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Bacterial Bioluminescence. Comparisons of Bioluminescence Emission Spectra, the Fluorescence of Luciferase Reaction Mixtures, and the Fluorescence of Flavin Cations*

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ABSTRACT: The fluorescence emission spectra of flavin cations in several rigid solvents were measured. Solvent polarity conditions were found in which these fluorescence spectra closely match the emission spectra of bacterial bioluminescence for the *in vivo* systems, the *in vitro* reaction initiated with various reduced flavins, and a chromophore which contributes in addition to free flavin to the total fluorescence of the reaction mixture.

The luciferase-bound flavin cation is proposed as the bacterial emitter.

It has long been known that the requirements for cell-free bacterial bioluminescence are bacterial luciferase (Strehler, 1953), reduced FMN[1] and oxygen (Strehler *et al.*, 1954), and a long-chain fatty aldehyde (Cormier and Strehler, 1953). Studies on pure crystalline luciferase isolated from *Photobacterium fischeri* (Kuwabara *et al.*, 1965; Cormier and Kuwabara, 1965; Hastings *et al.*, 1969) have not established any additional components for the reaction. Therefore the emitter must be derived from one or a combination of these reaction components. Despite many investigations into the subject the molecular identity of this emitter has remained hypothetical (Harvey, 1952; Cormier and Totter, 1964; Hastings *et al.*, 1965; Lee and Seliger, 1965; Seliger and McElroy, 1965; Cormier *et al.*, 1969; Hastings, 1968; Mitchell and Hastings, 1969). Although FMN[1] has been up to now the sole identified reaction product, the distinct difference between the species-dependent *in vivo* bioluminescence emission maximum, $\bar{\nu}_B$, which is in the range 21000 to 19500 cm^{-1} (λ_B 478–505 nm), and the fluorescence maximum of FMN ($\bar{\nu}_F$ 18400 cm^{-1}, λ_F 525 nm) appears to exclude FMN as the bioluminescence emitter. In addition any possibility of utilization of FMN in the reaction through degradation to a bluer fluorescing product such as lumichrome ($\bar{\nu}_F$ 20400 cm^{-1}) is eliminated since the quantum yield of bioluminescence with respect to FMN disappearance is greater than unity and as high as fifty[2] (Cormier and Totter, 1957; Lee and Seliger, 1965).

In this paper we show the fluorescence of the flavin cation in

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[1] Abbreviations used are: FMN, riboflavin 5′-monophosphate; $FMNH^+$, N-1 protonated FMN; FAD, flavin adenosine diphosphate; iso-FMN, 6,7-methyl isomer of FMN; 2-thio-FMN, C-2 keto oxygen atom of FMN replaced by a sulfur atom; $FMNH\cdot$, FMN semiquinone; $FMNH_2\cdot^+$, FMN semiquinone cation; $FMNH_2$, reduced FMN; $FMNH^-$, reduced FMN anion; $FMNH_3^+$, reduced FMN cation; EPA, ethyl ether–isopentane–ethyl alcohol.

[2] The quantum yield of bioluminescence with respect to a particular substrate is the total number of photons emitted divided by the total change in the number of these substrate molecules used in the reaction.

rigid glasses, the similarities of this fluorescence with the *in vivo* bioluminescence emission of two species of luminous bacteria, and the appearance of a similar fluorescence in the *in vitro* reaction product mixture. The luciferase-bound $FMNH^+$ is a good candidate for the bacterial emitter.

Materials and Methods

Chemicals Used. FMN and FAD were obtained from Sigma Chemical Company. Iso-FMN, 2-thio-FMN, lumiflavin, and isolumiflavin were synthesized by the methods of Föry and Hemmerich (1967). Some flavins were dissolved in melted boric acid (205°) which forms a glass at room temperature. For measurements at 77°K the solvents are indicated in Table I. For the reaction mixture FMN was purified by DEAE-cellulose column chromatography using ammonium carbonate for elution which was subsequently removed by sublimation. $FMNH_2$ was generated by bubbling H_2 gas through a FMN solution with platinized asbestos as catalyst. All other chemicals used were the best grades commercially available.

Purification and Assay of Luciferase. Cells from a strain of *P. fischeri* originally derived from ATCC 7744 were grown in complete medium described by Farghaly (1950) and the luciferase extracted by lysis and purified by procedures similar to those already described (Kuwabara, *et al.*, 1965; Hastings *et al.*, 1965). The column purified luciferase was essentially homogeneous by acrylamide gel electrophoresis. Crystallization was subsequently carried out by dialysis against slowly increasing concentrations of ammonium sulfate. Assay of luciferase activity was by the initial flash height obtained on rapid injection of 0.25 ml of $FMNH_2$ (10^{-4} M) into 1 ml of pH 7 mixture of luciferase, bovine serum albumin, and sonicated dodecanal (Hastings *et al.*, 1965). Using the luminol chemiluminescence secondary light standard for calibration (Lee *et al.*, 1966) final luciferase activities in the range 4–8 $\times$ 10^{13} photons sec^{-1} mg^{-1} were obtained, representing 70- to 140-fold purification over the lysate.

Measurement of Emission Spectra. Three different instruments were used for the emission studies all being calibrated for absolute spectral sensitivity by reference to the fluorescence of quinine sulfate in 1 N H_2SO_4 (Lee and Seliger, 1970). Color filters were used to reduce stray radiation and polarization anomalies were corrected out.

The first instrument was a Cary 14 spectrophotometer with the Threeport Illuminator attachment. For excitation a Bausch and Lomb high intensity f/3.5 grating monochromator selected the output from a 200 W A.C. Hg-Xe or 150 W D.C. Xe lamp. The first lamp was used for the measurement of fluorescence spectra since the output is high at 366, 405, and 435 nm. The level was always maintained below that which would produce detectable photoreduction of FMN in the sample. The Xe lamp which has a smoother spectral output was used in combination with a Rhodamine B–propylene glycol quantum counter for measurement of excitation spectra.

The second instrument used Bausch and Lomb 500 mm f/4.4 grating monochromators for both excitation and fluorescence sides. The excitation source was a 400 W D.C. Xe lamp and the intensity was again monitored with the Rhodamine B–propylene glycol solution. The third instrument was an Aminco-Keirs spectrophosphorimeter used because of the convenient low temperature Dewar accessory. Results were consistent when compared between the various instruments.

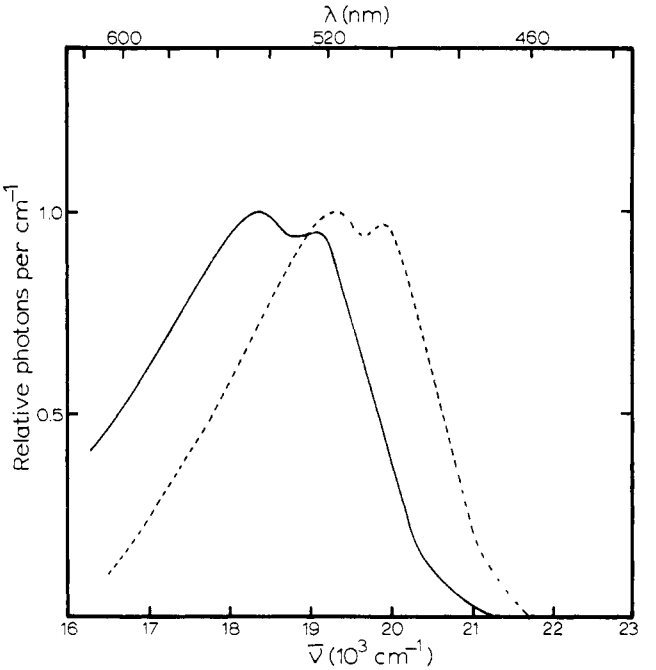

FIGURE 1: Fluorescence emission spectrum of FMN in fluid and rigid solutions. The solid line represents the spectrum in fluid aqueous solution at room temperature and the broken line represents the spectrum in a rigid glass of propylene glycol–water (1:1) at 77°K.

The *in vivo* emission spectra were made from cultures diluted about 100 times into 3% NaCl. *P. phosphoreum* (obtained from W. Terpstra, State University, Utrecht) was cultured in the medium described by Eley and Cormier (1968) and *P. fischeri* was cultured in the medium described by Farghaly (1950).

Analysis of Spectra. A standard FMN fluorescence spectrum was obtained by averaging results of excitation at three wavelengths in the range 350–450 nm. All spectra were corrected for self-absorption. All absorption spectra were measured on a Cary 14 recording spectrophotometer. An IBM 7094 computer program "FFIT" was devised to subtract the standard FMN from the reaction mixture by a least-squares overlap of each in the 18000- to 16700-cm^{-1} region of their fluorescence. At the high protein concentrations encountered in the reaction mixture, scattered and stray light make significant contributions to the apparent fluorescence spectra and must be accurately allowed for. Three techniques were employed to determine the "blank" fluorescence and each gave the same final results. These were by measurement of: (1) the luciferase solution before reaction; (2) a bovine serum albumin solution of the same protein concentration as the luciferase in the reaction mixture; (3) the above bovine serum albumin plus FMN to the same final concentration as the reaction mixture.

Results

Neutral Flavin and Flavin Cation Spectral Data. Upon careful examination structure is seen in the corrected fluorescence of FMN both under fluid and rigid aqueous conditions. A comparison of such spectra is illustrated in Figure 1. Absorp-

TABLE I: Absorption Maxima (λ_A) and Fluorescence Intensity Maxima (λ_F) of Neutral Flavins in Various Solvents.

Flavin	Absorption Maximum (25°C)		Fluorescence Intensity Maximum (77°K)[b]		
	Ethanol–H_2O (1:1) λ_A (nm)	EPA[a] λ_A (nm)	Propylene glycol–H_2O (1:1) λ_F (nm)	EPA λ_F (nm)	Ethyl Ether λ_F (nm)
FMN	447		500		
Lumiflavin	448	445		492	
FAD	450		502		
Iso-FMN	455		540		
Isolumiflavin	455	445	540	511	497
2-Thio-FMN	500		500–540		

[a] EPA, ethyl ether–isopentane–ethyl alcohol (5:5:2). [b] Fluorescence maxima were obtained from plots of fluorescence intensity per unit wavelength *vs.* wavelength and the maxima obtained from such plots will differ from that of wavenumber plots such as illustrated in Figure 1 (Seliger and McElroy, 1965).

TABLE II: Comparison of Intensity Maxima of Flavin Cation Fluorescence (λ_F) and Bioluminescence Emission (λ_B).

Flavin	Absorption Maximum (25°C) of the Cation		Flavin Cation Fluorescence, 77°K				Bioluminescence,[a] 9°C
	Ethanol–HCl λ_A (nm)	EPA–HCl[b] λ_A (nm)	Ethanol–HCl (1:1) λ_F (nm)	18 N H_2SO_4 λ_F (nm)	EPA–HCl λ_F (nm)	Ether–HCl[c] λ_F (nm)	λ_B (nm)
FMN	393		480	493			492
FAD	393		480	493			484
Iso-FMN	383		530	530			472
2-Thio-FMN	425		510–530	510–530			534
Lumiflavin	390	390	475		443		
Isolumiflavin	380	360	530		490	450	

[a] Bioluminescence data are uncorrected values (Mitchell and Hastings (1969)); λ_F represents corrected values as described in the text. [b] EPA–HCl, ethyl ether–isopentane–ethyl alcohol (5:5:2) saturated with HCl. [c] Ether–HCl, anhydrous ethyl ether saturated with HCl.

tion and fluorescence properties of several flavins in solvents of various polarities are shown in Table I. Table I should be compared to Table II in which are shown the absorption and fluorescence properties of these flavins in the same solvents under acidic conditions. The absorption changes correspond to the neutral to cation transition previously reported for aqueous solution (Dudley *et al.*, 1964). In rigid solvents the wavelengths of maximum fluorescence intensity (λ_F) for the cations listed in Table II are generally blue shifted up to 90 nm over the neutral species listed in Table I. In fluid solutions the flavin cations are not fluorescent. The fluorescence of 2-thio-FMN is very weak and accurate estimation of these λ_F are difficult. Such fluorescence could arise from slight contamination since the sulfur is extremely labile. The blank spaces in both Table I and Table II mostly arise from solubility difficulties. Table II also lists the λ_B obtained when reduced flavins were used to initiate the reaction with luciferase isolated from an unidentified luminous bacterium (Mitchell and Hastings, 1969).

No fluorescence was observed from the FMN anion, $FMNH\cdot$, $FMNH_2\cdot^+$, $FMNH_2$, $FMNH^-$, or $FMNH_3^+$ in either rigid or fluid media.

Bioluminescence and Fluorescence Spectral Comparison. Figures 2 and 3 compare the *in vivo* bioluminescence emission spectra of *P. fischeri* and *P. phosphoreum* with the fluorescence emission of $FMNH^+$ in two rigid solvents of slightly different polarity. Figure 2 shows the fluorescence of $FMNH^+$ in boric acid glass at room temperature. An excitation spectrum for the lower energy part of the curve shows that some free FMN contributes to the total fluorescence and this has been substracted to yield the dashed curve. In spite of the high temperature required for boric acid glass formation the spectra are quite reproducible. The absorption spectrum is typical of $FMNH^+$ having a λ_A at 393 nm ($\bar{\nu}_A$ 25450 cm^{-1}). When excited at λ_A the fluorescence quantum yield (Q_F) is about 20%. Figure 3 shows that the fluorescence spectrum of $FMNH^+$ at 77°K in ethanol–HCl glass is blue shifted about 100 cm^{-1} from the spectrum in boric acid at room tempera-

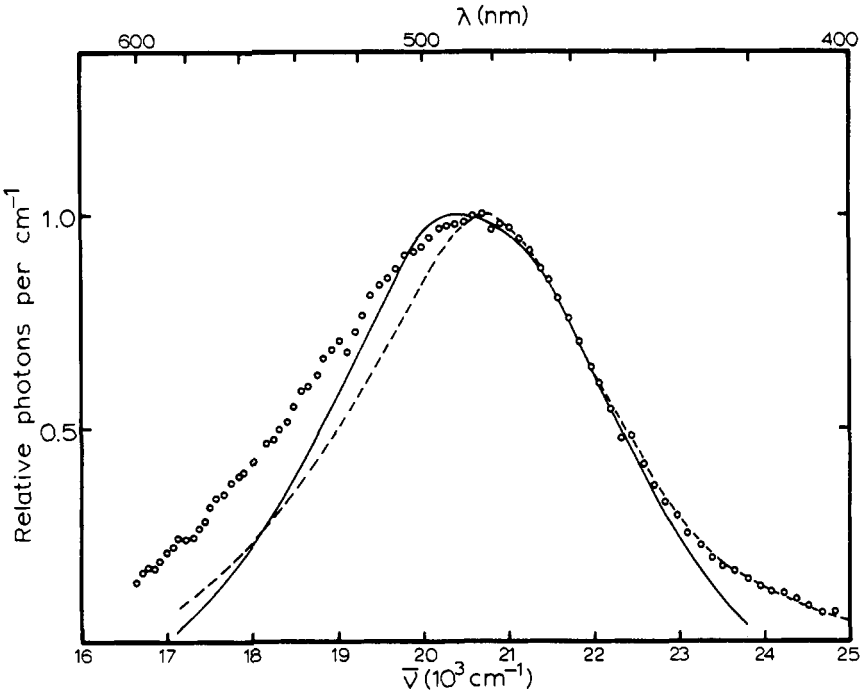

FIGURE 2: Comparison of the *in vivo* bioluminescence emission spectrum from *P. fischeri* (solid line; $\bar{\nu}_B$ 20450 cm^{-1}) and the fluorescence emission spectrum of $FMNH^+$ (broken line; $\bar{\nu}_F$ 20800 cm^{-1}). The fluorescence was measured in a boric acid glass at room temperature excited at 25500 cm^{-1}. A small amount of free flavin contributes to the total fluorescence emission (circles). This was determined by exciting at 22000 cm^{-1} and this has been subtracted to yield the broken line.

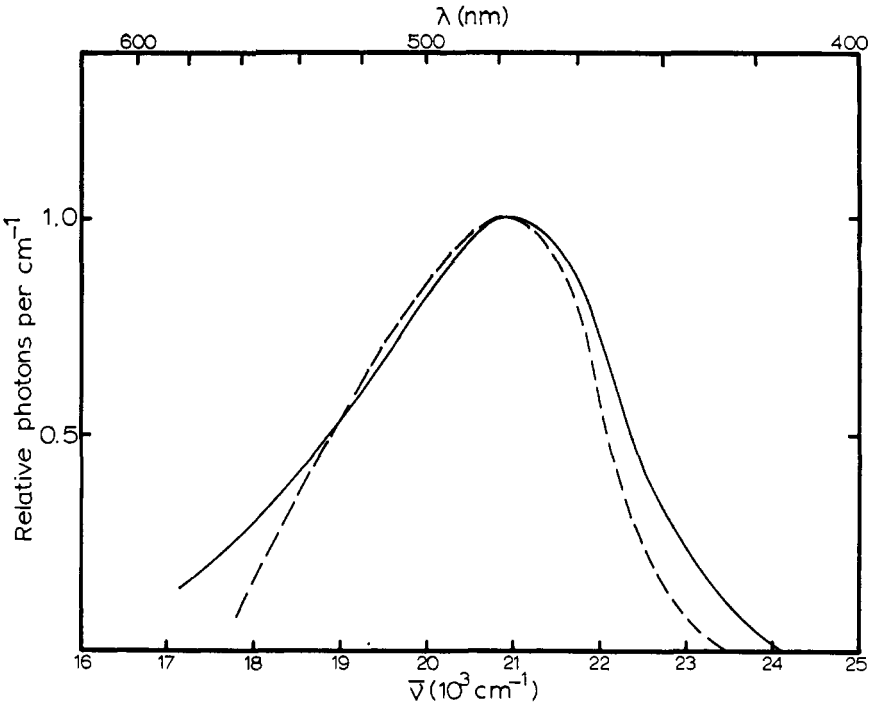

FIGURE 3: Comparison of the *in vivo* bioluminescence emission spectrum from *P. phosphoreum* (solid line; $\bar{\nu}_B$ 21000 cm^{-1}) and the fluorescence emission of $FMNH^+$ (broken line; $\bar{\nu}_F$ 20900 cm^{-1}). The fluorescence was measured in a glass of ethanol–12 N HCl (1:1) at 77°K excited at 25500 cm^{-1}.

ture. The excitation spectrum corresponds to the $FMNH^+$ absorption.

Fluorescence of Luciferase. The total fluorescence of a 30 mg/ml solution of crystalline luciferase (*P. fischeri*) lies in the range 16000–22000 cm^{-1} (460–600 nm) with a maximum at about 18300 and a shoulder at 19000 cm^{-1}. This structure is indicative of flavin (Figure 1) and Figure 4 shows that the excitation spectrum for the luciferase fluorescence at 18000 cm^{-1} is the same as the flavin absorption. The flavin is quantitatively characterized by the oxidized minus dithionite reduced absorption spectrum of the luciferase and is present at a level of 0.2–1.0% per mole of enzyme. In spite of intensive efforts to remove it this trace remains in all the crystalline preparations.

Figure 4 also shows the excitation spectrum for the luciferase fluorescence in the 21000-cm^{-1} region, where flavin would be expected to contribute negligible fluorescence. This is seen to be quite distinct from the flavin absorption and demonstrates that two independent chromophores contribute to the total fluorescence emission. Subtraction of the standard FMN

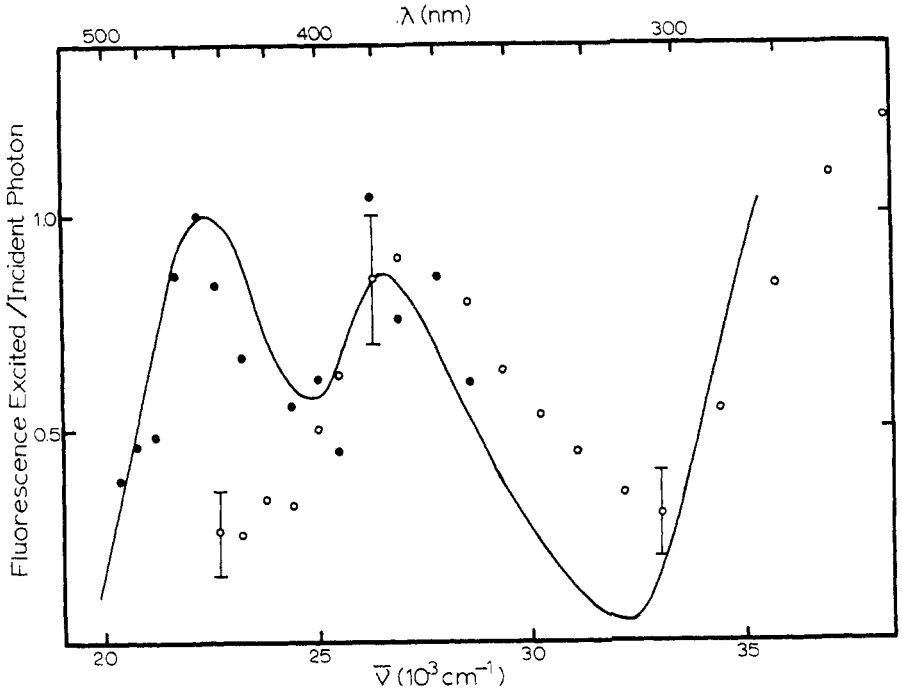

FIGURE 4: Excitation spectrum of a solution of crystalline *P. fischeri* luciferase (30 mg/ml) in 10 mM phosphate buffer, pH 7, with the fluorescence set at 18000 cm^{-1} (closed circles) and at 21000 cm^{-1} (open circles). The absorption spectrum of FMN (solid line) corresponds to the lower energy fluorescence excitation spectrum.

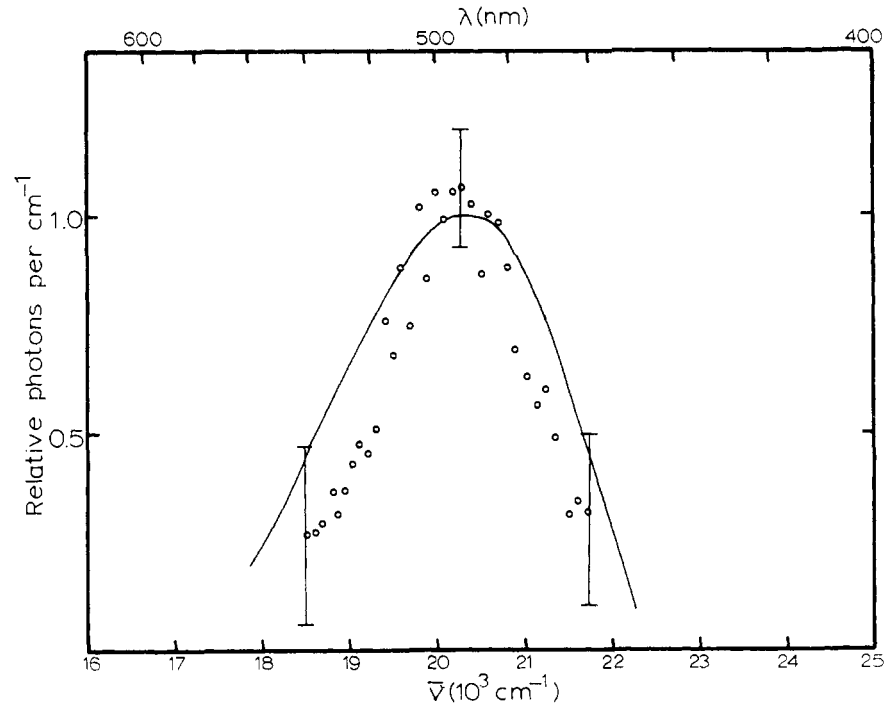

FIGURE 5: Comparison of the average difference spectrum (F490) of the total fluorescence of luciferase reaction mixtures (III-1 to V-3) less free flavin and scattered light, and the fluoresence of $FMNH^+$. The luciferase (open circles) was excited at 27300 cm^{-1} and the $FMNH^+$ (solid line) at 25500 cm^{-1} in 18 N H_2SO_4 (77°K).

fluorescence spectrum from the total fluorescence of the luciferase results in the second chromophore having a maximum intensity at about 20000 cm^{-1} ($\pm$500 cm^{-1}).

Fluorescence of Luciferase–FMN Reaction Mixtures. The total fluorescence of a bioluminescence reaction mixture of luciferase, oxygen, aldehyde, and $FMNH_2$ is dominated by FMN. If the standard FMN spectrum is subtracted a difference spectrum results as shown by the circles in Figure 5. Since λ_F is in the region 490–500 nm we have labeled this chromophore F490. The excitation spectrum for the 21000-cm^{-1} region of the fluorescence of the reaction mixture is distinct from FMN and preliminary data indicate that it is not the same as for the 21000-cm^{-1} region of luciferase (Figure 4). The circles in Figure 5 are the average of the F490 difference spectra for the reaction mixtures III-1 to V-3 (Table III) obtained with the Cary instrument. The uncertainty at the higher end of the spectrum arises mainly from the lack of precise control of the final FMN concentration, thus resulting in an imprecise correction for self-absorption. At the lower energies the very large contribution from the free flavin makes

the difference uncertain. Figure 5 also compares F490 with the fluorescence of $FMNH^+$ in 18 N H_2SO_4 glass at 77°K.

Table III lists the λ_F of the F490 difference spectra obtained for several reaction mixtures having differing luciferase concentrations. Those numbered III-1 to V-3 were measured on the Cary instrument with excitation at 366 nm and 1–3 on the Bausch and Lomb 500 mm instrument with excitation at 350 nm. Excitation at longer wavelengths up to 430 nm gives similar F490. The F490 intensity is about 15% of the free FMN contribution. Before reaction the nonflavin part of the total florescence contributes about 20% over the observed flavin fluorescence.

The omission of aldehyde from one of the reactions in Table III does not affect the appearance of F490 in the reaction mixture even though the bioluminescence quantum yield with respect to $FMNH_2$ is reduced to less than 0.01%. The F490 also appears on the addition of FMN to luciferase and quantitative studies of this interaction are in progress.

In the complete reaction mixture the quantum yield of bioluminescence with respect to $FMNH_2$ is in the range of 1–2%. This is five to ten times lower than that reported by previous workers after adjusting for the difference in spectral intensity standards (Hastings *et al.*, 1965; Lee *et al.*, 1966; Hastings and Reynolds, 1966). They had however photoreduced the FMN *in situ* and presumably carried out the light reaction at very low oxygen concentrations. In the present work and in spite of the high enzyme concentrations used, most of the $FMNH_2$ must be reacting by nonenzymatic paths and only 10–20% by the light path.

Finally the intensity of the FMN part of the total fluorescence of the reaction mixture is less than would be expected in the absence of luciferase. This is indicated in Table III as the difference between the expected and observed FMN fluorescence. In the case of reaction V-1, for instance, 43% of the added FMN has disappeared as judged by fluorescence assay. This is like the loss of fluorescence of FMN on addition of bovine serum albumin caused by nonspecific binding. An example of this is indicated in Table III. If all the components of the reaction mixture are separated by passing through a Sephadex G-25 column, the FMN is recovered quantitatively as judged by fluorescence and absorption assay.

TABLE III: Difference Fluorescence F490 Which Results on Subtraction of FMN Fluorescence and Scattered Light from the Total Fluorescence of the Bioluminescence Reaction Mixture.

Reaction	Luciferase Concn (mg/ml)	Final Concn of FMN (μM)	FMN Fluorescence Reduction[b] (%)	F490 λ_F (nm)	F490 Intensity (% obsd FMN)
III-1	17	9	1.3	502	18
III-2	30	15	1.3	492	22
IV	37.5	64	33	489	16
V-1	20	60	43	500	13
V-2[a]	20	31	2	497	10
V-3	20	12	5	503	13
1	2.5	37			<2
2	30	17		505	5
3	10	48		500	16
Bovine serum albumin	10	5	15		<2

[a] No aldehyde. [b] (Expected − observed)/expected.

Discussion

Figure 1 shows that a rigid environment maintains the structure of FMN fluorescence and does not shift it to sufficiently high energies to overlap bioluminescence emissions and thus favor a perturbed FMN as the emitter in bacterial bioluminescence. On the other hand the flavin cation fluorescence is a good match for the bioluminescence emission spectra (Figures 2 and 3). Table II also shows that the solvent polarity change in going from 18 N H_2SO_4 to ethanol–HCl shifts the λ_F of $FMNH^+$ over a range similar to the range of wavelengths of maximum bioluminescence intensity (λ_B) for *in vivo* bioluminescence from various species which is 478–505 nm and for *in vitro* bioluminescence in the presence of FMN which is 490–500 nm (Seliger and Morton, 1968).

The bioluminescence emission λ_B elicited by reduced flavin derivatives is significantly different from that by $FMNH_2$ (Mitchell and Hastings, 1969). In the case of isoflavins a solvent polarity between that provided by ether–HCl and EPA–HCl glasses is required to shift the λ_F of iso-$FMNH^+$ to correspond to the λ_B initiated with reduced iso-FMN. Such fluorescence shifts are shown by using isolumiflavin since iso-FMN is insoluble in these nonpolar solvents. In ethanol–HCl both isolumiflavin and iso-FMN cations give the same λ_F. In spite of the very weak fluorescence of the 2-thio-FMN cation its λ_F appears to correspond roughly with the observed λ_B.

The possibility that the F490 difference spectrum could arise as an artifact either from the distortion of the shape of the FMN emission by its binding to the luciferase or by an unexpected change of scattering or stray light produced by the luciferase under the conditions of the reaction mixture is not supported by the observations. Although nonspecific binding occurs with bovine serum albumin the FMN emission is *identical* with free FMN after scattered light subtraction. Of more importance is the excitation spectrum of F490 which is distinctly different from that of FMN and the quantitative computer matching of the reaction mixture with FMN in the region 16600–18000 cm^{-1} where the F490 does not contribute.

The scattered light argument is made unlikely by the fact that the F490 appearance is unaffected by variations in luciferase concentration, excitation wavelengths, or type of instrument used.

Although the data do not allow quantitative relations to be established it is apparent that the F490 intensity is higher at the higher luciferase concentrations and that it is always in about the same ratio to the FMN intensity. It is not clear whether the decrease in FMN fluorescence intensity in the presence of luciferase is related to the appearance of F490. This could simply be nonspecific binding.

Figure 5 compares the fluorescence emission of $FMNH^+$ in H_2SO_4 glass with that of F490. Within the experimental error these appear to be the same. The nondependence of

F490 on the presence of aldehyde and its appearance even on addition of FMN alone to luciferase suggest that a flavin cation-like chromophore is formed on the enzyme before the light reaction. A corresponding chromophore should be formed with luciferase and iso-FMN but the much lower fluorescence yield with this flavin would render its detection difficult. The nonflavin part of the luciferase fluorescence does not appear to be the same as F490 since it is both tightly bound to the luciferase and has a different excitation spectrum, similar to lumichrome although authentic lumichrome does not interact with luciferase (J. Lee, unpublished observations). Experimental difficulty has prevented a reliable estimate of the F490 excitation peak position. It would be expected to be at 393 nm, the same as the $FMNH^+$ absorption.

Under the acid glass conditions the fluorescence emitter is the cation protonated at N-1 (Hemmerich, 1960; Dudley *et al.*, 1964). It is conceivable that in the bioluminescence reaction luciferase could interact with FMN to protonate it and rigidly bind it in an environment having a polarity necessary to account for the color of bioluminescence observed. The environment provided for iso-$FMNH^+$ must be of significantly lower polarity than that for FMN implicating the importance of the flavin 8-methyl position in binding to luciferase. Species differences in the *in vivo* emissions of luminous bacteria can now be accounted for on the basis of differences in the *in vivo* environment of luciferase coupled with observed differences in amino acid compositions of the enzymes (Hastings *et al.*, 1969).

The suggestion that the enzyme-bound flavin cation represents the bacterial emitter is attractive from the points of view that flavin is not degraded in the reaction (Cormier and Totter, 1957; Lee and Seliger, 1965), that a flavin cation-like fluorescence is formed reversibly in the reaction mixture containing FMN and luciferase, and that the Q_F of 20% is more than adequate to account for the observed bioluminescence quantum yield (Hastings *et al.*, 1965) which is less than 10% on adjusting for spectral intensity standards (Lee *et al.*, 1966). This proposal parallels the firefly bioluminescence system in which it has been demonstrated that the emitting species is the luciferase-bound product dianion, a species normally present in free solution only under strongly basic conditions (Hopkins *et al.*, 1967).

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