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CELL BOUND BIOLUMINESCENCE FROM *PONTODRILUS BERMUDENSIS*, AND ITS SIMILARITIES TO OTHER EARTHWORM BIOLUMINESCENCE

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Abstract—1. *Pontodrilus bermudensis* (Acanthrodrilidae: Oligochaeta) is a small, bioluminescent, marine littoral earthworm species.

2. When properly isolated, the luminescence system is contained within (14.4 μm mean diameter) granule-filled coelomic cells.

3. These cells were previously characterized as mucocytes (Jamieson B. G. M., Wampler J. E. and Schultz M. C. (1981) Preliminary ultrastructural description of coelomocytes of the luminescent oligochaete. *Pontodrilus bermudensis* (Annelida). In *Bioluminescence and Chemiluminescence* (Edited by Deluca M. and McElroy W. D.), pp. 543–559. Academic Press, New York.)

4. The bioluminescence of these cells can be stimulated by agitation, by addition of hypotonic hydrogen peroxide and by addition of hypotonic synthetic earthworm luciferin, but hypotonicity alone stimulates little luminescence.

5. The spectrum of the bioluminescence (λ_{max} = 540 nm) matches the fluorescence spectrum of the cells and a one-to-one correlation exists between bioluminescent and fluorescent cells.

6. The data suggest that luciferin activity in *P. bermudensis* is packaged in a subcellular organelle.

7. Comparison with other bioluminescent earthworm species shows that the bioluminescent system of *P. bermudensis* is very similar to the others studied to date.

INTRODUCTION

Pontodrilus bermudensis, Beddard, is a widely distributed marine littoral species of bioluminescent earthworm. Its synonymy, world distribution and bioluminescence have been reported previously by Jamieson and Wampler (1979) and comparative biochemical data (Wampler and Jamieson, 1980) show that its bioluminescence system is very similar to that of other species. There are, however, two major differences in these previous data: *P. bermudensis* luminesced at longer wavelengths than the other species examined and it did not appear to have a cell bounded luminescence system. Previously, another luminescent species of this genus was discovered in 1936 by Kanada and Haneda (as recorded by Harvey, 1952) and studied by Richard Lynch (personal communications).

In other species the bioluminescence system is contained within large, granule-filled coelomic cells (for reviews see Jamieson, 1981; Wampler, 1981). Detailed morphological studies of the coelomocytes of various earthworm species as well as those isolated from *P. bermudensis* (Jamieson *et al.*, 1981) and this work show that the luminescent cell of *P. bermudensis* is smaller, but is similar to the bioluminescent cell of other species (Rudie and Wampler, 1978; Wampler, 1982). This cell has been previously identified and characterized as a mycocyte (Jamieson *et al.*, 1981). A careful re-examination of our previous procedures and measurements of the cytoplasmic osmolarity of *P. bermudensis* explains the failure to demonstrate cell bounded luminescence in *Pontodrilus* species. *P. bermudensis* is a marine littoral species. It is extremely salt tolerant and has high coelomic osmolarity (Jamieson, unpublished data). Previous isolation procedures used solutions which were hypotonic to the *P. bermudensis* coelomic cells, lysing most cells so that the luminescence components were then found free in solution.

This paper reports the properties and characteristics of the luminescent coelomocyte from *P. bermudensis*. When properly isolated, these cells contain the major proportion of the luminescence. The biochemical and physiological properties of this bioluminescence system are compared to those of other luminescent earthworms.

MATERIALS AND METHODS

P. bermudensis were collected at St Marks Wildlife Refuge, Florida, during April and May. The worms are found in wet sand at the high tide line under plant debris. They can be maintained in the laboratory in their native sand substrate moistened with artifical sea-water.

Preparation of *D. longa* luciferase and luciferin, spectral measurements, and the assay equipment and procedures were as previously described (Wampler and Jamieson, 1980).

To prepare suspensions of the luminescent exudate from these worms, individual specimens were placed in Instant Ocean brand artifical sea-water (34% salinity) and shocked with a hand-held magneto generator. For preparation of the washed particulate fraction, the exudate suspension was centrifuged, the pellet resuspended in artificial sea-water, recentrifuged and resuspended in a volume equal to the original volume. The supernatant fraction from the first centrifugation was saved for testing, as was the resuspended pellet.

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Stimulation experiments involved placing a 0.1 ml aliquot of the solution to be tested into a small test tube, placing the tube in the photometer housing and opening the shutter. Subsequent additions were made by injection. Agitation required that the tube be removed, agitated by a vortex mixer and then replaced. Volumes added by injection were as follows; 0.05 ml of distilled water, 0.05 ml 0.3% hydrogen peroxide in artifical sea-water, 0.05 ml of *D. longa* luciferase (0.1 units/ml) and 0.05 ml of *D. longa* luciferin (0.25 mg/ml in distilled water).

Light microscopy studies were carried out using the low-light video microscope system (Rich and Wampler, 1981) as described by Wampler (1982).

RESULTS AND DISCUSSION

Like the 12 other luminescent species examined so far by our laboratories (Jamieson and Wampler, 1979; Wampler and Jamieson, 1980; Wampler, 1982), the luminescence system of *P. bermudensis* is contained in exuded coelomic fluid. As reported previously, it is biochemically similar to that of other species, with cross-reactivity with the pure components of the *Diplocardia longa* system. However, the spectrum of the bioluminescence, even when stimulated by addition of pure *D. longa* luciferin, is unique with an emission maximum near 540 nm.

With another species, *Microscolex phosphoreus*, having a long wavelength emission maximum (538 nm), the bioluminescence spectrum is similar to the fluorescence spectrum of the suspension of washed coelomic cells. This is also the case for *P. bermudensis* (Fig. 1); however, unlike *M. phosphoreus* this spectral similarity is maintained throughout the course of a luminescence reaction. In *M. phosphoreus*, the spectrum can be changed dramatically if the luminescent cells are lysed and emission is stimulated *in vitro* by addition of dilute hydrogen peroxide (Wampler, 1982). Our previous studies of *P. bermudensis* (Wampler and Jamieson, 1981) showed that the spectral shape of peroxide and luciferin stimulated emission of *in vitro* extracts is identical and very similar to the *in vivo* spectrum.

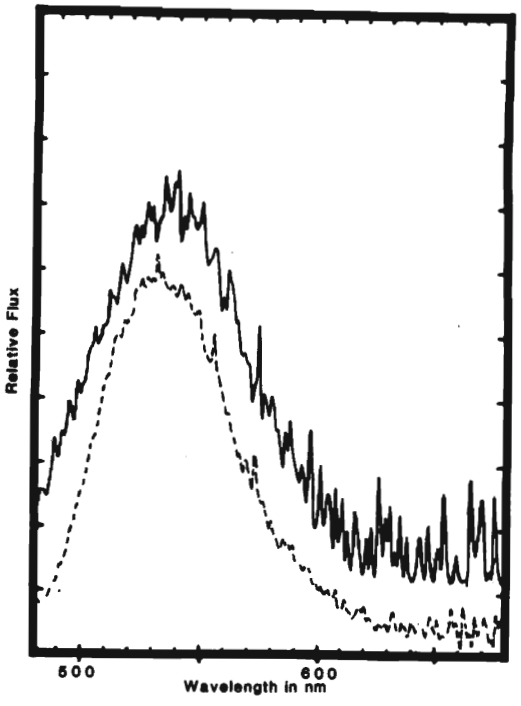

Fig. 1. Solid line—bioluminescence of fresh exudate from *Pontodrilus bermudensis* (offset for clarity). Dashed line-fluorescence emission spectrum of a suspension of freshly exuded coelomic fluid from *P. bermudensis* in artificial sea-water (excitation at 465 nm).

When the luminescing exudate is examined microscopically, much of the luminescence originates from an ill-defined viscous mass and clumps of cells held by strands of mucus. This is also the case with whole exudate in sea-water when it is stimulated to emit by addition of hypotonic hydrogen peroxide (Fig. 2). In a very few instances (Fig. 2), luminescence seems to be specifically associated with individual, granule-filled coelomic cells. However, in this case there is little or no spontaneous luminescence. This is similar to the result obtained with other species when they exude their coelomic fluid into 0.1 M potassium phosphate buffer (Wampler and Jamieson, 1980). In many of these cases, however (e.g. with *Spencerilla* and *Diplocardia* species), luminescence can be initiated simply by lowering the osmolarity of the solution by adding distilled water. With *P. bermudensis* this stimulates no luminescence even though microscopic examination shows that the granule-filled cells are indeed lysed. On the other hand, if the suspension of exudate in sea-water is agitated vigorously, strong luminescence is stimulated. Even the luminescence of the exudate suspension stimulated by addition of isotonic hydrogen peroxide can be further stimulated by agitation. When a hypotonic solution of *D. longa* luciferin is added to a small aliquot of the exudate suspension, strong luminescence occurs with only modest further stimulation by agitation. These results are shown in the temporal luminescence profiles of Fig. 3. Note the log scale on the *Y* axis.

The stimulation behavior of the sea-water suspension of *P. bermudensis* exudate contrasts with that of other species in that luciferin stimulation has, in general, been only moderate compared to stimulation by hypotonic shock and by hypotonic peroxide with these other species.

With the proper isolation conditions described above, a major portion of bioluminescence in the exuded fluid can be isolated in the pellet containing the coelomic cells by a repetitive cycle of gentle centrifugation and washing. As pointed out in the Introduction, the salinity of the isolation buffer in our previous attempt was too low. Since *P. bermudensis* is found at the high tide line of sandy ocean beaches, its cells have much higher osmolarity than those of other terrestrial earthworms. Thus, isolation of coelomic fluid using buffers appropriate for these other species presumably results in lysis of a large portion of the *P. bermudensis* coelomocytes by osmotic shock and the bioluminescent components are then found in the soluble fraction. When worms are stimulated in artificial sea-water, there is little or no bioluminescence, even though the water becomes opaque with exuded fluid and cells. As discussed above, luminescence of this suspension can be subsequently stimulated slightly by adding isotonic peroxide, greatly stimulated (around 10-fold) by agitation and equally well stimulated by addition of hypotonic

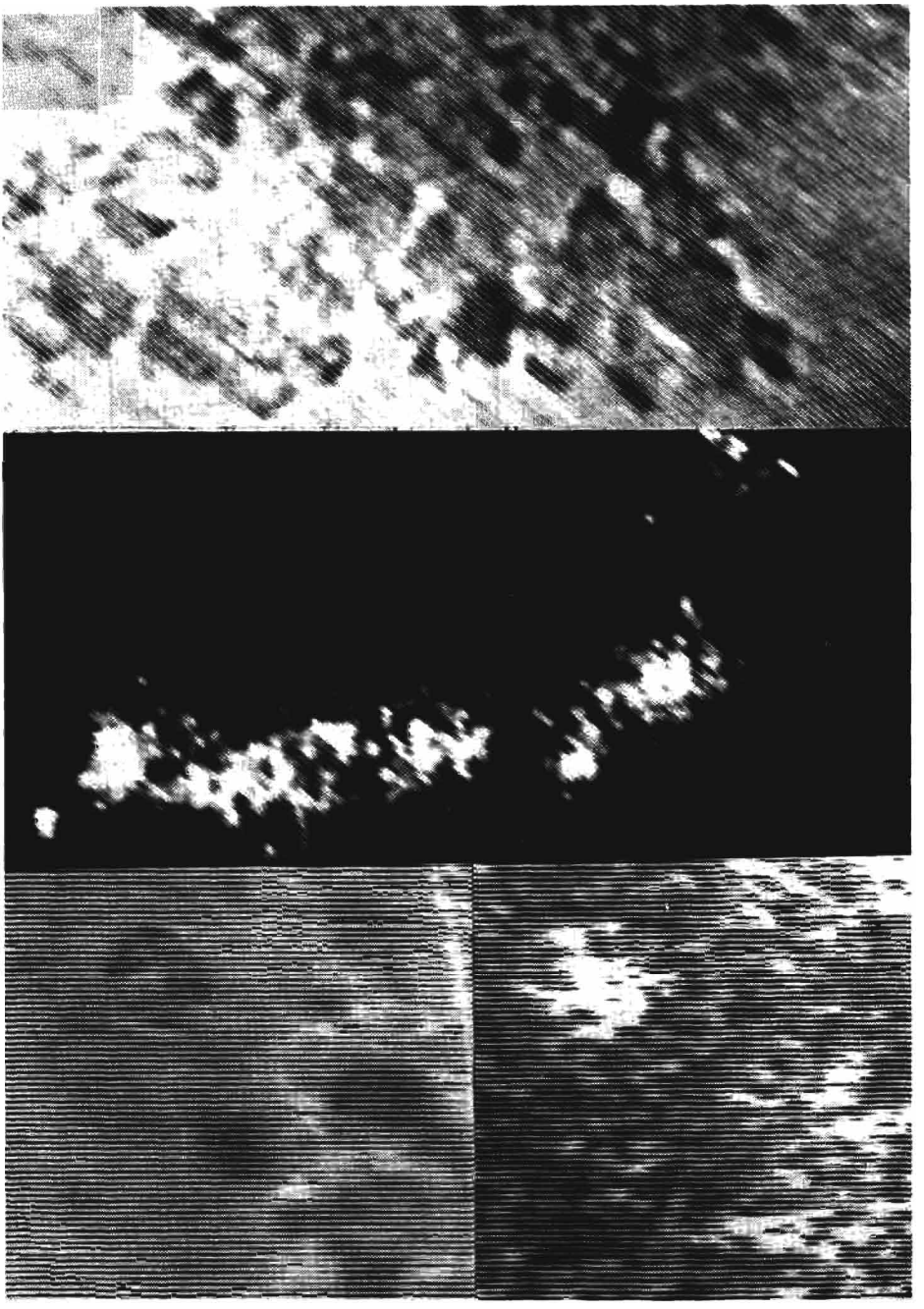

Fig. 2. Exudate in sea-water stimulated by addition of a drop of 0.3% hydrogen peroxide in artifical sea-water. The upper panel shows a bright field image and the center panel shows the bioluminescence of the same area. Lower left and right show the bright field image and bioluminescence (respectively) of an isolated, intact coelomocyte from the same preparation.

luciferin. These results are summarized in the bar graph of Fig. 4. When the particulate fraction is removed by centrifugation, the remaining supernatant is only mildly luminescent and is stimulated primarily by peroxide and very little by luciferin additions. The washed pellet, on the other hand, is highly bioluminescent, stimulated in a similar way to the exudate suspension (Fig. 4).

One implication of the experiments of Fig. 4 is that luciferin activity in *P. bermudensis* coelomic fluid is either in the soluble fraction or that it is contained in some subcellular component which is not affected by osmotic shock. If it were free in the soluble fraction, then a dilution effect would lower the stimulable bioluminescence level, and repeated washing would inhibit stimulation by agitation or peroxide addition. Osmotic shock should also give some luminescence, since the other ingredients must be present, as shown by addition of hypotonic luciferin. However, if luciferin were sequestered in some subcellular component, its release could depend more on chemical or physical alteration of that cell component than on

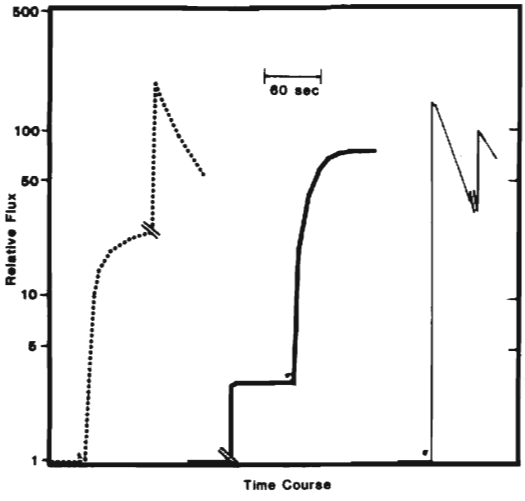

Fig. 3. Time course of bioluminescence of the exudate of *P. bermudensis* in sea-water. Dotted line—stimulation by addition of 0.3% hydrogen peroxide followed by agitation. Bold line—agitation followed by addition of 0.3% hydrogen peroxide. Thin line—stimulation by dilute luciferin (0.25 mg/ml) followed by agitation. Asterisks mark additions; double diagonal lines mark agitation.

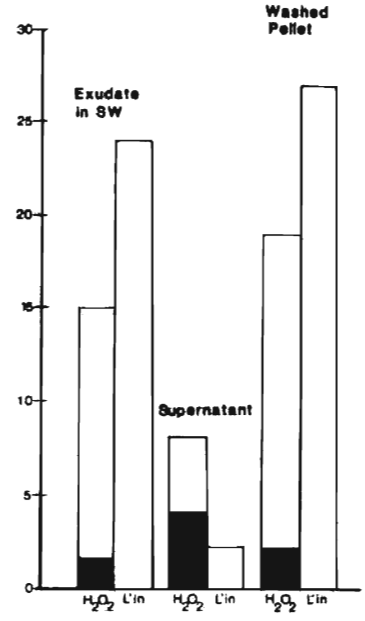

Fig. 4. Peak stimulation of sea-water suspension of *P. bermudensis* exudate. Filled bars indicate addition of 0.3% hydrogen peroxide alone. Clear bars represent peak light flux following addition of the dilute reagents followed by vigorous agitation. H_2O_2 = dilute hydrogen peroxide, L'in = synthetic *D. longa* luciferin, SW = artificial sea-water.

lysis of the cell membrane. Our tentative hypothesis, then, is that all of the components of the bioluminescence system are contained in the particulate fraction associated with *P. bermudensis* coelomic

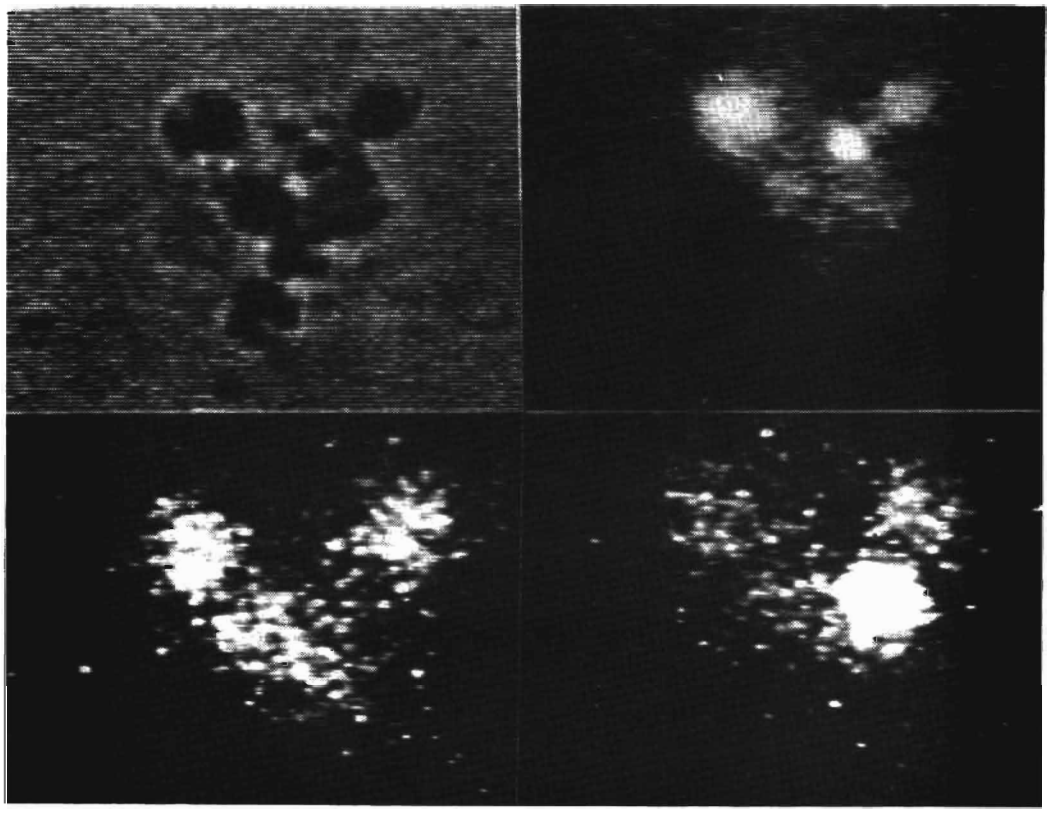

Fig. 5. Resuspended washed pellet material from *P. bermudensis* exudate showing a clump of coelomic cells. Upper left shows the bright field image while the upper right shows the fluorescence (emission at 520 nm; excitation at 465 nm). The two lower panels show two frames from the video tape record of the bioluminescence stimulated by addition of a drop of dilute luciferin to the slide.

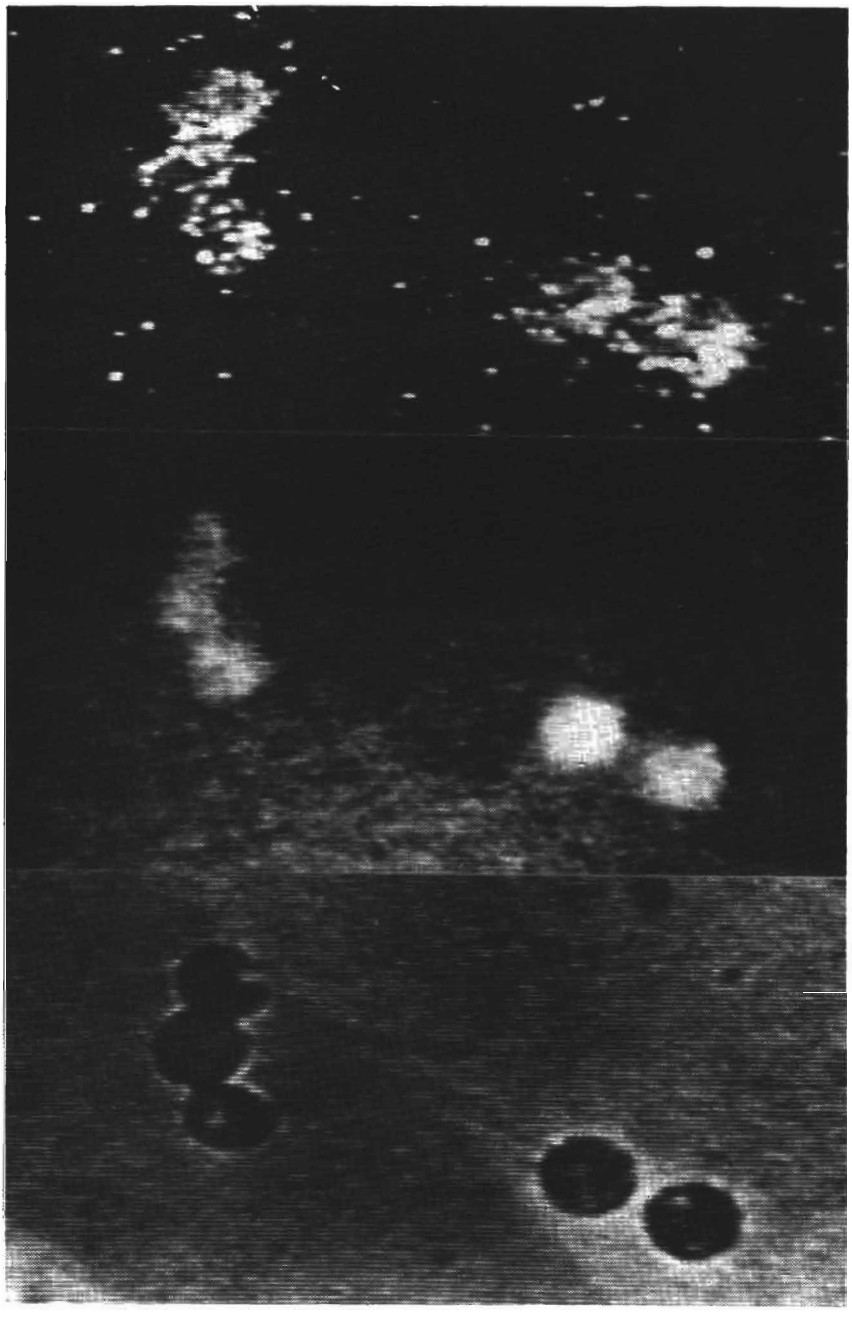

Fig. 6. Five fluorescent coelomocytes from *P. bermudensis* isolated by micromanipulation into a drop of artificial sea-water. Top—bright field image. Middle—fluorescence excited at 465 nm; emission at 520 nm. Bottom—single frame from the video tape record of their bioluminescence.

cells, and that luciferin is contained in a subcellular component which is little affected by osmotic shock.

The fluorescence of the cell suspension (Fig. 1) suggests that *P. bermudensis*, like *M. phosphoreus*, may have a fluorescent chromophore associated with the bioluminescent system. If this is the case, then bioluminescent cells should also be fluorescent. As shown in Fig. 5, this is indeed the case. The bioluminescent cells in the cell aggregate shown, which are fluorescent, are also bioluminescent when stimulated by hypotonic luciferin, but the non-fluorescent cells are not. In this figure the backlit image marks the positions of each cell in the group (frame A); the six fluorescent cells in the group are each bioluminescent when stimulated by addition of hypotonic luciferin. Each of the six lumineses at slightly different times. Two frames from the video record are shown.

Fluorescent cells can be isolated by micromanipulation, washed with artificial sea-water and then stimulated to luminesce by addition of hypotonic luciferin. This result is shown in Fig. 6. Here

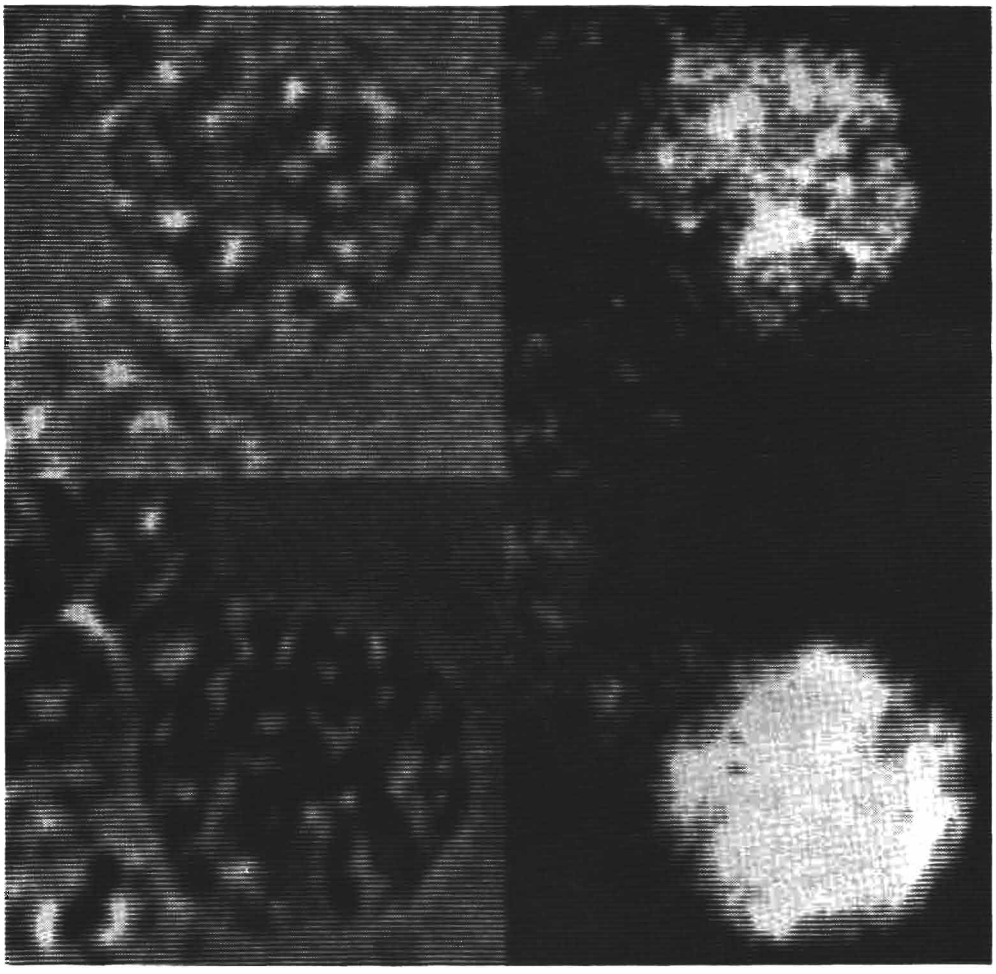

Fig. 7. Two fluorescent coelomocytes (~14 μm dia.) at high magnification. Left panels are bright field images, right panels are their fluorescence at 520 nm; excitation at 465 nm.

five fluorescent cells were isolated, and then stimulated. The correlation between fluorescence and bioluminescence is obvious.

Single cell studies confirm the cell suspension results, with luminescence stimulated more easily and more vigorously by hypotonic luciferin than by hypotonic hydrogen peroxide. In preliminary experiments we have also observed that when cells are slowly crushed by the cover slip, discrete, bright luminescence spots can be observed which are much smaller than the individual cells. This supports the hypothesis stated above that luciferin in *P. bermudensis* coelomocytes is contained in a subcellular organelle.

The luminescent coelomocyte can be easily identified by its fluorescence. Under slight cover slip pressure the cell appears to be spherical and granule-filled. The mean diameter of fluorescent, granule-filled cells is 14.4 μm. This compares with the measured diameters of mucocytes from Jamieson *et al.* (1981) of 13.9 μm. The higher resolution video micrographs of Fig. 7 show the typical fluorescent mucocyte.

The combined results of this study and our previous efforts (Jamieson and Wampler, 1979; Wampler and Jamieson, 1980; Jamieson *et al.*, 1981) show that the bioluminescence system of *P. bermudensis* is very similar to that of the other species examined to date.

Table 1. Comparative data

	D. longa	*M. phosphoreus*	*P. bermudensis*
Luminescence response	Exuded fluid from dorsal pores	Exuded fluid from mouth and anus	Exuded fluid from mouth and anus
Source of fluid	Coelome	Coelome	Coelome
Localization	30 μm granule-filled cell	27 μm granule-filled cell	14 μm granule-filled cell
Cross reactivity	H_2O_2, L'in, L'ase	H_2O_2, L'in, L'ase	H_2O_2, L'in, L'ase
TLC Rfs, L'in†			
25% EtOH in hexane	0.47	0.54	0.50
3% MeOH in $CHCl_3$	0.32	0.38	0.38
Emission maximum (nm)	500	538	540

*Abbreviations: L'in = synthetic *D. longa* luciferin, L'ase = purified *D. longa* luciferase.

†Thin layer chromatography on Eastman 6060 silica gel sheets of luciferin activity isolated by extraction of exudate with organic solvents. Activity was detected using *D. longa* luciferase and hydrogen peroxide. Spots were detected with I_2.

Comparative data for this species, *M. phosphoreus* and *D. longa* are summarized in Table 1.

Spectral differences between the various species do not appear to be caused by different luciferins but by the presence of different fluorescent entities which act as the emitter. Since no fluorescent component is found in *D. longa* coelomocytes, which matches its bioluminescence spectrum, its spectrum may be due to emission from the primary excited state species or a transient fluorophore formed during the reaction. In the case of *M. phosphoreus*, the emitter *in vivo* appears to be a fluorophore associated with the luminescent cell but one which is only loosely associated with the enzyme, luciferase. Thus when cells are lysed the loose association is lost and the emission spectrum shifts to reflect the primary excited state species with a spectrum near to that of *D. longa*. With *P. bermudensis* the emitting fluorophore appears to be more strongly associated with luciferase, so that even *in vitro* the longer wavelength emission spectrum is seen.

In all species examined to date by our laboratories, the bioluminescence system is contained in coelomic cells and is specifically associated with a large, granule-filled cell type. In at least two species this same cell type exhibits fluorescence which is spectrally quite similar to the bioluminescence. A careful study of the various types of coelomic cells in *P. bermudensis* (Jamieson *et al.*, 1981), a careful review of the coelomocyte literature and morphological characteristics (Jamieson, 1981) and a reinvestigation of the EM sections and photographs from our previous study of *D. longa* (Wampler and Jamieson, unpublished data) suggest that the luminescent cells in these various species are morphologically similar and that they are not free chlorogen cells, as was previously reported (Rudie and Wampler, 1978). Instead, this cell type is identified as a mucocyte, an acidophil cell containing β-glucuronidase and PAS positive granules.

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