Toad Urinary Bladder: Intercellular Spaces

Abstract. *Vasopressin causes dilation of the intercellular spaces of the mucosal epithelium in toad bladder, an effect previously thought to result from enhanced net transepithelial water transport. Under conditions of zero net fluid transport, vasopressin exerted the same effect in seven tissues, which indicates that the width of the intercellular spaces cannot be taken as a reliable index of net transepithelial fluid transport.*

Under a variety of conditions, the size of the intercellular spaces in rabbit gallbladder epithelium depends upon the rate of fluid transport (*1*). In toad urinary bladder, the intercellular spaces are also enlarged after vasopressin is administered under the condition of a dilute mucosal medium (*2–4*); this effect has been thought to arise from increased net fluid transport (*2*). It has been observed, however, that application of vasopressin in the absence of an osmotic gradient, when no net transfer of water occurs (*5*), results in an enlarged or open configuration of the intercellular spaces (*4*). This finding suggests that factors other than rate of water flow are involved in the regulation of size of the intercellular spaces.

Urinary hemibladders were excised from doubly pithed toads; they were rinsed in Ringer solution and mounted in Lucite double chambers (*6*) so that there were adjoining experimental and control halves for each preparation. Short-circuit current was monitored in experiments with Na^+ Ringer solution (Na^+, 113.4; K^+, 3.5; Ca^{2+}, 0.9; Cl^-, 116.3; HCO_3^-, 2.4 m*M*; *p*H 7.4 to 8.2; tonicity, 216 to 226 milliosmoles per kilogram of water). Transepithelial electrical potential was followed in experiments with choline$^+$ Ringer solution (quantitative replacement of Na^+ with choline$^+$; 2 m*M* phosphate rather than bicarbonate). In three of these experiments, net water flow was measured with a volumetric technique (*6*) so that no net transfer occurred under the isotonic conditions imposed.

After the physiologic measurements, the tissues were fixed with glutaraldehyde and prepared (*7*) for examination with a Philips EM-200 electron microscope. To provide objective in-

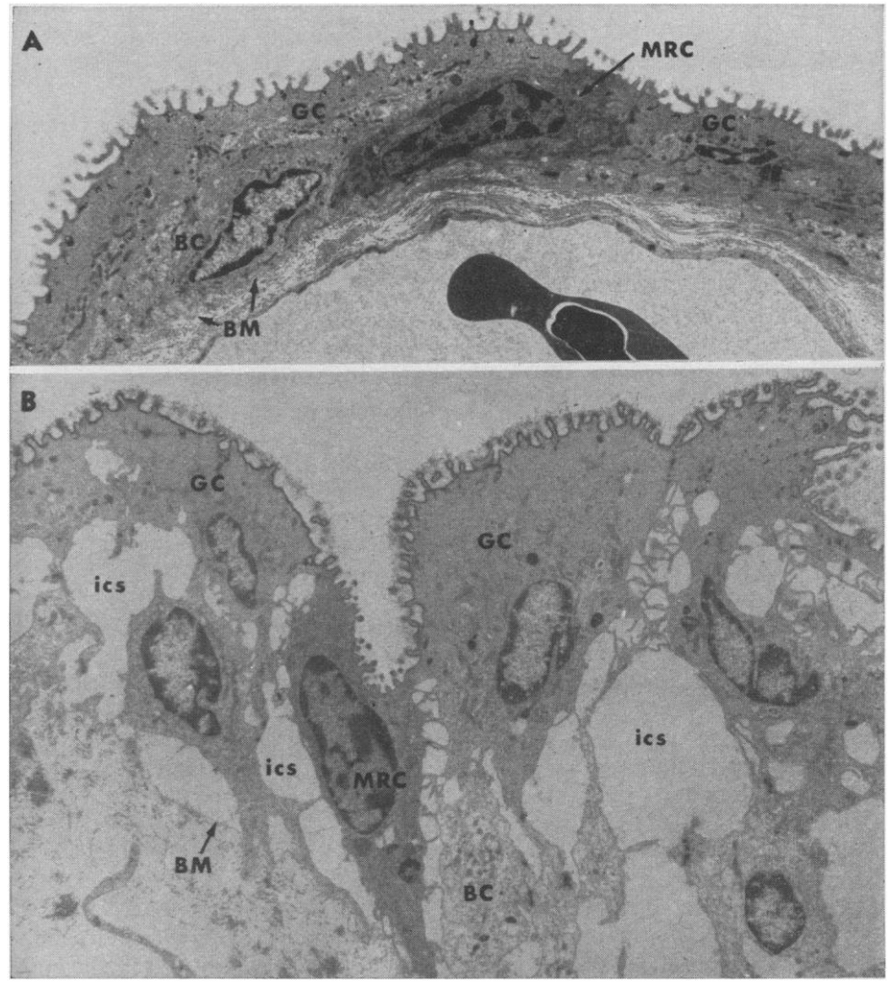

Fig. 1. Electron micrographs of toad bladder epithelium showing samples from the control and experimental portions of the same preparation. (A) Control tissue. Tissue was bathed on both surfaces with isotonic Na^+ Ringer solution; no vasopressin was added. At this magnification, there is no visible separation between neighboring cells or between cells and the basement membrane (*BM*); *GC*, granular cells; *MRC*, mitochondria-rich cells; *BC*, basal cells (× 4000). (B) Experimental tissue. Tissue was bathed on both surfaces with isotonic Na^+ Ringer solution. Vasopressin was added to the serosal bath 15 minutes prior to fixation; short-circuit current increased but net transfer of water was not measurable. The intercellular spaces (*ics*) are now markedly enlarged making cell margins clearly visible at low power (× 4000).