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Sensitivity to Stimulation, a Component of the Circadian Rhythm in Luminescence in *Gonyaulax*[1]

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ABSTRACT

A new method for the stimulation of bioluminescence in the dinoflagellate *Gonyaulax polyedra* is described. With this technique, in which cells flow through a capillary coil, it is possible to graduate the intensity of the stimulus by varying the flow rate. In continuous darkness, the threshold stimulus for cells in the middle of the day phase is greater than that for cells in the middle of the night phase. Some evidence suggests heterogeneity of sensitivity to stimulation among either cells or individual luminescent sources within a cell. At stimulus intensities much above threshold, the luminescence of both day- and night-phase cells is proportional to the number of cells within the capillary coil. Night-phase cells emit about 14 times as much light as do day-phase cells in continuous darkness.

Single bioluminescent flashes from cells were recorded with a high speed camera. No significant difference in flash kinetics was found between cells in the day and the night phase in continuous darkness. Cells in the night phase emit a flash three to five times brighter than that from day-phase cells. About twice as many flashes are recorded in a given time from a population of night-phase cells.

The activity of both luciferin and luciferase have been shown to vary rhythmically. The differences in threshold and number of flashes are evidence for a second component of the circadian rhythm in luminescence, a rhythm in sensitivity to stimulation.

The luminescence induced by either mechanical (11) or chemical (6, 8–10) stimulation of the dinoflagellate *Gonyaulax* shows a typical rhythm, the brightest luminescence occurring about 6 hr after the onset of darkness in a light-dark cycle, and the dimmest about 12 hr later. This rhythm continues under constant environmental conditions. In continuous darkness, the luminescence may be as much as 14 times brighter during the bright or night phase than during the dim or day phase. When cell-free extracts are prepared at different times of day, both the soluble luciferin and luciferase are three to five times more active in night extracts (2–4). The particulate fraction of *Gonyaulax* homogenates can also emit light. McMurray (6) reports that such luminescence is greater in night than in day extracts, but quantitative data are not given. Whether these differences observed in extracts account for the luminescence rhythm *in vivo* is in doubt, since extracts emit only a relatively small fraction of the light emitted by living cells. It has been suggested several times that a change in sensitivity to stimulation may contribute to the rhythm in luminescence in *Gonyaulax* (2, 6, 11). That there may be another component of the luminescent rhythm was also postulated on the basis of observations on the mode of excitation. In the day phase, cells stimulated chemically by the addition of acetic acid emitted more light than those stimulated mechanically, while the two methods of stimulation produced the same amount of light at night (8). When *Gonyaulax* cells are illuminated, the luminescence is inhibited and the flash characteristics are changed (1, 2, 5). However, cells in continuous darkness still show a larger amplitude rhythm when stimulated mechanically than when light is elicited chemically. It was postulated (8, 10) that biochemical differences are responsible for the rhythm when stimulation is chemical, and that the extra amplitude on mechanical stimulation represents another rhythmic component. The purpose of the experiments described in this paper was to investigate further whether or not sensitivity to stimulation varies rhythmically and contributes to the circadian rhythm of luminescence in living cells.

Cell populations of *Gonyaulax* were used in all the experiments described above and luminescence was measured under conditions such that single flashes could not be resolved. Recently, McMurray (6) has examined dilute cell populations with resolution sufficient to detect and count the number of individual flashes contributing to the luminescence in the night and day phases. She finds that the number of flashes, as well as their maximum intensity, varies with phase in constant darkness. About twice as many flashes are recorded at night as from the same population during the midday phase. Her equipment did not allow graduation of the stimulus intensity. Hence, measurements of the threshold were not made, nor was a comparison of the kinetics of night- and day-phase flashes attempted.

The present paper reports results obtained with a new method of stimulating the luminescence of *Gonyaulax*. This method permitted control over the degree of stimulation, as well as recording of the kinetics of individual flashes without distortion. The difference in the characteristics of luminescence in different phases in continuous darkness was measured with regard to the threshold stimulus needed to trigger luminescence flash kinetics, the number of individual flashes, and their intensity.

MATERIALS AND METHODS

Gonyaulax polyedra strain 60 was maintained at 22 C in enriched sea water medium containing KNO_3 (2 mM), KH_2PO_4 (0.2 mM), $FeCl_3$ (10 mM), Na_2EDTA (0.01 g/1), and soil extract (2%). All cultures were grown in 2.8-liter Fernbach flasks containing 1 liter of medium. Illumination was provided by four

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cool white fluorescent lamps giving 215 ft-c at the level of the culture. The light regime during growth was 12 hr of light and 12 hr of darkness. For experiments, cultures were transferred to continuous darkness at the beginning of a regular dark period. Under these conditions the rhythm in luminescence will be expressed for three to five cycles (11). In this paper, the time period when luminescence is bright is referred to as the "night phase," while the time of low luminescence is termed the "day phase." The cells were used at a concentration of 2000 to 3000 cells per ml. For experiments with the high speed camera, cultures were diluted 1/100 2 days before use to give 20 to 35 cells per ml.

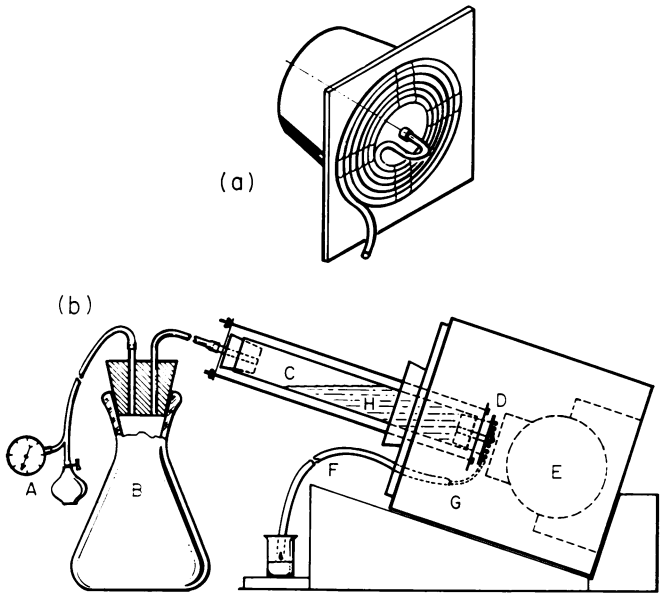

FIG. 1. Apparatus for stimulating bioluminescence in *Gonyaulax polyedra*. Included are (a) the capillary stimulation coil, (b) side view of apparatus showing sphygmomanometer and bulb (A), pressure reservoir (B), cell reservoir (C), stimulation coil (D), photomultiplier in water-tight housing (E), waste tube (F), light-tight enclosure (G), cell suspension in reservoir (H).

Since mechanical stimulation of luminescence by bubbling or stirring could not be varied quantitatively, another method for triggering luminescence was devised. Preliminary experiments in this laboratory indicated that *Gonyaulax* is not excited to flash when cells are accelerated by a sudden motion or in a centrifuge. However, the luminescence of a single cell of *Gonyaulax, Noctiluca,* or *Pyrocystis* can be stimulated by a shock wave generated from a ceramic cylinder with piezoelectric properties (7). In this case the stimulus may be deformation of the cell membrane. It is not possible to stimulate cell populations piezoelectrically, however. Observations suggest that the stimulus for luminescence may be a differential velocity across a cell or shear. To produce such a shear on free cells in large numbers, advantage was taken of the properties of flow within capillary tubes. In such tubes the rate of flow is fastest in the center and approaches zero next to the walls. Consequently, any cells within the capillary are subjected to shear, the magnitude of which can be varied by varying the flow rate through the capillary. Translucent polyethylene tubing (Intramedic No. PE 100), with an inside diameter of 0.86 mm, outside diameter of 1.5 mm, length of 609 mm, and volume of 0.36 ml served as the capillary. In order that the cells might emit light within the view of a photomultiplier tube (GE 1P21), the tubing was wound into a spiral of 5.8 turns with an outside diameter of 40 mm (Fig. 1a). The coil was backed with cardboard and attached to a rubber stopper which formed the bottom of the reservoir (Fig. 1b). The cell suspension entered the coil at the center from the reservoir through a short segment of hypodermic needle. The air space at the top of the reservoir was continuous with a 2000-ml flask in which pressure could be generated by means of an Erma sphygmomanometer calibrated in millimeters of mercury. Pressure in the system could be held constant to within 5 mm of mercury. The apparatus was inclined at an angle (Fig. 1b) to minimize the

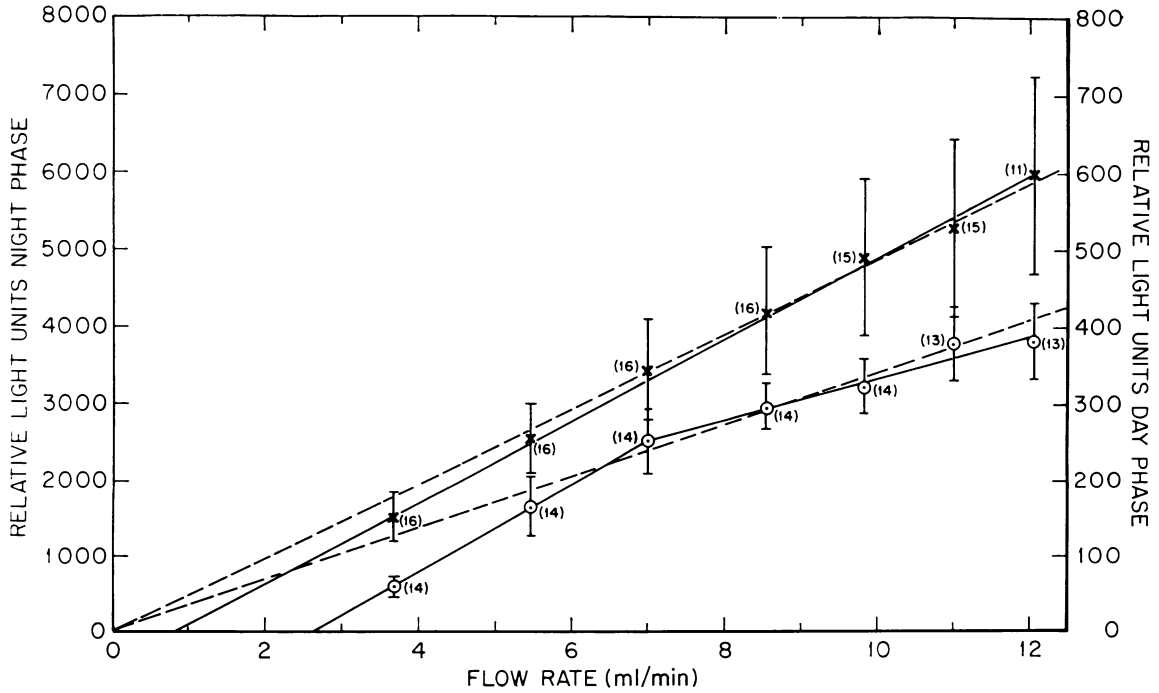

FIG. 2. Luminescence of *Gonyaulax polyedra* within the stimulation coil as a function of flow rate in the day phase (○) and night phase (×) of the luminescent rhythm in continuous darkness. The dashed lines represent the luminescence expected from the number of cells present in the coil at each flow rate. The vertical bar at each point is the standard deviation and the number is the number of determinations averaged.

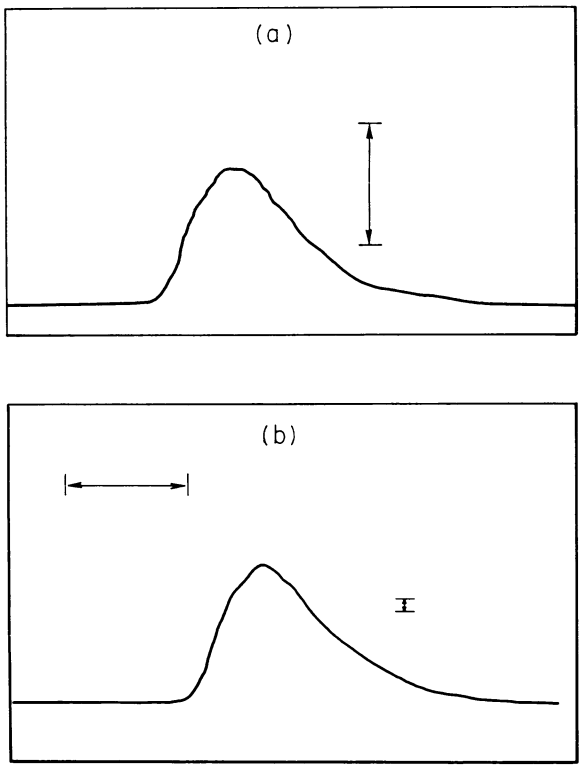

FIG. 3. Single flashes of *Gonyaulax polyedra* photographed with the high speed camera, (a) day phase, (b) night phase. The vertical bars represent ten light units for each flash. Note that the night phase flash is much larger than the day phase flash. The horizontal bar represents 40 msec. The same time scale applies to both curves.

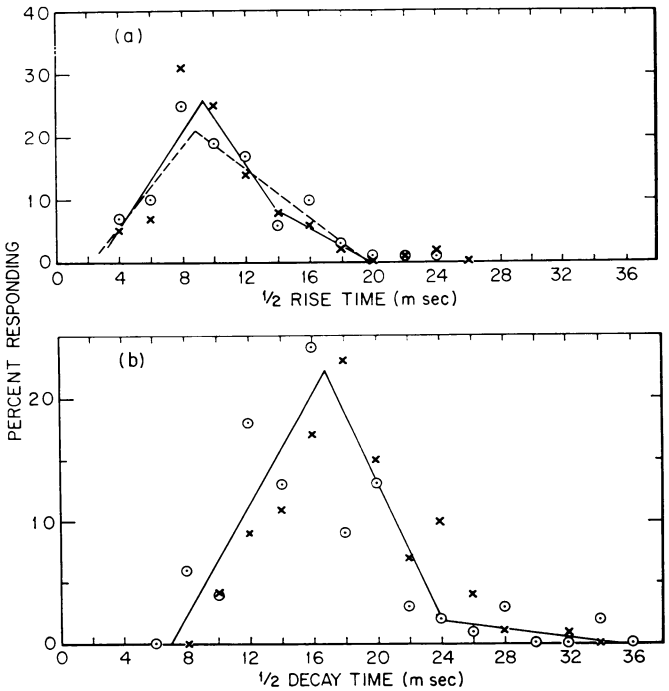

FIG. 4. Kinetics of the bioluminescent flash in *Gonyaulax polyedra*. Shown are (a) distribution of one-half rise times, (b) distribution of one-half decay times. Day phase flashes (○), night phase flashes (×).

Table I. *Characteristics of Bioluminescent Flashes of Gonyaulax polyedra at Different Phases*

Data are from four experiments with the high speed camera at two flow rates.

	Date	Intensity of Individual Average Flashes, Light Units	No. of Flashes Measured	Duration of Record
				sec
Flow rate: 6.3 ml/min				
Night phase	2/25/71	5.7	182	37
Day phase		1.8	77	
Ratio: night phase/day phase		3.2	2.4	
Night phase	5/28/71	55.4	116	61
Day phase		10.8	52	
Ratio: night phase/day phase		5.1	2.2	
Night phase	8/19/71	15.3	44	152
Day phase		3.3	16	
Ratio: night phase/day phase		4.6	3.6	
Average ratio: night phase/day phase		4.3 ± 0.6	2.7 ± 0.4	
Flow rate: 11.0 ml/min				
Night phase	2/25/71	15.4	262	37
Day phase		2.4	108	
Ratio: night phase/day phase		6.4	2.4	
Night phase	5/28/71	57	133	61
Day phase		11.6	115	
Ratio: night phase/day phase		4.9	1.2	
Night phase	8/18/71	57	54	61
Day phase		3.7	36	
Ratio: night phase/day phase		15.4	1.5	
Night phase	8/19/71	39.4	107	152
Day phase		2.7	63	
Ratio: night phase/day phase		14.6	1.7	
Average ratio: night phase/day phase		10.3 ± 2.7	1.7 ± 0.2	

effect of gravity on the flow through the coil. The relationship between pressure and flow rate was determined experimentally. Luminescence was observed to take place principally within the coil as the cells passed through the capillary. Since the cell suspension was continuously renewed from the reservoir, luminescence was constant with any given flow rate.

Using the measured values for the maximum flow rate and the dimensions of the capillary, the Reynolds number for the system can be calculated according to the equation:

$$R = pUd/\mu$$

where p = density of the fluid (1.025 g/cm³ for sea water), U = average velocity of flow (37.2 cm/sec), d = inside diameter of tube (0.086 cm), μ = viscosity of the fluid (0.0109 g/cm sec for sea water), R = Reynolds number. Since $R = 279$ and hence much less than 2000, the flow in the capillary is laminar at all flow rates. The fluid shear force can be calculated since flow is laminar, and will vary from zero at the center of the tube to a maximum at the edge. Calculations by Dr. Alex Charter give a value of 35 dynes/cm² for the maximum fluid shear force at the maximum flow rate in our apparatus.

Luminescence was recorded with a photomultiplier photometer described previously (11). Minor modifications were made to allow the photomultiplier to face upward and to make the system light-tight. For recording, 100 ml of cell suspension was gently transferred to the reservoir, which was then closed, clamped, and darkened. Recording was begun immediately. Luminescence was recorded in arbitrary light units.

Two methods of recording the luminescence from the coil were employed. In the first method, the signal from the photomultiplier was recorded as intensity in relative units on a Beckman Linear Potentiometric Recorder No. 93500. The second method of recording, which allowed the visualization of individual flashes, employed a high speed kymograph camera (Model C4G, Grass Instrument Co.). The signal from the photomultiplier actuated a Tektronix Model 502 dual beam oscilloscope, moving the beam in one plane while the paper film moved at right angles to this plane. The paper film (Kodak photographic paper Kind 1832, Specification 183) moved through the camera at a rate of 25 cm per sec. Single flashes were recorded with good detail by this method, provided the cell suspension was sufficiently dilute to prevent the superposition of flashes. Recordings were made in this way from both day and night phase cells from the same cell suspension. Since day-phase cells were in darkness as mentioned above, light inhibition of luminescence was not a complicating factor.

RESULTS AND DISCUSSION

When a cell suspension of *Gonyaulax* is passed through the capillary, light is emitted, provided that the flow rate is above a threshold value (Fig. 2). The flow rate which just triggers luminescence is lower for cells in the night phase than for day-phase cells (0.8 ml per min as compared to 2.6 ml per min), indicating that night phase cells are more sensitive to mechanical stimulation than are day-phase cells. No such difference in threshold with phase is noted in cells chemically stimulated to luminesce by the addition of different concentrations of acetic acid (10).

The amount of luminescence is directly proportional to flow rate over a considerable range of flow rates (Fig. 2) during both day and night. Brighter luminescence at faster flow rates is accounted for in part by the greater number of cells passing through the coil in a given time. At flow rates just higher than threshold, however, luminescence increases more rapidly than is accounted for by the increased flow rate alone. The range of flow rates which show this phenomenon are about the same during the day phase as at night (threshold to 7 ml per min). The existence of this region in the curves for luminescence as a function of flow rate indicates either that stimulation is not an all or none phenomenon or that cells or luminescent sources within a single cell may be heterogeneous with regard to sensitivity to stimulation. The greatest phase-dependent difference, however, is in the amount of light emitted. Night-phase luminescence is about 14 times brighter than that from cells in the day phase over the linear range of response to flow rate.

Since the method of recording used in the experiments of Figure 2 does not permit the observation of individual flashes, it was not possible to distinguish whether the greater amount of light recorded from populations of night-phase cells was the result of brighter flashes, longer flashes, a larger number of flashes, or some combination of these factors. To make this distinction, it was desirable to resolve individual flashes and examine their kinetics and number from the same population during the day and night phases. To accomplish this, we employed the high speed camera technique which allowed us to record the time course of day- and night-phase flashes (Fig. 3). The examination of many such flashes did not reveal any significant difference between day- and night-phase cell flashes with respect to the one-half rise time (mean 9–9.5 msec, Fig. 4a) or one-half decay time (mean 16–18 msec, Fig. 4b). The kinetics appear essentially similar in both phases (Fig. 3). This is in agreement with the conclusions of Biggley *et al.* (1) for the stimulated luminescence of a population of *Gonyaulax polyedra* under similar conditions. The brightness of individual flashes was compared at different phases (Table I). In the linear range of stimulation for both phases (6.25 ml per min), night-phase flashes are three to five times brighter at the peak intensity of the flash than are day-phase flashes. Also, 2.7 times more flashes are recorded in a given time interval during the night phase than during the day phase (Table I). In combination, the increased flash intensity and greater number of cells which flash account for the greater light emission during the night phase recorded for populations in the experiments of Figure 2.

Cells of *Gonyaulax* show a rhythm in sensitivity to stimulation, manifest both in the lower threshold, and the larger number of flashes at night compared to the day phase in darkness. The increased brightness of night-phase flashes reflects a biochemical rhythm. The rhythm in sensitivity constitutes a second component contributing to the rhythm in luminescence in *Gonyaulax*.

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