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EARTHWORM BIOLUMINESCENCE: COMPARATIVE PHYSIOLOGY AND BIOCHEMISTRY

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Abstract—1. The comparative physiology and biochemistry of twelve species of bioluminescent earthworms from six genera (Diplocardia, Diplotrema, Fletcherodrilus, Octochaetus, Pontodrilus and Spenceriella) from the U.S., Australia and New Zealand indicated considerable similarity in the mechanism of bioluminescence.

2. With the exception of *Pontodrilus bermudensis* where the fluid is exuded from the mouth, all species exude bioluminescent coelomic fluid from their dorsal pores.

3. Microscopic observation, isolation of active particulate fractions from seven species and comparison to the previous study of *D. longa* (Rudie & Wampler, 1978) suggests that the site of luminescence is the free chloragogen cell.

4. The emission spectra (eight species) were broad, unimodal with maxima ranging from 500 nm to greater than 570 nm.

5. The spectral and biochemical data supports the hypothesis that earthworm bioluminescence involves the reaction of hydrogen peroxide with *N*-isovaleryl-3-aminopropanal or its close analog, and that spectral distribution is determined by the luciferase or other luciferase-associated components.

INTRODUCTION

Bioluminescent earthworms (Oligochaeta) are found world wide (see Harvey, 1952; Jamieson, 1977) but the physiology and biochemistry of the bioluminescence has not been extensively studied. A review of the literature reveals considerable similarities between species however and suggests similar, but not identical, chemical and physiological systems. With few exceptions luminescence is reported in the fluided exuded when worms are stimulated (Gilchrist, 1918; Gates, 1925; Johnson *et al.*, 1965; Benham, 1899; Komarek, 1934; Skowron, 1926; Pickford, 1937; Rudie & Wampler, 1978; Jamieson & Wampler, 1979). Some workers have described large, granular cells as the source of this luminescence, (Benham, 1899; Gilchrist, 1918; Skowron, 1926; Komarek, 1934), similar to the free chloragogen cell source of *Diplocardia longa* (Rudie & Wampler, 1978). However, in Pontodrilus species no evidence for a particulate luminescence system has been obtained (Lynch, personal communication; Jamieson & Wampler, 1979). Others report that the *in vivo* luminescence is oxygen dependent (Gilchrist, 1918; Skowron, 1926), while in other species this is not the case (Komarek, 1934).

Recent discoveries of additional luminous earthworms (Jamieson, 1977; Jamieson & Wampler, 1979) and of *D. longa* luciferin-like activity in worms other than *D. longa* (Bellisario, 1971), led the authors to investigate the biochemical and physiological similarities between other species and the purified system from *D. longa* (Bellisario *et al.*, 1972; Rudie *et al.*, 1976; Ohtsuka *et al.*, 1976). A study of the stimulation of *in vitro* bioluminescence by the components of the *D. longa* system is reported here as well as a comparison between the general physiological features of the different species. Data are presented which suggest that *D. longa* luciferin *N*-isovaleryl-3-amino propanal or a close analog of it, is the common substrate for bioluminescence in earthworms. This data and data from the literature are brought together in the discussion to give an overview of the comparative physiology and biochemistry of earthworm bioluminescence.

MATERIALS AND METHODS

Earthworms were collected in the United States, New Zealand and Australia as part of a comparative study under the U.S.–Australian Cooperative Science Program. *D. longa* from the southern coastal plain of Georgia was routinely collected in the summer months by various members of this laboratory. *D. mississipiensis* were purchased from bait dealers in Athens, GA, as "Louisiana Pinks." *Anynthas sp.* (*hawayanus* group) was collected on Sapelo Island, Georgia, by J. E. Wampler and N. G. Rudie (November, 1974). *D. eiseni*, annnd *D. alba* were collected at Tall Timbers Research Station (August, 1978), Tallahassee, Florida by V. Vail and J. E. Wampler. *Octochaetus multiporus* was collected by J. E. Wampler and J. M. Anderson on the farm of T. Whitaker near Upper Hutt on the North Island of New Zealand (March, 1975). Spenceriella and Fletcherodrilus species were collected by B. G. M. Jamieson, B. Barnes, G. Dyne and J. E. Wampler in January and February, 1978, from Southern Queensland, Australia (see Jamieson & Wampler, 1978). *Diplotrema heteropora* was collected near Townsville, Australia by W. Nash

(May, 1977). *Pontodrilus bermudensis* was collected on Peel Island, Australia, by O. Kelly (January, 1978); and subsequently by V. Vail, J. E. Wampler, M. Mulkerrin and N. Rich at St. Marks Wildlife Refuge, Florida (June, 1979).

D. longa luciferase was prepared using a procedure which yields homogeneous, high activity enzyme (Rudie, 1977). *D. longa* luciferin, *N*-isovaleryl 3-amino propanal,, was synthesized as previously described (Ohtsuka *et al.*, 1976). Hydrogen peroxide was reagent grade and all organic and inorganic reagents were of the best grade available. Extractions, reagents, and assays were prepared using a buffer of 0.1 M potassium phosphate (pH 7.5) with 0.125 g/l. sodium azide and 0.075 g/l. dithiothreitol. To prepare *in vitro* reaction exxtracts, worms were shocked with a hand-held magneto generator and the exuded coelomic fluid homogenized with buffer in a Ten Broek glass homogenizer. Extracts were kept at ice temperatures during testing.

In vitro reactions were carried out with 0.1 ml of extract in a small test tube. The tube was placed in the photometer (Wampler, 1975) and the shutter opened. After observation on the endogenous level of bioluminescence, additional reagents were injected into the test tube while a continuous record was being maintained. Reagents were injected using

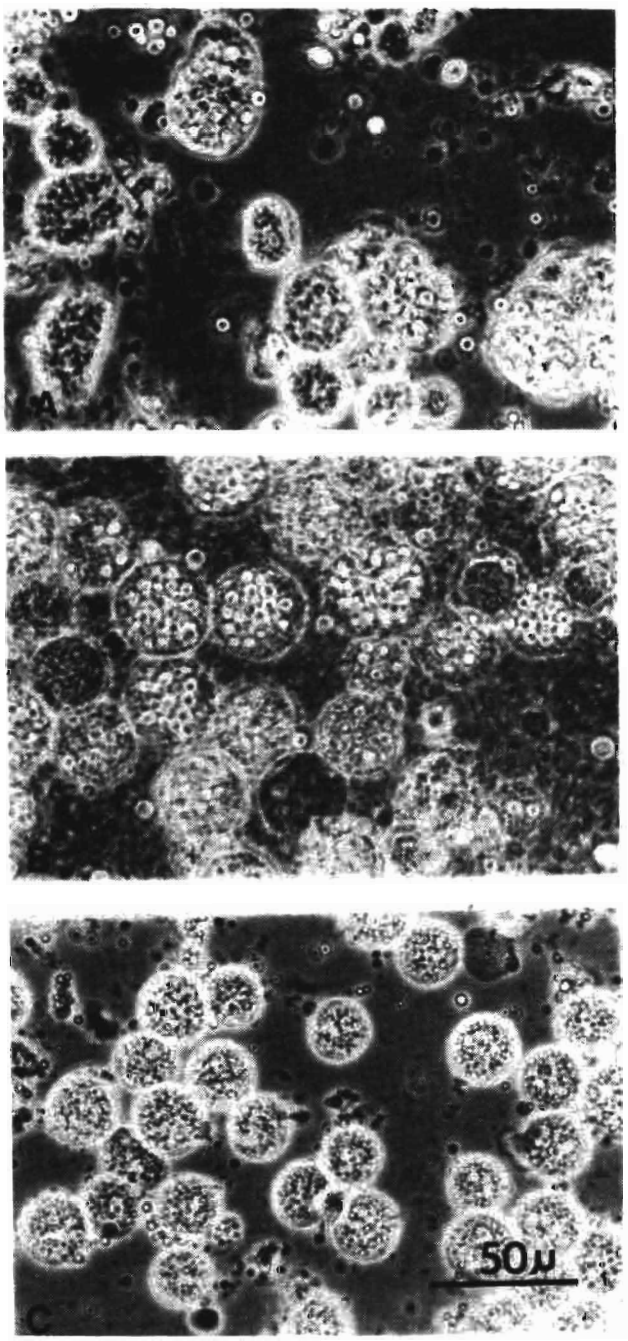

Fig. 1. Phase contrast photomicrograph of untreated exudate, obtained during electral stimulation, from the dorsal pores of luminescent Diplocardia species from the southeastern United States, showing live coelomocytes. A—*Diplocardia longa*. B—*D. alba*. C—*D. eiseni*. All to same scale.

the following volumes and concentrations: 0.05 ml of 0.3% hydrogen peroxide, 0.05 ml *D. longa* luciferase (0.1 units per ml), and 0.05 ml of *D. longa* luciferin (0.25 mg/ml).

Coelomic cells were isolated by stimulating worms in the extraction buffer, centrifuging and washing the cells with extraction buffer. After repeated centrifugation and washing, cells were prepared for microscopic examination and assayed for bioluminescence activity as previously described (Rudie & Wampler, 1978). Some cells were examined and measured in untreated exudate.

Bioluminescence spectra were recorded using the on-line computer system and techniques previously described (Wampler, 1978). For field work (1975), this system was carried aboard the R. V. *Alpha Helix*. (Scripps Institute of Oceanography, California)

Thin layer chromatograms were run using Eastman 6060 Silica gel sheets. Spots on the chromatographs were detected with I_2, luciferin-activity was tested by extracting strips of gel with methanol and assaying using *D. longa* luciferase and hydrogen peroxide.

RESULTS

In all cases examined the bioluminescence occurred when the earthworm exuded a viscous fluid upon excitation by tactile or electrical stimulation. With the exception of *Pontodrilus bermudensis* which has no dorsal pores, the exudate appeared from the dorsal pores and thus is assumed to be coelomic fluid. *P. bermudensis* exudes copious yellow fluid from the mouth. This exudate contains cells similar to coelomocytes. Further investigations are underway to discern the mechanism of exudation for this species.

In *D. longa* a more intensive investigation (Rudie & Wampler, 1978) has localized the bioluminescent system in the free chloragogen cells of the coelomic fluid. In *Diplocardia eiseni*, *D. alba*, *Spenceriella* (*S.*) *cormieri*, *S.* (*S.*) *curtisi*, *S.* (*S.*) *noctiluca* and *Fletcherodrilus unicus* the luminescence source has also been isolated in a particulate fraction which, when examined by light microscopy is seen to contain a large number of granule-filled, spherical cells (Figs 1 and 2). The mean diameters of the cells range interspecifically from 18 to 31 μm (Table 1). The diameters of the live coelomocytes, under slight coverslip pressure, in untreated exudate (Figs 1 and 2) have respective ranges, means and standard deviations for seven species as follows (number of cells measured in parentheses): *Diplocardia longa* 25–35 μm, 30 μm $\pm$ 0.7 (24); *D. eiseni* 14–23, 18 $\pm$ 0.3 (40); *D. alba* 27–35, 31 $\pm$ 0.5 (22); *Fletcherodrilus fasciatus* 14–31 μm, 23 μm $\pm$ 5.2 (19); *Spenceriella* (*S.*) *cormieri* 19–36 μm, 26 μm $\pm$ 4.2 (29); *S.* (*S.*) *curtisi* 20–27 μm, 24 μm $\pm$ 1.9 (18); *S.* (*S.*) *noctiluca* 10–27 μm, 18 μm $\pm$ 3.5 (83).

A preliminary study of non-luminescent heteropore species (Heteroporodrilus and Plutellus) from the Lamington and Tamborine National Parks, South Queensland, Australia, reveals exuded coelomocytes differing from those of luminescent species described here in being less numerous and in their more finely granular appearance. Similarly, a study of *D. mississippiensis* coelomic cells using the electron microscope (Rudie, unpublished) revealed considerably more internal structure (golgi, mitochondria, nucleus etc.) and a lower content of granules.

When the exuded fluid is left undisturbed, slow onset of luminescence occurs accompanied by an increase in viscosity and tenacity. Agitation stimulates both emission and the changes in fluidity. Similar changes occur when distilled water is added to the fluid.

If a worm is shocked while submerged in high ionic strength buffer (0.1 M potassium phosphate, pH 7.5), the fluid readily solubilizes, whole cells settle to the bottom of the container, and the appearance of the slime and luminescence is retarded. In the cases listed (Table 1), the settled particles were washed and their luminescence excited by addition of distilled water; addition of dilute hydrogen peroxide further stimulated the emission.

Extracts of the exuded fluid, prepared by using a glass homogenizer, were either not spontaneously bioluminescent or only very weakly emitting. In the latter cases, with for examples *D. longa* and *O. multiporus*, the low level emission may have been due to remaining suspended particles. Addition of dilute hydrogen peroxide to these extracts gave immediate stimulation of bioluminescence in all cases. Subsequent additions of either *D. longa* luciferase or *D. longa* luciferin gave further stimulation (Table 1). Figure 3 shows typical light responses to those ordered additions.

The degree of chemical similarity in these systems is indicated by the luciferin like activity found in the species listed in Table 1 when tested using the *in vitro* reaction with H_2O_2 and *D. longa* luciferase. *D. longa* luciferase is quite specific being active with only a few simple analogues of luciferin (Mulkerrin *et al.*, unpublished results). For example, *N*-isovaleryl-2-aminoacetaldehyde and *N*-acetyl-3-aminopropanal are active but none of the straight chain aldehydes are. TLC chromatography of the luciferin from *Pontodrilus bermudensis* showed that the luciferin activity had R_f values of 0.50 with a solvent of 25% ethanol in n-hexane and 0.38 in 3% methanol in chloroform. These values compare with values of 0.46 and 0.33 respectively for *D. longa* luciferin. Similarly close R_f values for luciferin activity from various non-luminescent earthworms have also been obtained (Mulkerrin *et al.*, unpublished).

Recorded spectra (Fig. 4) show that while the chemistry of these species appears quite similar, the spectral distributions of the bioluminescence vary considerably. Spectral data for *O. multiporus* was obtained, but the emission was for the most part at wavelengths beyond the correctable range of the photomultiplier used (EMI 9750) with the maximum at a wavelength greater than 570 nm. All of the species of the genus Diplocardia have similar spectra, but the spectra are quite different between genera.

Spectral data also supports the hypothesis that luciferin is a common structure in bioluminescent earthworms (Fig. 5). When the light reaction of a *Pontodrilus bermudensis* extract which had been stimulated by addition of hydrogen peroxide was allowed to decay to a negligible signal level and was then reinitiated by addition of synthetic Diplocardia luciferin, little, if any, spectral changes occurred. On the other hand, the emission spectrum obtained when the same kind of spent reaction is stimulated by addition of *D. longa* luciferase is definitely blue shifted toward the *D. longa in vivo* spectral region (λ_{max} = 485 nm). Similar experiments in the field using Octochaetus extract gave an equivalent result. The yellowish emis-

Table 1. Biochemical similarities in earthworm bioluminescence*

Species	Spontaneous level of extract hν sec^{-1}	Active particles	Mean cell diameter (μm)	Stimulation of extracts by: H_2O_2	H_2O_2 + L'ase	H_2O_2 + L'in	Emission maximum (nm)
Diplocardia							
D. longa	10^{12}	yes	30	+	+	+	500 ± 5
D. eigeni	10^{9}	yes	18	+	+	+	505 ± 5
D. alba	10^{9}–10^{11}	yes	31	+	+	−	501 ± 5
Diplotrema							
D. heteropora	10^{10}	yes	—	+	+	−	545 ± 10
Fletcherodrillus							
F. unicus	10^{8}	yes	−	+	++	−	—
F. fasciatus	10^{7}	X	23	+	?	?	—
Octochaetus							
O. multiporus	X†	X†	−	+	+	++	>570
Pontodrillus							
P. bermudensis	10^{9}	no	−	+	+	+	550 ± 10
Spenceriella							
S. cormieri	10^{11}	yes	26	+	?	+	—
S. curtisi	10^{8}–10^{9}	yes	24	+	++	+	535 ± 5
S. minor	10^{9}–10^{10}	X	—	+	+	+	531 ± 5
S. noctiluca	10^{9}	yes	18	+++	+	?	—

* In this table + indicates one order of magnitude stimulation: − indicates no stimulation; ? indicates variable results; X indicates that this item was not tested or examined. Light levels are in photons per second assuming a spectrum similar to *D. longa*.

† Octochaetus data was obtained in the field and was only qualitative. The spontaneous level of emission from the copious fluid exuded by these worms was very bright, easily seen by the non-dark-adapted eye. There was obviously an active particulate fraction in the fluid which could be separated by gravity and stimulated to emit by mechanical and chemical means.

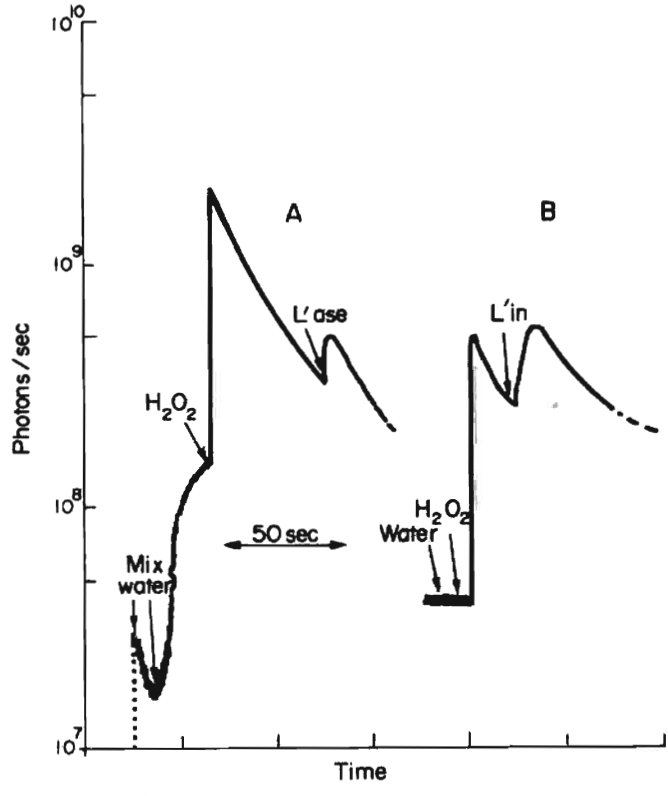

Fig. 3. Measured photon flux from exudate of bioluminescent earthworms. A. Washed particles from a *Diplotrema heteropora* which was shocked in 0.1 M potassium phosphate buffer (pH 7.5). Particles were centrifuged, washed with buffer and 100 μl of suspended particles were added to a cuvette with mixing. The light responses when water, hydrogen peroxide and *D. longa* luciferase (L'ase) were injected are shown. B. A phosphate buffer (0.1 M, pH 7.5) extract of *Spenceriella* (*S.*) *minor* exudate is stimulated by injection of hydrogen peroxide and *D. longa* luciferin (L'in).

Table 2. Common characteristics of earthworm bioluminescence

Species	Locality	Source of luminescence	Chemical effects*	Color (λ_{max})	Reference
Digaster keasti	Australia	Exuded fluid	$H_2O_2(+)$	Whitish	Jamieson (1977)
Diplocardia longa†	Southern United States	Free Chloragogen cells in coelomic	$H_2O_2(+)$, Lin(+) L'ase(+)	Blue–green (500)	Bellisario *et al.* (1972); Rudie & Wampler (1978)§
Diplotrema heteropora	Australia	Particles in coelomic fluid	$H_2O_2(+)$ L'ase(+)	Green (545)	Dyne (1979)§
Eisenia submontana	Poland	Granular cell in exuded fluid	—	Green–blue	Komarek (1934)
Euthyphoeus peguanus†	Rangoon	Exuded fluid	—	Whitish	Gates (1925)
Fletcherodrilus fasciatus†	Australia	Particles in coelomic fluid	$H_2O_2(+)$; L'ase(+)	Whitish	Jamieson & Wampler (1979)§
Microscolex phosphoreus	Poland	Granular cell in exuded fluid	—	Yellow–green	Skowron (1926; 1928)
Octochaetus multiporus	New Zealand	Milky, coelomic fluid	$H_2O_2(+)$; D. L'in(+) D. L'ase(+)	Yellow (>570)	Johnson *et al.* (1965)§ Benham (1899)
Parachilota elgonensis	South Africa	Particles in exuded fluid	$O_2(+)$	Green	Gilchrist (1918)‡
Pontodrilus bermudensis	Australia, United States	Exuded fluid	$H_2O_2(+)$; D. L'ase(+)	Whitish (550)	Jamieson & Wampler (1979)§
Spenceriella curtisi†	Australia	Particles in exuded fluid	H_2O_2; D. l'ase(+) D. l'ase(+)	Green (535)	Jamieson & Wampler (1979)§

* + for stimulation, − for inhibition of luminescence, Lin for *D. longa* luciferin, L'ase for *D. longa* luciferase.

† Other bioluminescent species of this genera have also been reported, see references.

‡ Gilchrist (1918) identified his specimen as a *Chilota* species. Dr Pickford (1937) records further information on it and the parachilota assignment.

§ Including also data presented in this work.

emission spectrum is determined by a non-substrate solute species, presumably by luciferase itself or an associated protein.

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