The ultrastructural localization of luciferase in three bioluminescent
dinoflagellates, two species of *Pyrocystis*, and *Noctiluca*, using anti-
luciferase and immunogold labelling

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Summary

In order to discover the intracellular location of luciferase in dinoflagellates, sections from a number of species were treated with a polyclonal anti-luciferase and the bound antibody was visualized at the electron-microscope level by indirect immunogold labelling.

In two species of *Pyrocystis* and in *Noctiluca*, as in *Gonyaulax*, antibody became bound to dense vesicles, which correspond in size and position to light-emitting bodies detected in previous work. These vesicles resemble microsomes, are bounded by a single membrane and sometimes project into the vacuole.

Unexpectedly, the trichocysts of *Gonyaulax* and *Noctiluca* and the related mucocysts of *Pyrocystis* also bound the antibody. This cross-reaction seems quite independent of bioluminescence, since the trichocysts of the non-luminous *Cachonina* also reacted positively. The possibility is discussed that a protein, different from luciferase but having some antigenic similarity, is present in trichocysts and related organelles.

Key words: bioluminescence, dinoflagellates, immunocytochemistry, *Noctiluca*, *Pyrocystis*.

Introduction

The fact that bioluminescence in the dinoflagellate *Noctiluca miliaris* is emitted from intracellular microsources has been known since the observations of Quatrefages (1850). Eckert & Reynolds (1967) showed that these microsources were identical to small fluorescent particles in the peripheral cytoplasm of this organism. Recently these observations have been extended to *Pyrocystis fusiformis* by Widder & Case (1982) and to *Gonyaulax polyedra* by Johnson et al. (1985), using the light microscope.

Further investigation of the bioluminescent microsources requires the use of the electron microscope because of their small size. Theoretically, the microsources could be identified by binding of anti-luciferase and secondary immunogold labelling. A preliminary paper describing the use of this technique in *G. polyedra* (Nicolas et al. 1985b) reported labelling of two organelles, dense vesicles and the capsule of the trichocyst. Trichocyst labelling was unexpected and intriguing since the recognized function of trichocysts is the rapid ejection of a protein filament. Furthermore, only the capsule of the trichocyst was labelled, never the core protein.

In order to investigate this problem further at the electron-microscope level, we have used the same anti-luciferase and general method as Nicolas et al. (1985b) in four other dinoflagellates: *P. fusiformis* of which the morphology and microsources have been studied at the light-microscope level by Sweeney (1982a,b) and Widder & Case (1982), another species of *Pyrocystis*, *P. noctiluca*, the non-photosynthetic, bioluminescent dinoflagellate *N. miliaris*, and a small non-bioluminescent dinoflagellate structurally different from any of the bioluminescent species discussed above, *Cachonina ildefina* (Herman & Sweeney, 1976). Here we report the results of these studies and discuss possible interpretations.
Materials and methods

Materials

Pyrocystis fusiformis (W. Thomson) Murray was isolated by one of us (BMS) from the Halmahera Sea, S.E. Asia. P. noctiluca Murray and Cachonina ildefonsina Herman and Sweeney were isolated from the Santa Barbara Channel by BMS. A culture of bioluminescent Noctiluca miliaris Surire was kindly supplied by Dr Gotram Uhlig of the Meeresstation, Helgoland, West Germany.

P. fusiformis and P. noctiluca were cultured in 1/2, an enriched sea water medium (Guillard & Ryther, 1962), omitting silicon, in a 12 h light, 12 h dark cycle at 20 °C and 800 µW cm⁻² white light (G.E. Cool White fluorescent lamps). Cachonina was grown in 1/2 in constant light, 1200 µW cm⁻², 23 °C or in the same conditions as Pyrocystis. Noctiluca was cultured at 20 °C and low irradiance (80 µW cm⁻²) in 85 % salinity sea water sterilized by heating to 80 °C for 1 h on 2 successive days, to which was added as food a marine Chlamydomonas sp. isolated in New Guinea by B. M. Sweeney. Irradiances were measured with an Optron (United Detector Technology, Inc.).

Both Pyrocystis species were harvested by filtration through Nitex nylon mesh (25 µm pores) in the middle of the light period and the middle of the dark period. One culture of P. fusiformis was irradiated (1200 µW cm⁻²) for 2 h before harvesting at midnight to inhibit mechanical stimulation of bioluminescence during harvesting and the consequent loss of luciferin (Bode et al. 1963). Noctiluca was harvested by picking up individual cells with a micropipette in the middle of the light period. Cachonina was harvested by centrifugation at 540 g for 1 min. All cells were washed once with filtered sea water before fixation.

Fixation for light microscopy

After harvesting, the cells were resuspended in 5 ml of culture medium to which 1 ml of 4 % OsO₄ in distilled water was quickly added. Cells were fixed at room temperature for 1 h. Aldehydes were not used as fixatives (Herman & Sweeney, 1976). After washing in distilled water, the samples were dehydrated in 50 %, 70 %, 90 % and 100 % ethanol. Cachonina cells were pre-embedded in 1:5 % agarose before dehydration. After an overnight incubation in resin, the samples were embedded in L. R. White (hard grade) acrylic resin embedding medium in closed gelatin capsules and polymerized under N₂ at 55 °C during 3 days.

Antibody labelling and electron microscopy

An immunogold staining method was used for antibody labelling of sections (De Mey et al. 1981). The sections were collected on nickel grids, dried and prepared for antibody labelling. All the different steps of the labelling procedure were carried out in the following buffer: 10 mM-Tris·HCl (pH 8.2), 150 mM-NaCl, 0·1 mM-EDTA, 3 % bovine serum albumin. The grids were put successively in the following solutions at room temperature: 10 min in buffer, 20 min in buffer containing normal rabbit serum (20 µl ml⁻¹), then in the buffer containing the anti-luciferase antibody at a concentration of 20 µl ml⁻¹. The antibody was DEAE-purified from serum (7 mg ml⁻¹). The incubation was carried out at 4 °C overnight in a humid atmosphere.

After thorough washing with the buffer, the grids were incubated in the second antibody solution, a goat anti-rabbit antibody labelled with colloidal gold particles, 40 nm in diameter (Janssen Pharmaceutica, GAR-G40) (De Mey, 1983). This gold-labelled antibody was diluted 1/50 or 1/20 with the antibody buffer and applied to the grids for 2 h at room temperature. After washing with buffer, the grids were rinsed with distilled water and dried before staining with uranyl acetate in water for 20 min, then with lead citrate for 20 min. Sections were observed with a Philips EM 300 microscope.

Control sections were treated in the same way except that the primary antibody against luciferase was replaced with normal rabbit serum.

Testing for luciferase in Cachonina

In order to test for the presence of luciferase in Cachonina, 10° cells were harvested by centrifugation at 2130 g at 4 °C and broken in 50 mM-Tris·HCl buffer (pH 8.4) containing 10 mM-EDTA and 5 mM-mercaptoethanol by vibrating with a Science Products deluxe mixer for 1 min with glass beads 0.45–0.5 mm in diameter obtained from B. Braun Melsungen (Samuelsson et al. 1983). Cellular debris was removed by centrifugation at 120 g for 2 min. The supernatant was assayed without additions and in combination with luciferin extracted from G. polyedra or P. fusiformis and heated at 95 °C for 1 min to inactivate luciferase. Assay mixtures were those described by Schmitter et al. (1976) for measuring soluble and particulate bioluminescence (see heading of Table 2).

Results

It is clear from our results that the technique of anti-luciferase binding visualized by a gold-labelled secondary antibody can be used to identify intracellular luciferase and visualize its location. Clear-cut labelling of specific organelles was seen by electron microscopy in sections of all four dinoflagellates treated with anti-luciferase, while labelling was slight and random in control sections in the absence of anti-luciferase. In P. fusiformis, many dense vesicles bounded by a single membrane were labelled (Fig. 1 A, C), while control
sections were not labelled (Fig. 1B). These vesicles were not always spherical, sometimes appearing oval or pear-shaped. The longest dimension was about 0.9 μm in cells fixed both at noon and at midnight (Table 1). Some of the labelled dense vesicles protruded into the vacuole (Fig. 1D) and others clearly did not (Fig. 1C). These dense vesicles resemble microsomes. Their size and position in the cell suggest that they are the bioluminescent microsources.

In *P. fusiformis*, mucocyst organelles were also clearly labelled, particularly in the neck region of the capsule (Figs 2A, 3A). Mucocysts in both species of *Pyrrocystis* closely resemble the mucocysts observed in *Kofoidinium* (Cachon et al. 1974; Hausmann, 1978) or

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**Fig. 1.** Immunogold labelling of dense vesicles and mucocysts in *P. fusiformis*. A. General view. ×15 000. B. Detail of a dense vesicle. ×38 000. C. Control section treated with normal rabbit serum, no anti-luciferase (note gold particles on the cell wall). ×28 000. D. Dense vesicle projecting into the vacuole. ×38 000. d, Dense vesicles; g, gold particles, 40 nm in diameter; t, mucocysts; v, vacuole. A,C, cell fixed at noon; B,D, cell fixed at midnight; D, exposure to 2 h light before fixation. Bars, 0.5 μm.
Table 1. Dimensions of gold-labelled dense vesicles in bioluminescent dinoflagellates

<table>
<thead>
<tr>
<th>Organism</th>
<th>Diameter (µm)</th>
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<tbody>
<tr>
<td><em>P. fusiformis</em></td>
<td></td>
</tr>
<tr>
<td>Day</td>
<td>0-90 ± 0-15 (n = 16)</td>
</tr>
<tr>
<td>Night</td>
<td>1-11 ± 0-47 (n = 10)</td>
</tr>
<tr>
<td>Night*</td>
<td>0-91 ± 0-14 (n = 12)</td>
</tr>
<tr>
<td><em>P. noctiluca</em></td>
<td></td>
</tr>
<tr>
<td>Day</td>
<td>0-79 ± 0-10 (n = 11)</td>
</tr>
<tr>
<td><em>P. lumula</em>†</td>
<td></td>
</tr>
<tr>
<td>Day and night</td>
<td>0-74 ± 0-20 (n = 6)</td>
</tr>
<tr>
<td><em>G. polyedra</em></td>
<td></td>
</tr>
<tr>
<td>Day and night</td>
<td>0-55 ± 0-14 (n = 16)</td>
</tr>
<tr>
<td>Extracts</td>
<td>0-49 ± 0-08 (n = 8)</td>
</tr>
</tbody>
</table>

*These night cells were exposed to light (1200 µW cm⁻²) for 2 h before harvesting in order to inhibit mechanical stimulation of bioluminescence and thus prevent loss of luciferin during harvesting.
†M.-T. Nicolas (unpublished data).

In the stalked euglenid, *Colacium calvum* (Willey, 1984), and are clearly related in structure to trichocysts (Sweeney & Bouck, 1966), which are labelled with anti-luciferase in *G. polyedra*, as reported previously (Nicolas et al. 1985a, b).

In *P. fusiformis*, bioluminescence can be seen during the day as a glow in the centre of the cell on acid stimulation of cells (Sweeney, 1982a). The source of this glow has been identified as a spherical group of particles, orange in colour. At the electron-microscope level, the individual particles appear to be blunt and rodlike in shape. These particles were found in the centre of the cell in immunogold-labelled sections of *P. fusiformis* fixed at midday and in the periphery in cells fixed at night, but were clearly not labelled (Fig. 2B).

In *P. noctiluca*, both dense vesicles and the neck part of mucocyst capsule were specifically labelled after treatment with anti-luciferase, as in *P. fusiformis* (Fig. 3A,B). The mucocysts closely resembled those of *P. fusiformis* (Fig. 2A). No other internal structures were decorated with gold particles.

In *N. miliaris*, we found labelled dense vesicles in the peripheral cytoplasm, very much like those in *Pyrocystis*. They were somewhat rarer than in *Pyrocystis*, as might be expected from the fact that there is very little peripheral cytoplasm in *Noctiluca* (Sweeney, 1978). Trichocyst capsules (Fig. 3C) were also labelled, as they are in *G. polyedra*.

Since trichocysts and mucocysts retained colloidal gold particles in all the bioluminescent dinoflagellates that have been studied by immunogold labelling, it was of interest to examine by this technique a non-bioluminescent dinoflagellate containing trichocysts. For this purpose we chose *C. illedefina*, a small marine dinoflagellate the ultrastructure of which has been studied (Herman & Sweeney, 1976). The trichocysts of *Cachonina* resemble those of *Gonyaulax*. Their

![Fig. 2. Other structures in *P. fusiformis*. A. Mucocyst with gold label on the neck of the capsule. ×22 400. B. Unlabelled orange particles in a cell fixed at noon. ×22 400. D, Dense vesicle; m, microtubules; o, orange particles; t, mucocysts. Cells fixed at noon. Bars, 0-5 µm.](image-url)
capsules were clearly labelled in anti-luciferase-treated cells (Fig. 4A). Vesicles containing filaments thought to be flagellar hairs (Fig. 4C) were also consistently labelled with gold particles in Cachonina. No specific labelling was found in control sections treated with normal rabbit serum (Fig. 4B).

Cachonina cells were tested for bioluminescence during the night phase, from cultures either growing in a light–dark cycle or maintained in constant light. No bioluminescence was detected following acid stimulation. The possibility that Cachonina contains luciferase but not luciferin was investigated by supplementing extracts of Cachonina with luciferin-containing heated crude extracts of P. fusiformis or Gonyaulax. No bioluminescence was detected (Table 2), although the luciferin-containing preparations did augment the bioluminescence of crude extracts of Pyrocystis.

Fig. 3. Gold-labelled structures in P. noctiluca and N. miliaris. A. P. noctiluca, labelled dense vesicles, and mucocyst capsules. ×23 100. B. Two labelled dense vesicles in P. noctiluca, one projecting into the vacuole. ×17 100. C. Trichocyst gold-labelled in N. miliaris. ×36 100. d, Dense vesicle; t, mucocyst or trichocyst; v, vacuole. Cells fixed at noon. Bars, 0.5 μm.
Discussion

There is now good evidence that bioluminescence in dinoflagellates in vivo is localized in microsources seen in the light microscope in *N. miliaris* (Eckert & Reynolds, 1967), in *P. fusiformis* (Widder & Case, 1982) and in *G. polyedra* (Johnson *et al.*, 1985). The most reasonable interpretation of our results is that the dense vesicles, gold-labelled in *P. fusiformis* and *P. noctiluca* as in *G. polyedra*, are the microsources seen in the light microscope. Their size is in the right range, 0.5–1 μm (Table 1). Their peripheral location, usually on or near the vacuolar membrane, is consistent with this interpretation. Microsources are known to flash in response to decreased pH, as well as to mechanical stimulation (Reynolds *et al.*, 1969; Sweeney, 1982a; Widder & Case, 1982). The vacuole of *Noctiluca* has a pH of about 3.5 (Nawata & Sibaoka, 1979), and that of *Pyrocystis* may also be acidic, thus there is a potential source of hydrogen ions in the vacuole. Furthermore, particles from *G. polyedra* that are found in the active bioluminescent fractions of

Fig. 4. *C. ildefina.* A. Gold-labelled trichocyst. ×36 100. B. Control section treated with normal rabbit serum, no antiluciferase. ×36 100. C. Gold-labelled hair filaments. ×36 100. *h*, Hairs in vesicle; *t*, trichocyst. Bars, 0.5 μm.

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Organism  | Volume (ml) | Additions (ml) | Bioluminescence (relative) |
--- | --- | --- | --- |
Assay mixture I for activity of soluble fraction: | | | |
_Cachonina_ | 0-2 | — | <0-001 |
0-25 | 0-25, _Pyrocyslis_, heated | — | <0-001 |
_Pyrocyslis_ | 0-25 | — | 5-5 |
Heated | 0-25 | — | <0-001 |
Assay mixture II for activity of particulate fraction: | | | |
_Cachonina_ | 0-2 | — | <0-001 |
0-5 | — | — | <0-001 |
0-5 | 0-5, _Pyrocyslis_, heated | — | <0-001 |
_Pyrocyslis_ | 0-25 | — | 0-15 |
0-25 | 0-25, _Pyrocyslis_, heated | — | 0-43 |

Luciferin was supplied from a crude extract from _P. fusiformis_ heated at 95°C for 1 min to inactivate luciferase, then cooled at once on ice. Assay mixture I contained 0.2 M-sodium phosphate buffer, pH 6.4, 0.25 mM-EDTA and 0.1 mg ml⁻¹ bovine serum albumin. Assay mixture II contained 0.2 M-sodium citrate, pH 5.2.

Sucrose gradients are about the same size as the dense vesicles from this species (Table 1), are fluorescent and label when fixed and treated with anti-luciferase and gold-labelled goat anti-rabbit antibody (Nicolas et al. 1985a). It is not surprising that antibody raised against luciferase from _G. polyedra_ should recognize luciferase from other species of dinoflagellates, because the luciferases from all the dinoflagellates tested cross-react with all the luciferins.

The labelling of trichocysts in all the dinoflagellates studied is intriguing. Is luciferase present in more than one organelle? The labelling of the trichocysts in the non-bioluminescent _Cachonina_ indicates that if present, luciferase is not active in trichocysts. It seems unlikely that contamination by soluble luciferase can explain such specific and consistent labelling of the trichocysts in all dinoflagellates. However, it is interesting to note in this connection that two previous cytochemical studies of the trichocysts of a dinoflagellate, _Prorocentrum micans_, showed labelling of the core for DNA (Gautier & Fakan, 1980) and for actin (Livolant & Karsenti, 1982), which these authors attributed to soluble proteins adsorbed to the core protein during preparation of sections.

The antibody used in these studies recognizes only a protein or proteins of _M_, 130 000 as shown by Western blots (Johnson et al. 1984). In a recent study (C. Johnson, unpublished) antibody was bound to the isolated luciferase band, released from binding and retested by immunogold labelling of sections of _G. polyedra_. The antibody retained its affinity for both trichocysts and dense bodies in electron-microscope sections. This procedure eliminated from the antibody the determinant to the faint contaminant (_M_, 118 000) originally present in the luciferase (Dunlap & Hastings, 1981). This result thus indicates that there is not a second and completely different determinant in the antibody but that the two organelles either both contain luciferase or there is a second anti-luciferase cross-reacting protein in the trichocysts and mucocysts. However, the presence of a second different determinant, even in the affinity-purified antibody, has not been completely ruled out. This might be possible by using monoclonal antibodies against luciferase.

As mentioned above, trichocysts and mucocysts are known to be non-bioluminescent, ejectile organelles, yet their labelling is consistent in all the species studied using this particular antibody against luciferase. Although _Cachonina_ does not appear to contain active luciferase, the labelling of its trichocysts indicates that it possesses an antigenically similar protein, possibly like luciferase in structure, but lacking the active site for bioluminescence. Antigenic similarity has been demonstrated between ribulose bisphosphate carboxylase oxygenase in the chloroplasts of _Ochromonas_ and a mitochondrial protein by immunogold labelling (Lacoste-Royal & Gibbs, 1985). Evolutionary relatedness between luciferase and a trichocyst protein is a reasonable possibility that requires further investigation.

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References


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