Reference: *Biol. Bull.* **162:** 423–448. (June, 1982)

DISTRIBUTION OF SUBCELLULAR BIOLUMINESCENT SOURCES IN A DINOFLAGELLATE, *PYROCYSTIS FUSIFORMIS*

EDITH A. WIDDER AND JAMES F. CASE

Department of Biological Sciences, University of California, Santa Barbara, California 93106

Abstract

Pyrocystis fusiformis exhibits rhythmic changes in the distribution and mechanical excitability of subcellular bioluminescent sources. Bioluminescence in night-phase cells can be stimulated either mechanically or by low pH and originates from microsources found throughout the cytoplasmic layer surrounding the large central vacuole. Microsources are weakly fluorescent and probably correspond to 0.5 μm or smaller cytoplasmic inclusions. With the onset of day phase, bioluminescence becomes mechanically inexcitable but responds to acid stimulation. Microsources disappear from the cell periphery during early day phase and all luminescence originates from the perinuclear region. In late day phase, bioluminescence originates both from the perinuclear region and from microsources in the periphery. However, luminescence remains mechanically inexcitable until the onset of night phase. Cells maintained in darkness exhibit the same rhythmic changes in mechanical excitability and development and disappearance of the perinuclear luminescence, except that microsources do not disappear from the periphery of early day-phase cells without a light induction period. Mechanisms which might underlie the rhythmic changes in bioluminescence distribution and mechanical excitability are proposed.

Introduction

Identification of particulate light sources (microsources) in bioluminescent cells is needed to understand mechanisms and regulation of light emission. Microsources have been tentatively identified in a number of bioluminescent cells (Sweeney, 1980), but confirmation at the ultrastructural level is lacking except in the firefly (Hanson *et al.*, 1969; Case and Strause, 1978) and the scaleworm (Bassot and Bilbaut, 1977). No dinoflagellate microsource has been positively identified. The phagotrophic dinoflagellate, *Noctiluca miliaris* has figured heavily in such studies because its individual microsource luminescence is readily seen by image intensifier microscopy (Eckert and Reynolds, 1967). However, correlation of *Noctiluca* microsources, as seen by image intensification, with subcellular structures, as identified by other techniques, has not proceeded beyond their correlation with similarly distributed, 1.5 μm or smaller, fluorescent particles in the peripheral cytoplasm (Eckert and Reynolds, 1967).

Unlike *Noctiluca*, which has no daily cycle of luminescence capacity, *Pyrocystis fusiformis* exhibits a circadian oscillation in stimulable bioluminescence (Sweeney, 1981). Night-phase cells luminesce brilliantly upon either mechanical or acid stimulation. Each mechanical stimulus elicits a single flash, the summation of all the

Received 11 January 1982; accepted 24 March 1982.

Abbreviations: CT, circadian time; d, largest cell diameter; DD, 24 h dark cycle; FF, first flash; IIT, image intensifier; l, length; LD, light-dark cycle; PMT, photomultiplier tube; SF, subsequent flash; SRU, stimulus recording unit; TMSL, total mechanically stimulated luminescence.

microsource microflashes. During a first flash (FF) the microsources flash synchronously, while they flash asynchronously during subsequent flashes (SFs) (Widder and Case, 1982). Total light output from acid-stimulated night-phase cells is a prolonged glow representing the summed activity of asynchronously flashing microsources. Day-phase cells are only weakly luminescent upon acid stimulation and are essentially unresponsive to mechanical stimuli (Sweeney, 1981; Widder and Case, 1981). Since our preliminary image intensifier studies revealed that the daily cycle of luminescence capability was associated with the appearance and disappearance, or movement, of microsources, we concluded that a detailed study of this phenomenon might be a step towards identification of microsources. In this paper, the properties of microsources as seen by image intensifier microscopy are described.

Materials and Methods

Organisms

Cultures of *Pyrocystis fusiformis* Murray (No. 5M6, UCSB Algal Culture Collection), and *P. noctiluca* Murray were isolated by Dr. B. M. Sweeney. Unialgal cultures were grown at 20°C under cool white fluorescent lamps at about 500 $\mu W \cdot cm^{-2}$ on a 12:12 LD cycle. The culture medium consisted of sterilized, filtered seawater plus soil extract and "f/2" nutrient (Guillard and Ryther, 1962) minus silicate.

Cells for image intensification microscopy were loaded into glass holding tubes (18 cm long × 2 mm I.D., tapering to 1 mm I.D.) connected by polyethylene tubing to a 1 cc micrometer-controlled syringe (Fig. 1). This system permitted delivery of single cells into the experimental chamber without mechanical stimulation of their bioluminescence. Cells were loaded into the holding tubes during their inexcitable day phase and then returned to the culture chamber and maintained under the standard light and temperature conditions until needed. For cells maintained in darkness (DD), the holding tubes were placed in light-tight containers in the same culture chamber. The effects of light exposure on DD cells were tested by exposing the cells in the holding tubes to the incubator lights (500 $\mu W \cdot cm^{-2}$) for 5, 30 or 60 min beginning at circadian time (CT) 0000 (daybreak) or CT 0200.

Recording methods

Luminescence of a single cell was imaged with an EMI 2001 four stage image intensifier system capable of a radiant power gain of 10^6 (Fig. 1). The cell was held by a glass suction pipet (I.D 80 μm) against the cover slip bottom of a 2 cc Plexiglas chamber filled with filtered seawater and attached to the stage of a Zeiss IM 35 inverted microscope equipped with Plan-Neoflaur 25/0.8 and Planapochromat 100/1.3 oil immersion and 4/0.14 objectives. Images were focused on the image intensifier tube (IIT) photocathode via a Zeiss photochanger (magnification factor 1.6×) and a 6.3× or 16× ocular.

The IM 35 microscope was also equipped for Nomarski interference contrast microscopy with a Planachromat 100/1.25 objective. Nomarski micrographs were taken of a portion of the cell surface (65 μm × 95 μm) using the microscope 35 mm camera for later comparison with bioluminescence and fluorescence recordings made of the same field with the aid of the IIT. Fluorescence excitation was achieved with a 100 W mercury lamp and a Zeiss (48 77 03) filter/reflector combination for reflected violet-selective excitation. This filter set provides narrow band exci-

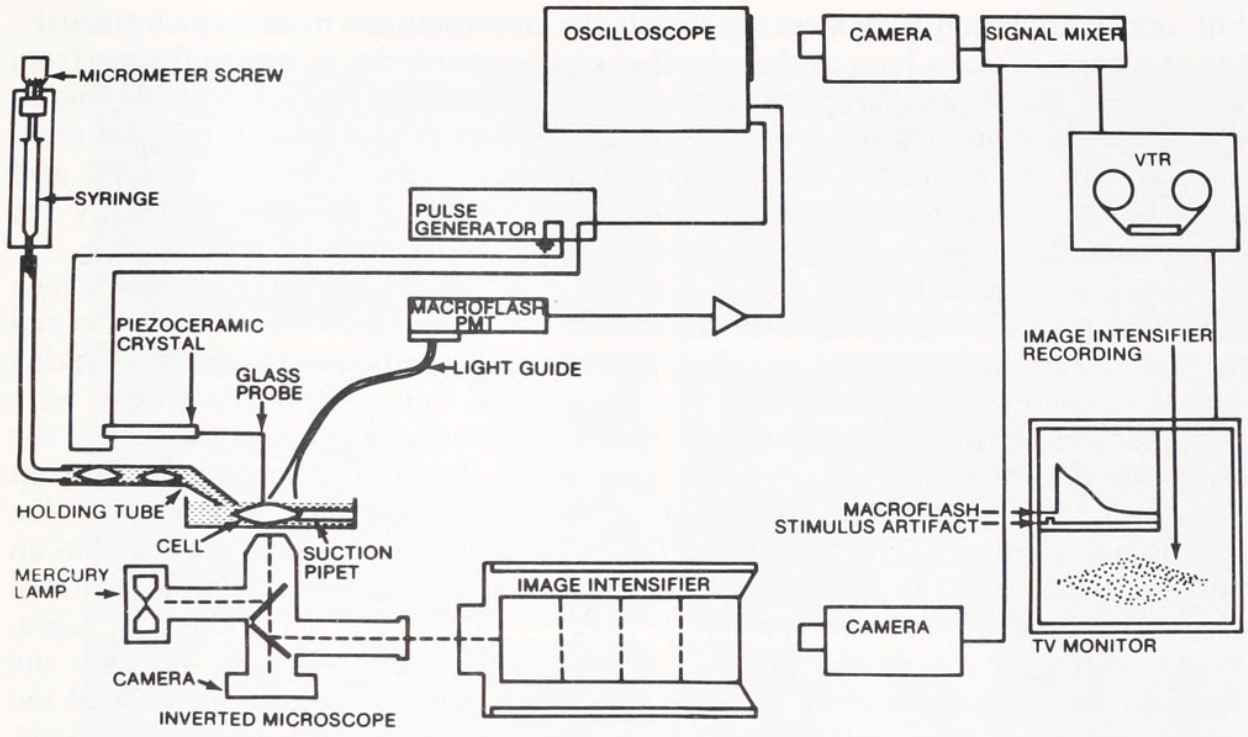

FIGURE 1. Experimental apparatus used to localize subcellular bioluminescent and fluorescent sources. A single cell from the holding tube was directed to the suction pipet without stimulating it. Bioluminescence resulting from mechanical stimuli delivered via the stimulator-controlled piezoceramic crystal or by the addition of acid to the seawater bath was recorded by a side window photomultiplier tube (PMT) via a light guide situated in the bath. PMT output was displayed on a storage oscilloscope and the display was recorded on video tape. The image of the cell was focused on the photocathode of the image intensifier tube (IIT) via the inverted microscope. Video recordings of the IIT output phosphor provided spatial and temporal records of subcellular bioluminescent activity which could be simultaneously displayed with the oscilloscope recordings of total light output. Photomicrographs of whole cells (as for Fig. 2) and Nomarski micrographs of cytoplasmic features were made with the internal camera before and/or after IIT recordings of bioluminescence and fluorescence.

tation at the 404.7 nm mercury line and also includes an FT 420 chromatic beam splitter with the associated barrier filter replaced by an interference filter (BP 520–560) to eliminate the intense red chlorophyll fluorescence.

Mechanical excitation was provided by a manipulator-held glass probe (tip diameter 50 μm) driven by a piezoceramic bender (Gulton Industries) controlled by a Grass S44 pulse generator. Acid stimulation of bioluminescence was achieved either by local micropipet application of 0.1 *M* acetic acid to the cell surface, or by addition of 0.05 cc of 4 *M* acetic acid or 23 *M* or 6 *M* formic acid to the 1.5 cc seawater bath.

A fiber-optic light guide built into the chamber wall led to an EMI 9781A photomultiplier tube (PMT) to register total light output on a storage oscilloscope. The image intensifier output was recorded with a video camera equipped with a Newvicon® tube and an f 0.95 lens. A second video camera recorded a video frame counter and the oscillographic display of the PMT recording of luminescent activity. A composite image of the two video recordings was formed by a Shintron Model 366 Special Effects Generator and recorded on a Sony VO-2610 videocassette recorder. A detailed description of the apparatus is presented elsewhere (Widder and Case, 1982). The time resolution of the video records was 16.7 ms/field.

To compare light micrographs with the video recordings of the same field, Polaroid micrographs were made from a video screen during real time playback.

For acid stimulation sequences, in which the microsources flash asynchronously, the exposures were as long as the stimulus sequence in order to record flashes from as many of the microsources as possible. The Polaroid photographs of the bioluminescence and fluorescence were then enlarged to 8 × 10 inch prints for comparison with the Nomarski photographs of the same field. A hemocytometer grid was photographed by both methods to correct for distortion introduced by the image intensifier and to adjust the enlargements to the same magnification.

Microsource counts were made from the video recordings using a video screen with a magnification of 9000 at the highest magnification used (100× objective and 6.3× ocular). Spots identified as microsources were traced on a transparent overlay as the video record was advanced one frame at a time. Night-phase cells were stimulated either mechanically or with acid and the sequence was studied until the luminescence was exhausted or the cell had moved. Study of prolonged luminescent activity made it possible to distinguish single from clumped microsources as the microsources flashed asynchronously. The number of microsources seen within an area (2500 μm^2 or 1849 μm^2) at the cell surface was related to the total cell surface area calculated as $0.5 \cdot \pi \cdot d \cdot [0.596 \cdot l + (d^2 + 0.493 \cdot l^2)^{0.5}]$ (Widder & Case, 1981). This formula fits the shape of stage I and II cells. In stage III cells the plasma membrane pulls away from the cell wall to form a constriction around the center (nuclear) region of the cell which complicates the surface area calculations. Therefore we only used stage III cells in which the central constriction was minimal.

Mechanical excitability of LD and DD cells was tested with the stimulus recording unit (SRU) described in Widder and Case (1981). Briefly, single stage II cells were loaded into fluoroplastic tubes during their mechanically inexcitable day phase. The cell-containing tube was placed in fixed relation to a photomultiplier tube and suprathreshold mechanical stimuli of fixed strength were applied directly to the tube at a frequency of 0.33 pps with a stimulator-controlled solenoid. The cell was stimulated to exhaustion and the integrated light output of the first flash (FF), the total mechanically stimulated luminescence (TMSL), and the total number of flashes produced were recorded.

The cell cycle of *P. fusiformis*, originally described by Swift and Durbin (1971), takes about 5 days in our cultures. Stages in the life cycle, identified according to the morphological criteria of Widder and Case (1981), are illustrated in Figure 2. Note that "stage" refers to cell morphology and "phase" refers to time of day.

Results

Distribution of bioluminescence during the daily cycle

To determine the intracellular distribution of microsources in a normal light-dark environment, 192 cells from different stages in the life cycle were examined with the IIT through the 24 h cycle. The five morphological stages of the life cycle in night phase and early day phase are shown in Figure 2, along with the luminescence patterns in response to acid stimulation. Changes in the pattern of luminescent activity over the 24 h cycle were considered in 5 phases: 1) night 2) early day 3) late day 4) day to night transition ("dusk") 5) night to day transition ("dawn"). Results are summarized in Table I and are briefly discussed below.

Night-phase cells (CT 1400–2200)

Both mechanical and acid stimulation revealed generally uniform distribution of microsources in the peripheral cytoplasm (Fig. 2). The cytoplasm forms a thin

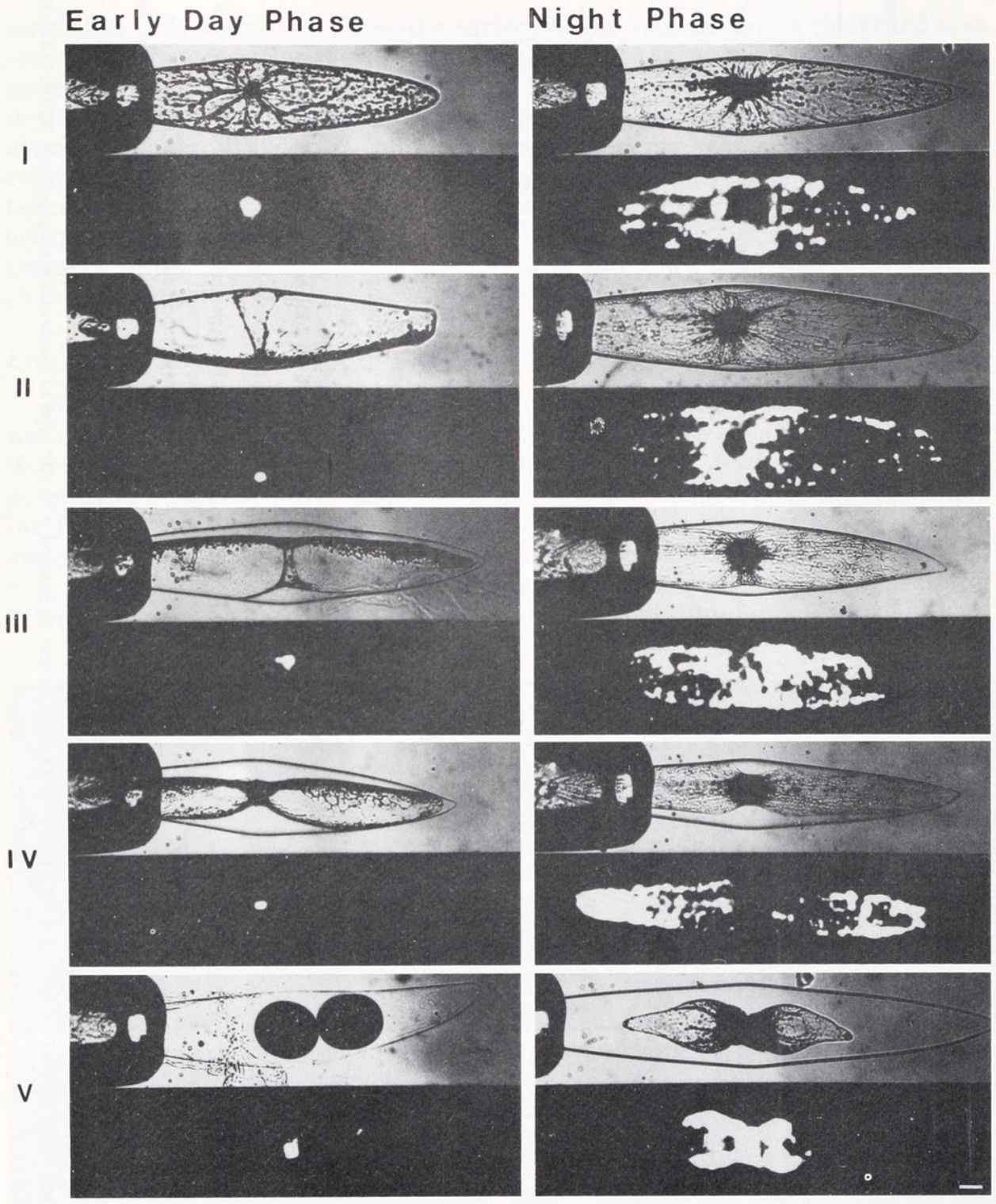

FIGURE 2. Distribution of subcellular luminescence in the five morphological stages (I–V) of cells in early day phase and night phase. Each of the 10 cells shown was positioned on the suction pipet, seen on the left, and photographed. The cell was then stimulated with acid and a video-IIT record of the resulting bioluminescence was made. Polaroid photographs of the video screen made during real-time play back are shown below each of the photomicrographs. In the night-phase stage I and II cells shown here, the nucleus was on the side of the cell closest to the objective and corresponds to the dark region seen in the IIT micrographs of bioluminescence. In the night-phase stage III cell, the nucleus was on the far side of the cell and no dark region was apparent. The night-phase stage V cell exhibits the morphology seen before aplanospore formation. For the two early day-phase aplanospores shown, the one on the left had its nucleus closest to the objective, while the nucleus of the right-hand aplanospore was on the far side of the cell, which accounts for its apparently dim perinuclear glow. Note that the chloroplasts which cover the surface of day phase cells migrate into a tight clump in the perinuclear region during night phase (Swift & Durbin, 1971; Sweeney, 1981). Scale, 50 μm.

TABLE I

Distribution of bioluminescence in cells maintained in normal LD cycle, stimulated by 4 M acetic acid.

Circadian time	N	Perinuclear luminescence	w/ss*	Cytoplasmic microsource luminescence
0000–0100 (Dawn)	12	12/12 Stage I & V—diffuse Stages II–IV—well defined	1/12	12/12 Stages I & V—evenly dispersed Stages II–IV—in bands and bridges
0100–0200	17	17/17 Stages I & V—diffuse Stages II–IV—well defined	3/17	10/17 Became rare, disappearing near end of hour Stages I & V—evenly dispersed Stages II–IV—in bands and bridges
0200–0600	40	40/40 Stages I & V—diffuse Stages II–IV—well defined	17/40	0/40
0600–0900	26	26/26 Well defined	8/26	21/26 Rare microsources around perinuclear zone; generally in cells w/o ss
0900–1000	9	9/9 Well defined but large	0/9	9/9 More, mostly around nuclear zone
1000–1100	11	11/11 Glow extended into bridges in 3 cells	0/11	11/11 More
1100–1200 (Dusk)	16	16/16 Glow spread into bridges and bands	0/16	11/16 Stage I—higher concentration around nucleus Stages II–IV—in bands and bridges
1200–1300	16	16/16 Diffuse	0/16	16/16 Higher concentration around nucleus. Otherwise evenly dispersed over cell surface
1300–1400	3	3/3 Very diffuse	0/3	3/16 Evenly dispersed
1400–2200	28	0/28		28/28 Evenly dispersed
2200–2300	7	0/7		7/7 Evenly dispersed w/mech stim w/acid more concentrated around center
2300–2400	7	6/7 Diffuse	0/7	7/7 Evenly dispersed except for one cell at end of hour with microsources concentrated in bands

* With microsource-like substructure.

Ratios in Tables I–III indicate what proportion of the cells tested (N) in a given time period responded with the type of luminescence designated by the column heading.

layer (less than 5 μm thick) over the surface of the vacuole with a thickened area around the nucleus (Sweeney, 1980; Sweeney, 1981). In stages II and III, two prominent cytoplasmic bridges extend from the perinuclear zone across the vacuole to the cytoplasm on the opposite side of the cell. Focusing through an acid-stimulated stage II cell (Fig. 3) showed microsources only within the thin layer of cytoplasm around the vacuole and in the cytoplasmic bridges. The cytoplasm between the nucleus and the cell wall was not luminescent (Figs. 2 and 3), possibly because the chloroplasts migrate into this area at night (Sweeney, 1981). Luminescence in night-phase cells appeared to originate only from the microsources, since no diffuse luminescence was seen at $10^6\times$ image intensifier gain.

Early day-phase cells (CT 0200–0600)

Microsources were not visible in the peripheral cytoplasm and all luminescence was confined to the perinuclear zone (Fig. 2). Cells were mechanically inexcitable throughout the day phase except rarely when one or two flashes occurred in the perinuclear zone (Fig. 4c). These were extremely dim (about 20,000 times less than the TMSL from a night-phase cell), brief (about 60 ms with rise time of about 10 ms, as compared with an FF which has a similar rise time but lasts more than 200 ms), and appeared as a diffuse emission around the nuclear region, lacking a clearly defined boundary. Unlike mechanical stimulation, acid stimulation consistently in-

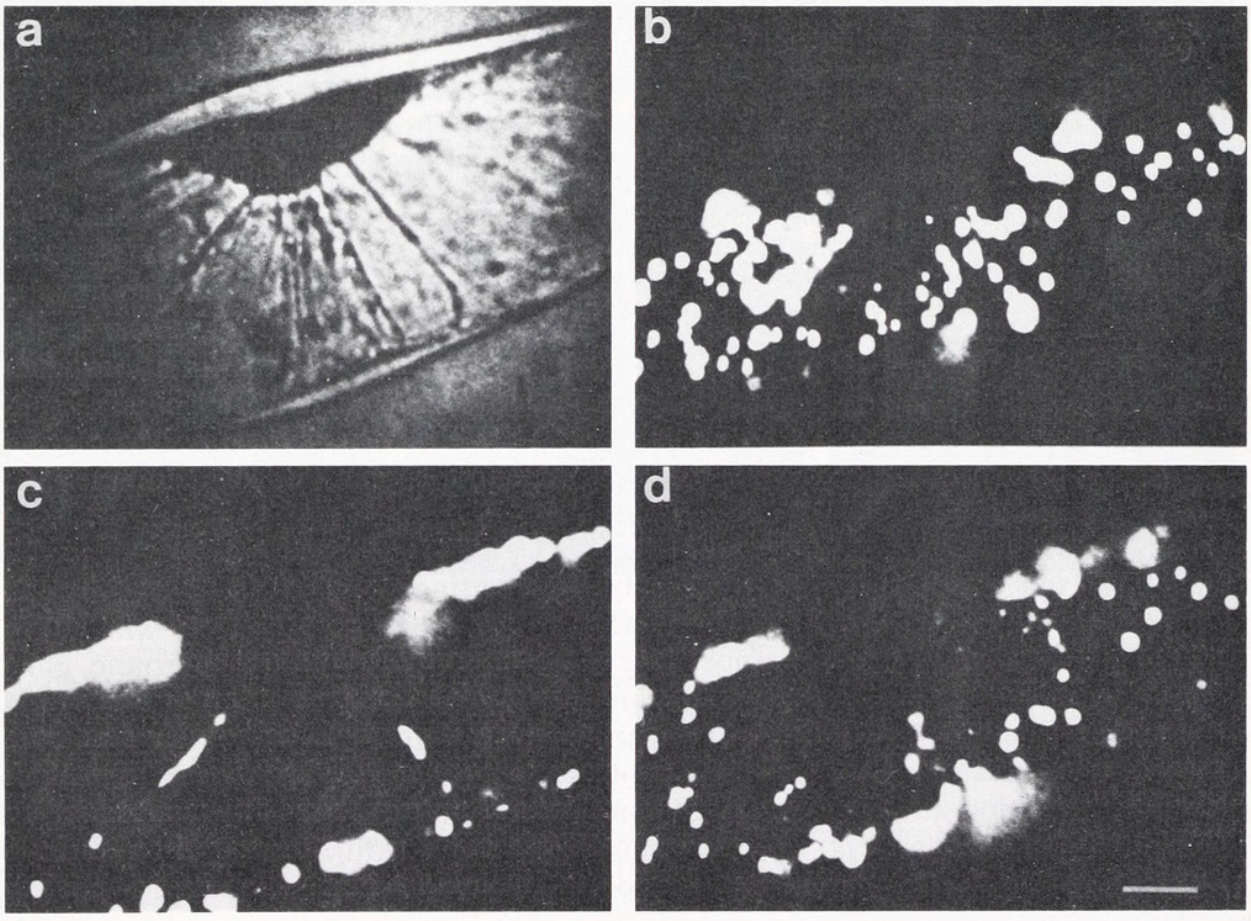

FIGURE 3. Three levels of focus in a stage II night-phase cell during acid stimulation. The cell was backlit (a) and photographed through the IIT prior to stimulation with acid. In (b) the focus is on the near side of the cell. Focusing to midpoint of the cell demonstrates the absence of microsources in the central vacuole except for those seen in the two cytoplasmic bridges which cross the vacuole (c). In (d) the focus is on the far side of the cell. Scale, 50 μm.

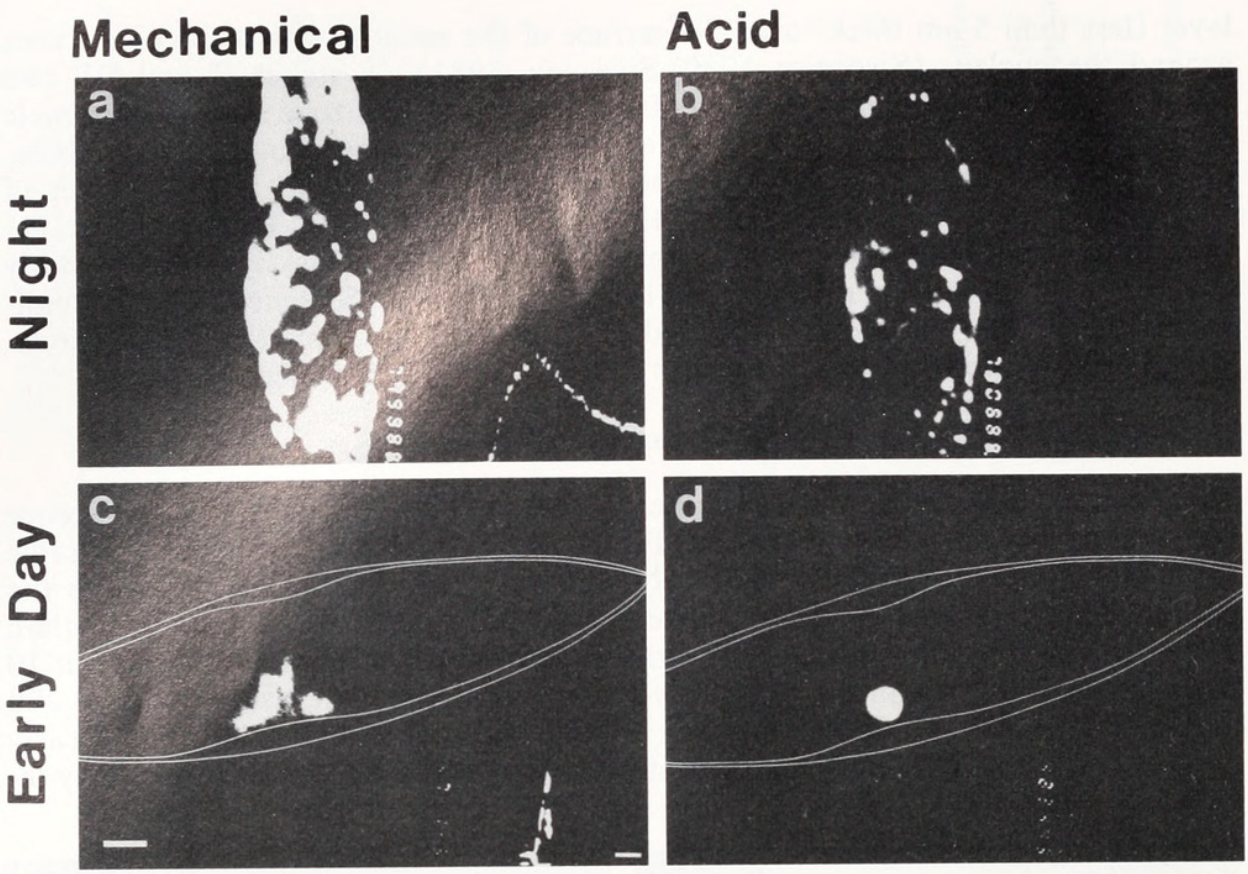

FIGURE 4. Mechanical and acid stimulation of a stage II night-phase (CT 1415) cell (a and b) and a stage III early day-phase (CT 0530) cell (c and d). The outline of the day-phase cell was traced in white from a photograph of the same backlit cell. The night-phase cell received 12 mechanical stimuli and responded with one FF and 11 SFs. The response shown (a) was the eleventh SF. It was immediately followed by acid stimulation (b), which appears dimmer because the luminescence was partially fatigued. The day-phase cell received 5 mechanical stimuli, but apparently responded only to the first stimulus (c). Acid stimulation (d) followed mechanical stimulation as in the night-phase cell. The PMT recordings of the mechanically stimulated flashes are shown at the bottom right of (a) and (c). The time bar in (c), 50 ms, applies to both oscillographic records. Scale, at lower left, 50 μm.

duced luminescence from day-phase cells. This was about 10 times dimmer than from night-phase cells (Sweeney, 1981), and appeared as a bright, clearly defined glowing spot near the nuclear zone. The perinuclear glow appeared either round, diameter about 30 μm (Figs. 5c and f), elliptical or saddle-shaped, about 60–90 × 40–70 μm (Figs. 5a and d). Depending on the orientation of the cell, the ellipse surrounded one or two dark areas probably representing the horseshoe-shaped, non-luminous nucleus. In the round form, the glow frequently exhibited an active, microsource-like substructure, appearing to "boil" (Figs. 5g–i). The round glow with substructure or the elliptical perinuclear glow appeared in all stages and frequently occurred sequentially in the same cell, with the elliptical glow preceding the appearance of substructure as in Figures 5a and c, or *vice versa*. At the onset of acid stimulation, individual sources could be seen that were easily distinguished from background glow (Fig. 5g) and their size and flash duration appeared equivalent to microsources seen in night-phase cells.

Late day-phase cells (CT 0600–1000)

Microsources appeared in the periphery with the greatest density around the perinuclear zone. As microsources appeared, fewer cells exhibited substructure in

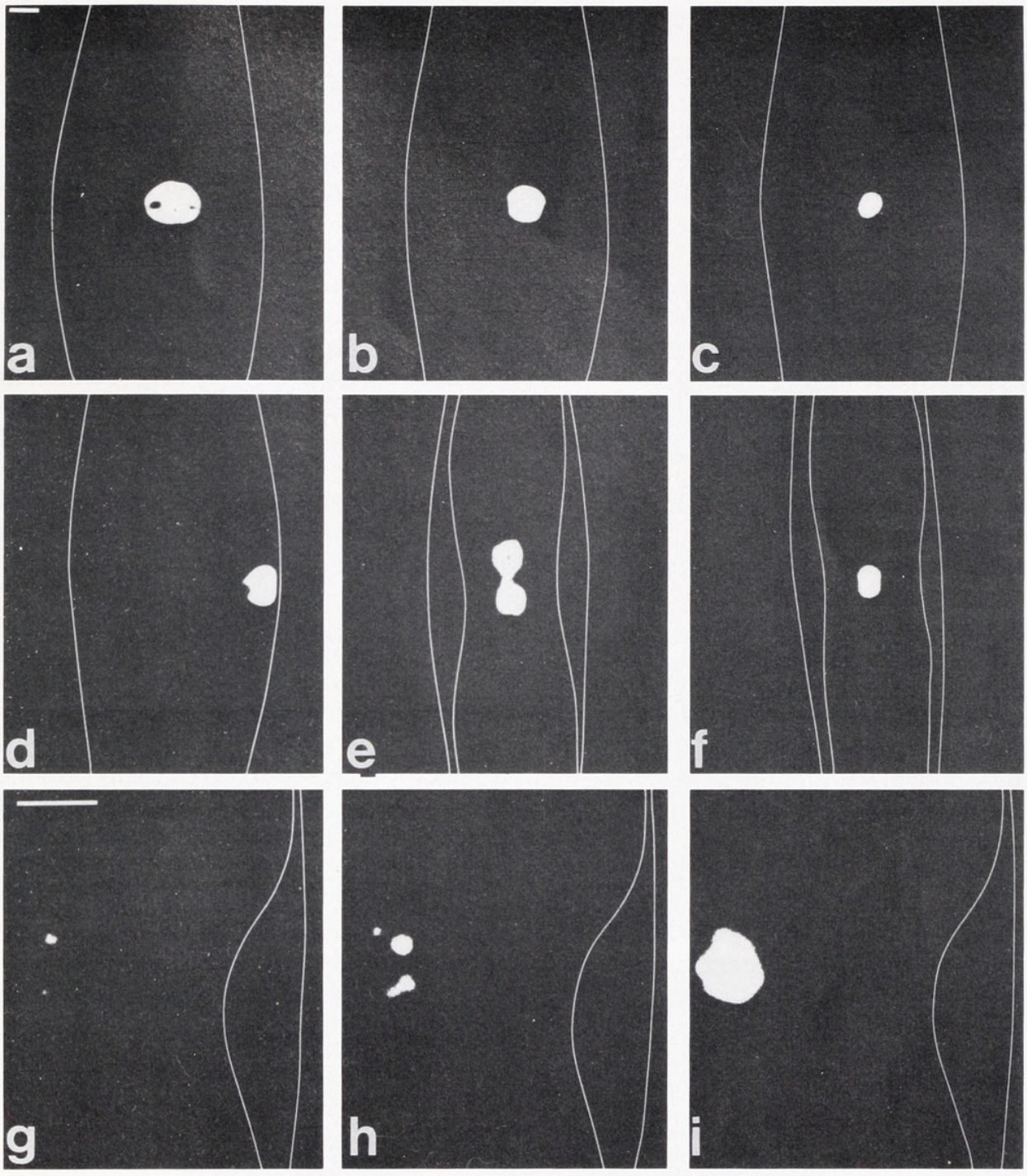

FIGURE 5. Forms of perinuclear glow observed in day-phase cells. White outlines are traced from backlit imaging of each cell prior to acid stimulation. (a–c) Transition from the elliptical perinuclear glow to the round perinuclear glow with substructure in an acid-stimulated stage II cell. Time between (a) and (b), about 45 sec; between (b) and (c), about 25 sec. When a cell was positioned with the nucleus to one side, only one dark area was seen in the elliptical perinuclear glow (d) compared to the 2 dark areas seen when the cell was positioned so the nucleus was in the center (a). The perinuclear glow in stage IV cells appeared as either a double (e) or single spot (f). (g–i) Development of the perinuclear glow in a stage III cell. Initially luminescence appeared as individual microflashes (g) and within 15 sec developed into round glow with substructure (i), which, in this case, persisted for more than 10 minutes. Scale, 30 μm. Upper scale bar applies to (a–f), lower to (g–i).

the perinuclear glow. The acid-stimulated microflashes had the same appearance as those from night-phase cells except that they occurred only briefly, while the nonstructured perinuclear glow persisted for 10 min or more.

Day-to-night transition phases (CT 1000–1400)

During the two hours (CT 1000–1200) before night phase the nonstructured central glow spread into the cytoplasmic bands and bridges (Fig. 6) and numerous microsources were visible. In stages II, III, and IV they were not evenly distributed over the cell surface as in night-phase cells, but were confined to the cytoplasmic bands. Microsources were also numerous in the cytoplasmic bridges crossing the large central vacuole from the perinuclear cytoplasm to the cytoplasmic band, running along the opposite side of the cell. Microsources of stage I cells covered the whole cell surface with the highest concentration around the nucleus. The microsources became mechanically excitable with the onset of night phase, and the perinuclear glow also appeared to be mechanically excitable. Occasionally, the glow appeared during an FF, an effect perhaps produced by the extremely bright emission of densely packed microsources. The glow was generally not seen in the SFs immediately following the FF. With repeated stimulation, however, a glow persisted around the perinuclear region for several seconds after stimulation ceased. This luminescence arose from densely packed microsources, as in the perinuclear glow with substructure, seen in early day-phase cells. The perinuclear glow was much more apparent with acid than with mechanical stimulation.

Night-to-day transition phases (CT 2300–0200)

From CT 1400 to CT 2300, luminescence from either mechanical or acid stimulation originated only from the evenly distributed microsources. Perinuclear glow became visible 1 h before sunrise (CT 2300) and was also occasionally seen with mechanical stimulation as in day-to-night transition-phase cells. As the cells became mechanically inexcitable during the first hour of light, acid stimulation revealed the microsources to be concentrated in the cytoplasmic bands and bridges of stages II, III, and IV and evenly distributed over the cell surface in stage I cells (Fig. 7). During the second hour of daylight (CT 0100–0200), the microsources became rare and dim and the perinuclear glow became brighter and well defined, except in stage I cells. In these, the microsources remained visible longer and the perinuclear glow assumed an astral appearance without well-defined boundaries. Since stage V cells (reproductive bodies) expand to form the stage I cells (Swift & Durbin, 1971), they might be expected to share many characteristics. Specifically, the perinuclear glow was diffuse, and the microsources remained visible over a longer period.

Effects of DD on microsource distribution

The rhythm of chloroplast migration and mechanically excitable bioluminescence in *Pyrocystis fusiformis* persists for more than 96 hours in darkness (Sweeney, 1981). In contrast, our image intensification analysis revealed that microsources did not disappear from the peripheral cytoplasm if the dark period continued through the ensuing subjective day. This was only apparent with acid stimulation, since the microsources were mechanically inexcitable. Although the microsources did not disappear from the periphery as in normal day-phase cells, the perinuclear glow still developed normally (Table II). Microsource distribution was not as in normal night-phase cells, but rather was identical to that assumed by normal LD cells during the first hour of daylight (CT 0000–0100) (Fig. 8). Acid stimulation revealed microsources in the cytoplasmic bridges and bands of stages II, III, and IV along with a nonstructured perinuclear glow. In stages I and V, the microsources

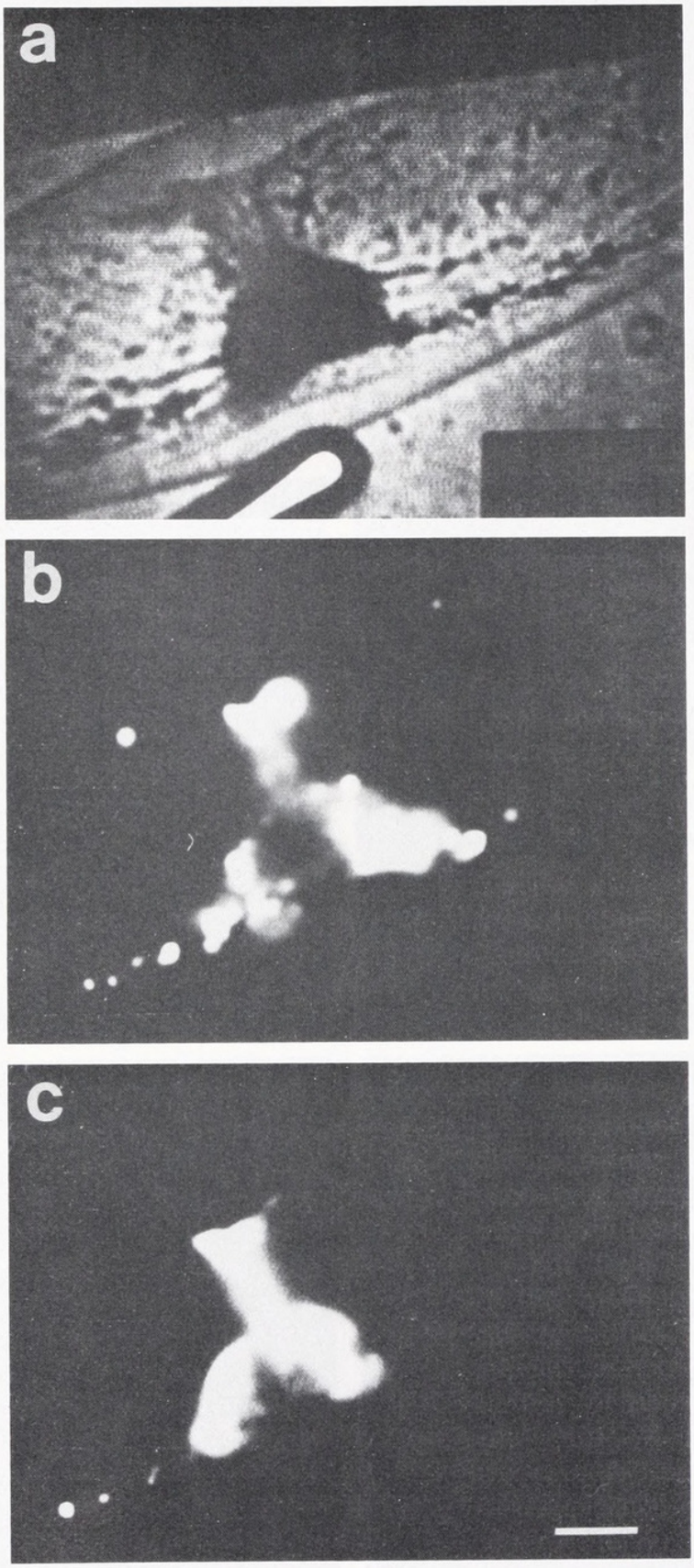

FIGURE 6. Light-to-dark transition phase, stage III cell at CT 1255. (a) Backlit, showing mechanical stimulator probe. (b) Fortieth SF. (c) Response to acid stimulation following 41 mechanical stimuli. Scale, 50 μm.

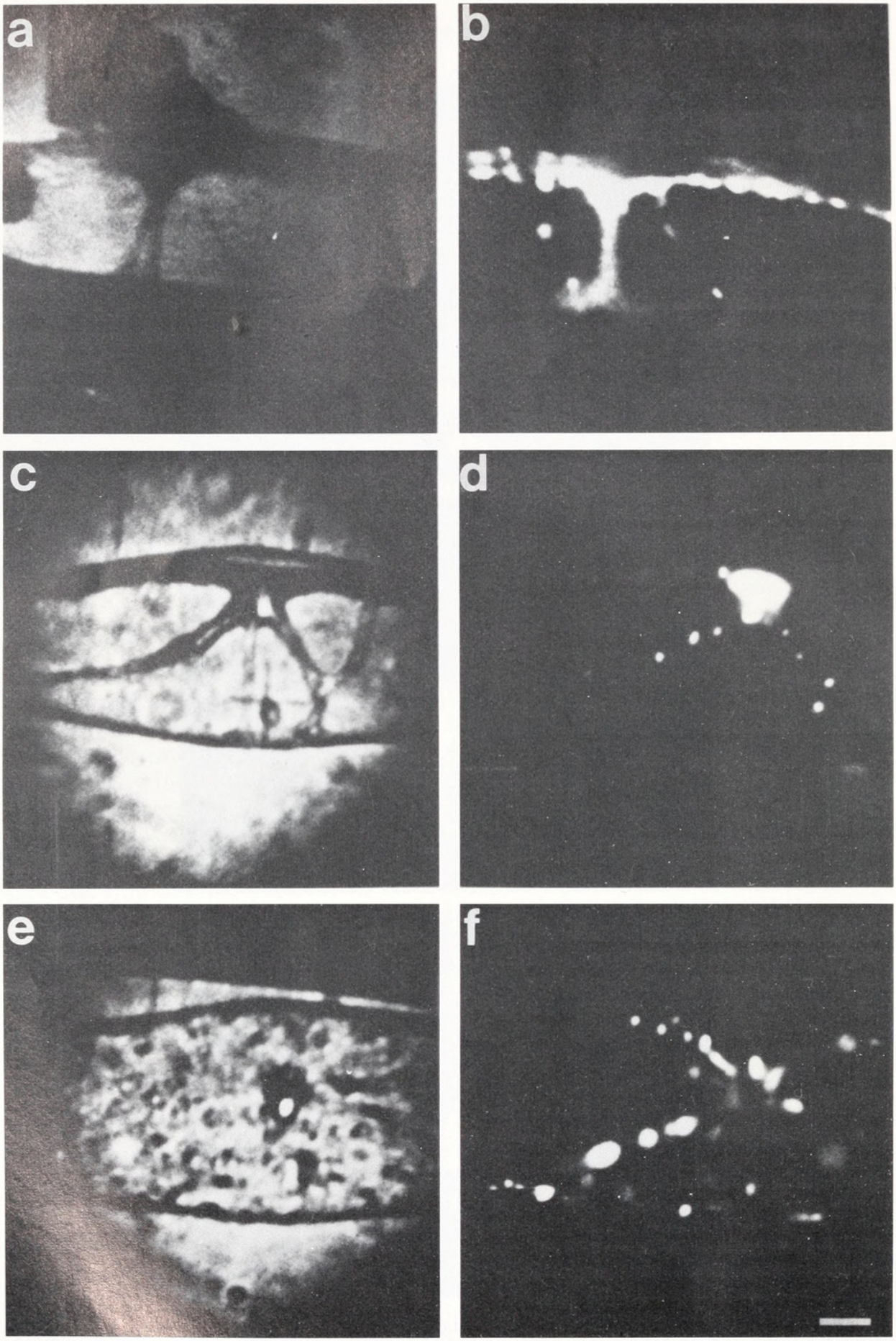

FIGURE 7. Three dark-to-light transition phase cells. Acid stimulated (a) and backlit view (b) of stage II cell at CT 2355. Acid stimulated (c) and backlit view (d) of another stage II cell at CT 0040. Acid stimulated (e) and backlit view (f) of a stage I cell at CT 0110. Microsources in the stage II cells were confined to the cytoplasmic bands and bridges, but were more generally distributed in the stage I cell. Scale, 50 μm.

TABLE II

Distribution of bioluminescence in cells maintained in DD, stimulated by 4 M acetic acid.

Circadian time	N	Perinuclear luminescence		w/ss*	Cytoplasmic microsource luminescence	
0000–0100	15	13/15	Spread into bands and bridges in 5 cells	4/15	15/15	Stage I—evenly dispersed Stages II–IV—in bands and bridges
0100–0700	46	36/46	Stages I & V—diffuse Stages II–IV—well defined	0/46	46/46	Stages I & V—evenly dispersed Stages II–IV—in bands and bridges
1100–1300	2	2/2	Stage I—very diffuse Stage IV—spread into bands and bridges	0/2	2/2	Stage I—evenly dispersed Stage IV—in bands
1500–1800	21	0/21			21/21	Evenly dispersed

* With microsource-like substructure.

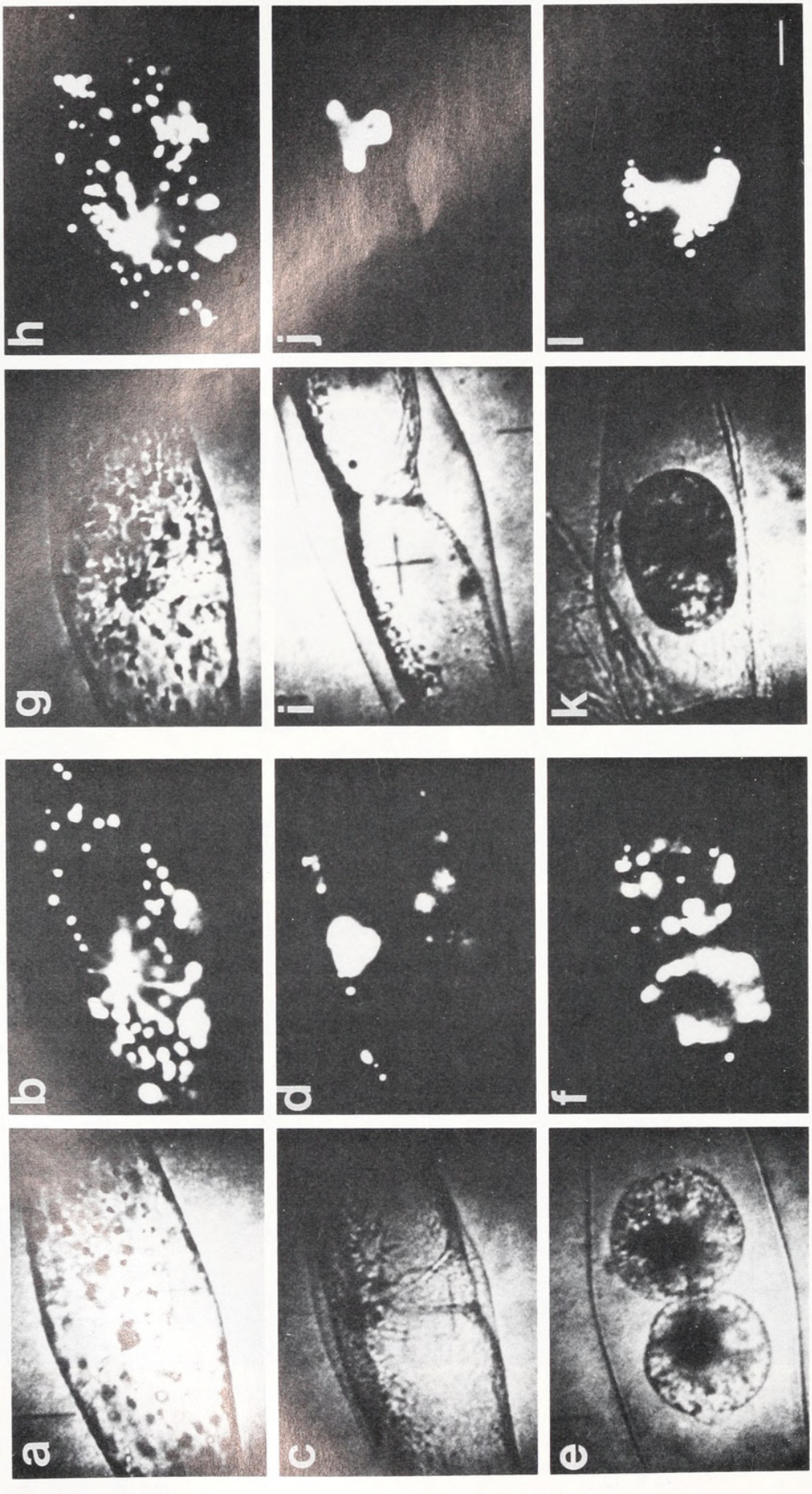

FIGURE 8. Distribution of subcellular luminescence in DD cells (a–f) and cells that received 1 h of light exposure from CT 0000 to CT 0100 (g–l). In each group the backlit cell is shown on the left and its response to acid stimulation is shown on the right. (b) and (h) are typical of the distribution seen in stage I cells under these light regimens; (d) and (j) are typical of the distribution seen in stages II, III, and IV. (e) and (f), a double aplanospore (stage V) and (k) and (l) a single aplanospore. Cells were tested between CT 0300 and CT 0500. Scale, 50 μm.

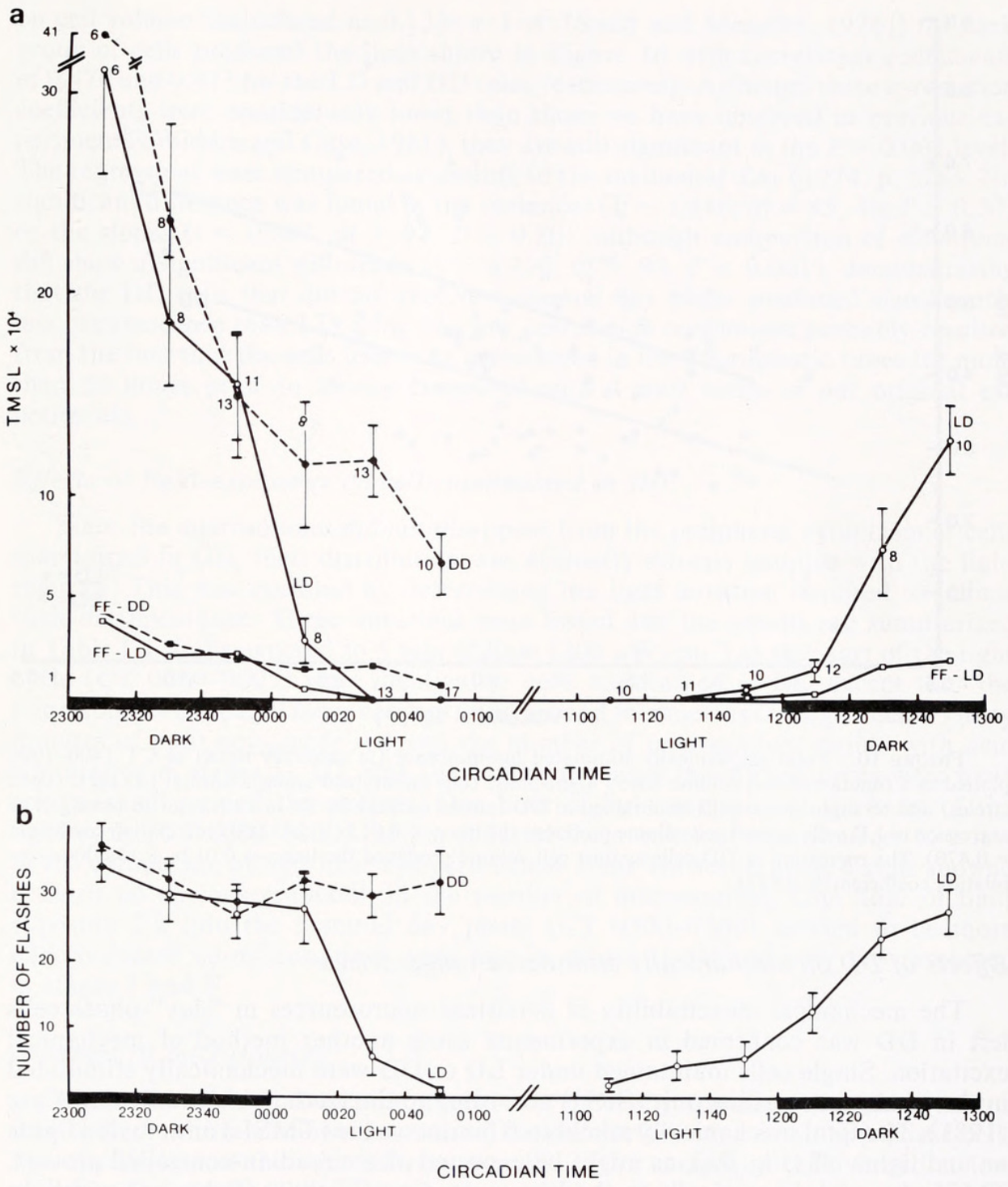

FIGURE 9. TMSL (a) (in arbitrary units) and total number of flashes (b) from transition-phase cells that were mechanically stimulated in the SRU. Dotted lines connect means of DD cells (closed circles) and solid lines connect means of LD cells (open circles). Cells were tested during 6 different dark-to-light and light-to-dark transitions and then means were taken of all cells tested within each 20 min interval. Bars represent standard error. Note that no correction was made for variations in cell size. N values are shown with each mean in (a) and also apply to (b).

remained evenly distributed in the peripheral cytoplasm and the perinuclear glow was diffuse and not located within a well-defined boundary, as in the other stages. During the following night phase of cells left in darkness, the cells became mechanically excitable again, the microsources were again evenly dispersed in the peripheral cytoplasm, and the perinuclear glow was no longer visible.

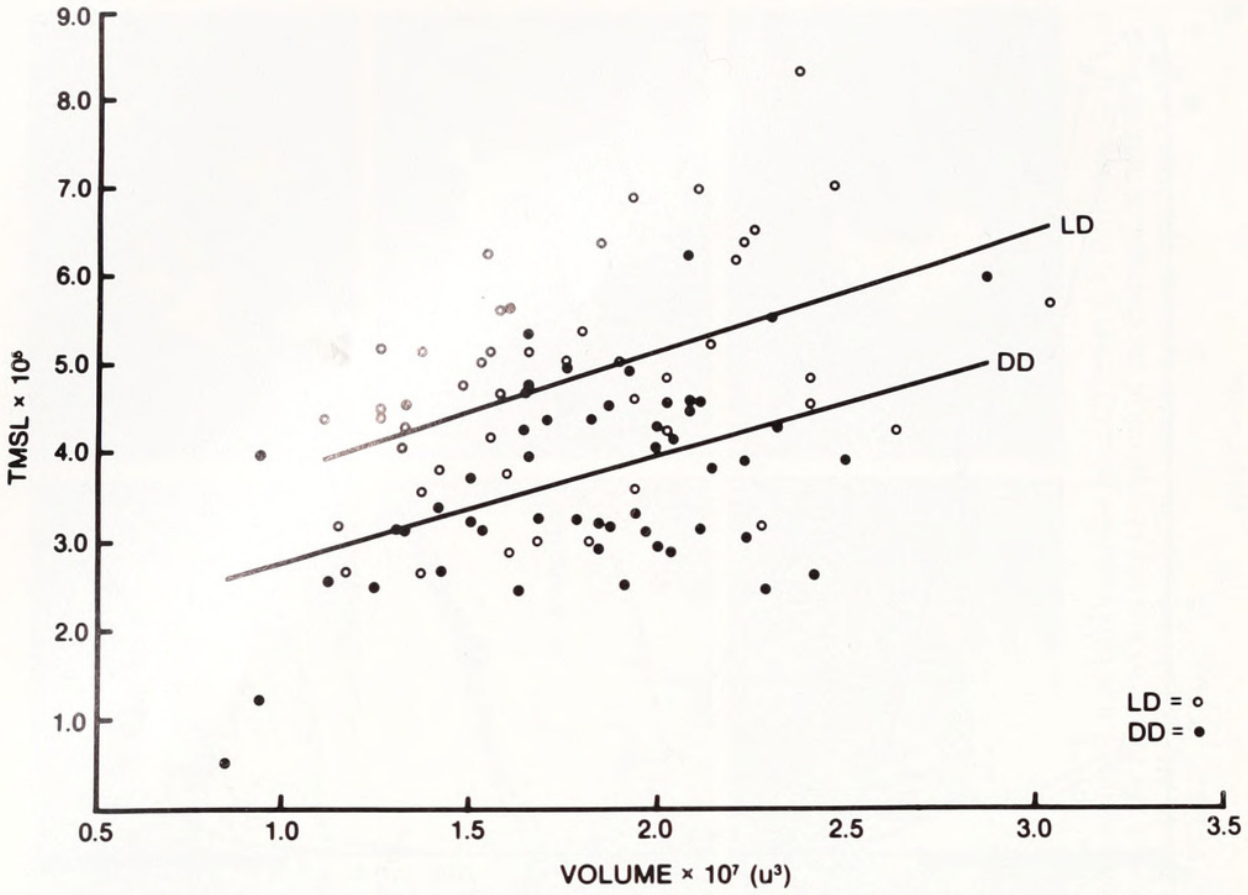

FIGURE 10. Total mechanically stimulated luminescence (in arbitrary units) at CT 1400–1900 plotted as a function of cell volume for 55 night-phase cells maintained under a normal LD cycle (open circles) and 63 night-phase cells maintained in DD (closed circles) for 28 to 33 h prior to testing. The regression of LD cells against cell volume produced the line $y = 0.013x + 245{,}000$ (correlation coefficient = 0.470). The regression of DD cells against cell volume produced the line $y = 0.012x + 159{,}000$ (correlation coefficient = 0.427).

Effects of DD on mechanically stimulated luminescence

The mechanical inexcitability of persistent microsources in "day"-phase cells left in DD was confirmed in experiments using another method of mechanical excitation. Single cells maintained under LD or DD were mechanically stimulated in the stimulus recording unit (SRU) according to the method of Widder and Case (1981). The total mechanically stimulated luminescence (TMSL) anticipated lights on and lights off (Fig. 9a), as might be expected of a circadian-controlled process. TMSL dropped dramatically in the hour preceding CT 0000 (lights on) and light output ceased less than one hour later. TMSL dropped less dramatically after CT 0000 for cells in DD, but no luminescence was recorded from mechanically stimulated LD or DD cells that were tested between CT 0500 and 0600. Interestingly, the number of flashes a cell could produce did not decrease in anticipation of dawn as did the TMSL. Also, while the TMSL of DD cells decreased in the hour following CT 0000, the number of flashes remained constant (Fig. 9b).

Mechanical excitability of day-to-night transition phase cells was only tested in LD cells which were loaded less than 3 h before testing. DD cells would have had to be maintained in the tubes more than 24 h which greatly increased the variability of the response. However, we did measure TMSL between CT 1400 and CT 1900 for 55 LD cells and 63 DD cells that were maintained in the fluoroplastic tubes between 28 to 33 hours prior to testing (Fig. 10). The regressions of TMSL

on cell volume (calculated as $0.133 \cdot \pi \cdot l \cdot d^2$ [Swift and Meunier, 1976]) for each group of cells produced the lines shown in Figure 10 with correlation coefficients of 0.470 and 0.427 for the LD and DD cells, respectively. Although these correlation coefficients were considerably lower than those we have observed in previous experiments (Widder and Case, 1981), they are still significant at the $P = 0.001$ level. The regressions were compared according to the method of Zar (1974, p. 228). No significant difference was found in the variances ($F = 1.216$, df = 45, 49, $P > 0.50$) or the slopes ($t = 0.297$, df = 92, $P > 0.50$), although comparison of elevations did show a significant difference ($t = 4.236$, df = 93, $P < 0.001$), demonstrating that the DD cells that did not receive a normal day phase produced significantly less luminescence than LD cells. The low correlation coefficients probably resulted from the fact that the cells had to be maintained in the fluoroplastic tubes for more than 24 hours prior to testing compared with 4 to 9 hours in our original experiments.

Effects of light exposures on cells maintained in DD

Since the microsources did not disappear from the peripheral cytoplasm of cells maintained in DD, their distribution was evidently directly coupled with the light regimen. This was explored by determining the light duration required to induce their disappearance. Three durations were tested and the results are summarized in Table III. Cells exposed to 5 min of light (500 $\mu W/cm^2$) at the start of the light cycle (CT 0000–0005) were identical to cells maintained in DD except that the microsources appeared more concentrated around the nucleus in stage I cells. Thirty minutes of light noticeably reduced the number of microsources visible with acid stimulation in stages II, III, and IV, and in all stages the microsources were most concentrated around the nucleus. One hour of light exposure at the start of the light cycle (CT 0000–0100) further reduced the number of microsources to a few in the cytoplasm, mostly near the perinuclear zone. However, stage I cells showed little or no obvious reduction in the number of microsources. One hour of light exposure 2 h into the nominal day phase (CT 0200–0300) seemed to be more effective since no microsources were seen in stages II–IV and very few remained in stages I and V.

Numbers of microsources

Estimates of microsource numbers were made by counting the microsources seen to flash within a known fraction of the cell surface area of a night-phase cell. The distribution of the microsources over the entire surface of cells viewed at low magnification showed no obvious regions of high or low concentration except for the small dark area near the perinuclear zone. At the high magnification used to count microsources, however, the microsources appeared in strings and clumps (Fig. 11b). During a first flash (FF) when the microsources flash synchronously (Widder and Case, 1982), it is difficult to distinguish the number of microsources in a clump. During subsequent flashes (SFs) in response to continued mechanical stimulation or during acid stimulation, the microsources flash asynchronously. By observing a sequence of asynchronous flashes, it was possible to distinguish the number of active microsources within a clump. However, the microsources may move along the cytoplasmic strands so that the same microsource may be counted more than once. Counts were made of both mechanically stimulated and acid-stimulated cells. As indicated in Tables IV and V, counts were quite variable and ranged between 1,000 and 13,000 microsources per cell with an overall average of 4,500 (SD

TABLE III

Light effects on bioluminescence distribution in cells maintained in DD, stimulated by 4 M acetic acid.

Time of L exposure	N	Perinuclear luminescence	w/ss*	Cytoplasmic microsource luminescence
CT 0000–0005	7	7/7 Stage I—diffuse Stages II–IV—well defined	3/7	7/7 Stage I—higher concentration around nucleus Stages II–IV—in bands and bridges
CT 0000–0030	23	23/23 Stages I & V—diffuse Stages II–IV—well defined	6/23	22/23 Stage I—higher concentration around nucleus Stages II–IV—in bands and bridges showing obvious reduction in the number of microsources present with the highest concentration in the nuclear region
CT 0000–0100	21	21/21 Stages I & V—diffuse Stages II–IV—well defined	4/21	18/21 Stages I & V—evenly dispersed Stages II–IV—dramatic reduction in number of microsources, sometimes only 3 or 4 per cell
CT 0200–0300	18	18/18 Stages I & V—diffuse Stages II–IV—well defined	8/18	6/18 Stages I & V—dramatic reduction in number of microsources Stages II–IV—no microsources

* With microsource-like substructure.

= 2,600, N = 56). Linear regressions of numbers of microsources per 2,500 μm^2 against cell surface area indicated no correlation between the density of microsources within a given area and cell size. However, there is significant correlation between the total number of microsources per cell and cell size (correlation coefficient for all cells stimulated with acetic acid = 0.395, N = 42, $P < 0.01$) and, therefore, comparisons between groups in Tables IV and V were made for microsources per 2,500 μm^2. Table IV lists results from the three different stimulus modes: mechanical, and formic and acetic acids. With mechanical stimulation, counts were made from the first flash (FF) alone as well as in conjunction with subsequent flashes (SFs). SFs were only counted for as long as enough microsources appeared to stay in register from one flash to the next to indicate that no cell movement had occurred.

Acetic acid was usually employed to stimulate the cells in making counts, since it is commonly used to stimulate bioluminescence in dinoflagellates and physiological evidence indicates that it is relatively harmless (Widder & Case, 1981). It also had the advantage of being less likely than mechanical stimulation to cause cell movement.

Formic acid was also tested because it induced a more rapid onset and decay of luminescence that we hoped would reduce microsource movement during the stimulus period. Image intensification analysis of formic acid-stimulated cells revealed that clumps of microsources flashed in near synchrony and that frequently a microsource flashed only once or twice, making it difficult to distinguish the number of microsources in a clump.

Two-sample *t*-test comparisons of the different stimulus modes indicated no statistical difference between counts made from the mechanically, formic acid- or acetic acid-stimulated cells. Counts made from FF and SFs, however, were statistically different from all three groups.

Microsource counts using acetic acid stimulation were also made on cells that were maintained in darkness (DD) for more than 24 h and then tested in their subjective night phase (CT 1400–1900) (Table V) after being in the holding tubes between 28 and 33 hours. These counts were compared with night-phase cells that had received a normal light-dark (LD) cycle while being maintained in a holding tube for the same period of time. Microsource counts from the two groups were not statistically different. There was also no difference between the LD cells maintained in the holding tube for 28 to 33 hours before acetic acid stimulation (Table V), and those that were loaded into the tubes only 4 to 9 h before acetic acid stimulation in the same part of the night phase (CT 1400–1900) (Table IV).

Fluorescence of bioluminescent sources

Since the bioluminescent sources in *Noctiluca* are brightly fluorescent (Eckert and Reynolds, 1967), we looked for the same phenomenon in *Pyrocystis fusiformis.* It could be very useful in identifying microsources for counting and measurements, as it was in *Noctiluca.* A number of filter combinations including broad-band excitation filters (BG 3, BG 12, UG 1, and UG 5) with barrier filters (410, 440, 460, 470, 500, 510, and 560), American Optical filter clusters 2072 and 2073, and the Zeiss filter cluster described in Materials and Methods failed to reveal fluorescent particles that visually correlated with the bioluminescent microsources without image intensification. When viewing the periphery of a night-phase cell (using the Zeiss filter cluster), uv excitation revealed large green fluorescent vesicles occasionally, but these were never correlated with bioluminescent activity. After approximately 15 sec of illumination small fluorescent sources began to brighten

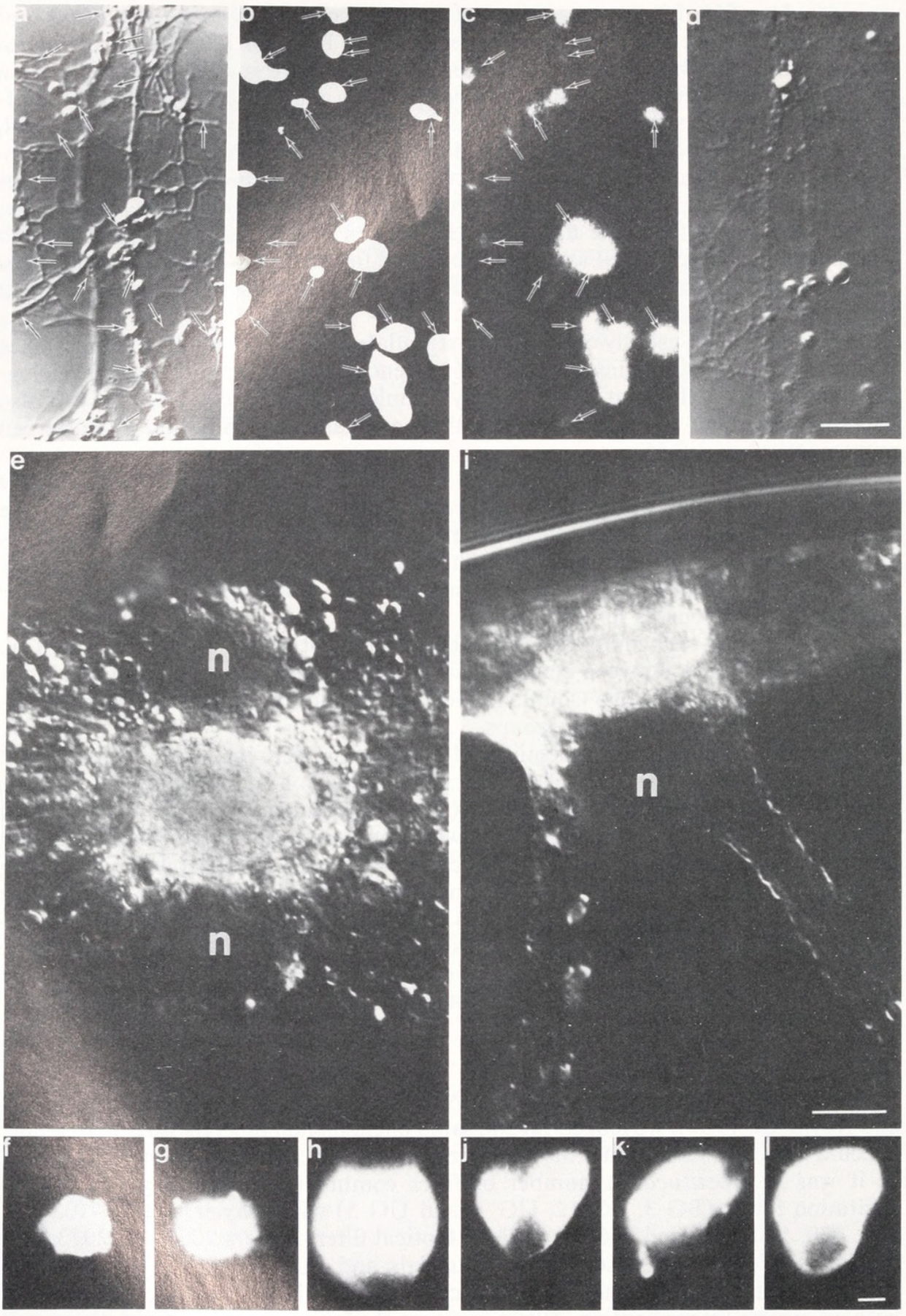

FIGURE 11. Correlation of bioluminescent and fluorescent sources with cytoplasmic structures in night-phase (a–d) and day-phase (e–l) cells. A Nomarski micrograph taken approximately 1 min prior to acid stimulation (a). Following 18 sec of acid stimulation (b) and 6 sec of uv exposure (c), another

and their appearance was associated with considerable movement and clumping, apparently due to the destructive effects of uv on the cell. After another 15 sec these fluorescent sources rapidly disappeared and after uv illumination it was generally impossible to induce bioluminescent activity from the irradiated portion of the cell.

The image intensifier revealed that the bioluminescent microsources were apparently dimly fluorescent. Night-phase cells that were stimulated to bioluminescence with either mechanical or acid stimulation and then viewed with ultraviolet illumination frequently exhibited correlations between the bioluminescent and fluorescent sources (Fig. 11b and c). When mechanical or acid stimulation occurred during the first 10 sec of irradiation, many of the fluorescent spots brightened for the time period of a normal microflash.

Nomarski micrographs were taken of the field before and after each image intensifier series. The time between recording the initial light micrograph and the first bioluminescent image of the same field recorded on video was generally about 1 minute. A typical acid stimulation sequence lasted between 15 and 20 sec and was followed by 5 to 10 sec of ultraviolet illumination. A single mechanical stimulus was used to produce a first flash. Comparison of bioluminescent sources from photographs of video recordings with corresponding loci in the Nomarski micrographs revealed no unequivocal microsource candidates. Frequently, luminescence originated from a dense clump of cytoplasmic material containing a number of different inclusions and in some instances it came from a point where no structure was apparent except for a cytoplasmic strand, or a point lacking even a cytoplasmic strand. Figure 11a–d represents one of our best series of such photographs. The most obvious microsource candidates in this and other Nomarski micrographs were the small, approximately 0.5 μm inclusions seen in many strands, although many such structures were apparently neither luminescent nor fluorescent. Other spherical or irregular inclusions larger than 0.5 μm were visible in the Nomarski micrographs, but these had no clear correspondence with the luminescent sources.

Nomarski micrographs of early day-phase cells in which the only luminescence was an acid-stimulated glow in a region near the nucleus revealed a dark yellow ovoid body (about 10×25 μm) surrounded by the horseshoe-shaped nucleus (Fig. 11e and i). This body has already been described and its rhythmic appearance in "day"-phase cells observed during 96 hours of continuous darkness (Sweeney, 1981). We confirm that the yellow body appears to be the source of much or all of the acid-stimulated bioluminescence and it is also strongly fluorescent (Fig. 11f–h and j–l) (Sweeney, 1980).

Nomarski micrograph was taken of the same field 1 min later (d). Arrows in (a–c) indicate equivalent loci. The fluorescent sources in (c) show good correspondence with the bioluminescent sources in (b). The brightest sources in the bottom right-hand corner of (c) were still flashing during uv exposure. In (a) the top three arrows point to the approximately 0.5 μm spherical structures mentioned in the text. In day-phase cells positioned so the nuclear region is seen in the center of the cell (e), a dark yellow spheroid is seen between the two arms of the horseshoe-shaped nucleus (n). When this cell was acid stimulated, the yellow body exhibited the round glow with substructure (f). It was also brightly fluorescent (g) and after several minutes of acid stimulation exhibited an eliptical glow with the 2 dark areas corresponding to the 2 arms of the nucleus (h). When the perinuclear region is located at one side of the cell, an optical section of the nucleus (n) shows the branch point of the 2 cytoplasmic strands, which cross the central vacuole (i). The yellow body lies between the nucleus and the cell wall. Initial response of the cell in (i) to acid stimulation (j) followed by uv excitation (k) and further acid stimulation (l) is shown. Scale, 10 μm. Scale bar in (d) applies to (a–d), in (i) applies to (e) and (i), and in (l) applies to (f–h) and (j–l).

TABLE IV

Effects of stimulus mode and cell size on numbers of microsources seen in night-phase cells.

Stimulus	Stage	MSs†/2500 μm^2	cell surface area ($\times 10^5$ μm^2)	MSs/cell ($\times 10^3$)
Mechanical				
FF	I	41	1.4	2.3
FF + 1 SF		45		2.5
FF	II	25	1.4	1.4
FF + 7 SFs		35		2.0
FF	III	23	1.5	1.4
FF + 3 SFs		59		3.5
FF	II	14	2.0	1.1
FF + 3 SFs		67		5.4
FF	II	24	2.2	2.1
FF + 6 SFs		75		6.6
FF mean + SD		25 ± 10		1.7 ± 0.5
FF + SFs mean ± SD		56 ± 16		4.0 ± 2.0
Formic Acid				
6 *M*	II	38	1.6	2.4
6 *M*	II	26	1.6	1.6
6 *M*	II	26	2.0	2.1
23 *M*	II	22	2.5	2.2
mean ± SD		28 ± 7		2.1 ± 0.3
Acetic acid (4 *M*)				
	I	26*	2.6	2.7
	I	31*	2.8	3.5
	III	74	3.4	10.0
	III	37*	3.5	5.2
	II	37*	3.7	5.5
	III	27*	4.0	4.3
	II	31*	4.3	5.3
	II	34*	4.5	6.1
	I	34*	4.7	6.4
	III	35*	4.8	6.7
mean ± SD		37 ± 14		5.6 ± 2.0

† MSs = microsources.

* Counts were made from 1849 μm^2 area and normalized to 2500 μm^2.

Cells maintained in holding tube less than 9 h on normal LD cycle and tested between CT 1400 and CT 1900.

DISCUSSION

The distribution of bioluminescence undergoes striking changes during the daily cycle of *Pyrocystis fusiformis*. Most apparent were the total disappearance of microsources from the periphery of early day-phase cells and the development of the perinuclear glow. While the appearance and disappearance of the perinuclear glow and the rhythm of mechanically excitable luminescence persisted in cells in DD, a light induction period was required before the microsources disappeared from the periphery. Since the perinuclear glow seemed to develop normally even when the microsources remained in the periphery, these two phenomena may not be causally linked. However, DD cells did not seem to develop the microsource-like substructure in the perinuclear glow, characteristic of some early day-phase cells. This observation, along with the apparent concentration of the microsources

TABLE V

Effect of a 24 h dark cycle (DD) on numbers of microsources seen in night-phase cells.

Stage	MSs†/2500 μm	Cell surface area ($\times 10^5$ μm^2)	MSs†/cell ($\times 10^3$)
LD			
II	33	2.7	3.6
I	24	2.7	2.6
II	11*	2.8	1.2
II	45*	3.1	5.6
I	14*	3.5	2.0
I	33*	3.6	4.8
I	53*	3.8	8.1
II	32	3.9	5.0
II	34	4.0	5.4
I	62*	4.0	9.9
II	22*	4.1	3.6
II	21	4.2	3.5
III	31*	4.6	5.7
III	24*	4.7	4.5
mean ± SD	31 ± 14		4.7 ± 2.3
DD			
II	36	2.2	3.2
I	57	2.6	5.9
I	20	2.6	2.1
II	40	2.7	4.3
III	22*	2.7	2.4
I	30*	2.9	3.5
II	36	2.9	4.2
I	31	3.0	3.7
II	50*	3.0	6.0
III	55*	3.0	6.6
I	51*	3.3	6.7
I	28	3.7	4.1
II	63	3.9	9.8
II	14	4.2	2.4
III	61*	4.4	11.0
I	26*	4.5	4.7
III	70*	4.6	13.0
II	14*	4.8	2.5
mean ± SD	39 ± 18		5.3 ± 3.1

† Microsources.

* Counts were made from 1849 μm^2 area and normalized to 2500 μm^2.

Cells in both LD and DD were maintained in holding tubes between 28 h and 33 h before testing between CT 1400 and CT 1900. Stimulus mode was addition of 0.05 cc of 4 *M* acetic acid to 1.5 cc SW bath.

in the bands and bridges of stages II–IV during the transition phases, suggested that the microsources might have been following a migration pattern opposite to that observed for the chloroplasts (Sweeney, 1981). If so, the nonstructured perinuclear glow that developed, whether or not the microsources disappeared from the periphery, might be attributed to the synthesis of bioluminescent substrates prior to their packaging in microsources.

If the microsources are normally broken down and resynthesized in the perinuclear region every day, then in DD cells, when the microsources seem to remain

in the periphery, we might expect to see twice the normal density of microsources in the periphery following the synthesis and outward migration of new microsources in the following night phase. Comparison of microsource density in LD and DD cells (Table V) indicated this was not the case. Furthermore, there was no greater TMSL of DD over LD night-phase cells; DD cells actually produced less light than LD cells (Fig. 10).

The apparent spreading of the perinuclear glow seen in the transition periods at dawn and dusk might indicate that the bioluminescent substrates, rather than the microsources themselves, migrated, or perhaps both the microsources and the substrates migrated independently.

Whatever the basis for the appearance and disappearance of the microsources and the perinuclear glow, the same rhythmic changes probably occur in *Pyrocystis noctiluca*, since our preliminary observations have shown the presence of a perinuclear glow and the absence of microsources in early day-phase cells and microsources spread over the surface of night-phase cells.

It has been suggested that the decrease in mechanically excitable luminescence in day-phase cells of *Pyrocystis* and several other dinoflagellates is due to an increase in the threshold for mechanical stimulation (Hamman & Seliger, 1972; Hamman *et al.*, 1981). Yet, we have recorded action potentials from both day- and night-phase cells in response to mechanical stimulation (Widder & Case, 1980). The action potential triggers a bioluminescent flash in night-phase cells, but a flash does not follow a similar mechanically inducible action potential in day-phase cells. Our present findings indicate that the lack of mechanically excitable luminescence of day-phase cells is not due to an absence of microsources, because acid stimulation revealed microsources in late day-phase cells as well as in the early "day" phase of cells in DD. Therefore, a link between the mechanically stimulated action potential and the microsource in night-phase cells is absent or refractory in day-phase cells. Since acid stimulation circumvents mechanical inexcitability in day-phase cells, it may be that protons are required to trigger bioluminescent activity, as suggested previously (Hastings, 1978). Therefore, the rhythm of mechanically stimulable luminescence in *Pyrocystis fusiformis* might be due to rhythmic changes in proton concentrations or in membrane proton gates within the cell. Plants that perform Crassulacean acid metabolism (CAM) exhibit cyclic changes in the level of malic acid concentration found in the vacuole (Lüttge and Higinbotham, 1979). Acid concentration reaches a peak towards the end of the night phase and decreases by an order of magnitude at the end of the day phase. If similar changes occur in *P. fusiformis*, it would offer a possible explanation for a number of our observations. Besides accounting for the inexcitability of day-phase cells compared with night-phase cells, changing proton concentration would also explain the sequence of events during the transition phases. At dawn a decrease in TMSL occurs, while the number of flashes a cell can produce remains fairly constant. This is a clear indication that the cells remain mechanically excitable as their luminescent output diminishes. At dusk, when mechanical stimulation begins to evoke dim luminescence, acid stimulation reveals the presence of many brilliantly luminescent microsources. Therefore, the reduced luminescence of the transition phases is not due to a decreased mechanical excitability or an absence of luminescent substrates.

Apparently microsource number varies with cell size, while microsource density is more constant. Despite the difficulties caused by the apparent clumping of microsources and the difficulty of counting asynchronously active microsources, counts made using three different methods of stimulation (acetic acid, formic acid, and

the mechanically stimulated FF) were not significantly different. Possibly the higher microsource numbers observed when SFs were included may have been due to greater movement of the microsources. The only other apparent explanation is that multiple mechanical stimuli more effectively reveal microsources in the field. Presently we do not see why that should be true.

Microsources in *P. fusiformis* were only dimly fluorescent with the filter combinations employed and only visible with the aid of the IIT. Their fluorescence was established by observing a field of bioluminescent sources corresponding to fluorescent sources at the same loci. These fluorescent sources were often seen to brighten briefly for the duration of a microflash when mechanical or acid stimulation was applied during the first few seconds of uv irradiation. The best focus for the fluorescent sources also corresponded to the best focus for the bioluminescent sources.

Since microsource identification was only possible with the IIT, attempts at correlating luminescent sources with cytoplasmic structures required a time delay of one minute or more. The possibility that cytoplasmic streaming could displace microsources during this delay complicated the comparisons. In general, luminescence seemed to originate from the junctions of cytoplasmic strands where several different cytoplasmic inclusions were clumped together. In cases where no cytoplasmic structure was seen at the location of a luminescent source we must assume either that the source was smaller than the resolving power of the light microscope, or that some movement had occurred. Our observations suggest that the microsources are 0.5 μm or less in diameter.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of David C. Smith and the continuing advice and interest of Dr. B. M. Sweeney. This work was supported by a grant from the Office of Naval Research (ONR contract N00014-75-C-0242).

LITERATURE CITED

BASSOT, J. M., AND A. BILBAUT. 1977. Bioluminescence des elytres d'*Acholoe*. IV. Luminescence et fluorescence des photosomes. *Biol. cellul.* **28:** 163–168.

CASE, J. F., AND L. G. STRAUSE. 1978. Neurally controlled luminescent systems. Pp. 331–366 in P. J. Herring, Ed., *Bioluminescence in action.* Academic Press, New York.

ECKERT, R., AND G. T. REYNOLDS. 1967. The subcellular origin of bioluminescence in *Noctiluca miliaris*. *J. Gen. Physiol.* **50:** 1429–1458.

GUILLARD, R. R. L., AND J. H. RYTHER. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. *Can. J. Microbiol.* **8:** 229–239.

HAMMAN, J. P., W. H. BIGGLEY, AND H. H. SELIGER. 1981. Photoinhibition of stimulable bioluminescence in marine dinoflagellates. *Photochemistry and Photobiology* **33:** 909–914.

HAMMAN, J. P., AND H. H. SELIGER. 1972. The mechanical triggering of bioluminescence in marine dinoflagellates: chemical basis. *J. Cell Physiol.* **80:** 397–408.

HANSON, F. E., J. MILLER, AND G. T. REYNOLDS. 1969. Subunit coordination in the firefly light organ. *Biol. Bull.* **137:** 447–464.

HASTINGS, J. W. 1978. Bacterial and dinoflagellate luminescent systems. Pp. 129–170 in P. J. Herring, Ed., *Bioluminescence in action.* Academic Press, New York.

LÜTTGE, U., AND N. HIGINBOTHAM. 1979. *Transport in plants.* Springer-Verlag, New York, Heidelberg, Berlin. 468 pp.

SWEENEY, B. M. 1980. Intracellular source of bioluminescence. *Int'l. Rev. Cytol.* **68:** 173–195.

SWEENEY, B. M. 1981. The circadian rhythms in bioluminescence, photosynthesis and organellar movements in the large dinoflagellate, *Pyrocystis fusiformis.* Pp. 807–814 in H. G. Schweiger, Ed., *International Cell Biology 1980–1981.* Springer-Verlag, New York, Heidelberg, Berlin.

SWIFT, E., AND E. G. DURBIN. 1971. Similarities in the asexual reproduction of the oceanic dinoflagellates, *Pyrocystis fusiformis, Pyrocystis lunula* and *Pyrocystis noctiluca. J. Phycol.* **7:** 89–96.

SWIFT, E., AND V. MEUNIER. 1976. Effects of light intensity on division rate, stimulable bioluminescence and cell size of the oceanic dinoflagellates *Dissodinium lunula, Pyrocystis fusiformis* and *P. noctiluca. J. Phycol.* **12:** 14–22.

WIDDER, E. A., AND J. F. CASE. 1980. Bioluminescence excitation in a dinoflagellate. Pp. 125–132 in K. H. Nealson, Ed., *Bioluminescence: current perspectives.* Burgess Publishing Company, Minneapolis.

WIDDER, E. A., AND J. F. CASE. 1981. Two flash forms in the bioluminescent dinoflagellate *Pyrocystis fusiformis. J. Comp. Physiol.* **143:** 43–52.

WIDDER, E. A., AND J. F. CASE. 1982. Luminescent microsource activity in bioluminescence of the dinoflagellate, *Pyrocystis fusiformis. J. Comp. Physiol.* **145:** 517–527.

ZAR, J. H. 1974. *Biostatistical analysis.* Prentice-Hall, Englewood Cliffs, New Jersey. 620 pp.