Structured Bioluminescence. Two Emitters during both the *in Vitro* and the *in Vivo* Bioluminescence of the Sea Pansy, *Renilla**

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ABSTRACT: Luciferase, a low molecular weight energy conversion enzyme, isolated from the sea pansy, *Renilla reniformis*, catalyzes the bioluminescent oxidation of *Renilla* luciferin to produce light. At low enzyme concentration the *in vivo* reaction produces a blue structureless emission with a maximum at 20,500 cm^{-1} (488 nm). A product of the bioluminescent reaction having fluorescence properties similar to that of the blue emission has been isolated. Light from living animals, however, is green and exhibits a very narrow, structured emission having a maximum at 19,640 cm^{-1} (509 nm). We have been able to reproduce this unusual green emission under *in vitro* conditions at high enzyme concentration. We have also found that about 1% of the luciferase protein contains a tightly bound "green" chromophore whose fluorescence is identical with that of the green bioluminescent emission and whose presence is related to the contribution of the green emission during bioluminescence. A detailed spectral study of this chromophore is reported. These spectral studies suggest that the two chromophores would be a good donor–acceptor pair for excitation energy transfer although the present results do not allow an interpretation of the details of the mechanism.

Renilla luciferase has been recently isolated and found to be a low molecular weight energy conversion protein (Karkhanis and Cormier, 1971). It consists of a single subunit whose molecular weight is 12,000 and it catalyzes *in vitro*

$$\text{luciferin} + O_2 \longrightarrow \text{light } (h\nu) + \text{products}$$

where ν is the emission frequency and h is Planck's constant.

It has been previously reported (Karkhanis and Cormier, 1971) that the *in vitro* reaction results solely in a blue structureless emission with a maximum at 20,500 cm^{-1} (488 nm).[1] We report here, however, that the *in vivo* emission from whole animals which consists of a very narrow, structured green luminescence having a maximum at 19,640 cm^{-1} (509 nm) can be accurately matched by the *in vitro* bioluminescent reaction at high enzyme concentration. A product having fluorescence properties similar to that of the broad blue emission has been isolated. In addition a protein-bound chromophore associated with luciferase preparations has been shown to have fluorescence properties *identical* with the narrow green bioluminescence emission. Hence the results indicate two distinct emitting species. An analogy to the firefly keto–enol tautomerization (Seliger and McElroy, 1965) can be made, but in contrast in the *Renilla* system the contribution of the narrow "green" emission is dependent on the initial level of the green fluorescent chromophore in the protein preparation.

Methods and Materials

The following materials were obtained from the suppliers indicated: Sephadex G-75 and Sephadex G-25 (Sigma); urea, glycerol, and methanol (Fisher Scientific); Blue Dextran 2000 (Pharmacia). The solvents used were Spectrograde. Quinine hydrogen sulfate (Reagent grade) was from British Drug House, Poole, England, and was recrystallized several times (0.01 N H_2SO_4).

Isolation of Luciferase. The luciferase from *R. reniformis* was isolated by the procedure of Karkhanis and Cormier (1971).

Measurements of Quantum Yield and of Bioluminescence Emission Spectra. The *in vitro* bioluminescence reaction was carried out in potassium phosphate buffer (pH 7.2, 0.1 M) unless otherwise indicated. For these reactions the substrate, luciferin, was prepared by the procedure of Cormier *et al.* (1970) and stored in known amounts in individual sealed vials under an argon atmosphere. When a reaction was to be carried out, a vial was broken and the contents dissolved in 0.1 ml of degassed, absolute methanol, and transferred into a cuvet under an argon atmosphere. The vial was washed twice with 0.05 ml of methanol, and the washings were added to the cuvet. This solution was then flash-dried under a flow of argon. The reaction was initiated by injection of an air- or oxygen-saturated enzyme solution.

In vitro quantum yield measurements were carried out as outlined in the above paragraph, using the luminol chemiluminescence reaction as a standard (Lee *et al.*, 1966). The values presented represent the ratio of einsteins of light emitted per total moles of lucifierin utilized.

The *in vivo* bioluminescence spectra of *Renilla* were measured in the instrument described below using a sample holder constructed from glass plates (5 × 10 cm) mounted parallel to each other with a separation of 1 cm and perpendicular to the emission monochromator. This arrangement did not affect the spectral characteristics of various bioluminescence and chemiluminescence systems when compared to spectra obtained using a cuvet and the standard cell holder. Live *Renilla* were frozen with Dry Ice and placed between these plates. On warming bioluminescence was observed, and it continued for several minutes. The same spectrum was observed when the live animals were stimulated with ethyl acetate or methanol, but in these cases bioluminescence lasted less than 1 min.

* From the Department of Biochemistry, University of Georgia, Athens, Georgia 30601. *Received February 5, 1971.* This work was supported in part by the National Science Foundation and the U. S. Atomic Energy Commission. M. J. C. is a Career Development Awardee, No. 1-K3-6M-3331-06, of the U. S. Public Health Service. Contribution No. 219 from the University of Georgia Marine Institute, Sapelo Island, Ga. This is No. IX of a series entitled: Studies on the Bioluminescence of *Renilla reniformis*.

[1] The previously reported value for the maximum of emission (475 nm) was not corrected for photomultiplier response.

Reabsorption Corrections. The *in vitro* bioluminescence spectra at high enzyme concentrations were corrected for reabsorption by the enzyme-bound chromophore using eq 1

$$S(\bar{\nu}) = \frac{2.303a}{1 - \exp(-2.303a)} \quad (1)$$

(Parker and Barnes, 1957). In this equation the correction factor (S) at wave number $\bar{\nu}$ was determined from the absorbance (a) of the solution corrected for light scattering. The light-scattering contribution was determined using *Renilla* luciferin sulfokinase as a blank (J. C. Mathews *et al.*, unpublished data).

On-Line Computer System for Collection of Luminescence Data. Luminescence measurements were carried out using a component spectrofluorimeter described previously (Cormier and Prichard, 1968). This instrument has been adapted for use in an on-line, data acquisition system consisting of a Nova minicomputer (Data General) and ancillary hardware. For bioluminescence, an EMI 9558 Q/A photomultiplier tube was used as a reference. It viewed the total emission from the sample by means of reflection from a quartz cover slip mounted at a 45° angle to the axis of center from the cell to the emission monochromator. The wavelength drive of the monochromator was coupled to the computer by means of a programmable stepping motor (Slo-Syn, type HS50). The sample and reference photomultiplier outputs were preamplified using a FET operational amplifier system (Analogue Devices) and the resulting signals were fed into a 12-bit A/D converter (Minidac) through two channels of an eight-channel multiplexer (Signettics, Inc.). The design of this system and the associated computer program will be described in detail in a forthcoming publication. The program allows rapid, repetitive scans of high-resolution spectra from low-level and/or short-lived sources. For example, the bioluminescence spectra shown here were obtained using 1 mm slits unless otherwise indicated and with a total source intensity of between 10^{10} and 10^{12} photons per sec. Each spectrum was in general the average of five or more scans; the average time per scan was 8 sec, and each spectrum consisted of 500 points taken at intervals of 5 Å or less. Each point in each spectrum was in turn computed from an average of 16 samplings of both the reference and sample inputs with subtraction of the photomultiplier dark currents. The sample average was divided by the reference average to correct for fluctuations in the excitation source in fluorescence or for decay of the total emission in bioluminescence. The computer program allows storage of the raw data on punch tape, display during collection of both the current and average spectra, and conversion of the final average into units of relative quanta per unit wave number or wavelength with correction of the data using factors obtained from a standard fluorescence substance (Parker and Rees, 1960). In addition, this instrumentation allows plotting of the corrected data on an X–Y recorder and the spectral data presented in this paper are an example of its capabilities.

Fluorescence Measurements. For this work the correction factors were determined for quinine in 1 N sulfuric acid. The average spectrum of quinine from literature references (Lee and Seliger, 1970) was compared with a 71 scan average of the quinine spectrum obtained on-line using 1-mm excitation and emission slits and exciting with 348-nm light. The individual correction factors were evaluated by the program. These factors were found to be independent of slit width from 3 to 0.5 mm, and of photomultiplier supply voltages from 700 to 1800 V. They were periodically checked over the course of these experiments.

Temperature and viscosity dependence of polarization of fluorescence was determined with a component fluorimeter described previously (Cormier and Prichard, 1968). The temperature was maintained by a Lauda constant-temperature circulating water bath (Model K-L/R). The polarization excitation spectrum of *Renilla* luciferase was determined using the component fluorimeter described by Churchich (1967). These measurements were made at 23° in a mixed-solvent system of glycerol buffer (90:10, v/v).

In order to minimize scattering in determining the blue contribution from the protein-bound "green" chromophore, its emission was excited at 19,640 cm^{-1} on the emission peak. This demonstrated that there is no emission from this chromophore above 20,100 cm^{-1}.

Excitation spectra were determined using fluorescein in 1 N NaOH as a standard according to the method of Parker and Rees (1960).

Fluorescence quantum yields were determined using quinine in 1 N sulfuric acid as a standard. The quantum yield of quinine was taken as 0.55 (Melhuish, 1955).

Spectral results are reported in this paper on a wave-number scale with intensity in relative quanta per unit wave number. For convenience the wavelength position is also shown in angstroms in parentheses. It must be pointed out, however, that the spectral maximum of a wavelength plot and that of a wave-number plot do not necessarily coincide due to a difference in intensity units, *i.e.*, quanta per unit wavelength as opposed to quanta per unit wave number (Parker and Rees, 1960).

Absorption Measurements. Absorption spectra were measured using a Cary Model 14 recording spectrophotometer. The spectrum of luciferase in the visible region was determined as the difference spectrum using *Renilla* luciferin sulfokinase as a blank, since luciferin sulfokinase has very similar light-scattering properties to those of luciferase, but contains no visible absorbing chromophores (J. C. Mathews *et al.*, unpublished data).

Lifetime Measurements. Measurements of the fluorescence lifetime of the luciferase-bound chromophore were attempted using a TRW lifetime apparatus (TRW systems, El Secundo, Calif.) in conjunction with a Tektronix 556 oscilloscope. In use this instrument has demonstrated the ability to detect fluorescence decay times as short as 2 nsec (R. Irwin and J. E. Churchich, unpublished results).

Results

Luciferase-Bound Chromophore. As has been pointed out previously (Karkhanis and Cormier, 1971) purified luciferase from *R. reniformis* contains a green chromophore with an absorption band in the visible region, 20,000 cm^{-1} (500 nm), and a fluorescence with a maximum at 19,640 cm^{-1} (509 nm) (see Figure 1). This is a rather interesting chromophore in that it has narrow structured absorption and emission bands—the half-bandwidth of the fluorescence spectrum is 800 cm^{-1}—and exhibits only a small Stokes shift (360 cm^{-1}). The fluorescence excitation spectrum shown in Figure 1 is quite similar to the absorption band. There is clearly a mirror image relationship between the absorption and emission spectra, and the shape and position of the emission spectrum is excitation wavelength independent over the whole excitation spectrum. When the polarization of the emission was determined as a function of excitation (Figure 1), the values were high over the

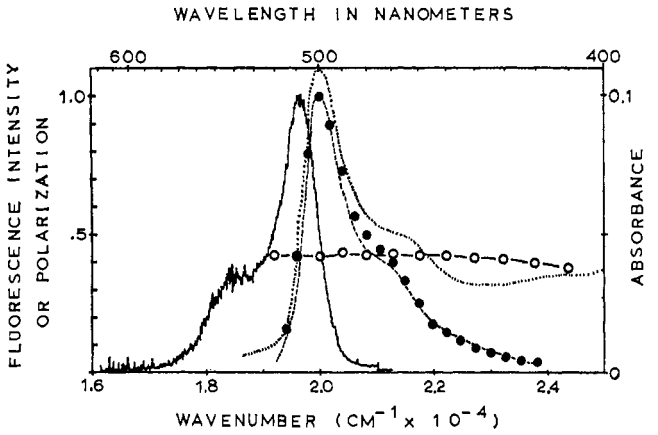

FIGURE 1: Spectral characteristics of the luciferase-bound green chromophore: (solid line) the fluorescence emission spectrum (12 scan average) exciting at 470 nm with 1-mm excitation and emission slits; (dotted line) absorption spectrum of luciferase (10 mg/ml in 0.1 M potassium phosphate buffer, pH 7.2); (dashed line) absorption spectrum of luciferase (10 mg/ml in same buffer) using luciferin sulfokinase (10 mg/ml in the same buffer) as a blank; (●) fluorescence excitation spectrum with emission at 540 nm and 1-mm excitation and emission slits; (○) fluorescence polarization excitation spectrum of luciferase (3 mg/ml in the mixed solvent glycerol–buffer (90:10, v/v)).

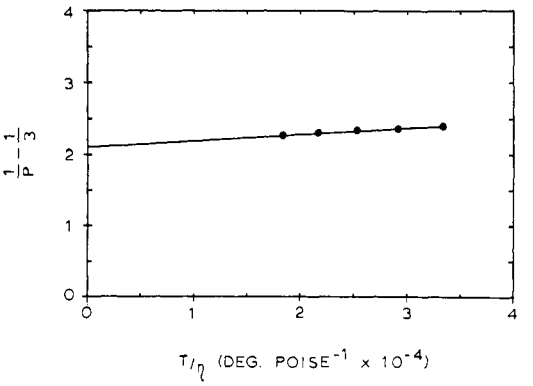

FIGURE 2: Variation of the polarization of fluorescence (P) of the enzyme-bound green chromophore as a function of temperature (T) and viscosity (η) for the luciferase-bound chromophore.

entire excitation spectrum. The quantum yield of this chromophore determined relative to quinine was 0.30. Taken together these data suggest that the absorption and emission transitions investigated here arise from the same electronic transition and that the structure seen is of a vibrational nature and not due to electronic or species differences.

The green chromophore is very strongly bound to protein. Whether this chromophore is indeed bound to luciferase or to an "associated" protein that accompanies the enzyme throughout the purification process is not known. It is not removed by dialysis or by Sephadex G-75 chromatography in the presence of 8 M urea, and it exhibits a highly polarized fluorescence when excited with polarized light. At 20° in potassium phosphate buffer (0.1 M, pH 7.2) the value of polarization is 0.37, suggesting that either the enzyme molecule to which it is attached is very large or that its fluorescence lifetime is short. Sephadex G-75 chromatography of the purified native enzyme gave a molecular weight of 34,000 (Karkhanis and Cormier, 1971). From amino acid analysis the partial specific volume of the enzyme is 0.732 cm^3 g^{-1} (Karkhanis and Cormier, 1971), giving a molar volume of 25,000 cm^3 mole^{-1}. These data suggest that the green chromophore has a short fluorescence lifetime.

Attempts to measure the fluorescence lifetime of the enzyme-bound green chromophore directly indicate that the lifetime is shorter than 2 nsec. In these measurements the fluorescence decay curve was indistinguishable from the lamp decay curve and was distinctly faster than a computer simulated 2-nsec decay curve.

By studying the variation of the polarization of fluorescence as a function of temperature and viscosity (Figure 2), the fluorescence lifetime of this chromophore can be estimated using Perrin's equation (Perrin, 1929), where P is the polarization of fluorescence, P_0 is the limiting value of polarization as the ratio of temperature (T) to viscosity (η) goes to zero, τ is the fluorescence lifetime, R is the gas constant, and V is the molar volume of the enzyme. These data allow estimation of the fluorescence lifetime of the chromophore as about 1 nsec.

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{R\tau}{V}\frac{T}{\eta}\right) \qquad (2)$$

Using 2 nsec as the upper limit for the fluorescence lifetime and 1 nsec as the lower limit, the range for the radiative lifetime (τ_0) can be calculated from eq 3, where Q is the quantum yield (0.30). This gives a radiative lifetime between 3 and 7 nsec.

$$\tau = Q\tau_0 \qquad (3)$$

The importance of this chromophore is indicated by a comparison of its fluorescence with the *in vivo* bioluminescence of the two species of *Renilla* examined (Figure 3). In both cases the spectra are identical and it is concluded that this enzyme-bound green chromophore is the *in vivo* emitter.

In other systems (Eley *et al.*, 1970; Seliger and McElroy, 1965) the difference between *in vivo* and *in vitro* emission has

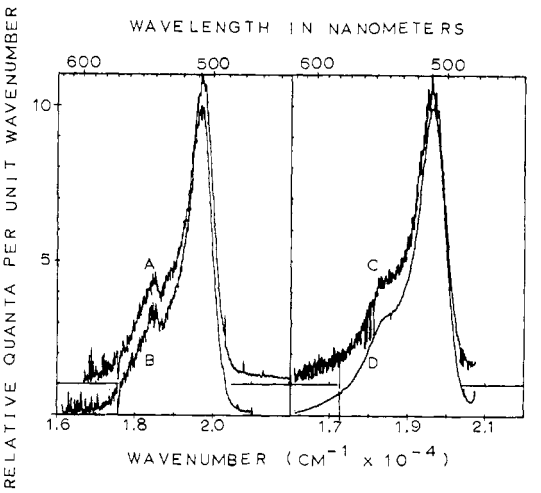

FIGURE 3: Comparison of the *in vivo* bioluminescence of *R. mülleri* (A) and *R. reniformis* (C) to the fluorescence of the luciferase bound chromophore (B and D) isolated from *R. reniformis*. The *R. mülleri* bioluminescence spectrum (8 scan average) and the luciferase fluorescence spectrum (12 scan average) to which it is compared were obtained with 1-mm emission slits. The *R. reniformis* bioluminescence spectrum (4 scan average) and the luciferase fluorescence spectrum (4 scan average) to which it is compared were obtained with 3-mm emission slits.

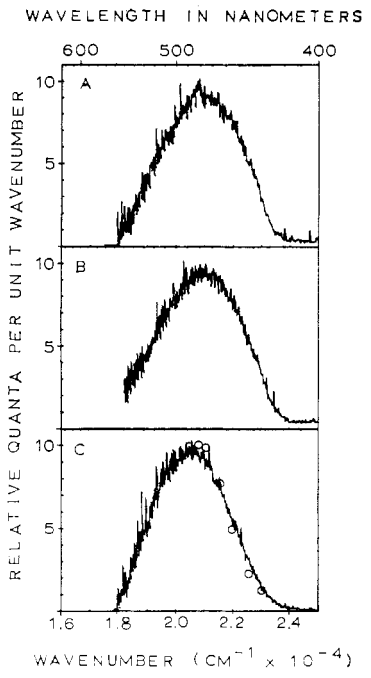

FIGURE 4: The fluorescence spectra of the reaction product isolated from the *in vitro* reaction (A) and from the *in vivo* reaction (B) compared to the *in vitro* bioluminescence spectrum (C). Spectrum A (9 scan average) and spectrum B (16 scan average) were obtained with excitation at 380 nm and 1-mm excitation and emission slits using methanol as the solvent. The *in vitro* bioluminescence spectrum (C) is the average of 13 scans with 1-mm emission slits. The reaction was initiated by injection of 1 ml of an air-saturated solution (0.1 M potassium phosphate buffer, pH 7.2) containing 10 nmoles of luciferase into a cuvet containing 5 mμmoles of luciferin. The open circles indicate the difference spectrum obtained from a *R. reniformis in vivo* bioluminescence spectrum and a 20 scan average of the fluorescence of the luciferase-bound chromophore using excitation at 510 nm (see Methods).

been explained as an environmental change affecting the same emitter species. In order to investigate this possibility, *Renilla* luciferase was incubated for 24 hr in 8 M urea–phosphate buffer (pH 7.2). The resulting denaturation of the enzyme and increased exposure of the enzyme-bound green chromophore to an aqueous environment did not change its spectral characteristics. Even digestion of the enzyme by subtilisin (10% w/w, pH 8, 12 hr) had no effect on either the shape or band position of this emission.

In Vivo Bioluminescence. The *in vivo* bioluminescence spectra of two species of *Renilla* were examined, *R. reniformis* and *Renilla mülleri.* In both cases the spectrum was identical with the fluorescence spectrum of the enzyme-bound green chromophore (Figure 3) with the exception in both cases of a shoulder in the region of 20,500 cm^{-1} (488 nm). The structure in the *in vivo* bioluminescent emission is easily detected with our on-line high-resolution spectrofluorimeter but the vibrational structure at 18,500 cm^{-1} is completely missed when commercial instruments such as the Amino-Keirs spectrofluorimeter are used. In this figure the bioluminescence spectra are offset one unit from the corresponding fluorescence spectrum in each case, since without offset the major part of the spectra are identical and indistinguishable. This identity held true when individual animals were compared with each other, again with the exception that the relative intensity of the shoulder at 20,500 cm^{-1} (488 nm) varied from animal to animal by as much as twofold. The difference spectrum of Figure 4C shows the similarity of this region of the spectrum to the *in vitro* bioluminescence. Hence in both *R. reniformis* and *R. mülleri* there are two emitting species, the enzyme-bound chromophore described above and structureless blue emission from a species which is probably related to or identical with the *in vitro* product of the bioluminescence reaction (see Figure 4).

TABLE I: *In Vitro* Bioluminescence at Low Enzyme Concentration.

Enzyme Concn (μM)	Luciferin Concn (μM)	pH	Gas Satn	Bioluminescence Quantum Yield
10	0.5	7.2	Air	0 032
100	0 5	7.2	Air	0.040
10	0.5	7.2	Oxygen	0.036
100	2.8	7.2	Air	0.028
100	5.0	7.2	Air	0.039
10	0.5	6.2	Air	0.0033
10	0.5	6.7	Air	0.039
10	0.5	7.1	Air	0.032
10	0.5	7.5	Air	0.032
10	0.5	7.8	Air	0.036
10	0.5	8.3	Air	0.030

In Vitro Bioluminescence at Low Enzyme Concentration. The *in vitro* bioluminescence is characterized by a broad structureless spectrum (Figure 4C) with a maximum at 20,500 cm^{-1} (488 nm) and with a half-bandwidth of 3250 cm^{-1}. The quantum yield of this reaction with respect to substrate was 0.04. It was found to be relatively independent of pH (Table I) and neither oxygen concentration nor luciferin concentration affected the quantum yield under the conditions of these experiments (Table I). It was also independent of enzyme concentration except at very high concentrations where the quantum yield was seen to increase. Similarly the spectral shape remained constant under all of these conditions, again with the exception of high enzyme concentration.

In Vitro Bioluminescence at High Enzyme Concentration. At high enzyme concentration the results indicate the presence of two emitting species. Figure 5 shows the spectra of the *in vitro* bioluminescence using high concentrations of two separate enzyme preparations. These spectra have been corrected for reabsorption and indicate the presence of two emitter species. The spectra obtained with the same enzyme preparations at low concentration are also shown in this figure. They have been normalized to the high wave-number sides of the high enzyme concentration spectra to show the contributions from the two emitters. The difference spectra (Figure 5) indicate that the second emitter is the enzyme-bound green chromophore. Table II shows the appropriate characteristics of these experiments at high and low enzyme concentrations. The data show that the contribution of emission from the enzyme-bound green chromophore to the total bioluminescence spectrum depends strongly on the content of green chromophore in the enzyme preparation as indicated by the ratio of optical densities at 280:500 nm. Any correlation between the enzyme concentration, the chromophore content, and the contribution of green emission to the total spectrum must await further experimentation with large amounts of enzyme from a range of preparations. These experiments are being pursued in this laboratory.

TABLE II: *In Vitro* Bioluminescence at High Enzyme Concentration.

Luciferase Prepn	OD_{280}/OD_{500}	Enzyme Concn (μM)	$I_{19,640\,cm^{-1}}/I_{20,500\,cm^{-1}}$	Bioluminescence Quantum Yield[a]
I-17	330	10	0.86	0.032
		1000	1.25	0.041
I-23	94	10	0.86	0.036
		330	1.77	0.042
		680	5.26	0.11

[a] These data were not corrected for decreased red sensitivity of the integrating photomultiplier tube. Thus the values shown at high enzyme concentration are minimum ones.

Product Fluorescence. A blue fluorescent material has been isolated from the *in vitro* bioluminescence reaction and from the surrounding medium following repetitive stimulation of *R. reniformis* to induce the *in vivo* bioluminescence. Both samples had emission maxima at 20,900 cm^{-1} (478 nm) excited at 24,000 cm^{-1} (417 nm). The position and shape of these spectra were independent of excitation. This material has spectral properties similar to those of the *in vitro* bioluminescence (Figure 4), and since it is the major *in vitro* reaction product, it appears to be directly related to the *in vitro* emitter. As noted in the figure legend, the product fluorescence was observed in methanol solution, whereas the *in vitro* reaction was carried out in aqueous solution. The solvent difference may account for the small spectral differences observed between the product fluorescence and the *in vitro* bioluminescence. It should be noted that purified luciferin is not available in large quantities, hence the reactions discussed in this work are carried out with nanomolar quantities of substrate. This level of substrate is quite adequate for light measurements, but is not sufficient for detailed chemical studies of the reaction product. Luciferin (100 nmoles) was used to produce the product for the spectra reported here. Unfortunately the amount of product produced was too small for a more detailed spectral study. Chemical and physical methods are now being used to elucidate the structure of this compound and to investigate any relationship between it and the enzyme-bound green chromophore.

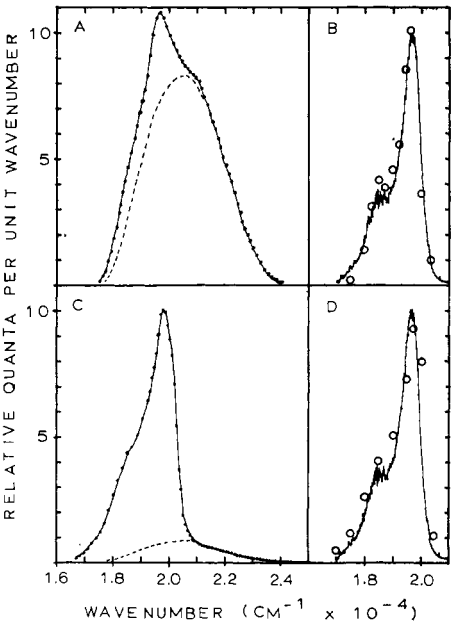

FIGURE 5: *In vitro* bioluminescence spectra at high luciferase concentrations. (A) (-●-●-) *In vitro* bioluminescence (8 scan average) corrected for reabsorption using a luciferase concentration of 34 mg/ml (1000 μM) for an enzyme which contained a low amount of enzyme-bound green chromophore (OD_{280}/OD_{500} = 330); (- - - -) *in vitro* bioluminescence spectrum (12 scan average) for a reaction using the same enzyme at 10 μM concentration. (B) (○) The difference spectrum between the curves of part A compared to the fluorescence spectrum of the enzyme-bound green chromophore (solid line). (C) (-●-●-) *In vitro* bioluminescence (8 scan average) corrected for reabsorption using a luciferase concentration of 24 mg/ml (710 μM) for an enzyme which contained a larger amount of enzyme-bound green chromophore (OD_{280}/OD_{500} = 94) compared to the enzyme of part A; (- - - -) *in vitro* bioluminescence spectrum (8 scan average) for a reaction using the same enzyme at 10 μM concentration. (D) (○) The difference spectrum between the curves of part C compared to the fluorescence spectrum of the enzyme-bound green chromophore (solid line).

Discussion

The results presented in this work suggest that the *Renilla* bioluminescence system involves two excited-state species, both of which can act as the major emitter under specific conditions. The unique character of the *in vivo* emission—a structured, narrow spectrum with a maximum at 19,640 cm^{-1} (509 nm)—and the precise similarity of the fluorescence of the enzyme-bound green chromophore to it leave little doubt that the same chromophore is responsible for both emissions. While it might be argued that the *in vitro* emission also arises from this chromophore due to the reaction taking place in a more aqueous environment, exposure of this enzyme-bound green chromophore by urea denaturation or by subtilisin digestion did not result in any change in the shape or position of its fluorescence spectrum. The perturbation necessary for a blue shift for electronic states of singlet multiplicity would be a decrease in polarity. In fact the *in vitro* emitter appears to be the reaction product itself and distinctly different from the enzyme-bound green chromophore in its spectral characteristics. The spectrum in methanol of the small amount of product isolated (Figure 4) is similar in shape and position to that of the *in vitro* emission. The solvent difference could easily account for the small shift seen.

The unusual nature of the enzyme-bound green chromophore suggests an interesting electronic structure. While the nature of the transition involved is not known, the data presented here suggest a strongly allowed transition, most likely between states of singlet multiplicity. The strength of the transition as reflected by the extinction coefficient can be estimated from eq 4 (Strickler and Berg, 1962) and similar equations (Birks and Munro, 1967).

$$1/\tau_0 = 2.88 \times 10^{-9}\eta^2(N)\int_0^\infty \frac{\epsilon(\bar{\nu})}{\bar{\nu}}d\bar{\nu} \qquad (4)$$

where $\epsilon(\bar{\nu})$ is the molar decadic extinction coefficient at wave number $\bar{\nu}$, η is the mean refractive index of the solution, and (N) is equal to

$$\frac{\int f(\bar{\nu})d\bar{\nu}}{\int f(\bar{\nu})/\bar{\nu}^3 d\bar{\nu}} \qquad (5)$$

with $f(\bar{\nu})$ equal to the fluorescence intensity at wave number $\bar{\nu}$. By evaluating the integral in eq 4 using an absorption spectrum normalized so that the maximum has a value of unity, the maximum extinction coefficient can be evaluated. Using a radiative lifetime of 3 nsec from the indirect measurement of polarization of fluorescence, the molar decadic extinction coefficient, $\epsilon_{500\,nm}$, is about 5×10^4 l. mole^{-1} cm^{-1}; or using the upper limit of the lifetime evaluated directly (7 nsec), ϵ_{500} is about 3×10^4 l. mole^{-1} cm^{-1}.

A typical enzyme preparation has a ratio of optical densities at 280:500 nm of 100. Using the value of $\epsilon^{0.1\%}_{280\,nm}$ for luciferase reported by Karkhanis and Cormier (1971) of 1.04, a molecular weight of 34,000, and the above range of extinction coefficients for the enzyme-bound chromophore, it is estimated that this chromophore is present in such preparations at a level of between 0.6 and 1.2 mole %. Taken along with the variability of the level of this chromophore from preparation to preparation and the lack of any correlation between its level and the specific activity of the enzyme, this evidence suggests that the enzyme-bound chromophore is not directly related to enzyme function. It is certainly not a cofactor in the reaction.

It is of interest to note that a protein-bound chromophore whose fluorescence properties are similar to those described above has been isolated from the luminous jellyfish, *Aequorea* (Johnson *et al.*, 1962).

The most interesting result in this work involves the *in vivo* like emission recently observed in the *in vitro* system at high enzyme concentrations. Prior to this the *in vitro* bioluminescence had been observed only at the lower enzyme concentrations, and was seen as the structureless "blue" emission described above. Such observations lend even stronger support for two emitters in *Renilla* bioluminescence. Two questions immediately arise in the discussion of this phenomenon. (1) Why are there two different emitters? (2) By what mechanism are the excited states of these emitters populated?

In answer to the first question, one might argue that the *in vitro* reaction at low enzyme concentration is an artifact representing a reaction pathway not seen *in vivo*. This is not supported by the evidence, however, since the same reaction product has been isolated from both the *in vitro* and *in vivo* reactions, and since it is this product which appears to be the *in vitro* emitter. It is also important to note that even in the *in vivo* spectra there is a contribution from this same blue emission. One answer to the question of two emitters may be found in the difference in the fluorescence quantum yield of the enzyme-bound green chromophore ($Q_f = 0.30$) and the bioluminescence quantum yield of the *in vitro* reaction at low enzyme concentration ($Q_b = 0.04$). Certainly if light emission serves a purpose to the organism, an increase in efficiency through a higher quantum yield is desirable. In fact under conditions of high enzyme concentration and sufficient green chromophore content, it has been demonstrated that a large increase in bioluminescence quantum yield is observed which approaches the fluorescence quantum yield of the enzyme-bound green chromophore.

There is some preliminary data which suggest that the two emitters are chemically related compounds. For example, if the reaction mixture from an *in vitro* bioluminescence reaction is stored at 4° for several weeks, a twofold increase in fluorescence due to enzyme-bound green chromophore is observed. Note however no immediate increase in fluorescence of the green chromophore is observed after the reaction either at low enzyme concentrations or high enzyme concentrations. If this chromophore simply represents the covalent attachment of the product to the enzyme, then the existence of two different emitters is much more attractive from an evolutionary point of view. Under these conditions it would not be necessary to envoke an additional evolutionary event to account for the difference between *in vitro* and *in vivo* light.

In view of the spectral characteristics of these two species a superficial argument might be advanced for excitation energy transfer of the Forster type between them (Forster, 1949). Since the overlap of the product emission with the enzyme-bound green chromophore absorption is very good, and since this absorption appears to be a strongly allowed transition, the conditions for such a process are excellent. Because of the extremely small amounts of product isolated no direct demonstration of such a phenomenon was attempted. However, calculations have shown that energy transfer alone cannot explain the results, since the intermolecular distances necessary for such transfer are much smaller than those which would exist in a solution at the estimated chromophore concentrations used in these experiments. For instance, using Forster's theory (Forster, 1949, 1965; Karreman and Steele, 1957) and the data from expt 5 of Table II, the efficiency of transfer necessary to account for these results is 32%. From Forster's theory the concentration of enzyme-bound green chromophore necessary for this efficiency is about 4×10^{-3} M whereas the estimated concentration of this chromophore is around 5×10^{-6} M. Of course the possibility still remains that aggregation of the enzyme at these high concentrations could result in closer interaction distances and preferential orientation of the chromophores.

Several chemical mechanisms might be proposed to explain the data presented here. Among these would be alternate catalytic mechanisms for low and high enzyme concentrations, conformational changes in enzyme effecting a change in the emission properties of the product, etc. However, the experimental data would put severe restrictions on such mechanisms since no change in the level of green chromophore is observed after the reaction, since the product of the reaction is the same both *in vivo* and *in vitro*, and since the spectral properties of the enzyme-bound green chromophore are unaffected by denaturation and digestion. The present data do not allow clear distinction between these and other possibilities.

It is clear from the data that the *in vivo* bioluminescence can be duplicated by an *in vitro* system at high enzyme concentrations. Hence study of the *in vitro* system may provide additional insights into the nature of the *in vivo* environment of luciferase in *Renilla*.

Acknowledgments

The authors thank Dr. Jorge E. Churchich of the Department of Biochemistry, University of Tennessee, Knoxville, Tenn., for the use of the polarization spectrofluorimeter and the lifetime apparatus. We also greatly appreciate the skillful assistance of Mr. Richard Irwin in making the lifetime measurements.

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Spectral Properties, Respiratory Activity, and Enzyme Systems of Bovine Corpus Luteum Mitochondria*

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ABSTRACT: Mitochondria isolated from bovine corpora lutea were shown to oxidize a number of tricarboxylic acid cycle substrates coincident with the conversion of cholesterol into pregnenolone.

The oxidative and phosphorylative capacities of these mitochondria were similar to those observed with heart and liver. With malate as substrate, respiratory control ratios exceeded 4 and ADP/O ratios approached 3. By contrast, mitochondria isolated from ovarian interstitial tissue had very low respiratory activity which was not stimulated by electron-donor substrate, ADP, or uncouplers of oxidative phosphorylation. Difference spectra of corpus luteum mitochondria demonstrated the presence of cytochromes *b*, *c* + c_1, and *a* + a_3. The presence of a carbon monoxide binding pigment with absorption maximum at 448 nm (cytochrome P-450) was confirmed, and its molar ratio to cytochrome *a* (+a_3) was 0.8:1. Data on steroid biosynthesis by corpus luteum mitochondria support the concept of interaction of the conventional cytochrome-containing respiratory chain with the cytochrome P-450 containing cholesterol side-chain cleavage enzyme(s) (cholesterol oxygenase) system.

In the adrenal and corpus luteum, the conversion of cholesterol into pregnenolone is generally considered to be the slowest step in steroid synthesis and therefore an important site for expression or influence by control mechanisms. Both extra- and intramitochondrial controls are known. The former includes luteinizing hormone in the case of the corpus luteum and adrenocorticotropic hormone for the adrenal, both presumably acting through the intermediacy of 3′,5′-cAMP and perhaps the supply of cholesterol and/or its precursors. Intramitochondrial factors limiting the rate of steroid synthesis are the availability of oxygen, electrons for oxygen activation associated with steroid hydroxylation, suitable enzyme system(s) for supplying these electrons, and quite possibly the rate of efflux of pregnenolone (Koritz and Kumar, 1970). For the past few years we have studied several of the intramitochondrial factors controlling the conversion of cholesterol into pregnenolone and its inhibition. We have sought to understand the relationship between the mitochondrial respiratory chain enzymes and the steroid hydroxylation pathway as well as the influence of various inhibitors on the molecular mechanism of steroid hydroxylation. This is the first of a series of publications dealing with these topics.

In the corpus luteum, unlike the adrenal cortex, the only steroid mixed-function oxidase present in the mitochondrial fraction is cholesterol oxygenase which carries out side-chain oxygenation and cleavage (Ichii *et al.*, 1963). Although less well studied, the cholesterol oxygenase system in the corpus luteum appears to be identical with the corresponding enzyme complex in the adrenal (Sulimovici and Boyd, 1968a). Each requires a NADPH-specific flavoprotein dehydrogenase and oxygen, and is inhibited by carbon monoxide. Moreover, the components of this electron transport pathway are probably the same since, as Yohro and Horie (1967) have shown, bovine corpora lutea contain cytochrome P-450 localized to the mitochondria, and pig ovaries contain an adrenodoxin-like nonheme iron protein (Kimura and Ohno, 1968).

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