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# Two Bioluminescent Diptera: The North American *Orfelia fultoni* and the Australian *Arachnocampa flava*. Similar Niche, Different Bioluminescence Systems¶

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## ABSTRACT

***Orfelia fultoni* is the only bioluminescent dipteran (Mycetophilidae) found in North America. Its larvae live on stream banks in the Appalachian Mountains. Like their Australasian relative *Arachnocampa* spp., they build sticky webs to which their bioluminescence attracts flying prey. They bear two translucent lanterns at the extremities of the body, histologically distinct from the single caudal lantern of *Arachnocampa* spp., and emit the bluest bioluminescence recorded for luminescent insects ($\lambda_{max}$ = 460 nm *versus* 484 nm from *Arachnocampa*). A preliminary characterization of these two bioluminescent systems indicates that they are markedly different. In *Orfelia* a luciferin–luciferase reaction was demonstrated by mixing a hot extract prepared with dithiothreitol (DTT) under argon with a crude cold extract. Bioluminescence is not activated by adenosine triphosphate (ATP) but is strongly stimulated by DTT and ascorbic acid. Using gel filtration, we isolated a luciferase fraction of ~140 kDa and an additional high molecular weight fraction (possibly a luciferin-binding protein) that activated bioluminescence in the presence of luciferase and DTT. The *Arachnocampa* luciferin–luciferase system involves a 36 kDa luciferase and a luciferin soluble in ethyl acetate under acidic conditions; the bioluminescence is activated by ATP but not by DTT. The present findings indicate that the bioluminescence of *O. fultoni* constitutes a novel bioluminescent system unrelated to that of *Arachnocampa*.**

## INTRODUCTION

Among dipterans, bioluminescence has been reported only in Mycetophilidae (fungus gnats). Harvey listed three genera, *Arachnocampa*, *Orfelia* (previously *Platyura*) and *Keroplatus* (1). Of these, *Arachnocampa* is most widely known for the brilliant displays its larvae create on the roofs of caves in New Zealand and Australia. The almost unknown *Orfelia* of North America, discovered by Fulton only in 1941 (2), shares many of the outward characteristics of *Arachnocampa*. At first glance, the two brownish larvae look much alike, being on average 10–20 mm long and 1–2 mm in diameter. To catch prey, both build remarkable webs in similar environments, such as crevices on damp stream banks (3); but unlike *Arachnocampa*, *Orfelia* is seldom found on the roofs of caves and does not use ''fishing lines'' as does *Arachnocampa*. Both *Arachnocampa* and *Orfelia* are carnivorous, even cannibalistic; in spite of the name, there is little indication that fungi are part of the diets of either of these gnats.

Here, however, the similarities end. The bioluminescence of *Orfelia* is blue, and that of *Arachnocampa* is blue-green. The light-emitting organs are different. *Arachnocampa* has only one caudal lantern derived from the malpighian tubules (4), whereas *Orfelia* larvae have very unusual, bilateral, anterior and posterior lanterns (2). They are characterized by two rows of black bodies consisting of large binucleated cells filled with dark granules, seemingly budding out of mitochondria (5). This report also suggested that the chemistry of the bioluminescence of *Orfelia* differed from that of *Arachnocampa* in at least one significant aspect: adenosine triphosphate (ATP) enhances the luminescence of crude extracts of *Arachnocampa* (6,7), whereas it has no effect on that of *Orfelia* (5).

Considering the well-known dependence of the bioluminescence of Coleoptera on ATP (8), the difference between *Arachnocampa* and *Orfelia* with respect to ATP seemed significant and intriguing. Our results support the conclusion that the chemistries of bioluminescence of *Orfelia fultoni* and *Arachnocampa flava* from Australia are distinct; there are no cross-reactions of enzymes or substrates.

## MATERIALS AND METHODS

*Reagents*. Coenzyme A (CoA), dithiothreitol (DTT), ethylenediamine tetra-acetic acid (EDTA), D-luciferin, ATP, reduced flavin mononucleotide ($FMNH_2$), ascorbic acid, glutathione, nicotinamide adenine dinucleotide (reduced form) (NADH), carbonic anhydrase,

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*Abbreviations:* ATP, adenosine triphosphate; A.U., arbitrary units; CoA, coenzyme A; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid, disodium salt; $FMNH_2$, reduced flavin mononucleotide; NADH, nicotinamide adenine dinucleotide (reduced form); NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); SBF, substrate-binding fraction in the *Orfelia* system; TLC, thin-layer chromatography.

bovine serum albumin, alcohol dehydrogenase, β-amylase, apoferritin, tyroglobulin and pyrophosphate were obtained from Sigma Chemical Co. (St. Louis, MO). Triton X-100 was obtained from Fisher Scientific (Pittsburgh, PA), Sephacryl S-300 and S-400 from Pharmacia (Piscataway, NJ).

*Insects*. *Orfelia fultoni* larvae were collected from May through June in 1999, 2000 and 2001 at the Shenandoah National Park, (Virginia), from early April through August at the Highlands Biological Station (Highlands, NC), Cashiers (North Carolina) and Glenville (North Carolina), usually among moss on stream banks, in exposed cavities under stones along streams or under decaying logs. In Pickett State Park (Tennessee) they were found in May 2001 at the base of sandstone caves. Larvae were located at night by their bioluminescence and collected by hand; usually about 20–30 larvae per night. They were transferred to plastic jars with moist soil and moss from their habitat. Some larvae were kept alive for as long as 3 weeks on a diet of *Drosophila*. Most were immediately rinsed with water, transferred to Eppendorf tubes, frozen in liquid nitrogen, then stored at −80°C. *Arachnocampa flava* larvae were collected at Springbrook National Park (Queensland, Australia) during spring and summer of 2000 and 2001, on damp banks similar to those rich in *Orfelia* but with a high density of trapdoor spiders; the larvae were shipped to our laboratory in liquid nitrogen.

*Crude extracts*. Crude extracts of *Orfelia* were usually prepared from 1–4 larvae in 1 mL of cold extraction buffer (0.1 *M* phosphate, 1 m*M* EDTA, 1% Triton X-100, pH 7.0) with a Potter–Elvejem homogenizer. After homogenization the extracts were centrifuged at 15 000 ***g*** for 15 min at 4°C. The supernatants were used for most of the routine assays, whereas the pellets were resuspended in extraction buffer or stored at −80°C. For *Arachnocampa* the crude extracts were prepared from 20 larval lanterns in 27 m*M* Tricine, 7 m*M* $MgSO_4$, 0.2 m*M* EDTA, 10% glycerol and 1% Triton X-100, pH 7.4 (9), and centrifuged as described for *Orfelia*.

*In vitro assays*. Bioluminescence intensities were determined in a custom-built photometer (10) calibrated with reference to the Hastings–Weber light standard (11). For *in vitro* bioluminescence assays of crude extracts of *Orfelia*, 5–10 μL of crude extract was added to 90–95 μL of assay buffer (0.1 *M* Tris–HCl buffer, pH 8.0). The effect of different compounds on bioluminescence activity was assayed by mixing 10 μL of the chosen compound and 10 μL of crude extract with 80 μL of assay buffer. Luciferase activity was determined by mixing 5 μL of hot extract with 95 μL of assay solution containing 10 μL of luciferase and 85 μL of assay buffer.

For the crude extract of *Arachnocampa*, the bioluminescence assays were carried out by mixing 10 μL of crude extract and 5 μL of 40 m*M* ATP–80 m*M* $MgSO_4$ with 85 μL of assay buffer (0.1 *M* Tris–HCl, pH 8). Luciferase assays were performed by mixing 5 μL of hot extract with a mixture containing 80 μL of assay buffer (pH 8), 10 μL of luciferase and 5 μL of 40 m*M* ATP–80 m*M* $MgSO_4$.

*Luciferase–luciferin reaction*. To test if the bioluminescence of *Orfelia* is the result of a luciferin–luciferase reaction, crude extract was prepared as described above. After centrifugation the supernatant was divided into two aliquots of 500 μL each. One aliquot was labeled as cold extract and kept on ice for 1 h or less. The other aliquot was treated with 10 m*M* DTT (final concentration), then purged with argon for 15 min and heated at 95°C for 5–10 min in the presence of argon; this was labeled the hot extract. The reaction was performed by mixing 10 μL of cold extract with 90 μL of 0.1 *M* Tris–HCl buffer (pH 8.0) in a vial in the photometer sample compartment and then adding 10 μL of hot extract. In the case of *Arachnocampa*, crude extracts were prepared according to the method described by Wood (9). After centrifugation the supernatant was divided into two aliquots. The cold extract was prepared by adding 1 m*M* ATP to one of the aliquots (final concentration) and incubating on ice for 18 h. The hot extract was prepared by heating the second aliquot at 98°C for 5 min and adding ATP to a concentration of 1 m*M*. The test involving mixing equal amounts of cold and hot extracts resulted in light emission (9).

*Isolation of* Arachnocampa *luciferin*. Lanterns of *Arachnocampa* were homogenized in hot 0.1 *M* citrate buffer (pH 5), and the solution was incubated for 5 min at 95°C. The hot extract was then acidified to pH 2.5–3 with HCl and extracted with an equal volume of ethyl acetate. The ethyl acetate extract was dried under argon flow, and the whitish residue was dissolved in water. Thin-layer chromatography (TLC) of *Arachnocampa* luciferin was performed on silica-gel plates using the solvent systems ethyl acetate–ethanol–water (5:3:2 or 3:5:2) or ethanol 75%.

*Bioluminescence emission spectra*. Spectra were obtained with a SPEX Fluorolog spectrofluorometer with the excitation lamp turned off, using a quartz microcuvette. The spectra were scanned from 400–700 nm (4 nm/s, 1.0 mm emission slit) and run in triplicate. The *in vitro* spectrum of *Orfelia* was obtained from 100 μL of bioluminescent crude extract in the presence of 1 m*M* DTT, and that of *Arachnocampa* was obtained by mixing in the cuvette 10 μL of 20 mM ATP–40 m*M* $MgSO_4$ with 100 μL of crude extract.

*Column chromatography*. In order to isolate the components of the bioluminescent reactions of *Orfelia* and *Arachnocampa* and to determine the molecular mass of their luciferases, gel filtrations were performed at 4°C on Sephacryl S-300 (92 cm × 1 cm) or S-400 (5 cm × 0.7 cm) columns, with flow rates between 250 and 300 μL/min. The elution buffer used for *Orfelia* was 20 m*M* phosphate (pH 7.0) and 1 m*M* EDTA, and the buffer for *Arachnocampa* was 30 m*M* Tricine, 1 m*M* EDTA, 10% glycerol (pH 7.4). The void volumes were estimated using blue dextran, and the columns were calibrated with standard molecular weights (carbonic anhydrase, 29 kDa; bovine serum albumin, 66 kDa; alcohol dehydrogenase, 150 kDa, β-amylase, 200 kDa; apoferritin, 443 kDa and tyroglobulin, 669 kDa). After gel filtration, the fractions were analyzed for endogenous bioluminescence, luciferase activity and fluorescence. In the case of *Orfelia*, the endogenous bioluminescence was assayed by mixing 20 μL of each column fraction (300 μL) with 100 μL of 0.1 *M* Tris–HCl buffer (pH 8.0). Luciferase activities for both *Orfelia* and *Arachnocampa* chromatograms were determined as described above. In order to investigate the involvement of a second protein factor in the bioluminescence system of *Orfelia*, another experiment involved the mixing of 10 μL of each chromatographic fraction with 90 μL of assay buffer–10 m*M* DTT plus 10 μL of luciferase fraction (from a pool of fractions 27–32). The experiments were repeated four times with similar results.

## RESULTS

### *Orfelia* bioluminescence

*Visual observation of bioluminescence*. Larvae were more abundant in early spring, although they could still be found in August in North Carolina. Some active bioluminescent larvae were found at temperatures close to freezing during early April nights. A rough estimate of the larval population density by visual inspection indicated ~60 larvae/$m^2$ at the best locations, but 20–30 larvae was more usual. Although the luminescence originates from the anterior and posterior lanterns, a diffuse bioluminescence is often observable throughout the body. When larvae were manipulated, we sometimes noticed that bioluminescent material was released, either as a secretion or as the result of injury. A luminescent adult was found in a cavity close to a spring in Shenandoah National Park in July 2000. The bluish luminescence was diffuse throughout the body, but no distinct light organ was identified visually.

*Bioluminescence* in vitro. *Effect of reductants*. Crude extracts from whole larvae in buffer at pH 8 emit light without any addition ($\lambda_{max}$ 460 nm, Fig. 1), similar to the emission spectrum *in vivo*. The peak intensity from such extracts is estimated at *ca* $10^9$–$10^{11}$ photons per second per larva. If the extraction procedure is performed in a dark room, a readily visible blue luminescence is observed during the preparation. As expected, the emission from *Orfelia* extracts is abolished by argon purging but returns when air is readmitted. In a typical assay, the mixing of 10 μL of *Orfelia* extract with 90 μL of assay buffer results in an emission that rises promptly to maximum intensity and decays fast (Fig. 2).

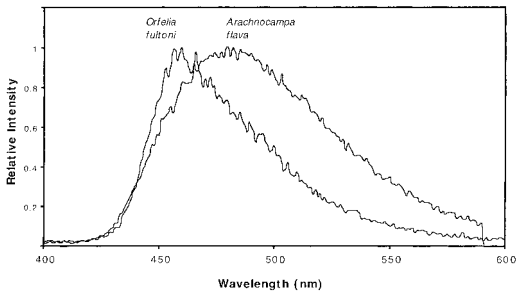


**Figure 1.** *In vitro* bioluminescence spectra of *O. fultoni* and *A. flava*.

DTT (10 m*M* in the assay buffer) increases significantly the emission intensity and photon yield from crude extracts of *Orfelia* (Fig. 2), whereas ATP has no effect, as previously reported (5). After the luminescence has decayed almost to the baseline, addition of DTT brings about a second burst of emission, but rising and decaying more slowly. An extract retains the capacity for DTT-stimulated emission even after the spontaneous emission from the sample has declined to less than 1% of the initial intensity. However, the peak intensity obtained from such a delayed addition of DTT never matches that obtained when DTT is present in a fresh extract from the start (Fig. 2; note the 10-fold difference in ordinate scale). Once an emission enhanced by DTT has decayed, a second addition is without effect. Among other reducing agents, ascorbate has the same effect as DTT, both qualitatively and quantitatively, whereas NADH and CoA have qualitatively similar but weaker effects. In contrast, $FMNH_2$ and reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) are inactive, as is 2-mercaptoethanol, contrary to the early report that it enhanced the luminescence of crude extracts (T. Hopkins, personal communication to J. M. Bassot [5]).

*Mixing hot and cold extracts indicates a luciferin–luciferase reaction.* After the emission of a crude extract containing DTT has decayed to ~10% of peak intensity, addition of a heat-treated extract (5–10 min at 95°C under argon, with 10 m*M* DTT) causes an immediate increase in emission. Successive additions result in similar responses with rapid onsets and slower decays (Fig. 3). The activity can be attributed to a heat-stable luciferin, and the decay of luminescence can be interpreted as resulting from luciferin loss *via* the bioluminescence reaction or auto-oxidation (or both). Although DTT must be added prior to the heat treatment, the activity of hot extracts is not simply the result of their DTT content because

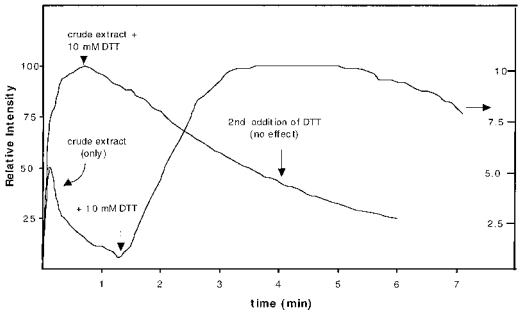


**Figure 2.** *Orfelia* bioluminescence *in vitro.* Luminescence obtained after adding 10 μL of crude extract to 90 μL of 0.1 *M* Tris–HCl buffer (pH 8) in the absence (right ordinate) or presence (left ordinate) of 10 m*M* DTT. In the assay started without DTT, this component was added later when the luminescence had decayed to less than 10% of the original intensity.

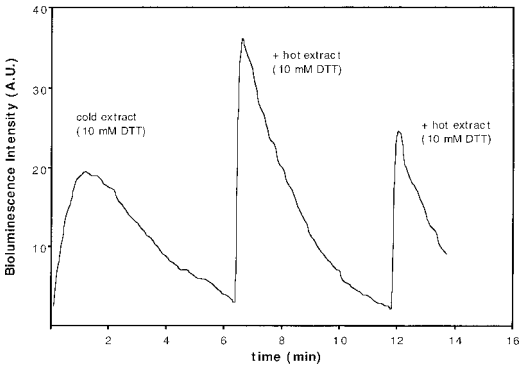


**Figure 3.** *Orfelia* bioluminescence *in vitro.* Effect of successive additions of 10 μL hot extract to 90 μL of the assay mixture containing cold crude extract and 10 m*M* DTT after the luminescence has decayed.

(1) DTT is already present in the cold extracts;
(2) the onsets of emission resulting from additions of hot extracts are immediate, in contrast with the slow-rising peaks that follow addition of DTT;
(3) successive additions of hot extracts bring about successive responses; and
(4) hot extracts prepared without DTT, but to which DTT is added after the heat treatment, are ineffective. These results suggest that DTT and other reducing agents act by releasing a heat-stable luciferin from a substrate-binding fraction, making it available to the luciferase.

*Two proteins may participate in the light reaction.* When crude extracts were passed through molecular filters of 3, 10, 30 or 100 kDa, the bioluminescence activity was always retained on the filter, and mixing the filtrate with the retentate did not stimulate its luminescence. In order to isolate and characterize the high molecular mass components, crude extracts were subjected to Sephacryl S-300 chromatography (Fig. 4; similar results were obtained with an S-400 column [data not shown]). Activity was always obtained by addition

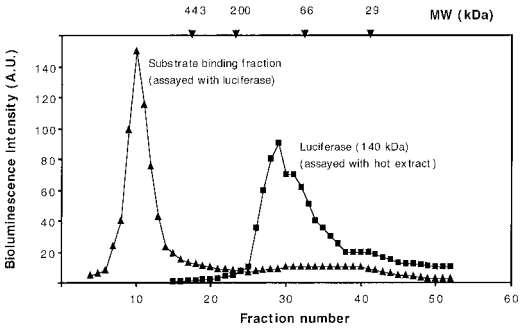


**Figure 4.** Gel filtration chromatogram of *Orfelia* crude extract using Sephacryl S-300. Luciferase-containing fractions were identified by light emission obtained by mixing with hot extract (substrate). A substrate binding fraction (SBF) was identified by mixing fractions with luciferase pooled fractions 20–40.

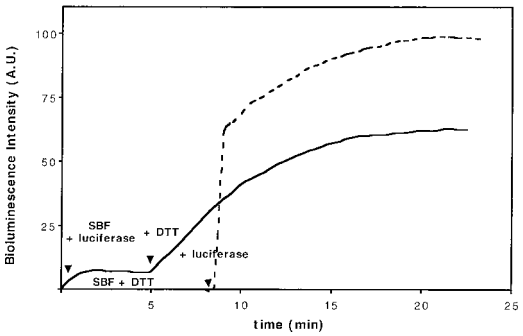


**Figure 5.** *Orfelia* bioluminescence *in vitro.* Effect of the time of addition of DTT on the kinetics of reaction of SBF and luciferase fractions. In the first assay (solid line), 10 μL of luciferase fraction and 10 μL of SBF fraction were added to 80 μL of assay buffer. After 5 min, 10 μL of 10 m*M* DTT was added. In the second assay (dashed line), 10 μL of SBF and 10μL of 10 m*M* DTT were added to 80 μL of assay buffer. After 9 min, 10 μL of luciferase was added.

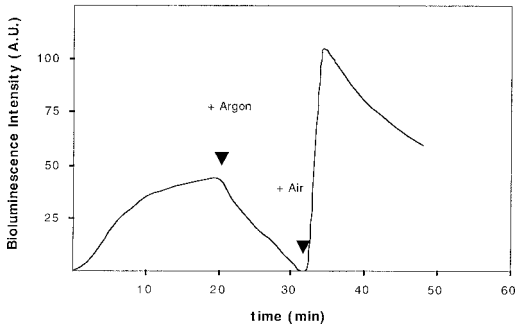


**Figure 6.** *Orfelia* bioluminescence *in vitro.* Effect of argon purging and air-readmission on the bioluminescence of a mixture containing 100 μL of column-chromatographed luciferase, 10 μL of hot extract and 10 μL of 10 m*M* DTT.

of hot extract to fractions between 20 and 40, with maximum activity corresponding to a molecular weight of ~140 kDa, suggesting that the protein in these fractions might be luciferase; sometimes a weak activity was observed from fractions between 10 and 50 without addition of hot extract. On the basis of the hypothesis that the luciferin might be protein-bound in crude extracts, all fractions were tested for activity in assays with luciferase pooled from fractions 20 to 40. This resulted in the identification of active fractions (~5–15) near the void volume, which we tentatively call the substrate-binding fraction (SBF).

On sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-Page), the luciferase fractions appeared as two bands of *ca* 70 kDa, not as a single 140 kDa band, suggesting that luciferase is a heterodimer. The kinetics of the emission that results from mixing the luciferase fractions with hot extracts are similar to those resulting from mixing cold and hot extracts. Also, as in that case, when the luminescence from such an assay has decayed, it can be restored by a new addition of hot extract even after 45 min. In contrast, adding more luciferase has no effect.

The kinetics of the luminescence resulting from mixing luciferase and SBF depends on the time of addition of DTT (Fig. 5). Its addition after the reaction has started gradually increases the luminescence in a kinetics pattern similar to that observed when DTT is added to a crude extract (Fig. 2). By themselves, SBF fractions emit no light, with or without DTT. But if SBF is allowed to stand at room temperature in the presence of DTT for 5–10 min, and luciferase is then added, an immediate and rapid rise of emission follows. These results are a clear indication that DTT, and most likely the other active reducing agents, act by releasing a luciferin from an association with a high molecular weight component, thus allowing it to react with luciferase. Characterization of luciferin is in progress; firefly D-luciferin and *Arachnocampa* luciferin (see later) were found to be inactive.

An intermediate complex between luciferase and luciferin appears to be formed and to accumulate in the absence of oxygen (Fig. 6). The reaction was started by adding hot extract containing DTT to purified luciferase. After 20 min, when the emission intensity was still increasing, the solution was purged with argon, causing a prompt drop in intensity. When the emission had decreased to the baseline, air was rapidly readmitted, bringing up an immediate high peak followed by a slow decay (Fig. 6). The slowly increasing intensity during the first 20 min of aerobic reaction must, therefore, reflect the slow buildup of a luciferase–luciferin complex, which can build up to an even higher level in the absence of oxygen. This complex is then poised for immediate reaction with oxygen, and the kinetics of the decay reflect the rate constant of that reaction or of a subsequent step.

### *Arachnocampa* bioluminescence

*Bioluminescence* in vitro. Although the bioluminescence of *Arachnocampa* appears brighter *in vivo* than that of *Orfelia*, the opposite is true in crude extracts. The peak intensity from the extract of one larva is of the order of $10^7$–$10^9$ h$\nu$/s, and thus one or two orders of magnitude lower than from *Orfelia* extracts. As mentioned in the introduction, the emission is redshifted from that of *Orfelia*, peaking at 484 nm (Fig. 1).

The weak blue-green emission of crude extracts is not enhanced by DTT or ascorbic acid, contrary to that of *Orfelia*, but is readily stimulated by MgATP. Ascorbic acid decreases the decay rate. A luciferin–luciferase reaction can be readily demonstrated by mixing hot and cold extracts, as previously described (9), or by mixing luciferase partially purified by ammonium sulfate precipitation with hot extracts in the presence of ATP.

*Luciferin isolation. Arachnocampa* luciferin was isolated from crude extracts by ethyl acetate extraction under acidic conditions. On TLC plates, this luciferin migrates with R*f* = 0.77 for the solvent mixture ethanol–ethyl acetate–$H_2O$ in the ratio 5:3:2 or R*f* = 0.68 for a ratio 3:5:2 of the same solvents. The fluorescence of the active fraction is purple (emission $\lambda_{max}$ 415 nm with excitation at 290 nm).

*Luciferase isolation: only one protein is involved in the light reaction.* Only one protein fraction, having an estimated molecular mass of 36 kDa, was isolated from crude extracts of *Arachnocampa* by gel filtration with Sephacryl S-400 (Fig. 7). Mixing this luciferase fraction with luciferin and ATP resulted in an emission with rapid onset and a kinetically complex decay of approximately first order only during the first phase (Fig. 8). Varying the luciferase concentration did not affect the decay rate, and the concentration

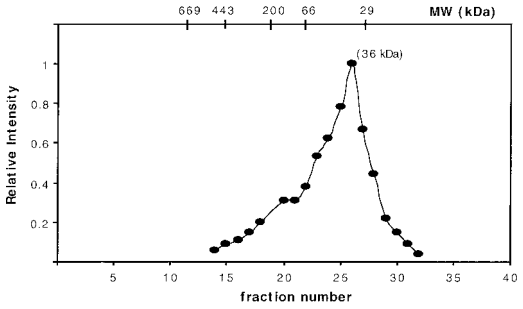


**Figure 7.** Gel filtration chromatogram of *Arachnocampa* luciferase on Sephacryl S-400. Ten microliters of each column fraction were mixed with 85 μL of 0.1 *M* Tris–HCl, 5 μL of 40 m*M* ATP–80 m*M* $MgSO_4$ and 5 μL of hot extract.

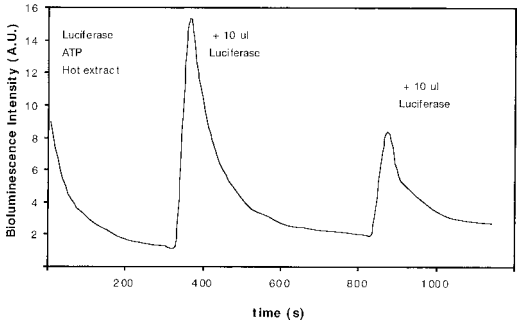


**Figure 8.** Effect of successive additions of 10 μL of *Arachnocampa* luciferase to 90 μL of the assay mixture containing 2 m*M* ATP–4 m*M* $MgSO_4$ and 5 μL of hot extract after the luminescence has decayed.

of ATP has been previously reported to have no effect on the decay rate either (7). However, further additions of fresh luciferase after the luminescence has decayed to about 10% of its maximum value result in luminescence responses similar to the initial one (Fig. 8). In contrast, adding fresh luciferin at the same timepoint has only a very slight stimulatory effect. This is exactly the opposite of what was observed in the case of *Orfelia*. *Orfelia* luciferase appears to turn over because more luciferin brings new emission (Fig. 3), whereas *Arachnocampa* luciferase apparently does not. CoA and $FMNH_2$ have no effect on the light reaction, whereas pyrophosphate inhibits it.

## DISCUSSION

In spite of the relatedness of these two Diptera and their similar appearances, habitat and behavior, it is clear that the bioluminescence systems of Orfelia and Arachnocampa are completely different, both from morphological and biochemical points of view.

*Orfelia's* system is novel. It involves a high molecular fraction (≫500 kDa), dubbed SBF, whose function is inferred to be the protection of luciferin both from autoxidation and from reacting with luciferase, itself a protein of *ca* 140 kDa, possibly a heterodimer. It may be mentioned that a luciferin-binding protein in the dinoflagellate *Lingulodinium polyedrum* plays such a dual function for dinoflagellate luciferin (12). In the case of *Orfelia*, DTT appears to release luciferin from SBF (see Figs. 2 and 5). How this is achieved is not clear. It cannot result from the cleavage of disulfide bridges because ascorbic acid and, to a lesser extent, other reductants have the same effect as DTT, whereas 2-mercaptoethanol does not. In any case, it is depletion of luciferin that causes the decay of luminescence because adding hot extract restores the luminescence (Fig. 3). This luciferin is very unstable and has not yet been characterized.

There are some intriguing similarities between *Orfelia* bioluminescence and that of *Pholas dactylus*, which also involves two proteins, a luciferase that contains two copper atoms and pholasin, a protein whose chromophore (of still unknown structure) can be regarded as the true luciferin (13). Pholasin alone can emit luminescence if exposed to superoxide. In the luciferase-catalyzed reaction of pholasin, ascorbic acid behaves as a strong activator (14), as it does in the case of *Orfelia*. This effect is attributed to a recycling mechanism involving oxidation–reduction of the copper ions of luciferase (15). However, it seems unlikely that reducing agents act *via* a similar mechanism in the case of *Orfelia*.

The bioluminescence system of *Arachnocampa* comprises a relatively small luciferase (*ca* 36 kDa) and a luciferin separable on TLC and fluorescing at 410 nm. Although ATP acts as an activator, as it does in beetle bioluminescence, there is no cross-reaction between the two systems, as already reported (7), or between the *Arachnocampa* and *Orfelia* systems. Attempts to demonstrate a cross-reaction of *Arachnocampa* luciferase with firefly antiluciferase on Western blots were unsuccessful. The bioluminescence of *Arachnocampa* is not activated by CoA, nor is it stimulated, as in the firefly case, by pyrophosphate after the luminescence has decayed. Addition of more luciferase after the luminescence of *Arachnocampa* has decayed restores it (Fig. 8), suggesting product inhibition of luciferase, as in the firefly system (16).

The bioluminescent systems of the millipede *Luminodesmus sequoiae* (17,18) and the firefly squid *Watasenia scintillans* (19) are also stimulated by ATP. In the millipede *L. sequoiae* the mechanism of ATP activation remains unknown. In the case of the firefly squid, ATP serves to phosphorylate adenylsulfate to give 3′-phosphoadenosine-5′-phosphosulfate, which in turn is used to sulfurylate coelenterazine and produce its active form. We suspect that ATP activation in the *Arachnocampa* system is not through the formation of an acyl-adenylate intermediate such as that which occurs in beetles.

To conclude, the anatomical and histological characteristics of the light organs of *Orfelia* and *Arachnocampa* already suggested that their bioluminescent systems are different, and indeed they involve different luciferases and luciferins and altogether different reaction conditions. To our knowledge this is the first case of two closely related species belonging to the same family that use distinct bioluminescence systems.

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