Compartmentalization of Algal Bioluminescence: Autofluorescence of Bioluminescent Particles in the Dinoflagellate *Gonyaulax* as Studied with Image-intensified Video Microscopy and Flow Cytometry

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ABSTRACT Compartmentalization of specialized functions to discrete locales is a fundamental theme of eucaryotic organization in cells. We report here that bioluminescence of the dinoflagellate alga *Gonyaulax* originates in vivo from discrete subcellular loci that are intrinsically fluorescent. We demonstrate this localization by comparing the loci of fluorescence and bioluminescence as visualized by image-intensified video microscopy. These fluorescent particles appeared to be the same as the previously described in vitro "scintillons." We attribute the endogenous fluorescence to that of the bioluminescence substrate, luciferin, because (*a*) the fluorescence excitation and emission characteristics are comparable, (*b*) the autofluorescence is lost after exhaustive stimulation of bioluminescence, and (*c*) the fluorescence of discharged particles in vitro can be restored by adding luciferin. The fluorescence in vivo exhibits a standard property of circadian (daily) rhythmicity: under constant environmental conditions, the intensity of the particle fluorescence fluctuates cyclically (it is maximal during the night phase and is low during the day). Thus, luciferin is localized within the cell at discrete loci from which the bioluminescence emanates; the cellular quantity of luciferin is rhythmically modulated by the circadian clock.

The ability of "red tide" algae to emit light has long fascinated biologists (16). Although much is known about the biochemistry of this algal luminescence, the cell biology of the phenomenon is less well understood. In the dinoflagellate *Noctiluca miliaris*, bioluminescent light is emitted from subcellular "microsources" in response to a membrane depolarization (9), which may activate a proton flux (25); but the triggering pathway is incompletely characterized and the ultrastructural identity of these microsources is unknown. In *Pyrocystis fusiformis*, the intracellular sites of light production have been visualized, but the transducing mechanism is even less well understood than in *Noctiluca* (34, 38, 39).

In a third bioluminescent dinoflagellate, *Gonyaulax polyedra*, fractionation of cell extracts revealed sedimentable bioluminescent particles which were called "scintillons" (6, 19). These scintillons were defined as self-contained particulate systems whose in vitro light production is specifically dependent upon protons:[1] light emission in vitro occurs as a flash kinetically similar to the flash in vivo and is triggered by a rapid pH change (e.g., pH 8 to 6; see references 7 and 18). Early studies by image-intensified microscopy suggested that flashes in vivo emanated from discrete subcellular sources that were not distributed uniformly throughout the cytoplasm (28). In vitro studies using image-intensified microscopy supported the idea that these sources were identical to scintillons (27).

Bioluminescence in *Gonyaulax* is of special interest because it and other cellular systems are controlled by the circadian clock. Major research efforts have characterized the biochem-

[1] Scintillons were originally hypothesized to be particles containing guanine crystals (6, 19). Later studies (12) excluded this proposal and defined scintillons as sedimentable subcellular particles that are capable of bioluminescent light emission in vitro.

ical means by which the clock controls rhythmic behaviors in this alga (22, 29).

In the work reported here, we confirm that *Gonyaulax* bioluminescence originates in vivo from discrete subcellular particles that are intrinsically fluorescent. This fluorescence is attributable to the bioluminescent substrate, luciferin, which is therefore compartmentalized within the cell. We found that luciferin content of *Gonyaulax* cells, measured by the intensity of this fluorescence, is rhythmically modulated by the circadian clock. The fluorescent particles were present in extracts of the cells and appeared to correspond to the previously described in vitro scintillons.

MATERIALS AND METHODS

Culture Conditions and Strains: Unialgal, but not axenic, cultures of *Gonyaulax* were grown at 20 ± 2°C in 2.8-liter Fernbach flasks containing 1 liter of f/2 medium (13), omitting the silicate and adding 0.5% autoclaved extract of soil. This medium consists of seawater supplemented with vitamins, nitrate, phosphate, and trace metals. Cultures were grown on a light:dark cycle of 12 h of light followed by 12 h of darkness (light intensity = 150 μ einsteins/m^2s).

G. polyedra strains GP70 and GP60 (both luminous) were used in this study as well as two strains of *Gonyaulax tamarensis* (formerly *G. excavata*), which were obtained from Dr. Donald Anderson (Woods Hole Oceanographic Institute, Woods Hole, MA): the luminous GT429 (*Gonyaulax tamarensis var. excavata*) and the nonluminous GTPP (*Gonyaulax tamarensis var. tamarensis*).

Fluorescence and Image-intensified Video Microscopy: *Gonyaulax* cells and extracts were observed with 40/0.75 dry or 100/1.30 oil immersion objectives attached to a Zeiss IM-35 inverted epifluorescence microscope (Carl Zeiss, Inc., New York). The epifluorescence excitation was generated by a mercury lamp (HBO 50W) using various sets of fluorescence filters: (*a*) EX (exitation) 365/EM (emission) 420 long pass (Zeiss filter set 487702), (*b*) EX 395–420/EM 450 long pass (Zeiss filter set 487718), and (*c*) EX 450–490/EM 520 long pass (Zeiss filter set 487709). In most cases, an additional short-pass barrier filter (Zeiss filter KP560) was interposed to filter out the red fluorescence from chlorophyll. For direct photography of the microscopic image, an Olympus OM-2 camera was attached to the camera port of the IM-35 microscope.

Image-intensification of the fluorescent and bioluminescent images was accomplished by coupling a Zeiss/Venus TV-3M three-stage image-intensified vidicon camera to the cine-port on the side of the IM-35. After insertion of time and date information (Model ET 202 [Cramer Video, Newton, MA]), the video signal was stored on a ¾ inch cassette with a Sony VO-4800 video tape recorder.

The temporal and spatial properties of *Gonyaulax* fluorescence and bioluminescence were analyzed by transcribing the relevant cassette sequence onto a video motion analyzer (Sony SVM 1010). We then analyzed field-by-field the entire 10-s sequence stored on the disk of the motion analyzer. Kinetic changes of the bioluminescence of single scintillons were measured by inserting a video analyzer (Model 321 [Colorado Video, Boulder, CO]) into the instrumentation network that allowed the intensity of single pixels to be recorded on a strip-chart recorder. In some cases, the comparison of fluorescence and bioluminescence distributions was facilitated by image processing using a system functionally identical to the IMAGR-I system from Interactive Video Systems (Concord, MA). Photography of the image-intensified images was done from single frames on the video monitor through a Ronchi grating to eliminate scan lines and improve resolution (21).

Preparation of Cells for Microscopy: For correlating the subcellular distributions of autofluorescence and stimulated bioluminescence, cells were immobilized by embedding them in 2% low-temperature gelling agarose (Sea Prep 15/45 agarose from FMC Corporation, Marine Colloids Division, Rockland, MN). 1 ml of *Gonyaulax* cells was added to 1 ml of 4% agarose in f/2 medium that had been heated to 90°C to melt the agarose and then cooled to 25°C. After gentle mixing of the *Gonyaulax*/agarose suspension, two-drop aliquots (~100 μl) of the suspension were placed in small wells on top of 24-×-60-mm cover slips (we used sections of 1.5 ml plastic centrifuge tubes to make the well). The preparations were then placed in a humidified chamber in the dark at 18–20°C until the agarose solidified. Agarose embedding was usually performed on *Gonyaulax* cells at the end of day phase (ct 12)[2].

[2] *Abbreviations used in this paper:* ct, circadian time (ct 0 is dawn, ct 12 dusk, in a light/dark cycle of 12 h of light, then 12 h of darkness); GT429, a luminous strain of *Gonyaulax tamarensis*; GTPP, a nonluminous strain of *G. tamarensis*; PAS, periodic acid-Schiff staining.

Gonyaulax cells thus embedded in agarose were observed 1–2 h later from beneath the cover slip through the IM-35 inverted microscope. Bioluminescence was stimulated by adding a drop of an ion solution into the well in darkness while observing a previously focused cell in the video monitor coupled to the image-intensified camera and video tape recorder. Because the ions diffuse through a layer (~3 mm) of agarose before reaching the cell in focus, high concentrations were used to reduce the time required to elicit luminescence: 3 M $CaCl_2$ or KCl, concentrated NH_4OH, or glacial acetic acid. Much lower concentrations are sufficient for cells in solution where mixing is rapid (14, 15).

"Spheroplasts" lacking cell walls (1) were prepared by centrifuging and resuspending the cells in the following medium: 400 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 10 mM Na phosphate, 7.5 mM EDTA, 60 mM Tris, pH 8.2. After 4–8 h of incubation in this medium, many cells shed their cell walls.

Preparation of Cell Extracts; Scintillon Assay: 1 liter of *Gonyaulax* culture was filtered at dusk (ct 12) through a Whatman 541 filter, resuspended in 5 ml extraction buffer (100 mM Tris, 10 mM EDTA, 5 mM 2-mercaptoethanol, pH 8.5, 1°C) and the cells were broken by a single pass through a Kirkland emulsifier (Brinkmann Instruments, Inc., Westbury, NY). More than a single extraction pass tended to decrease scintillon activity. Cell debris was removed by a 10-min centrifugation at 800 *g*. The pellet was re-extracted two or three times, each time by resuspension in 5 ml of extraction buffer with a glass homogenizer and centrifugation at 800 *g*. The supernatants were then pooled and spun at 27,000 *g* for 20 min. The pellet was resuspended with a glass homogenizer in 2–4 ml of extraction buffer and either loaded directly onto a sucrose gradient (see below) or incubated overnight at 1°C in the dark and then subjected to density gradient sedimentation.

Scintillon activity of 10-μl aliquots of extract was assayed by injecting acetic acid (12); the initial maximum light intensity was recorded.

Isodensity Sedimentation: Partial purification of scintillons was achieved by centrifugation through sucrose density gradients by a modification of the procedure of Fogel et al. (12). Gradients were formed from six layers (5.5 ml each) of 35%, 40%, 45%, 50%, 55%, and 60% sucrose and smoothed for 36–48 h at 4°C. The sucrose solutions contained 50 mM Tris, 10 mM EDTA, and 5 mM 2-mercaptoethanol at pH 8. 1- or 2-ml samples of extracts were layered onto the gradients and spun for 2 h at 17,000 rpm in an SV-288 vertical rotor in a Sorvall RC-5B centrifuge (DuPont Company, Newtown, CT) (0–1°C). 1- to 1.8-ml fractions were collected and assayed. The concentration of fluorescent particles in the fractions of sucrose gradients was measured with a hemacytometer under the fluorescence microscope (see Fig. 6). The distribution of chloroplast sedimentation was determined by measuring absorption at 675 nm in each of the fractions.

Recharging of Scintillons In Vitro: Recharging of scintillon activity was performed essentially as described by Fogel and Hastings (11). The peak scintillon fraction from a sucrose density gradient was acidified to pH 5.5 to discharge its scintillon activity. Then it was alkalinized back to pH 8 and luciferin purified as described by Fogel and Hastings (10) was added. After incubating at 20°C and/or 1°C, the fluorescent particles were photographed and scintillon activity re-assayed.

Flow Cytometry: A Becton-Dickinson FACS Analyzer (Becton-Dickinson FACS Systems, Sunnyvale, CA) was used to measure the endogenous fluorescence of populations of intact *Gonyaulax* cells. A mercury lamp provided the excitation light, which was filtered as described below. Cells were slowly infused through a 100-μm orifice using millipore-filtered (0.22 μm) seawater as the sheath buffer. Fluorescence intensity measurements from at least 1,000 *Gonyaulax* cells were collected with a logarithmic amplifier and the peak position of the resulting histogram (i.e., the log of fluorescence intensity vs. number of cells) was estimated by a Becton-Dickinson data analysis package (Consort 20 Application Program Version 2, 1983). This peak position on the log scale was then converted to a calibrated linear scale by comparing the log peak position to a standard curve generated by measuring the fluorescence of calibrated polystyrene beads (Curtis-Matheson fluorescent "microspheres") of different fluorescence with both logarithmic and linear amplifiers by the procedure of Muirhead et al. (24). *Gonyaulax* fluorescence as measured with either the logarithmic or linear amplifiers was equivalent after this conversion, but accumulation of the data obtained with the logarithmic amplifier allowed a more precise estimation of the peak position.

The interference filters used in the cytometer were as follows: (*a*) 350–375 excitation: 360 ± 10–nm band pass filter, 375-nm short-pass filter, and 375-nm short-pass dichroic mirror; (*b*) 475–495 excitation: 485 ± 10–nm band pass filter, 505-nm short-pass dichroic mirror; (*c*) 470–500 emission: 485 ± 15–nm band pass filter, 400-nm long-pass filter; (*d*) 505–545 emission: 525 ± 20–nm band pass filter; (*e*) 580-nm long-pass emission: 580-nm long-pass filter. Filters were obtained from Becton-Dickinson.

Cells were prepared for flow cytometry by placing 2-ml aliquots of a healthy, log-phase culture into clear polystyrene tissue-culture tubes (6 ml), which were loosely stoppered. These aliquots were dispensed one day or more before the experiment began from cultures which had been synchronized by a light/dark

cycle. At the beginning of the experiment, the tubes of cells were placed under constant temperature and continuous dim illumination (~1,000 lux). This light treatment resulted in preparations of *Gonyaulax* cells that were "photoinhibited" so that the turbulence within the flow cytometer would not mechanically discharge the bioluminescence (36). At each time point, the cytometer stability was checked with calibrated fluorescent beads and readjusted if necessary to avoid instrument drift during the long time course experiments. Then a 2-ml aliquot of cells was sampled until the data of at least 1,000 *Gonyaulax* cells was collected. Then calcium (or another) ion was added to the same tube to discharge the bioluminescence and a second sample of 1,000 cells was collected.

RESULTS

Autofluorescent Particles

When *Gonyaulax* cells are viewed under epifluorescent illumination, the cells display considerable endogenous fluorescence (Fig. 1), which falls into three classes: (*a*) red chlorophyll fluorescence (which has been blocked by a 560-nm short-pass filter in most of the photographs of Fig. 1, but is visible in Fig. 1, *d* and *l*; (*b*) yellow fluorescence from the large "PAS body" (see reference 31), of which only one or two are present per cell (most obvious in Fig. 1, *b*, *f*, and *j*, but is also visible in the other fluorescence micrographs); and (*c*) blue fluorescence from many small particles present in the cortex of *Gonyaulax* cells (Fig. 1, *a*, *e*, *h*, and *i*). It is these blue-fluorescent particles that are the subject of this paper.

The fluorescence of these particles is maximally excited by light of 395–420 nm. The fluorescence is dimmer if excited by 365-nm light, and is not detectable if excited with a standard fluorescein filter combination at 450–490 nm. The particulate fluorescence emission is blue-green. These characteristics suggested that this autofluorescence is emitted by the bioluminescent substrate, luciferin, which is maximally excited at 390 nm and fluoresces maximally at 475 nm (3, 26). Several other lines of evidence reported later in this paper will demonstrate that this fluorescence is indeed that of luciferin. The fact that 450–490 nm light does not excite the fluorescence of the bioluminescent particles is used as a criterion for distinguishing the relevant intracellular particles from nonspecific endogenous fluorescence in many of the experiments described below.

The particles are located near the cell membrane in the cortex of the cell, as can be seen by comparing cells focused

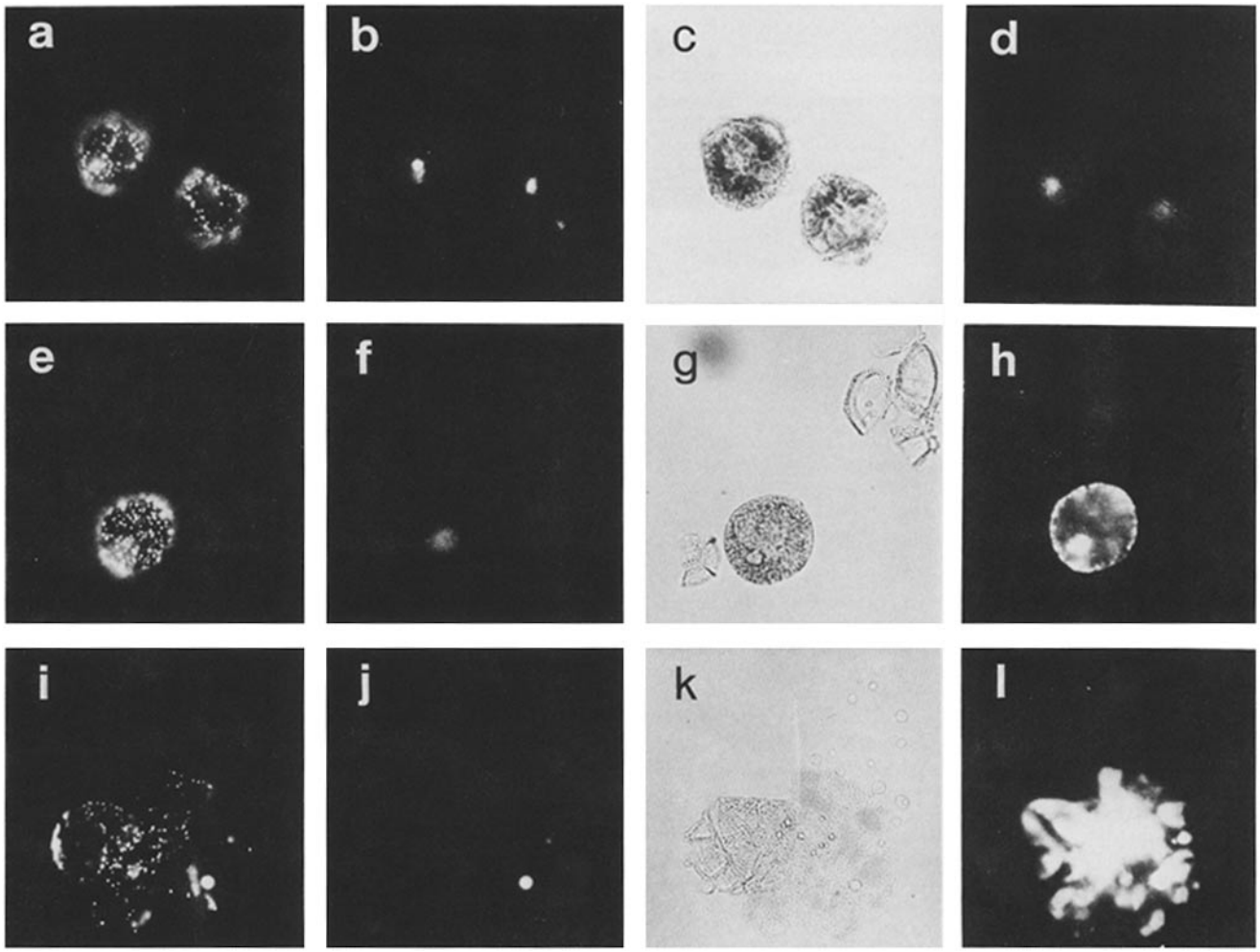


FIGURE 1 Endogenous fluorescence of *G. polyedra*, strain GP70 (luminous). (*a–d*) Two intact *G. polyedra* cells. (a) Fluorescent particles (EX 395–420; EM 450–560); (*b*) PAS bodies (EX 450–490; EM 520–560); (c) bright field; and (*d*) chloroplast fluorescence (EX 450–490; EM 520 long pass). (*e–h*) One *G. polyedra* spheroplast. (e) Fluorescent particles focused at cell surface (EX 395–420; EM 450–560); (*f*) PAS body (EX 450–490; EM 520–560); (*g*) bright field; and (*h*) fluorescent particles focused at cell periphery (EX 395–420; EM 450–560). (*i–l*) One *G. polyedra* cell lysed by gentle pressure on the cover slip. (*i*) Fluorescent particles (EX 395–420; EM 450–560); (*j*) PAS bodies (EX 450–490; EM 520–560); (*k*) bright field; and (*l*) chloroplast fluorescence (EX 450–490; EM 520 long pass). Tri-X film with Acufine development. 40x Neofluor objective.

at the surface vs periphery of the cells (Fig. 1 *e* vs *h*, and Fig. 10 *a* vs *b*). The appearance of discrete fluorescent particles in the *Gonyaulax* cortex is not an artifact due to optical distortions of the cell wall, which had been suggested (33) as an explanation for earlier observations of compartmentalized bioluminescence (28). For example, if cells are gently lysed by squeezing them between cover slip and slide, fluorescent particles of the appropriate size and shape are seen in the lysate (Fig. 1, *i–l*), confirming that the existence of these particles in vivo is not an artifact. This can also be shown by removing the cell wall; "spheroplasts" display the same fluorescent particles (Fig. 1, *e–h*). Nevertheless, there may be some relationship between the particles and the cell wall. Indeed, the fluorescent particles of cells with intact walls often

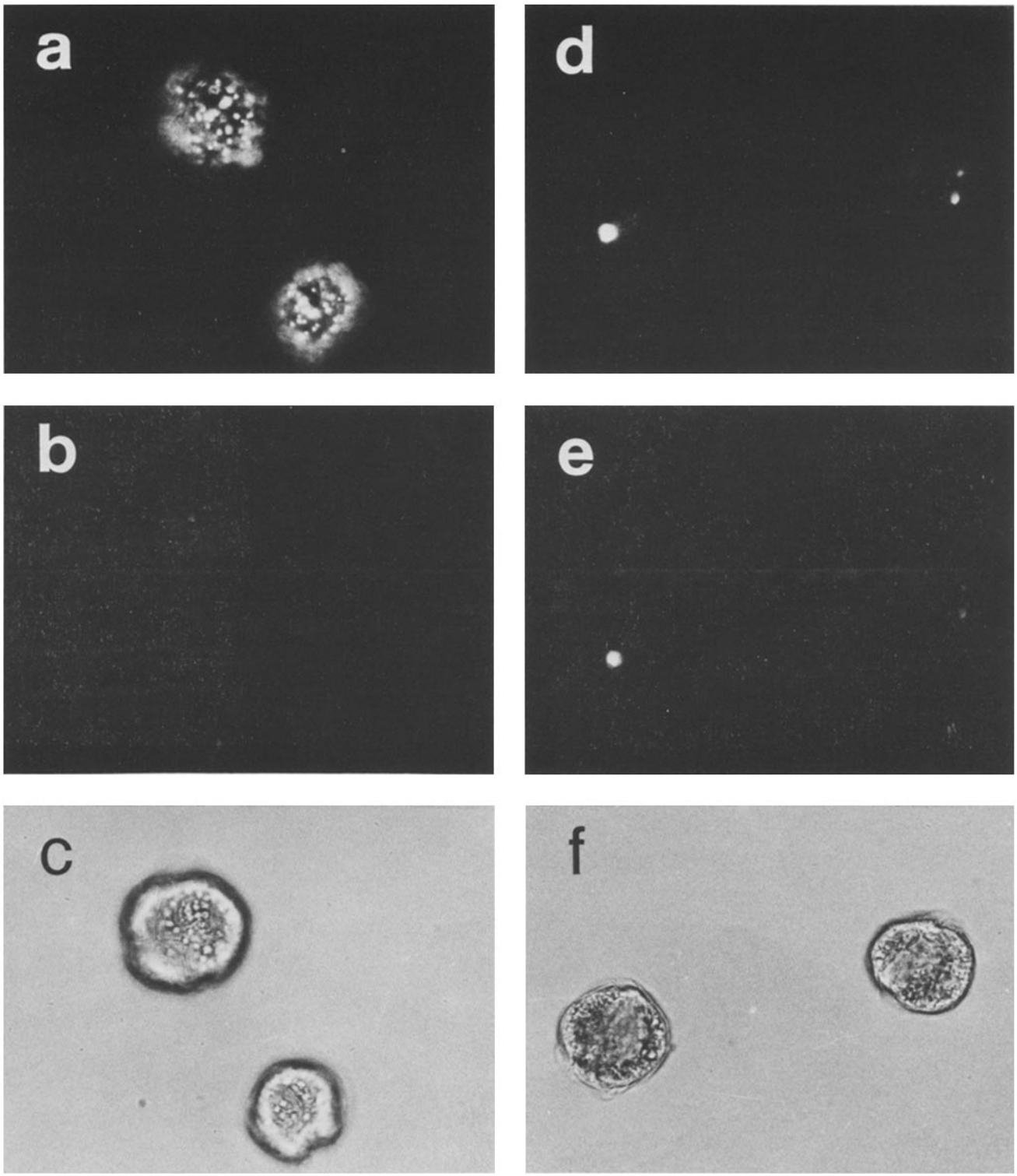


FIGURE 2 Endogenous fluorescence of *G. tamarensis*. (*a–c*) Two *G. tamarensis* cells, strain GT429 (luminous). (*d–f*) Two *G. tamarensis* cells, strain GTPP (nonluminous). (*a* and *d*, EX 395–420; EM 450–560; *b* and *e*, EX 450–490; EM 520–560; *c* and *f*, bright field. Tri-X film with Acufine development.) 40x Neofluor objective.

(but not always) tend to be organized in rows that correspond with the edges of cell wall plates or the cell "girdle," whereas the particles in spheroplasts appear to be dispersed more uniformly. This observation suggests that some underlying cytoskeletal organization reflecting the pattern of the cell wall plates can influence the positioning of the fluorescent particles.

The apparent size of the particles as visualized by their fluorescence is variable; in lysates they range from ~0.3 to 1.0 μm, with an average diameter of 0.64 μm ± 0.19 (SEM, n = 75; in vitro scintillon activity is trapped by a 0.45-μm millipore filter). In intact cells, the particles fall within the same size range.

Nonluminous *Gonyaulax* do not have the fluorescent particles. Fig. 2 shows fluorescence micrographs of two strains of *G. tamarensis*: the luminous one (GT429) has fluorescent particles (Fig. 2*a*), whereas they are absent from GTPP, a nonluminous strain that does not have luciferin, as previously reported (30). The absence of luciferin in extracts of GTPP has been confirmed for the strain used here (C. H. Johnson and J. Roeber, unpublished results). Both GT429 and GTPP have PAS bodies which, however, are not seen in Fig. 2, *a* and *b*, because they are on the far side of the cells.

Autofluorescent Particles Are the Source of Bioluminescence In Vivo

Gonyaulax cells were embedded in low temperature melting agarose so that they would be immobile while being stimulated to bioluminesce. The cells were viable for at least 4 h after this agarose embedding. The cells were viewed by image-intensified video microscopy during the stimulation procedure and the results were recorded on videotape for later analysis.

The bioluminescence of these immobilized cells was stimulated by any one of four different ionic treatments that had previously been found to elicit dinoflagellate luminescence: namely, high extracellular Ca^{++}, NH_4^+, K^+, or H^+ (15). Fig. 3 illustrates the response of a *Gonyaulax* cell to high Ca^{++}. Fig. 3*A* depicts the distribution of fluorescent particles before Ca^{++} was added. Then the fluorescence excitation was turned off, Ca^{++} added in darkness, and the bioluminescent flash triggered by this treatment was recorded. The picture in Fig. 3*B* was taken of the frame at the peak of the light flash. Clearly, the bioluminescence emanates from the same subcellular loci as does the fluorescence. Within a few seconds after the flash, the fluorescence-exciting lamp was turned on again and it was

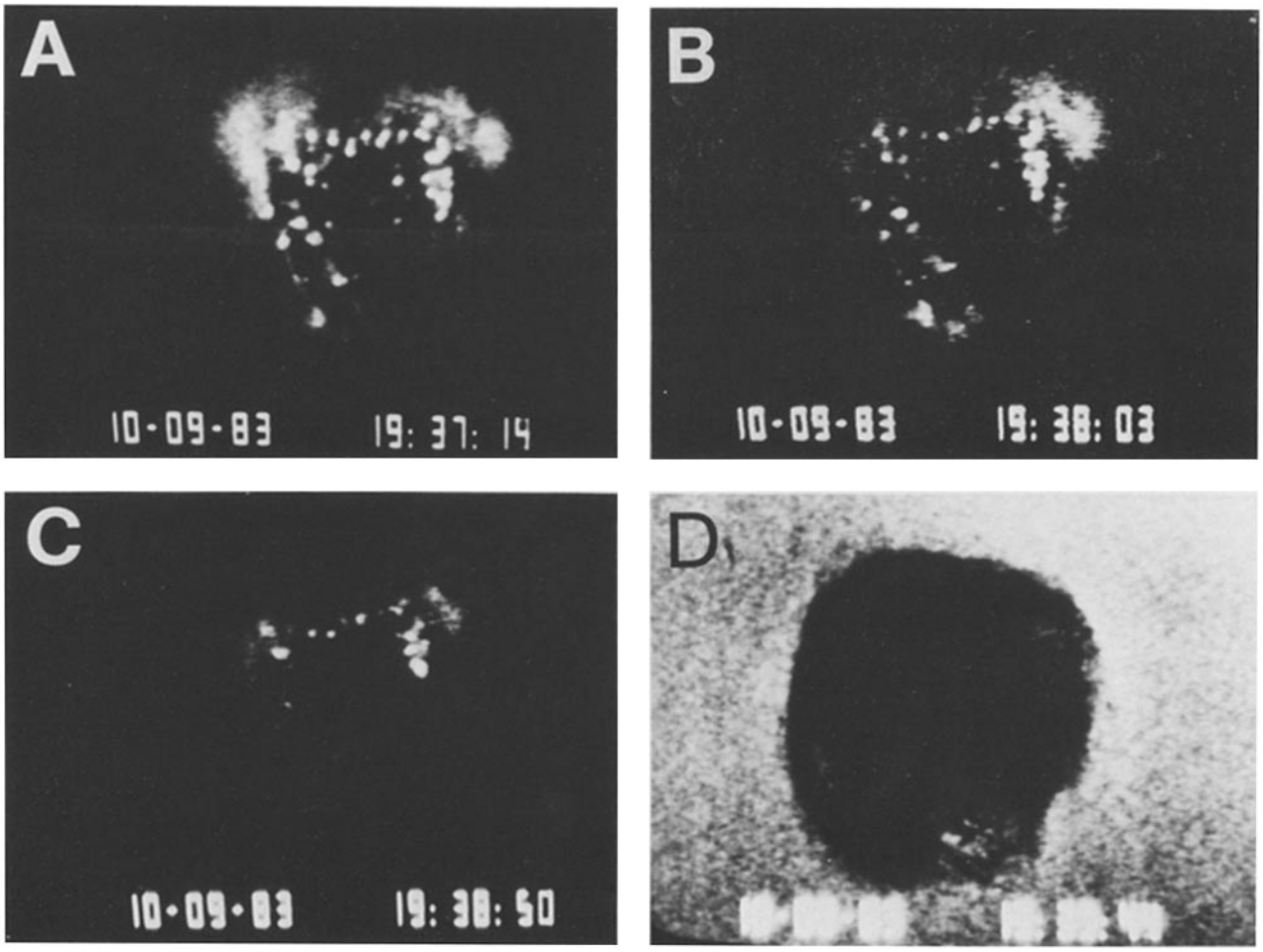


FIGURE 3 Co-localization of endogenous fluorescence with bioluminescence stimulated by calcium. (*A*) Fluorescent particles before stimulation of bioluminescent flash (EX 395–420; EM 450–560); (*B*) video frame at the peak of bioluminescent flash elicited by Ca^{++} (no fluorescence excitation); (*C*) fluorescent particles 45 s after the bioluminescent flash (EX 395–420; EM 450–560; this photo is overexposed as compared with *A*); and (*D*) bright field. (Images intensified with Zeiss/Venus TV-3 video camera, photographed from video monitor with Tri-X film developed with Acufine.) 40x Neofluor objective.

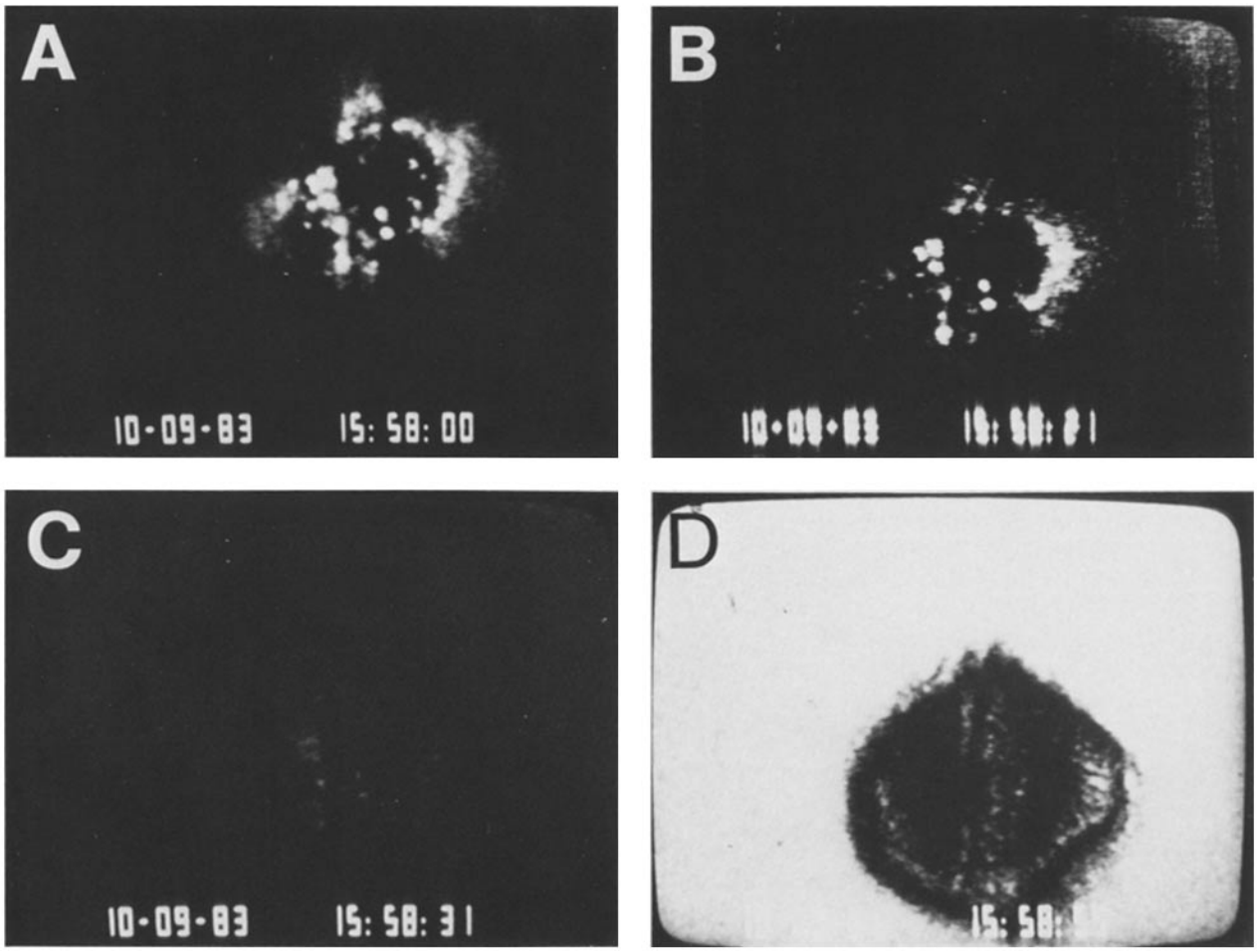


FIGURE 4 Co-localization of endogenous fluorescence with bioluminescence stimulated by NH_4^+. Legend and procedure as in Fig. 3, except that exposure times for *A* and *C* are comparable.

found that the intensity of the endogenous fluorescence is diminished after the flash but remains in the same loci (Fig. 3*C*), as demonstrated here in an overexposed print of this panel. Finally, the bright-field picture in Fig. 3*D* is included to orient the reader as to the size and position of the cell.

Fig. 4 illustrates the same experimental protocol performed upon a cell stimulated by NH_4^+ (in this case, Fig. 4, *A* and *C*, were photographed with comparable exposures). Again, the bioluminescence and fluorescence are co-localized. Similar results were obtained with high K^+ and acetic acid (data not shown).

The fact that the particulate fluorescence is considerably diminished after exhaustive chemical stimulation (Fig. 3*A* vs *C*; Fig. 4*A* vs *C*) supports the interpretation that luciferin is the fluorescent entity: the fluorescence of dinoflagellate luciferin is known to be lost in vitro after oxidation by luciferase (2) or autoxidation by air (8). Therefore, the in vivo fluorescence data corroborates the in vitro behavior of luciferin.

We were able to measure the kinetics of the flash from a single particle by using a video analyzer attached to a strip-chart recorder (see Materials and Methods). The duration of bioluminescent flashes in vivo and in vitro has been measured to be ~100 ms (19), whereas the flash duration illustrated in Fig. 5 is ~300 ms. This discrepancy is probably due either to the lag inherent in the video camera and/or to the different methods used to stimulate the cells (i.e., chemical vs mechanical stimulation). Nonetheless, the flash kinetics of the video record are similar to those of a bioluminescent flash. Fig. 5 also illustrates the decrease of endogenous fluorescence subsequent to the flash by the difference in the baseline (fluorescence under dim excitation) before and after the flash. Occasionally we have observed multiple flashes from immobilized cells after ionic stimulation. When this happens, it appears that most of the particles contribute to each flash. Therefore, multiple flashes from a cell are not due to recruitment of a different population of particles for each flash, but each individual particle can emit multiple flashes until the bioluminescence capacity of the particle is exhausted, as is also true for *Noctiluca* microsources (9).

Autofluorescent Particles In Vitro

Previous work had characterized the scintillon as a self-contained sedimentable particle that emitted a light flash upon rapid acidification (19). When *Gonyaulax* extracts were centrifuged into a sucrose gradient, the scintillon activity co-sediments with the fluorescent particles (Fig. 6). This experiment supports the hypothesis that these fluorescent particles, which also emit the bioluminescent flash in vivo, are identical with the previously described scintillons.

Another characteristic shared by scintillons and the fluorescent particles is their ability to be recharged by luciferin at pH 8. Fogel and Hastings (11) discovered that scintillons whose activity had been discharged by acidification could be

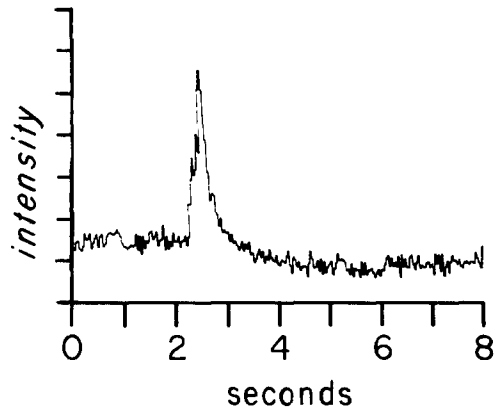


FIGURE 5 Kinetics of the bioluminescent flash from a single microsource (scintillon) in vivo (see Materials and Methods). This cell was excited with very dim light (EX 395–420) and the bioluminescent flash stimulated by Ca^{++} (20°C). Therefore, the baseline intensity before the flash is due to dim fluorescence upon which the flash is superimposed. Note that, after the flash, the intensity of the endogenous fluorescence has decreased.

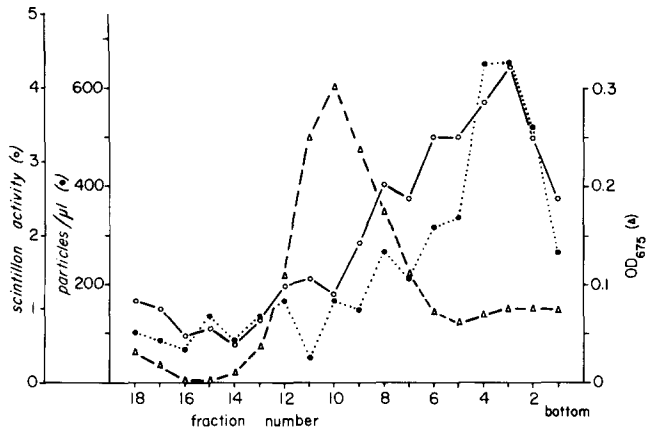


FIGURE 6 Co-sedimentation of fluorescent particles and scintillon activity in sucrose gradients. Scintillon activity (○); concentration of fluorescent particles (●); chloroplasts (△).

recharged by incubation with purified luciferin at pH 8. We find that the fluorescence of particles discharged in vitro can be similarly restored. Fig. 7 shows fluorescent particles in vitro that can be distinguished from other fluorescent material by the same criterion used with intact cells, namely fluorescence that is present under 395–420-nm excitation (Fig. 7, *b*, *d*, and *f*) but not 450–490-nm excitation (Fig. 7, *a*, *c*, and *e*). Fig. 7*b* shows two fluorescent particles in vitro (arrows). Acidification concomitantly discharges the fluorescence (Fig. 7*d*) and scintillon activity. But after readjusting the pH to 8 and incubating with luciferin, the scintillon activity was restored to 45% of the original level and fluorescent particles reappeared in the extract (two shown by arrows in Fig. 7*f*). In addition to further supporting the notion that fluorescent particles are the same as scintillons, this experiment constitutes the strongest evidence that the fluorescence of these particles is that of luciferin that is bound to the particles at alkaline pH.

Flow Cytometric Studies

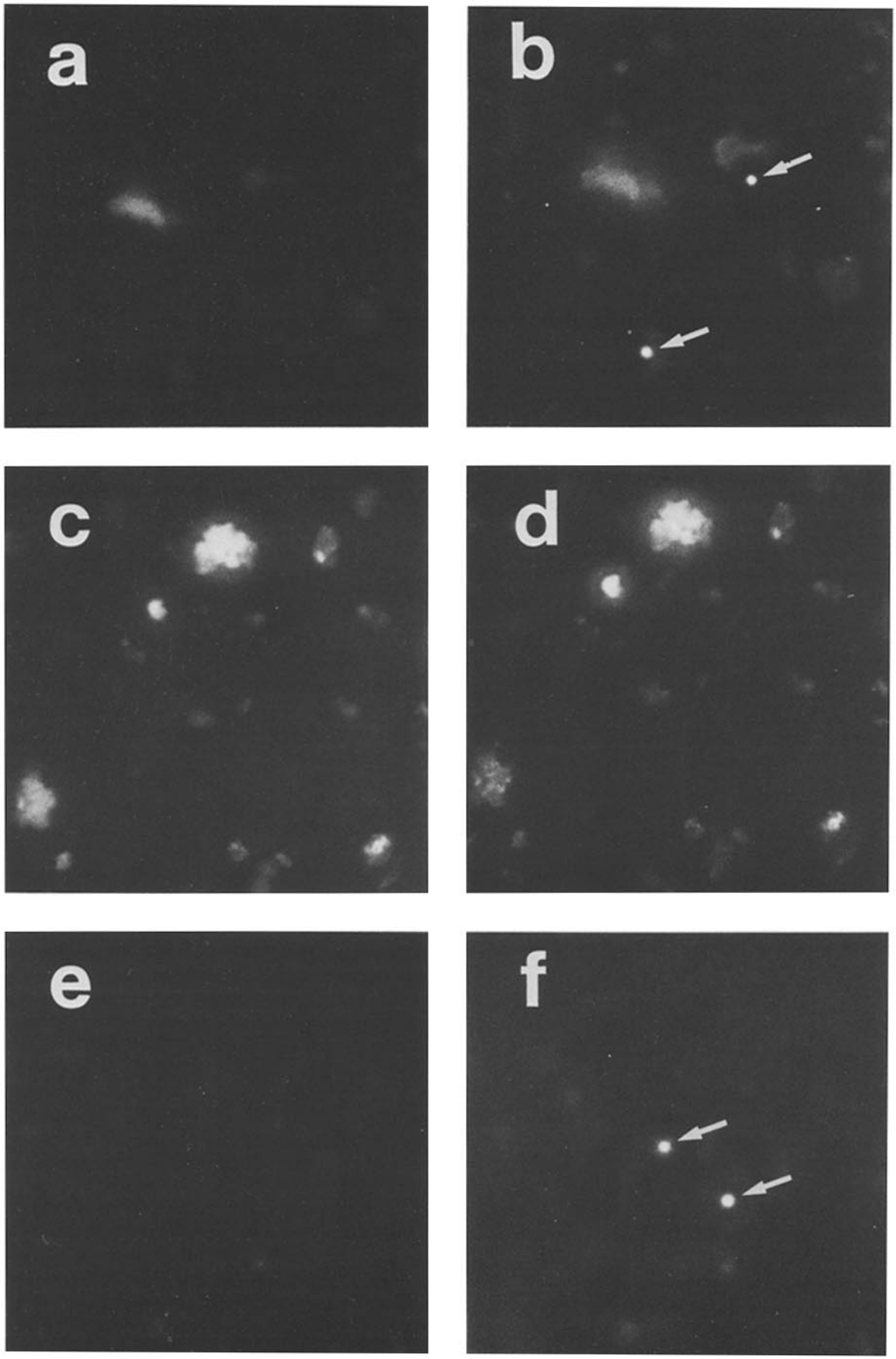
We employed flow cytometry to quantify the change of endogenous fluorescence in intact cells (*a*) after the discharge of bioluminescence and (*b*) over the course of the circadian cycle. A strict linear correspondence between cellular fluorescence and luciferin content might not be possible because of the optical properties of the cells and the possible quenching of luciferin fluorescence in the particles (scintillons). Nevertheless, measuring cell fluorescence excited by light of the appropriate wavelength should give data that reflects the luciferin concentration in vivo.

Fig. 8 illustrates "scatter plots" of cell volume vs fluorescence for *Gonyaulax* cultures. Fig. 8, *a* and *b*, are data from cultures whose fluorescent emission was monitored at 470–500 nm after excitation at 350–375 nm (a combination that is comparable to the micrographs of Figs. 1, *a*, *e*, and *h*, and 2*a*). Two fluorescent populations are observed in Fig. 8*a*: one group of small volume and low fluorescence and another group of larger volume and much greater fluorescence. The latter group is *Gonyaulax*, as can be demonstrated by filtering the culture through a 25-μm Nitex filter, which traps *Gonyaulax* cells (cell diameter, ~35 μm); only the smaller volume per fluorescence population remains. We believe these smaller fluorescent particles to be the bacteria that contaminate our *Gonyaulax* cultures. For our flow cytometry experiments, we excluded the signal from the bacteria by simply raising the triggering threshold for cell volume until no bacteria were recorded, as in Fig. 8*b*. We were thereby able to selectively measure *Gonyaulax* fluorescence in our standard cultures, and this is the way the data in Figs. 9 and 11 and Table I were collected.

Fig. 8, *c* and *d*, show two other control measurements. The cells in Fig. 8*c* were excited with 475–495-nm light and fluorescence emission monitored at 505–545 nm (comparable to the micrographs of Figs. 1, *b* and *f*, and 2, *b* and *e*). Again, both the bacteria (i.e., the cluster of many smaller volume cells) and *Gonyaulax* (i.e., the few large volume cells above the bacterial cluster) are fluorescent, but their intensities are not very different. This is consistent with the finding that *Gonyaulax* cells have negligible particulate fluorescence if excited at 450–490 nm (Figs. 1, *b* and *f*, and 2, *b* and *e*), and indicates that the fluorescence measured by flow cytometry is not an artifact of, for example, light-scattering. Fig. 8*d* depicts the volume/fluorescence plots for a culture excited by 350–375-nm light, but monitored for fluorescence at wavelengths >580 nm. This filter combination allows chlorophyll fluorescence of *Gonyaulax* to be recorded. As expected, the larger dinoflagellate cells display more red fluorescence than the smaller bacteria that do not have chlorophyll. These observations confirm that the cell fluorescence measured in Fig. 8*b* is that of *Gonyaulax*.

To quantify autofluorescence with the flow cytometer, we collected the data from 1,000 *Gonyaulax* cells with the amplifier in a logarithmic mode and arranged into a histogram (Fig. 9). The peak of this histogram as estimated by the data analysis program was then compared to a calibration curve calculated by measuring the relative fluorescence of polystyrene beads of calibrated fluorescence intensities by the procedure of Muirhead et al. (24). This "relative fluorescence" is the quantity used in Table I and Fig. 11.

Table I shows that stimulatory treatments that exhaust bioluminescence will discharge most of the autofluorescence as well. This effect is particularly significant for cells in their subjective night phase, when the bioluminescence capacity is greatest (and also autofluorescence; see below). Hydrogen, calcium, potassium, or ammonium ions at concentrations that discharge bioluminescence also reduce cellular fluores-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c
d
e
f

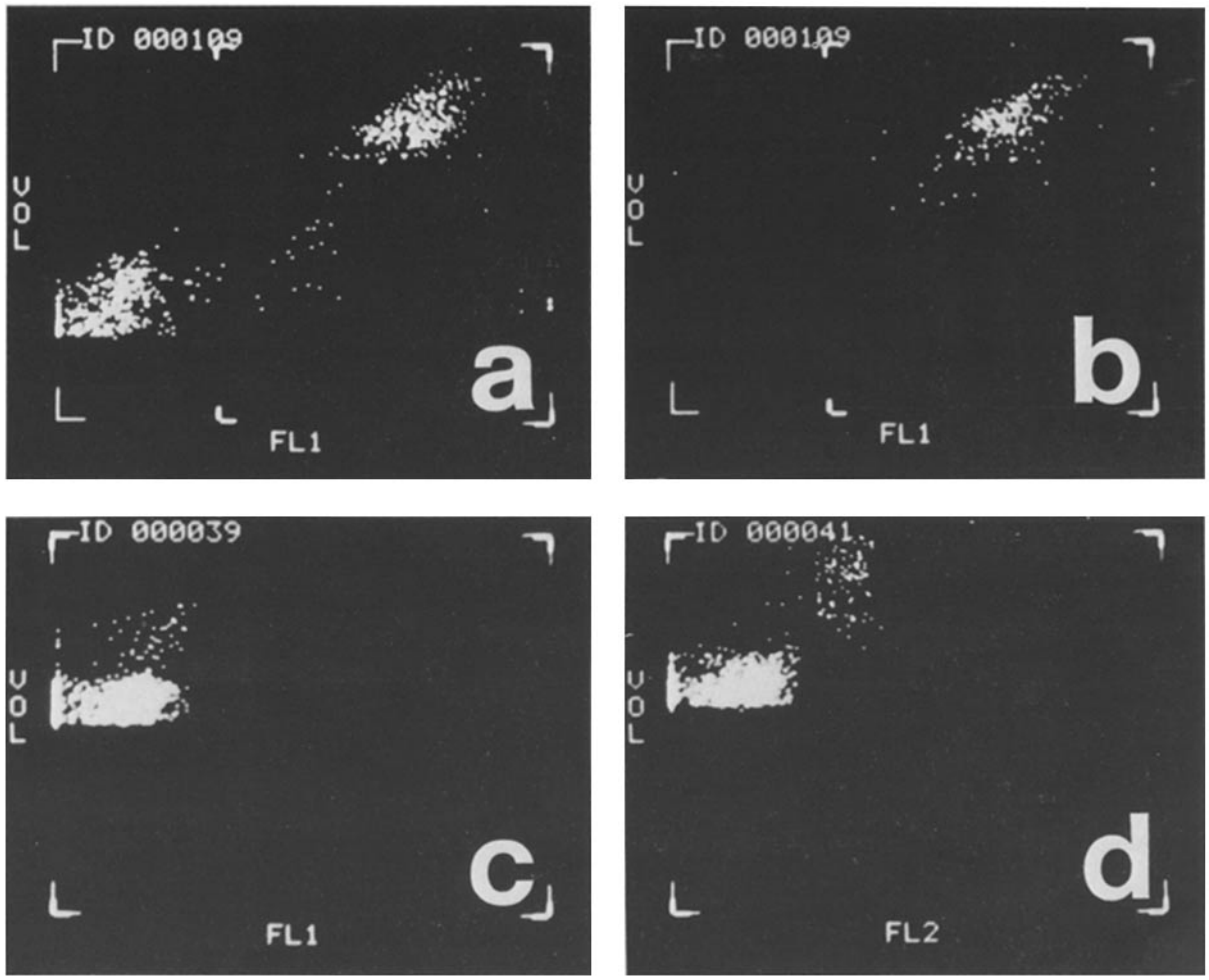

FIGURE 8 Flow cytometer scatter plots of cell volume (*VOL*) vs fluorescence (*FL*) of *Gonyaulax polyedra* cultures. *a* and *b* (EX 350–375; EM 470–500), scintillon fluorescence. *b* is the same as *a* except that triggering threshold for volume has been raised to exclude the bacteria. *c* (EX 475–495; EM 505–545), PAS-body fluorescence; the volume gain in this panel is slightly less than for the other three panels, resulting in an apparently smaller volume for the *Gonyaulax* cells, while the fluorescence gain has been adjusted to compensate for the difference in excitation intensity between EX 350–375 and EX 475–495 as can be seen from the fact that the bacterial fluorescence in *a* and *c* are comparable. *d* (EX 350–375; EM 580 long pass), chlorophyll fluorescence. The triggering threshold for volume is higher in *c* and *d* than in *a*, causing a "chopped off" distribution. Both volume and fluorescence scales are logarithmic scales.

cence by more than half, whereas equivalent concentrations of sodium ions, which do not trigger bioluminescence (15), do not affect *Gonyaulax* fluorescence. This result is comparable to that visualized in Figs. 3 and 4.

Since bioluminescence in vivo is stimulated by mechanical agitation (35), we were concerned that the measurements of luciferin fluorescence might be compromised by stimulation of the cells to emit bioluminescence during their passage through the orifice of the cytometer. This might differentially alter the amount of luciferin fluorescence at different times of day, because *Gonyaulax* cells are more sensitive to mechanical stimulation during the night phase (5). We circumvented this possible problem by using cells that had been "photoinhibited" by the exposure to continuous dim illumination; such cells are far less susceptible to mechanical stimulation of bioluminescence (4, 36). Preliminary results not included here confirmed the prediction that the autofluorescence of night phase cells as measured by flow cytometry is lower in nonphotoinhibited cells as compared with cells that have been photoinhibited as in the following experiments.

Table I includes data showing that ionic treatments have less effect upon the fluorescence of cells in their subjective "day" phase, as is also true for ionic effects on bioluminescence (14). Table I also shows that unstimulated cells in their day phase are substantially less fluorescent than those in night phase as is evident as well from the histogram plots of Fig. 9.

FIGURE 7 Restoration of fluorescence of discharged particles in vitro by luciferin. *a* and *b*, *c* and *d*, and *e* and *f* are representative, but not identical fields. *a* (EX 450–490) and *b* (EX 395–420), control particles in vitro (arrows) before discharge by acidification. *c* (EX 450–490) and *d* (EX 395–420), disappearance of fluorescent particles after acid discharge (pH readjusted to 8.0 for micrograph). *e* (EX 450–490) and *f* (EX 395–420), reappearance of fluorescent particles (arrows) after incubation of same extract with luciferin at pH 8 (incubated for 1.5 h at 20°C, then 2 h at 0°C). Fluorescence that appears under both EX 450–490 and EX 395–420 is mostly that of chloroplasts, while that visualized only under EX 395–420 is that of the scintillons. Tri-X film with Acufine development. 100x objective.

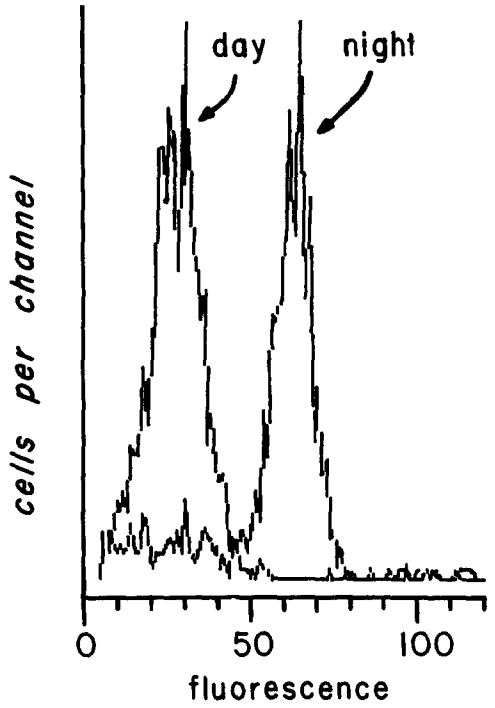

FIGURE 9 Histogram plots of *G. polyedra* fluorescence from cells at day and night phase in constant light. Ordinate is "cells per channel" in relative linear units, while "channel" on the abscissa refers to a given fluorescence intensity measured by the cytometer's amplifier in the logarithmic mode. 1,004 cells contributed to the "day" histogram, while the "night" histogram is the distribution of 998 cells. This experiment is the same as the day and night controls in Experiment 2 of Table I.

TABLE I

Effect of Different Treatments on Fluorescence of "Night" and "Day" Phase Cells in Constant Light

	Relative fluorescence	
Treatment*	Expt. #1‖	Expt. #2§
Night cells (ct 17)		
Control	100	100
Na^+	100	98
K^+	52	33
NH_4^+	44	42
Acid	44	29
Ca^{++}	47	42
Day cells (ct 7)		
Control	38	38
Na^+	39	38
K^+	28	27
NH_4^+	30	27
Acid	33	29
Ca^{++}	34	31

ct, circadian time.

* Final concentrations of ionic treatment: 150 mM NaCl, 150 mM KCl, 100 mM NH_4Cl, 33 mM acetic acid or 150 mM $CaCl_2$. Cell fluorescence was measured ~10 s after the ionic stimulation.

‖ Same experiment as depicted in Fig. 11.

§ Same experiment as depicted in Fig. 9.

The daily variation in cellular fluorescence is easily visualized in Fig. 10, which contrasts the intensity of particulate fluorescence from cells in the subjective night vs day, which were focused at the cell surface (Fig. 10, *a* and *c*) and the cell periphery (median optical section), where the night/day difference is most obvious (Fig. 10, *b* and *d*). As seen in Fig. 10, *a* and *c*, the number of particles per cell appears to remain rather constant throughout the circadian cycle, whereas their intensity changes. The daily modulation of *Gonyaulax* fluorescence correlates with that of extractable luciferin (4).

This daily change of autofluorescence is controlled by the endogenous circadian clock, as illustrated in Fig. 11. This experiment shows that *Gonyaulax* fluorescence is rhythmically modulated and persists for at least 48 h under constant environmental conditions. The times of greatest fluorescence coincide with greatest capacity to bioluminesce (subjective night, CT 12 corresponds with dusk). Calcium discharges fluorescence to a background level which might itself be rhythmic (but if so, its amplitude is very small). The magnitude of the oscillation in total cell fluorescence is at least 2.5-fold; if the background defined by the fluorescence remaining after Ca^{++} discharge is subtracted, the rhythm's amplitude may be as much as 25-fold. Thus, the circadian clock directs substantial changes in luciferin content as can be measured by the flow cytometer in vivo.

DISCUSSION

Compartmentalization of Bioluminescence Components

Bioluminescence of *Gonyaulax* originates from subcellular particles that are fluorescent. These particles appear to be the same as the in vitro scintillons because they co-sediment in sucrose gradients and have the same characteristics of recharging by luciferin in vitro. Consequently, scintillons do not seem to be an artifact of extraction, especially since the appearance of the fluorescent particles in vitro matches that in vivo.

The fluorescence of these particles can be attributed to luciferin because (*a*) their fluorescence excitation/emission characteristics are comparable to luciferin, (*b*) their fluorescence is lost after exhaustive bioluminescent emission, as is the fluorescence of luciferin, (*c*) the fluorescence intensity undergoes a daily oscillation (as does luciferin), and (*d*) the fluorescence of discharged particles can be restored in vitro by adding luciferin at pH 8. Therefore, as judged by its fluorescence, the vast majority of luciferin is associated with these cortical particles in vivo. This observation implies that luciferin binding protein (32) is also associated with the particles (20). As with many chromophores, the fluorescence quantum yield of luciferin may be different when protein bound (3), and this effect may differ depending on the protein.

Unlike luciferin, the intracellular distribution of the bioluminescence enzyme luciferase is unclear. Some portion of luciferase is definitely associated with scintillons (20), but the reported relative proportion of luciferase in the scintillon vs soluble pools varies considerably (19, 23, 37). This reported variability certainly derives in part from differences in extraction procedures and the time of the circadian cycle chosen for extraction, but it has been difficult to eliminate extraction artifacts when the only assay of the enzyme's localization has been its activity in vitro. Resolution of this issue may have to wait for immunocytochemistry of luciferase at the ultrastructural level. In the meantime, it is important to note that the localization of a single component of the bioluminescence reaction—namely, luciferin—will result in localized bioluminescent emission irrespective of whether luciferase is distributed throughout the cytoplasm or is restricted to the scintillons in vivo.

Cellular Physiology of Gonyaulax Flashing

Fogel and Hastings (11) have suggested that the mechanism of scintillon flashing involves a local acidification that both

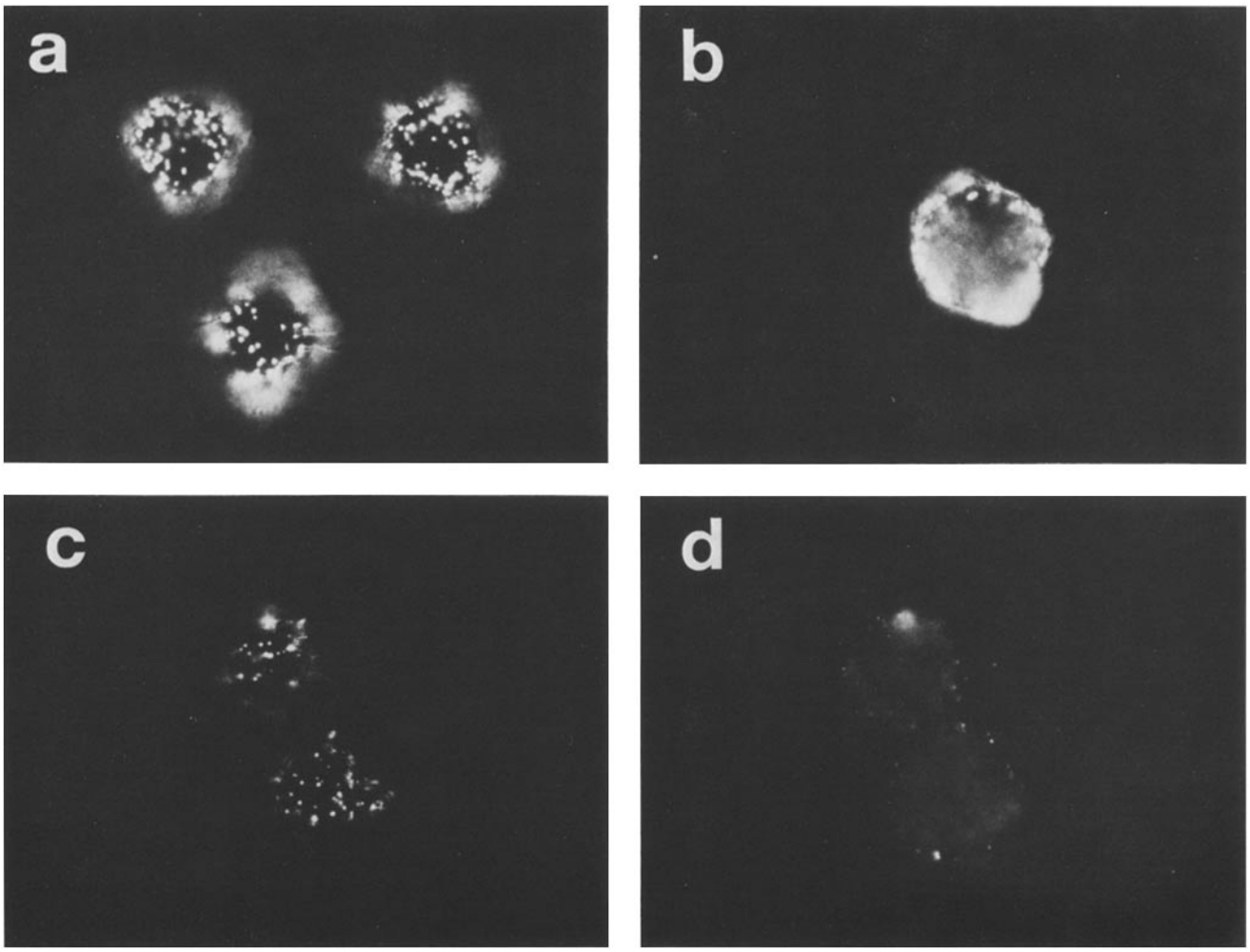

FIGURE 10 Endogenous fluorescence of *G. polyedra* cells in night and day phases of the circadian cycle (all photos: EX 395–420; EM 450–560). Cells previously synchronized in a light/dark cycle were placed in continuous dim light at dawn (ct 0). *a* (surface focus) and *b* (peripheral focus) are photos of night-phase cells after 18 h in dim light (ct 18). *c* (surface focus) and *d* (peripheral focus) are photos of day-phase cells after 27 h in dim light (ct 3). Ektachrome 160 film. 40x Neofluor objective.

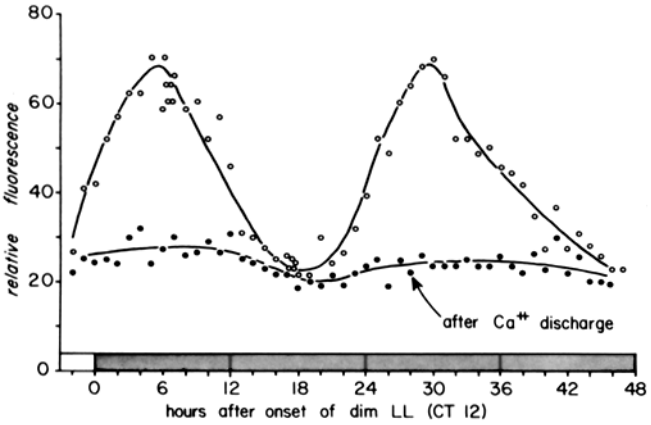

FIGURE 11 Circadian variation in endogenous fluorescence of *G. polyedra* as measured by flow cytometry. Cells previously synchronized in a light/dark cycle were placed in continuous dim light (LL) at dusk (ct 12). The relative fluorescence (EX 350–375; EM 470–500) of cells at 1-h intervals was measured before and after the discharge of bioluminescence with 150 mM $CaCl_2$. This experiment was performed at 25°C and is the same as Experiment 1 in Table I.

releases luciferin from its binding protein and activates luciferase, resulting in light emission. That protons are the active agent is indicated by the kinetic similarity of flashes in vivo and those elicited in vitro by a rapid pH drop and by the fact that no other ion provokes light emission from scintillons (19). A more specific model based on the properties in vitro of luciferase and luciferin binding protein (20) was later proposed (18).

How might such a proton flux result from mechanical stimulation (shear) of the cell surface? Hamman and Seliger (14, 15) have proposed that turbulence causes a depolarization of the plasma membrane—a signal that is ultimately transduced into the acidification. They marshal evidence that calcium ions are the depolarizing cation, while the subsequent steps in the triggering pathway are unclear. However, since neither calcium ions or Ca^{++}/calmodulin will evoke a flash from scintillons in vitro (C. H. Johnson, unpublished observations), unidentified intermediate steps must exist.

In the cases of the bioluminescent dinoflagellates *Noctiluca* and *Pyrocystis*, stimulation depolarizes the tonoplast, which appears to allow a proton flux from the central vacuole into the peripheral cytoplasm (25, 38). This cytoplasmic acidification probably triggers the flash from the luminescent microsources (9, 39) by the same mechanism proposed for *Gonyaulax* (11).

Although it is tempting to conclude that the scenario for flash stimulation will be the same for *Gonyaulax* as for *Noctiluca* and *Pyrocystis*, the organization of these cells is

quite different. In particular, *Noctiluca* and *Pyrocystis* have a large central vacuole, whereas *Gonyaulax* has a much smaller peripheral vacuole of unknown acidity. In none of these cells has the proximity of scintillons/microsources to vacuoles (which are the putative source of acid) been carefully documented. One alternative to the *Noctiluca* model would be that *Gonyaulax* scintillons might be membranous vesicles enclosing an acidic milieu that in vivo is the proton source that triggers luminescence. Therefore, caution is advised against a brash amalgamation of the evidence from these disparate dinoflagellates. Undoubtedly, ultrastructural identification of *Gonyaulax* scintillons and their relationship to other organelles will help refine our hypotheses concerning the cellular physiology of flashing.

Clock Control of Bioluminescence

The capacity of *Gonyaulax* cells to emit light is a function of the time of day; the endogenous circadian clock controls this modulation (17). Understanding the means by which the clock regulates this "driven" phenomenon is a major objective of our strategy to uncover the mechanism of the clock itself. In the case of bioluminescence capacity, the clock changes the intracellular content of each component of the reaction: luciferase (22), luciferin (this paper and reference 4), and probably luciferin binding protein as well (32). Although such a mechanism seems wasteful, it might be that for circadian clocks—a cellular event with a relatively long time-constant—turnover of components may prove to be a common mode of regulation (22).

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