The Spectral Distribution of Firefly Light. II

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ABSTRACT The in vivo peak emission wavelengths of bioluminescence are reported for 15 species of American fireflies. A spectrophotometric study of the dorsal light organs of 155 specimens of the Jamaican firefly *Pyrophorus plagiophthalamus* showed three distinct color distributions with peak emission wavelengths at 550.1 ± 1.5 mμ, 556.8 ± 1.4 mμ, and 562.4 ± 1.0 mμ. Similar spectral measurements of 35 ventral light organs of the same insects gave peak emission wavelengths ranging from 547 through 594 mμ. This is a wider distribution than the total range of all 34 species of firefly studied to date. There was no obvious correlation between the colors of the ventral and dorsal light organs. It appears that *P. plagiophthalamus* is a special case in which the luciferase enzyme is not only different among members of the same species, but it may be different for the dorsal and ventral light organs in a single individual. A minimum of six different luciferase molecules for *P. plagiophthalamus* ventral light organs is proposed. The statistical precision in making these spectrophotometric measurements is discussed.

INTRODUCTION

In a previous paper (1) a summary through 1963 of all measurements of the in vivo bioluminescence emission spectra of fireflies was presented. This included 20 species, both American and Jamaican, measured by photoelectric spectrometry (2, 3). The present paper extends the data to include an additional 15 American species. In addition we have completed an extensive study of the emission spectra of 301 specimens of the Jamaican firefly, *Pyrophorus plagiophthalamus*, a species which exhibits an unusual intraspecies variability in the color of light emitted. From a statistical analysis of these data, it might be possible to deduce that there exist structural differences in the "luciferase" enzyme molecules responsible for light emission in the firefly.

EXPERIMENTAL TECHNIQUE AND EXPERIMENTAL PRECISION

For the American species the spectrometer used and the method of spectral efficiency calibration were the same as in the original paper (1). We had found previously that

most flashing fireflies (as differentiated from glowing fireflies) could be stimulated, while alive, to continuous luminescence by exposure inside a small glass tube to ethyl acetate vapor. This technique was employed in the present experiments as well. However, we found that for a number of species it was necessary to puncture the light organ with a needle point in order to stimulate a luminescence which was reasonably constant over a period of several seconds, which was the time required for the measurements. In all cases the firefly light organ was placed in the plane of the entrance slit of the ½ m grating spectrometer so that the light to be measured was emitted normally through the transparent layer of cuticle overlaying the organ. The laboratory temperature was 22°C ± 1°C. The American fireflies were collected by one of us (J.E.L.) in Florida and New York during 1965.

For the Jamaican species, *Pyrophorus plagiophthalamus*, a ¼ m scale model of the larger spectrometer was designed and constructed and the in vivo measurements were performed in Jamaica, W.I., with freshly captured specimens. *P. plagiophthalamus*, a nocturnal click beetle, is known locally as the "Peenie Wallie" or "Kitty Boo" or the "automobile bug" because of the two light organs on the dorsal prothorax which shine brightly and continuously when the insect lands on the ground or when it is handled. In addition to these "headlights" there is a ventral light organ which is turned on only when the insect is in flight or when the organ is forcibly exposed. This ventral organ is in a cleft in the first abdominal segment and is neither visible nor shining when the insect's hard wing covers are closed. Since the dorsal organs shine continuously when the insect is handled, the insect was held so that the organ was positioned directly behind a 2 mm diameter mask at a fixed distance with respect to an external spherical mirror so that a 1:1 image of the light organ was projected onto the entrance slit of the spectrometer. The insect was held immobile and with the light organ in a plane parallel to that of the entrance slit. In this manner we were able to minimize any effect of cuticle self-absorption and consequent spectrum distortion. In order to record the ventral organ emission spectrum, it was necessary to gently break off the wing covers with a fine forceps, to bend the insect back on itself, and in a few cases to stimulate the exposed light organ with the point of a needle. The method of holding the light organ behind the 2 mm diameter mask was the same. To demonstrate that this technique did not introduce any measurable spectral distortion due to absorption by the light organ structure, we performed control experiments in which the organ was cut into and some of the glowing material was squeezed out onto the point of a needle from which the spectrum was run. In all these cases the observed spectra were identical with those of the unbroken organs. 155 dorsal organs from 155 separate insects and 35 ventral organs from among those 155 insects were measured spectrophotometrically. Including these, a total of 301 insects was examined visually in the dark. In these spectrophotometric measurements a minimum of six consecutive spectra for each organ was recorded and analyzed.

In order to establish the limits of accuracy in our assignment of peak wavelengths of emission, we performed the following control experiments: (*a*) A bright, constantly glowing, homogeneous, in vitro extract of *Photinus pyralis* in a test tube was mounted in front of the entrance slit of the spectrometer and a series of 15 consecutive spectra was run. These uncorrected spectra on Sanborn chart paper were then analyzed visually and their peak intensity positions recorded. The results of a statistical analy-

sis of these observations gave an individual standard deviation of ± 0.7 mμ. (*b*) A bright and constantly glowing specimen of *Photinus umbratus* was held fixed within a small glass tube at the entrance slit and again a series of consecutive spectra was run. The individual standard deviation of these measurements was ± 0.5 mμ.

The experimental conditions chosen for the control experiments were essentially ideal conditions since we avoided having to focus and to hold the sources manually and we chose bright, nonflickering samples. The major source of error in these control experiments occurs in the visual determination of the peak position because of the fairly broad shape of the emission spectrum. Superimposed on this uncertainty in the actual field measurements are: (*a*) There may be slight variations in intensity with time of the insect's light organ as it is held manually in position behind the mask. (*b*) The individual insects are held in position by sighting in the dark on the small hole through which the organ is visible. This position could be slightly different for different insects and this could conceivably illuminate the entrance slit nonuniformly, resulting in a slight distortion of the observed spectrum. (*c*) A slight deviation in the plane of the light organ with respect to the plane of the entrance slit might introduce a small self-absorption distortion. (*d*) A portion of the light emitted has also been diffusely reflected by the light organ. Depending on the size of the light organ and the amount of diffuse reflection, there could be a variable self-absorption distortion which would increase the spread of the data.

On the basis of these control experiments we established an arbitrary criterion for the identification of "separate" peak emission wavelengths. In all cases we assumed a minimum of only three emission spectral determinations. On the basis of control experiments with individual insects, we established that repetitive field measurements on single individuals gave standard deviations which fell within two times the maximum value of 0.7 mμ obtained in the control experiments. On this basis, the expected standard deviation of the mean, $\sigma_m = \frac{1.4}{\sqrt{3}} = 0.8$ mμ. We then arbitrarily specify that a difference in peak position is significant only if

$$\frac{\lambda_m^{(1)} - \lambda_m^{(2)}}{[\sigma_m^{2(1)} + \sigma_m^{2(2)}]^{1/2}} \geq 3$$

which is past the 99% confidence limit. Thus only when $\lambda_m^{(1)} - \lambda_m^{(2)} \geq 3.4$ mμ do we call the difference significant. Here λ_m is the average value of the peak intensity wavelength.

RESULTS AND DISCUSSION

A table supplemental to Table I of the original paper (1) is given in Table I of the present paper. For these measurements, a minimum of three complete emission spectra was recorded and calculated for each of 41 individual fireflies. In those cases in which identification could be made only as *Photuris* sp., the flash patterns were used as an identification criterion (4, 5). For example, for *Photuris* sp. the peak emission wavelength difference between 549 and 553 mμ is considered to be significant on the basis of the discussion

TABLE I

SUMMARY OF MEASUREMENTS OF EMISSION SPECTRA OF FIREFLY BIOLUMINESCENCE

Species	Collection location	Wavelength at peak intensity	FWHM*	50% Points	10% Points
		mμ	mμ	mμ	mμ
Photinus ardens (3 specimens)	Oneida, N. Y.	566	68	539-607	521-659
Photinus collustrans (3 specimens)	Gainesville, Fla.	558	72	531-603	513-657
Photinus consanguineus (4 specimens)	Gainesville, Fla.	563	66	537-603	520-660
Photinus consimilis (3 specimens) (slow flasher)	Gainesville, Fla.	562	67	535-602	518-656
Photinus consimilis (2 specimens) (rapid flasher)	Gainesville, Fla.	568	64	542-606	524-662
Photinus floridanus (1 specimen)	Gainesville, Fla.	565	67	538-605	520-662
Photinus marginellus (3 specimens)	Ithaca, N. Y.	565	66	536-602	519-654
Photinus sabulosus (2 specimens)	Ithaca, N. Y.	566	69	530-608	521-654
Photinus tanytoxus (4 specimens)	Gainesville, Fla.	555	67	529-596	510-648
Photinus umbratus (2 specimens)	Gainesville, Fla.	562	64	535-599	517-653
Photuris sp. (3 specimens) (double pulse during ½ sec, every 4 sec)	Gainesville, Fla.	549	67	523-590	502-640
Photuris sp. (3 specimens) (flicker lasting ½ sec every 2–3 sec)	Gainesville, Fla.	552	72	524-596	505-650
Photuris sp. (3 specimens) (single flash every 3 sec)	Wampsville, N. Y.	553	70	526-596	506-650
Pyractomena linearis (4 specimens)	Oneida, N. Y.	568	72	540-612	522-665
Pyrophorus physoderus (1 specimen) (ventral organ)	Coconut Grove, Fla.	553	77	525-602	505-659
Pyrophorus physoderus (1 specimen) (dorsal organ)	Coconut Grove, Fla.	553	75	525-600	505-650

* FWHM is the width in millimicrons between 50% intensity points.

in the previous section. This is also the case for the differently flashing *Photinus consimilis* species (6) where the peak positions were 562 and 568 mμ, respectively.

In Table I of the previous paper (1), data were presented for two specimens of *Pyrophorus plagiophthalamus*, showing peak wavelengths of 553 and

TABLE II

SUMMARY OF PEAK IN VIVO EMISSION WAVELENGTHS FOR ALL 35 FIREFLY SPECIES MEASURED TO DATE

Wavelength at peak intensity	*	Species
mμ		
594	*	
592	*	
590	*	
588		
586	*	
584	*	
582	*	
580	*	
578	*	
576	*	
574		*Photinus scintillans* ♂♀
572		*Photinus gracilobus*
570	*	*Lecontea* sp.; *Photinus* (*ceratus-morbosus, evanescens, lobatus, melanurus, nothus*)
568	*	*Photinus* (*consimilis,*‡ *leucopyge* sp., *xanthophotis* ♂, *Pyractomena linearis*
566	*	*Photinus* (*ardens, sabulosus, xanthophotis* ♀)
564	*	*Photinus* (*commissus* ♀, *floridanus, marginellus,*§ *pallens*)
562	*	*Photinus* (*consanguineus, consimilis,*‖ *pyralis* ♂♀,¶ *umbratus*)
560	*	*Photinus pardalis*
558	*	*Photinus collustrans*
556	*	
554	*	*Diphotus* sp.; *Photinus tanytoxus; Photuris jamaicensis* ♂♀
552	*	*Photuris* sp.;** *Photuris* sp.;‡‡ *Photuris pennsylvanica* (?); *Pyrophorus physoderus*§§
550	*	
548	*	*Photuris sp.*‖‖
546	*	

Where the sex is not indicated, it is assumed to be the male of the species.

* Emission observed from *P. plagiophthalamus*, measured from either a dorsal or a ventral organ.

‡ Flash pattern, rapid pulses.

§ In vivo emission spectra for three samples collected in 1965 were identical with those measured in 1963.

‖ Flash pattern, slow pulses.

¶ In vitro emission spectra from crude and crystallized enzyme extracts were identical with in vivo emission.

** Flash pattern, fast flicker lasting ½ sec each 2 to 3 sec.

‡‡ Flash pattern, single flash each 3 sec.

§§ In vitro emission spectra from crude extracts of both dorsal and ventral light organs were identical and also identical with in vivo emission.

‖‖ Flash pattern, double pulse during ½ sec every 4 sec.

582 mμ for the dorsal and ventral organs, respectively. However, on the basis of our present experiments, we recognize that there can be a wide range of emission colors for different individuals in this species and therefore *P. plagiophthalamus* has been omitted from the present Table I. This implies that in all the other species reported, both in the present paper and in the previous paper, we have observed no intraspecies differences in emission spectra. Over the past several years, routine in vivo emission spectral measurements of randomly selected (both in time and geography) specimens of *Photinus marginellus*, *Photinus pyralis*, and *Photuris pennsylvanica* (?) have always given the same peak emission wavelengths. It should be noted that in two

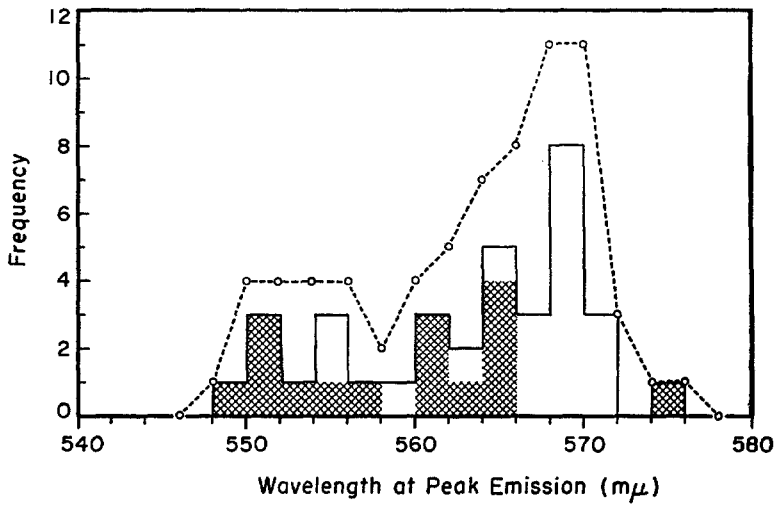

FIGURE 1. Histogram (solid line) of peak emission wavelengths for 34 species of fireflies The cross-hatched portions represent American fireflies and the open portions represent Jamaican fireflies. The broken line connecting the circles is an averaging curve where the number of samples plotted at wavelength λ mμ is given by $N_\lambda = N_{\lambda-1} + N_{\lambda+1}$.

cases reported in Table I there *are* differences in peak emission wavelengths for ostensibly the same species; e.g., *P. consimilis* and *Photuris* sp. However, in these cases we were able to observe a difference in flashing patterns between the specimens.

Table II is a summary of peak emission wavelengths for all firefly species measured to date, including the large intraspecies differences shown by *P. plagiophthalamus*.

We have examined the data of Table II in two separate ways. First, we have made the assumption that all the species with the exception of *P. plagiophthalamus* show no intraspecies color differences. We therefore have plotted a species histogram (solid lines) in Fig. 1, showing the distribution of peak emission wavelengths for the remaining 34 species of fireflies. We have fur-

ther separated the species in the histogram by geographical location into American and Jamaican species and represented these as cross-hatched and open portions respectively. The broken lines of Fig. 1 form a further averaging curve from the arbitrary formula

$$N_\lambda = N_{\lambda-1} + N_{\lambda+1}$$

It appears that on the average Jamaican fireflies have longer peak wavelengths than American fireflies. We assumed that 558 mμ divides the distribution of Fig. 1 into two portions. A χ^2 analysis of the Fourfold Table (7) for the number of American and Jamaican species measured to have peak emissions above and below 558 mμ gave, for one degree of freedom, $\chi^2 = 2.9$. If the peak wavelength distribution were completely random, the observed distribution would occur less than 10% of the time. We feel that more data are required before we can say definitely that Jamaican fireflies are different from American fireflies in spectal emission color.

More significantly in terms of the mechanism of enzyme action it is of interest to compare the spectral emission differences within the same species obtained at the same geographical location. This has been done for *P. plagiophthalamus* in Fig. 2. There are two histograms in Fig. 2, one of the peak emission wavelength positions measured spectrophotometrically from the dorsal organs of 155 separate specimens of *P. plagiophthalamus* selected at random, and the other of the peak emission wavelength positions of 35 ventral organs selected from among those 155 specimens. During our preliminary visual observations of ventral organ bioluminescence (of these insects in flight) we were able to find two specimens which exhibited a green bioluminescence and two specimens which exhibited an orange bioluminescence, markedly different from the generally yellow-green to yellow emission from most ventral organs. These 4 specimens were included in the histogram along with the other 31 in order to show the complete color range observed.

The colors of the dorsal organs summarized in Fig. 2 fell into three distinct and statistically significant distributions. These are:

550.1 ± 1.3 mμ	"Lime" color
556.8 ± 1.4 mμ	"Lemon-lime" color
562.4 ± 1.0 mμ	"Lemon" color

The standard deviations of the means are 0.21, 0.19, and 0.14 mμ, respectively so that in each case

$$\frac{\lambda_m^{(1)} - \lambda_m^{(2)}}{[\sigma_m^{2(1)} + \sigma_m^{2(2)}]^{1/2}} > 20$$

The bioluminescence emission colors of both left and right dorsal organs in

any individual insect are identical. This is based on extensive spectrophotometric measurements of 10 of the 155 insects examined, again selected at random, as well as on visual determinations on a total of 301 insects, including the 155 actually measured spectrophotometrically. To illustrate the accuracy of the visual observations, the preliminary visual color identification as to Lime, Lemon-lime, or Lemon of the 310 light organs of the 155 insects sub-

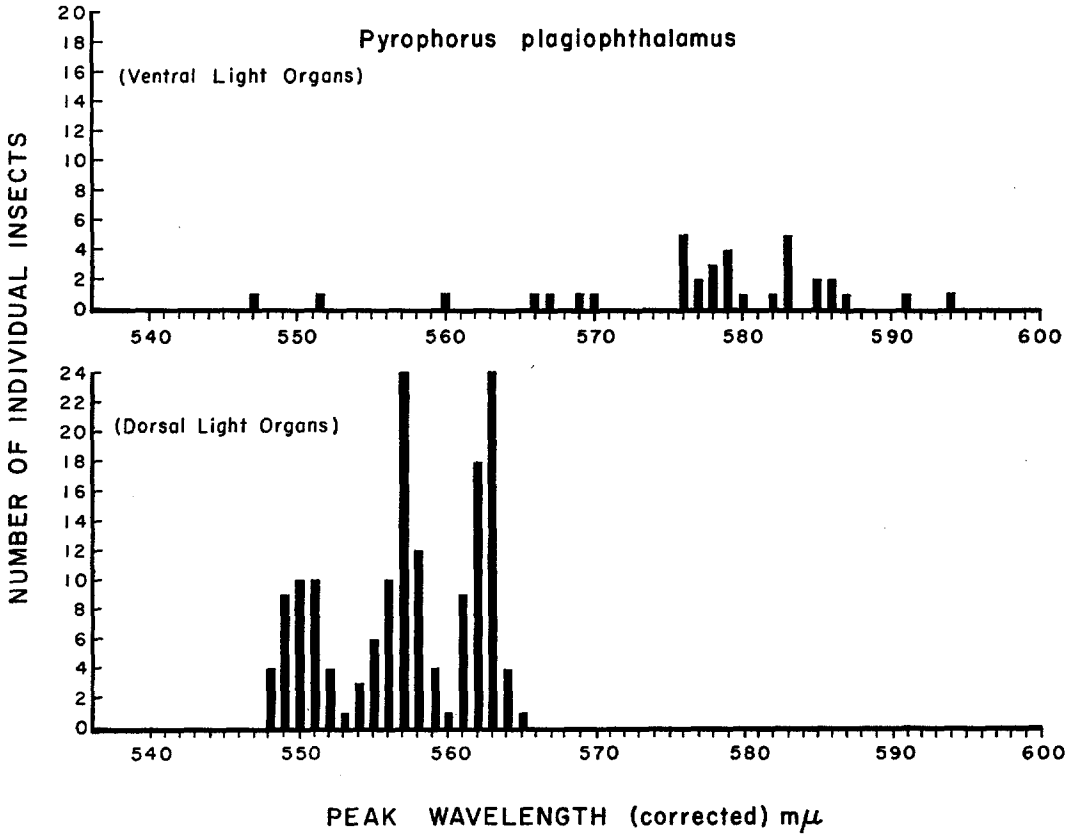

FIGURE 2. Histograms of peak emission wavelengths for 155 dorsal light organs and 35 ventral light organs of *Pyrophorus plagiophthalamus*.

sequently measured spectrophotometrically proved to be 97% correct. This is not too surprising in that it is possible to detect visually a color difference between the two sodium *D* lines which are separated by only 2 mμ.

The case is more complex for the ventral organ color distributions of Fig. 2. There is no obvious correlation between dorsal and ventral organ color. Most of the specimens emitted a yellow bioluminescence between 575 and 585 mμ. However, there is a total spread of 47 mμ in peak positions measured. In this species the distribution of ventral organ colors covers a wider

range than the colors observed for all the other 34 species investigated previously (see Table II).

In the following discussion we shall consider the *P. plagiophthalamus* data in the light of the mechanism of enzyme-substrate participation in the excited state produced as a result of the enzyme-catalyzed chemical oxidation of luciferin.

We have previously shown that *Photinus pyralis* luciferin, both natural and synthetic, is the same as luciferin isolated from *Photinus jamaicensis*, *Photuris pennsylvanica* (?), and *Pyrophorus plagiophthalamus* dorsal and ventral organs, as judged by paper chromatography, absorbance, and fluorescence spectra, and by the fact that the color of bioluminescence of these isolated luciferins when reacted with *P. pyralis* luciferase, was the same as with native *P. pyralis* luciferin (8). In addition the color of light emitted with *P. pyralis* luciferin as a substrate in an in vitro bioluminescent reaction depends on the species from which the luciferase was isolated and was identical with the species in vivo emission (references 1, 8, and Table II (*P. physoderus*)). While we have demonstrated this species specificity for only three species (four light organs), the further evidence of the reproducibility of peak wavelengths among many different specimens of the same species and the reproducibility of the in vivo peak wavelengths over the past several years of annual samples of *P. pyralis*, *P. pennsylvanica* (?), and *P. marginellus* leads us to infer that the color differences observed are due to luciferase differences among species and that, except for *P. plagiophthalamus*, the luciferase molecule is the same for all individuals in a given species. It would appear that the luciferase enzyme is not the same for all members of *P. plagiophthalamus*, and is not even the same for the dorsal and ventral organs in a given individual.

The concept that a difference in color of bioluminescence emission implies a difference in enzyme (primary, secondary, or tertiary structure) arises from the following experimental observations. In Table I of the previous paper (1) and in the present Table I we have reported in addition to the peak intensity wavelength values, the full width at half-maximum intensity (FWHM) for each of the firefly emission spectra measured. The average FWHM is calculated to be 690A $\pm$ 44A. The bioluminescence emission spectra as shown in Figs. 3 and 4 of the previous paper (1) are smooth, relatively wide bands with no obvious shoulders or fine structure. We have reported in addition that the *visually observed* color of emission of *P. pyralis* bioluminescence can vary from green through yellow to red, depending on the pH at which the in vitro light-emitting reaction is run, with purified *P. pyralis* luciferase and luciferin (2). We have further observed this effect as a function of concentration of metal cations such as Zn^{++}, Cd^{++}, and Hg^{++} (8) and as a function of temperature (9). In all these cases we have demonstrated that the shift in *visually observed* color is the physiological integration

of the relative intensities of a normal green-emitting excited state and a red-emitting excited state; the latter becomes prevalent as the pH decreases from pH 7.8, as the metal cation concentration increases from zero, or as the temperature is increased from 7°C. From the normalized emission spectra presented in these references it is obvious that a *visual yellow* emission is actually produced by a composite of both green and red emissions; a doubly peaked spectrum showing the presence of both bands and effectively as wide (FWHM) as both the bands. In none of the in vivo emission spectra measured have we observed any evidence of such composite spectra and in particular it was not observed for *P. plagiophthalamus* which is specifically under discussion in this paper. This is not to say that it is impossible for a slight admixture of a red-emitting excited state to shift the observed peak intensity wavelength by a maximum of 4 mμ without it being observable spectrally as a slight shoulder on the long wavelength side of the emission spectrum. In fact we showed that this could be done for the temperature effect in *P. pyralis* (9). Thus while we might be willing to concede that intraspecies pH or metal variations in *P. plagiophthalamus* light organs could produce a spread of about 4 mμ in observed peak intensity wavelengths, it is impossible to ascribe the measured spread of 47 mμ to this mechanism.

The fact that the visual colors observed from *P. plagiophthalamus* are due to different single emission spectra is evidence that the excited enzyme-substrate complexes giving rise to these spectra are different molecular species. Since we make the apparently reasonable assumption that the oxidation of luciferin by molecular oxygen should produce the same oxidation product of luciferin independent of the protein catalyst, it must be that the excited state molecular species are (enzyme-product)* complexes and that the different energy levels for the first excited states (which produce the different colors) are due to product complexes with functionally identical but structurally different enzymes or possibly isoenzymes.

Our criterion for establishing experimentally a significant difference in peak emission wavelength among all the other species was that

$$\lambda_m^{(1)} - \lambda_m^{(2)} \geq 3.4 \text{ m}\mu$$

This was assumed to be evidence of a "different" luciferase molecule participating in the light reaction. In order to apply a conservative criterion to the ventral data of Fig. 2, we observe that the maximum spread in the dorsal organ distributions is 7 mμ (553 to 560 mμ for the Lemon-lime distribution). The minimum number of ventral organ distributions which can be accommodated by the ventral organ data (assuming this maximum spread of 7 mμ) is therefore six. On this basis, we propose that there is a minimum of six structurally different luciferase molecules in *P. plagiophthalamus*.

There are mechanisms whereby the emission spectrum of the excited

enzyme-product molecule can vary from 547 through 594 mμ. A structural difference in the species enzyme molecule could produce a significant change in the polar environment of the bound chromophore, thereby causing a "solvent effect." Usually the more polar the solvent, the larger the red shift (10). In addition it is possible that weak complex formation between the chromophore and different amino acid groups on the protein can alter the energy levels of the excited state of the chromophore.

In all these cases, the bioluminescence observed in vivo is due to the interaction of the excited enzyme-chromophore complex (11) and therefore the structure of the enzyme at least in the vicinity of the chromophore must be changed in some way so as to affect the allowed energy levels. It would be interesting to understand, therefore, why strict control over luciferase synthesis appears to exist in all the species tested with the one exception of *Pyrophorus plagiophthalamus*.

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