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The Spectroscopic Properties of Firefly Luciferin and Related Compounds. An Approach to Product Emission*

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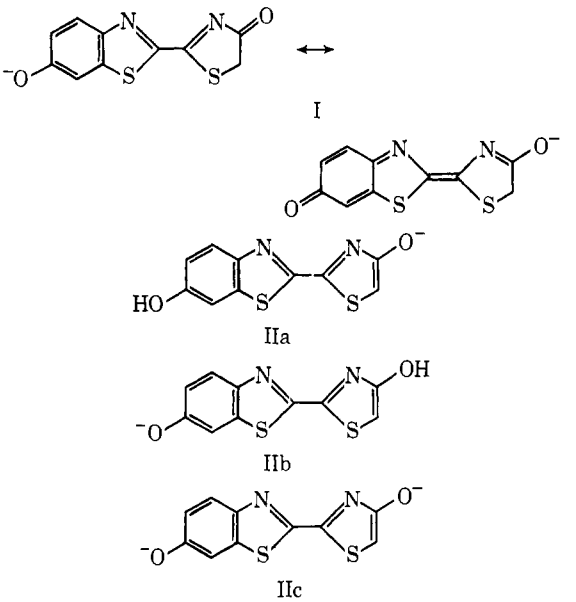
ABSTRACT: The spectroscopic properties of firefly luciferin, its adenylate, and several analogs were investigated in an effort to understand the excited state formed in firefly bioluminescence. It was found that in aqueous solutions, the 6′-hydroxyl group of firefly luciferin (phenol form) dissociates its proton in the excited state. Fluorescence is from the corresponding phenolate anion, the phenol form being relatively nonfluorescent in aqueous solutions. In addition, the fluorescence emission peak of the phenolate anion of luciferin is red-shifted by more than 3200 cm^{-1} in going from nonpolar solvents to water. In aqueous solutions, Zn^{2+} and Cd^{2+} ions produce a further red shift of the fluorescence emission peak. The spectra of the adenylates of luciferin and dehydroluciferin showed effects which could be interpreted in terms of an intramolecular interaction between the benzothiazole and adenine chromophores. All these perturbations of the luciferin chromophore result in significant shifts of the peaks but only small changes in the band width of luciferin emission. Finally, the relevance of these results to the bioluminescence is discussed.

The complete mechanism of firefly bioluminescence must ultimately include, in addition to the biochemical information normally sought for all enzymatic reactions, an understanding of the excited state which emits the visible light. Spectroscopy thus has a more central role than as a means for studying molecular structure. If the immediate oxidation product of the bioluminescent reaction could be extracted and identified, knowledge of its excited state(s) could be obtained by studying its spectroscopic properties. Recent identification of the product emittor in luciferyl adenylate red chemiluminescence as the monoanion of the decarboxylated, 4-keto derivative of luciferin (I) suggests that an anion of the enol form (IIa, b, or c) may be the bioluminescence emittor



I

IIa

IIb

IIc

(Hopkins *et al.*, 1967). These molecules, however, are not stable in aqueous solutions, and attempts to isolate the product of the enzymatic reaction have failed (Seliger and McElroy, 1966; Plant *et al.*, 1968). Neither have we been able to observe a molecular species in a

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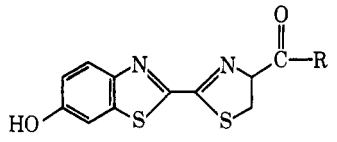
TABLE I: Quantum Yields of Firefly Luciferin and Related Compounds.

Compound	Condition	Absorption		Fluorescence[a]			
		Lowest Energy Band (cm^{-1})	Molar Extinction	$\bar{\nu}_m$ (cm^{-1})	$1/\bar{\nu}_m$ ($m\mu$)	λ_m	Quantum Yield
($^-$O)-Luciferin	H_2O, pH 11	26,020	1.82×10^4	18,450	541	537	0.62
(HO)-Luciferin	H_2O, pH 5.0	30,480	1.82×10^4	18,450	541	537	0.25
(HO)-Luciferin	EtOH (>99%) plus acetate buffer, pH 5.0	30,250	1.82×10^4	23,000	420	415	0.03
(HO)-Luciferyl adenylate	H_2O, pH 5.0	29,800	1.51×10^4	18,150	553	548	0.11
($^-$O)-Dehydroluciferin	H_2O, pH 11	25,250		17,850	561	555	0.62
(HO)-Dehydroluciferin	H_2O, pH 4.5	28,750		17,850	561	555	0.26
(HO)-Dehydroluciferin	EtOH (98%) plus acetate buffer, pH 4.5		2.4×10^4 [b]	23,000	435	430	0.20
($^-$O)-Dehydroluciferin	EtOH (90%) plus carbonate buffer, pH 11			18,200	549	546	0.62
($^-$O)-Dehydroluciferyl adenylate	H_2O, pH 11	24,550					0.01
(HO)-Dehydroluciferyl adenylate	H_2O, pH 4.5	28,250					0.01
(HO)-Dehydroluciferyl adenylate	Ethylene glycol (95%) plus acetate buffer, pH 4.5			21,900	457	452	0.25
6′-Methoxyluciferin	H_2O, pH 11.1	30,600	*c*	22,700[d]			0.03[d]
6′-Methoxyluciferin	H_2O, pH 5.23	30,600	*c*	22,700[d]			0.03[d]
6′-Methoxyluciferin	EtOH (99%) plus pH 5.2 acetate buffer	30,750	*c*	23,800[d]			0.01[d]

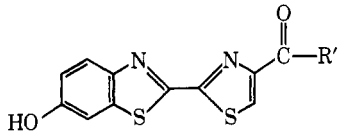
[a] λ_m is position of peak fluorescence ($m\mu$) on a $dN/d\lambda$ plot, $1/\bar{\nu}_m$ is the reciprocal ($m\mu$) of the peak position in a $dN/d\bar{\nu}$ plot. [b] Values in ethanol (White *et al.*, 1963). [c] The absolute extinctions were not measured. However, they were the same for acid and base. [d] Approximate value owing to extreme photosensitivity.

spent *in vitro* bioluminescence reaction whose fluorescence properties are identical with the bioluminescence emission. Furthermore, the available evidence indicates that the excited state from which light is emitted is an enzyme–product complex. For example, the colors of *in vivo* bioluminescence in various firefly species are different (Seliger *et al.*, 1964; Biggley *et al.*, 1967) even though the substrate (luciferin) appears to be the same (Seliger and McElroy, 1964), implying that the enzyme significantly perturbs the excited state of the product molecule.

We have, therefore, taken an alternative approach. We have examined certain properties of molecules that we hope are reasonable analogs of the product molecule. These are properties likely to be common to the electronic configuration of the emitting chromophore. In this paper we will describe and interpret such results for firefly luciferin (III), luciferyl adenylate (IV) (substrates of the bioluminescence reaction), dehydroluciferin (V), and dehydroluciferyl adenylate (VI) (potent inhibitors of the light reaction). Our primary interest is not with the spectroscopic properties themselves, but how they may be applied to the understanding of firefly bioluminescence, and in particular the role of the enzyme in determining the color of the emission.



III, R = OH
IV, R = AMP



V, R′ = OH
VI, R′ = AMP

Experimental Procedures

Materials. In anticipation of the possible ambiguities introduced in fluorescence analysis by minute amounts of contaminants, a considerable effort was made to ensure the purity of our preparations. We found that reagent grade chemicals were acceptable for preparation of solvents (and buffers).

Firefly luciferin was prepared according to the procedure of White *et al.* (1961). Samples of L- and DL-luciferin, dehydroluciferin, 6′-methoxyluciferin, and luciferin methyl ester were the gifts of Dr. Emil White. These compounds deteriorate considerably in storage, and usually additional purification was necessary. We found that excellent separation of impurities was obtained by elution from columns of G-25 Sephadex (Pharmacia Fine Chemicals, Inc.). For best resolution either fine or superfine grades must be used, and the eluting buffer must have an ionic strength of at least 0.05. Separation occurs due to adsorption, since there is no correlation between elution behavior and molecular weight. The column capacity is small (about 10^{-7} mole/ml of swollen Sephadex). G-10 Sephadex is not effective in the separation. Dehydroluciferin was recrystallized from *N,N′*-dimethylformamide–water before chromatography. Concentrations of solutions of these compounds were determined using the molar extinction coefficients shown in Table I.

We prepared the mixed carboxylic–phosphoric anhydrides (adenylates), IV and VI, essentially according to the procedure of Berg (1958), a technique differing slightly from that previously used for this purpose (Rhodes and McElroy, 1958). Briefly, LH_2 or L, AMP (Pabst Biochemicals), and dicyclohexylcarbodiimide (Aldrich Chemical Co.) were reacted in either aqueous, acidified pyridine at 4°, or in dimethyl sulfoxide at 25°. When the reaction was complete (about 20 min for luciferyl adenylate, 360 min for dehydroluciferyl adenylate) the product was both precipitated and washed with cold acetone (−20°), dried *in vacuo*, and stored in a freezer (−20°) under nitrogen. Within 1 day the precipitate was dissolved in sodium acetate buffer (0.01–0.04 M NaCl, pH between 4.5 and 5) and chromatographed on G-25 Sephadex. The LH_2AMP (or LAMP) separated on the column from unreacted material and fluorescent impurities. The purified product contained a minute amount of a fluorescent component which remained at the origin in paper chromatography using the solvent system of Airth *et al.* (1958). LH_2AMP hydrolyzes very rapidly in aqueous solution and it is therefore difficult to prepare it entirely free of LH_2. Our purified solutions of LH_2AMP contained about or less than 3–5% LH_2. LAMP solutions hydrolyze much less rapidly, and no contamination with L could be detected.

The active substrate in the bioluminescent reaction is the adenylate of the D isomer of LH_2 (Seliger *et al.*, 1961). The aqueous pyridine condensation reaction, while it gives high yields of mixed anhydride, produces mixtures of D and L diastereomers of luciferyl adenylate from the original D- or L-luciferin. In addition the optically active Sephadex effects a partial separation of D-LH_2AMP from L-LH_2AMP. The LH_2AMP samples used for the spectroscopic investigations contained mixtures of D and L diastereomers, between 50 and 90% D-LH_2AMP. We have recently been able to establish that when distilled (anhydrous) DMSO is used as the reaction solvent, practically pure D- or L-LH_2AMP can be prepared. Since the experiments in this paper refer only to spectroscopic properties, and since our comparisons revealed no differences between D- and L-LH_2AMP with respect to these properties, the use of isomeric mixtures should in no way affect the validity of our data.

D-LH_2AMP reacts directly with firefly luciferase to produce light while D-LH_2 requires the presence of ATP and Mg^{2+} for enzymatic light production (Rhodes and McElroy, 1958). It is therefore possible to assay for D-LH_2AMP with excess luciferase in the presence of LH_2, and to assay for the D-LH_2 afterward by further light produced upon additions of ATP and Mg^{2+}.

A brief description of the experimental techniques we used with adenosine might be useful. We dissolved adenosine in the appropriate buffer at about 80°, and allowed the solution to cool. For concentrations above 0.01 M the adenosine eventually precipitated out of solution. However, it was usually possible to complete the necessary measurements before this occurred. The LH_2 or L was not added until after the solution cooled to room temperature. For absorption spectra, the blank contained adenosine, although the spectra we report were run only over the region where there was no significant blank absorption.

Measurements of Spectra. Absorption spectra were measured on a Beckman Model DK 2 or Cary Model 14 spectrophotometer. Fluorescence emission spectra were obtained using either a 1-m f/3 grating spectrometer or a 0.5-m f/4.5 grating spectrometer designed by W. Fastie. Both RCA 7326 and EMI 9558 phototubes, having an S-20 spectral response, were used. These instrument–phototube combinations were calibrated for relative photon spectral efficiency using an NBS color temperature standard lamp (Lee and Seliger, 1965). The band width used with these instruments was 4 mμ.

For excitation spectra, we used a Perkin-Elmer prism monochromometer, calibrated for spectral intensity distribution using a solution of Rhodamine B, 3 g/l. in ethylene glycol, as a quantum counter. Band widths were between 3 and 5 mμ.

We determined quantum yields relative to quinine bisulfate in 1 N H_2SO (Melhuish, 1961). All fluorescence and absorption measurements were made at room temperature (about 24°) in air-saturated solutions of absorbance less than 0.05. Corrections for self-absorption were therefore negligible and since quantum yields are relative to quinine bisulfate ($\phi_f = 0.55$) corrections due to refractive index differences are cancelled. Over the range 2–38°, the fluorescence quantum yields of (HO)-LH_2[1] and ($^-$O)-LH_2[1] varied by less than -0.1%/deg, including -0.02%/ deg due to the volume expansion of water. This variation is well within the accuracy of our measurements of the absolute quantum yields. No deviations from Beer–Lambert behavior in absorption and no concentration-dependent self-quenching of fluorescence were observed.

Plotting of Data. All spectral data are plotted as a function of wave number (cm^{-1}). A wavelength scale (mμ) is given at the top of each graph for orientation. The ordinate for fluorescence emission spectra has the units photons per unit wave number ($dN/d\bar{\nu}$) and the curves are normalized to unity at the wave number of peak intensity. The $dN/d\bar{\nu}$ *vs.* $\bar{\nu}$ plot can be used for the determination of energy differences between absorption and emission shifts and especially in the examination of the shapes of absorption and fluorescence bands. This type of plot is not the same as a $dN/d\lambda$ plot where photons per unit wavelength are plotted as a function of wavelength (Seliger and Morton, 1968). It should be remembered that neither the peak intensity positions nor the full width at half-maximum intensity values of fluorescence data are reciprocally related between the two types of plots (Seliger and McElroy, 1966).

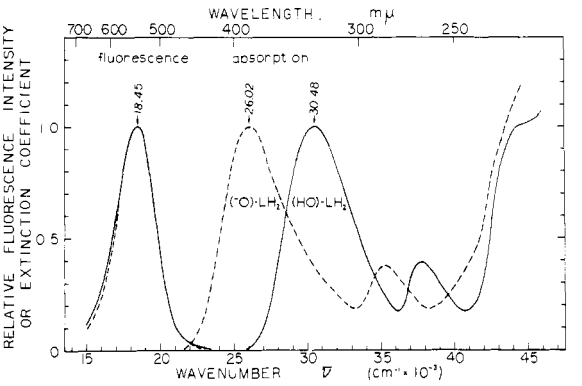


FIGURE 1: Absorption and fluorescence spectra of luciferin in 0.05 M sodium acetate buffer (pH 4.8) (----) and in 0.05 M sodium carbonate buffer (pH 10.5) (——). Spectra were normalized and fluorescence emission was corrected as described in the text.

Results and Interpretation

We investigated the effects of pH (state of ionization), solvent, heavy metal ions, and intramolecular complexing on the spectroscopic properties. A summary of all data is given in Table I.

The Effect of pH. EXCITED-STATE IONIZATION. The titration of LH_2 both potentiometrically and spectrophotometrically shows a single ionization between pH 4 and 11.5. This reflects the dissociation of the 6′-hydroxyl group to the phenolate ion with $pK_a = 8.7$. The ionization of this group has a large effect on the absorption spectrum (Figure 1). A similar effect is found with L and other analogous molecules having a 6′-hydroxyl group.

The shift of an absorption spectrum on ionization is both necessary and sufficient to conclude that the pK_a of ionization of the excited state is different from the ground state (Weller, 1961; Jaffé *et al.*, 1964). From formulas given in these references and the relevant absorption spectra, the phenolate pK_a of the lowest energy excited state of LH_2 is about -1 (the corresponding value for L is 1.3). The excited-state phenolate pK_a shift was previously suggested by White *et al.* (1963). It is similar to that found in naphthalene derivatives (*e.g.*, 1- and 2-naphtol).

From the calculated pK_a we would predict that, if equilibrium were established in the excited state, the predominant form between pH 4 and 12 would be the phenolate ion. The fluorescence emission spectrum of LH_2 at pH 4.5 (also shown in Figure 1) shows that if the phenol form is excited, proton transfer to the solvent occurs and emission is from the excited state of phenolate ion, ($^-$O)-LH_2*. The quantum yields of fluorescence due to absorption by the two ground-state forms

[1] The state of ionization of 6′-(benzothiazole)hydroxyl group of these compounds are indicated by the prefixed (HO) or ($^-$O); * denotes an excited-state species.

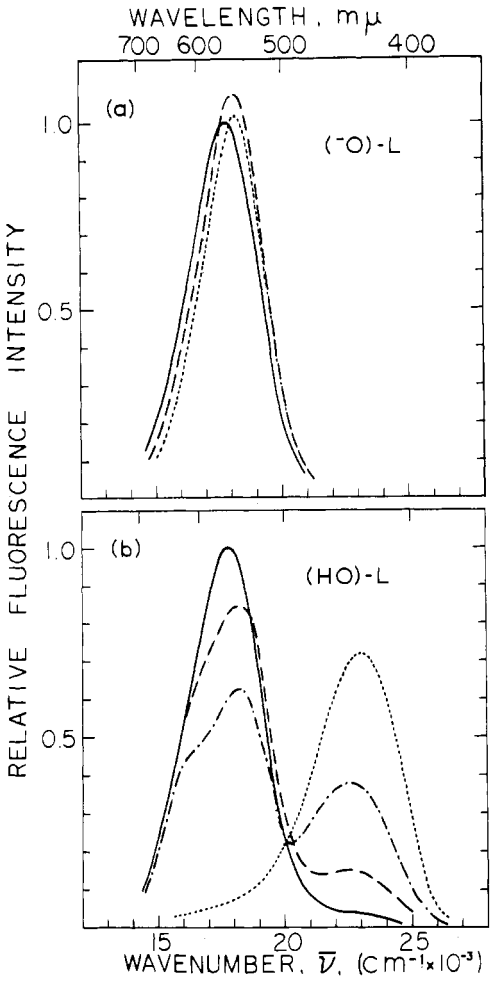


FIGURE 2: Fluorescence emission spectra of dehydroluciferin phenolate form, (⁻O)-dehydroluciferin (panel a), and phenol form (HO)-dehydroluciferin (panel b), in mixtures of water and ethanol containing 0% (————), 50% (----), 80% (—— · —— ·), 98% (----------) ethanol. Corrected spectra are plotted relative to the peak intensity in water (0% ethanol) as 1.0.

are not, however, the same (Table I). That of the phenolate ion is higher by a factor of about 2.5, implying only partial equilibrium in the excited state is obtained. Furthermore, the quantum yield for emission from the excited-state phenol form is very small in aqueous solution. It is, however, possible to detect a blue shoulder in the green fluorescence of both LH_2 and L at acid pH. In the case of LH_2 this shoulder is so weak that it cannot be unequivocally associated with the excited-state phenol.

In nonaqueous solutions three effects may occur: the emission spectrum shifts slightly, the quantum yield of the excited-state phenol form changes, and excited-state proton transfer may be inhibited. Figure 2 shows how these effects change the fluorescence emission. Panel a is the fluorescence of (⁻O)-L in ethanol plus water mixtures. Panel b shows the results for (HO)-L. In the former case sodium carbonate (10^{-3} M) was added, and in the latter sodium acetate buffer (0.05 M, pH 4.5) was mixed with the ethanol. In the case of (HO)-L, the yellow-green fluorescence (17,850 cm^{-1}) in aqueous solution is gradually replaced by a blue fluorescence (23,000 cm^{-1}) as the ethanol concentration is increased. We interpret the blue fluorescence as due to emission by the (HO)-L* ion. Proton transfer is inhibited in ethanol, and the quantum yield of fluorescence increases. In contrast, the quantum yield of the (⁻O)-L ion changes hardly at all in ethanol. Additional evidence that the yellow-green fluorescence at acid pH is due to emission by the (⁻O)-L* ion is that the same, small shift to higher energy occurs with increasing ethanol as for direct excitation of the (⁻O)-L ion (compare bands of about 18,000 cm^{-1} in panels a and b). In addition to these two fluorescence bands, there is a weak red shoulder in the emission of L at acid pH (approximately 16,000 cm^{-1}) which may be due to protonation of the pyridyl nitrogens.

The excitation spectrum of L fluorescence in 75% ethanol, 25% 0.05 M sodium acetate buffer, where two emission bands are present, shows essentially identical excitation spectra, corresponding to absorption by the (HO)-L ion.

In addition to these quantitative data, our qualitative observations of the fluorescence of LH_2 and its derivatives in various solvents are consistent with this interpretation of the excited-state ionization. Thus the fluorescence of LH_2 at neutral pH is blue in acetone, methanol, ethylene glycol, DMSO, and ice (Seliger and McElroy, 1960b; all of these solvents prevent proton transfer) as opposed to the yellow-green fluorescence in liquid water. In DMSO saturated with triethylamine (a proton acceptor), a yellow fluorescence is observed. The absorption spectrum in this solvent again indicates that the ground-state phenol form is present. Thus, proton transfer occurs in the excited state.

The fluorescence of 6′-methoxyluciferin (Table I) is weakly blue in both ethanol and water at pH 4.5 and 11. In this case, because of the extreme photosensitivity and corresponding difficulty in purification and analysis of 6′-methoxyluciferin, we have been unable to place reliable figures on fluorescence quantum yield and spectrum. This is indicated in Table I. The fluorescence quantum yield is, however, much smaller than for the yellow-green fluorescence of (HO)-LH_2 in water, and more like the yield of (HO)-LH_2 in ethanol.

Figure 3 summarizes the excited-state ionization of LH_2. A similar diagram could be drawn for L, the energy differences being correspondingly smaller. The highly fluorescent form in aqueous solution is the (⁻O)-LH_2 ion. When (HO)-LH_2 is excited in aqueous solutions the excited-state molecule transfers the proton of the 6′-hydroxyl group to the solvent. In nonaqueous media (no proton acceptors) the (HO)-LH_2 ion can fluoresce (blue) with low efficiency. As shown in Table I, the quantum yield of fluorescence of (HO)-L in nonaqueous media is much higher than that of (HO)-LH_2.

Solvent Perturbation. In contrast to the abrupt shift to lower energy in absorption and fluorescence emission observed in going from the phenol to phenolate form, a gradual shift in emission is found for the phenolate ion in a series of organic solvents. In addition, the peak

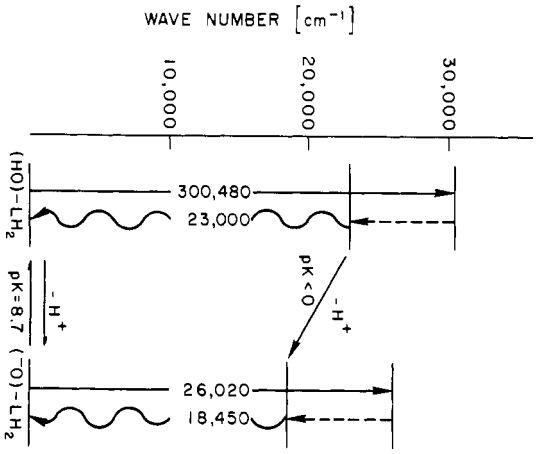


FIGURE 3: Diagram illustrating excited-state ionization of luciferin. Absorption transitions are shown by the solid vertical arrows. Internal transitions are shown by the dashed vertical arrows. We assume that the lowest excited state of (OH)-luciferin* becomes ionized and results in (⁻O)-luciferin*, which then undergoes a fluorescent transition indicated by the wavy arrow. Since the peak wave numbers shown in the figure are spectroscopic values both the (⁻O)-luciferin ground state and the (OH)-luciferin ground state are placed at zero, although as the result of ground-state ionization (⁻O)-luciferin would normally be slightly higher in energy than (HO)-luciferin.

broadens as its maximum moves to lower energies (longer wavelengths). For the phenolate (⁻O)-LH_2 $\bar{\nu}_M$ varies from 21,700 cm^{-1} in dioxane[2] to 18,500 in water. The full width at half-maximum of the phenolate (⁻O)-LH_2 form decreases from 3150 cm^{-1} in water to 2800 cm^{-1} in dioxane.

Theories relating the changes in $\bar{\nu}_M$ and the FWHM to solvent composition are generally very difficult to apply because of the many different factors which must be taken into account. However, the red shift in more polar solvents which we observe for (⁻O)-LH_2 is typical of $\pi \rightarrow \pi^*$ transitions in which the dipole moment increases on excitation (Lippert, 1957). For our purposes, it will be sufficient to simply compare the changes in these parameters with those observed in the bioluminescence. The ultimate cause of the latter may also depend on specific interactions between chromophore and protein to which the solute–solvent interactions are only a partial analogy.

Effect of Divalent Metal Ions. In the presence of Cd^{2+} or Zn^{2+} (0.5 M, as perchlorates) and at pH 5, the $\bar{\nu}_M$ of LH_2 fluorescence emission (from the phenolate excited state) shifts from 18,500 cm^{-1} (with uncorrected excitation maximum at 31,200 cm^{-1}) to 16,900 cm^{-1} (with uncorrected excitation maximum at 26,000 cm^{-1}) (as shown in Figure 4a). This shift is accompanied by little or no change in the band width (the FWHM = 2900 cm^{-1}) or fluorescence quantum yield (0.25). Mg^{2+} does not affect the fluorescence of LH_2. Mn^{2+} and Ca^{2+} shift the emission very slightly to lower energies. However, as shown in Figure 4b, the yellow-green to red shift in the *in vitro* bioluminescence upon addition of excess Zn^{2+} or Cd^{2+} or in acid buffer (Seliger and McElroy, 1960a, 1964) is accompanied by a significant decrease in the FWHM (2400–1600 cm^{-1}). This point will be discussed later.

The fluorescence emission spectra of both 6′-methoxyluciferin and luciferin methyl ester showed equivalent peak shifts to lower energies (the FWHM = 1300–1500 cm^{-1}) in the presence of Cd^{2+} and Zn^{2+} ions. Decarboxyluciferin exhibits the same behavior. Thus phenolate or carboxylate anions are not required for the interaction.

At concentrations between 0.01 and 0.5 M Zn $(ClO_4)_2$ luciferin fluorescence spectra cannot be analyzed in terms of only two contributors (the two emissions shown in Figure 4a). There is no absorption band narrowing or resolution into fine structure as seen in bidentate complexes of α,α'-dipyridyl with divalent metal ions (Sone *et al.*, 1955; Schlafer, 1956). An α,α'-dipyridyl-like structure in which the metal ion is bonded to both nitrogens of a single luciferin molecule is not the only possible chelating structure, although it is consistent with our results.

Adenylates of Luciferin and Dehydroluciferin; Intramolecular Complexing. The first step in the firefly bioluminescent reaction is the enzymatic formation of enzyme-bound D-LH_2AMP (Seliger *et al.*, 1961). Firefly luciferase also catalyzes the formation of adenylates of L-LH_2 and L.[3] As a second step in the examination of the spectroscopy of luciferin analogs, we have determined the properties of the chemically synthesized adenylates.

The absorption spectrum of LAMP is shown in Figure 5. Since it is much more stable toward base-catalyzed hydrolysis than LH_2AMP, we were able to obtain a reasonably accurate spectrum for the (⁻O)-LAMP ion. A similar spectral shift in absorption upon ionization of the 6′-hydroxyl group occurs for LH_2-AMP and LAMP just as with LH_2 and L. The band due to absorption by the adenine part of the molecule naturally does not change appreciably. The phenolate pK_a for LH_2AMP is about 8.7 as estimated from changes in the absorption spectra with pH.

No emission spectrum is shown with LAMP because of its low fluorescence yield (Table I). The value "$\leq$0.01" means that we feel the fluorescence observed, while probably due to the LAMP molecule, cannot be unambiguously associated with it because a small amount of a highly fluorescent impurity might still be present. In any case LAMP is essentially nonfluorescent in water. On the other hand, LH_2AMP is reasonably fluorescent. Its fluorescent yield at pH 4.5 is about 0.45 times that of LH_2. The yellow-green emission spectrum of (HO)-LH_2AMP indicates that the fluorescence is from (⁻O)-LH_2AMP*. LAMP and LH_2AMP

[2] A small amount of triethylamine (3%) was added to each solvent as a proton acceptor. No absorption spectrum shifts accompany these additions.

[3] It is incorrectly stated in Seliger *et al.* (1961) that L-LH_2 reacts with the enzyme, ATP, and Mg^{2+} to yield the same product as D-LH_2. McElroy and Seliger (1962) give data showing that L-LH_2 does not react with oxygen in the enzymatic system.

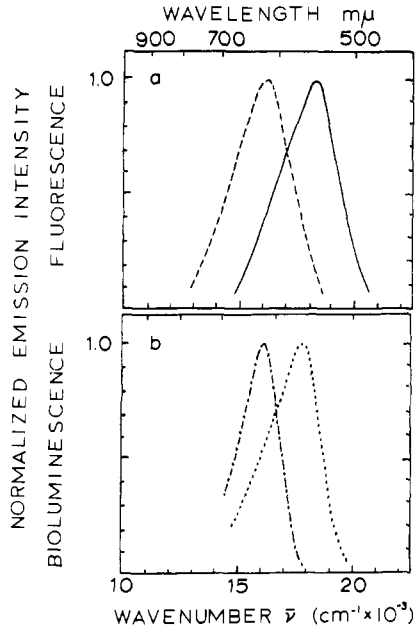


FIGURE 4: Fluorescence studies. (Panel a) Of luciferin at pH 5.0 (————), compared with LH_2 plus $Zn(ClO_4)_2$ at 0.5 M, pH 5.0 (----). (Panel b) The comparison of the *in vitro* bioluminescence spectrum without (·····) and with $ZnSO_4$ at 2×10^{-3} M (— · — · —).

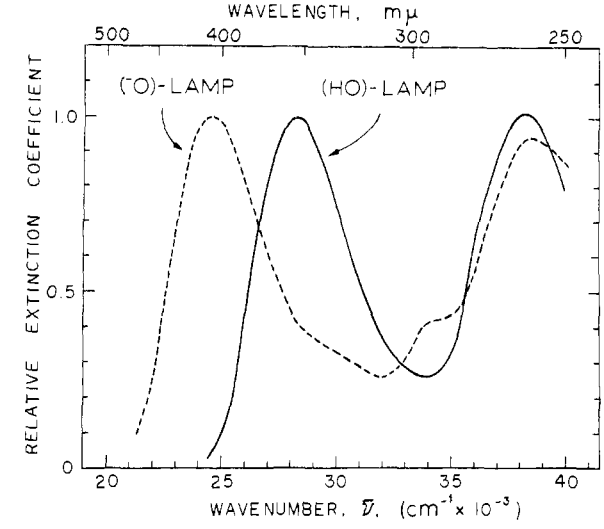


FIGURE 5: Absorption spectra (normalized) of dehydroluciferyl adenylate in 0.05 M sodium acetate buffer (pH 4.6) (————), and in 0.05 M sodium carbonate buffer (pH 10.5) (----).

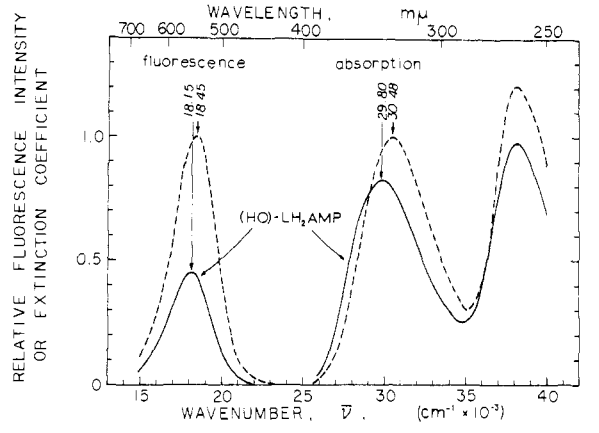


FIGURE 6: Absorption and fluorescence emission spectra for luciferyl adenylate (————), and hydrolyzed luciferyl adenylate (-----) measured at about pH 4.5 in sodium acetate buffer. Absorption spectra are normalized so that the extinction coefficient of luciferin equals 1.0. The fluorescence spectra have areas proportional to ϕ_f.

have the same ionic species dependence of fluorescence as L and LH_2. For example, in ethylene glycol (95%), (HO)-LAMP is blue fluorescent (Table I). Just as (HO)-L has a higher fluorescence quantum yield in nonaqueous solvents than (HO)-LH_2, so (HO)-LAMP fluoresces more efficiently than (HO)-LH_2AMP.

The absorption spectra of the adenylates are not strictly the sum of the LH_2 or L and AMP at pH 5. This is shown in Figure 6 where LH_2AMP absorption and fluorescence are compared to the base-hydrolyzed material.[4] The absorption spectra of the hydrolyzed adenylates are exactly the sum of a 1:1 mole ratio of LH_2 or L and AMP. LH_2AMP absorption and fluorescence are both red shifted relative to LH_2. There is a hypochromic effect, and the fluorescence is partially quenched. Qualitatively the same effects occur for LAMP, except that fluorescence quenching is nearly complete.

The spectral changes on hydrolysis of LH_2AMP or LAMP suggest an intramolecular interaction between the benzothiazole and adenine chromophores. This would be analogous to similar effects found for the pyridine and flavin nucleotides (Weber, 1950, 1957; Velick, 1961). We can present the following additional evidence for such an intramolecular association.

First, the excitation of LH_2AMP fluorescence shows that energy absorbed in the 260-mμ region is efficiently transferred from the adenine chromophore to cause the benzothiazole-type fluorescence. At 38,460 cm^{-1}, the extinction of (HO)-LH_2 is only 37% of the total extinction of (HO)-LH_2AMP. A similar situation holds for the pyridine nucleotides (Weber, 1957), and similar arguments can be used for intramolecular complexing and energy transfer in our case.

Second, both LH_2 and L interact with adenosine in solution to give spectral changes similar to those ob-

[4] The fluorescence emission spectrum for purified LH_2AMP differs from that given by Seliger and McElroy (1960b). The fluorescence quantum yields for LH_2 and L in basic and acidic solutions given in Table I also differ from those of Seliger and McElroy (1960b); however, only by about 10%. There are several factors responsible for these differences. We have used better methods of preparation and purification of the compounds. We have examined more thoroughly the conditions (especially pH and ionic concentrations) favorable to stability. We have more carefully determined the purity of samples used for fluorescence measurements and we have used more accurate spectral sensitivity calibrations.

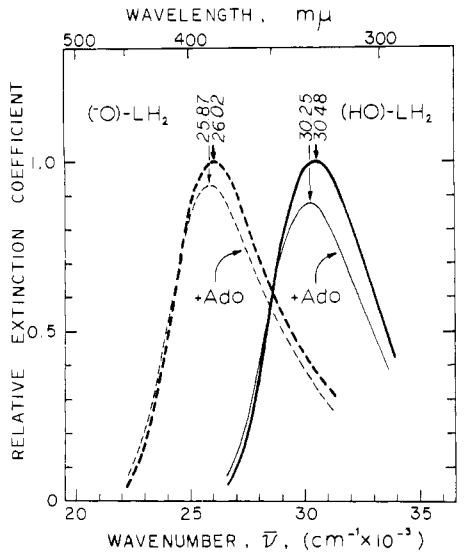


FIGURE 7: Absorption spectra of luciferin phenolate form (⁻O)-luciferin and phenol form (HO)-luciferin with and without added adenosine (0.045 M). Spectra in the absence of adenosine are normalized.

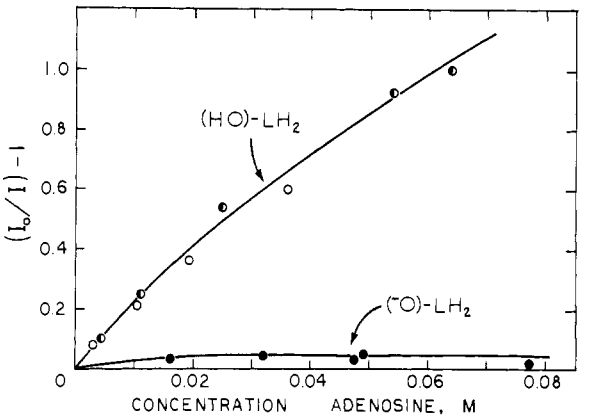


FIGURE 8: Quenching of luciferin fluorescence with added adenosine, at pH 7 (HO)-luciferin and pH 11 (⁻O)-luciferin. In both cases emission is from the excited phenolate, (⁻O)-luciferin*.

served in their respective adenylates. The changes are also very similar to those observed by Weber (1950) for solutions of riboflavin and adenosine. In Figure 7, the absorption spectra of (HO)-LH_2 and ($^-$O)-LH_2 are compared to those observed in the presence of about 0.045 M adenosine. For both ionic forms there are red shifts and hypochromic effects. The red shifts and hypochromic effects in mixtures of LH_2 and adenosine (and L plus adenosine) are smaller than those of LH_2AMP (or LAMP), but this may be due to incomplete complexing in the solutions examined because of the limited range of adenosine solubility.

The fluorescence of solutions of LH_2 plus adenosine is somewhat different from LH_2AMP. Figure 8 shows the quenching of LH_2 fluorescence by adenosine at pH 7 (0.02 M sodium phosphate buffer) and at pH 11 (0.05 M sodium carbonate buffer). In neither case is the spectral distribution of fluorescence appreciably different from LH_2. The fluorescence of the ($^-$O)-LH_2 ion is only very slightly quenched, in spite of the significant change in the absorption spectrum. For (HO)-LH_2 the quenching curve would lead us to expect a complex of significantly weaker but not negligible fluorescence. The lack of any appreciable change in the fluorescence spectrum of mixtures may indicate that the ground-state intermolecular complex dissociates in the excited state or, merely, that the complex has an emission indistinguishable from LH_2. The larger quenching of (HO)-LH_2 may be due to inhibition of excited-state ionization by hydrogen bonding with adenosine. The quenching of L fluorescence in the presence of adenosine is qualitatively similar, but less than LH_2. On the other hand, LAMP is essentially nonfluorescent.

We feel that these experiments demonstrate that LAMP and LH_2AMP exist in aqueous solution in the form of intramolecular complexes, the nature of which, while unknown, is such that the electronic properties of the benzothiazole chromophore are slightly perturbed. These perturbations are manifested as a red shift of absorption and fluorescence spectra, a hypochromic effect, and a quenching of fluorescence. The almost complete quenching of LAMP fluorescence is not due to inhibition of proton transfer in the excited state since both ionic forms are nonfluorescent (Table I). We have not found a testable hypothesis to explain the quenching of LAMP emission. Courtauld models show that LAMP can be more easily folded into a conformation allowing the benzothiazole and adenine rings to be placed closer together than in the case of LH_2AMP and this fact, together with the relatively high fluorescence yield of (HO)-LAMP in ethylene glycol, suggests that there is some quenching interaction.

Discussion

In this section we will discuss how the results can be applied to the firefly bioluminescent reaction.

Molecular Nature of the Light Emittor. The available information suggests that the chromophore responsible for bioluminescence emission is very similar to that of the molecules discussed in this paper. The fluorescence of spent reaction mixtures indicates that the electronic character of the 6′-hydroxylbenzothiazole chromophore is probably essentially unchanged by the reaction (Seliger and Morton, 1968). The bioluminescence emission differs from the fluorescence emission of LH_2, L, and LH_2AMP more in shape (the FWHM = 3000 cm^{-1} for fluorescence *vs.* 2400 and 1700 cm^{-1} for bioluminescence) than in peak position. It has been suggested that in bioluminescence, oxidation takes place in the same manner as in LH_2AMP chemiluminescence, with initial attack at the 4-carbon of luciferin α to the carboxyl group, to form the decarboxylated, 4-keto derivative (I) (Hopkins *et al.*, 1967). If this were the case, the emittor would have a chromophore essentially the same as that of LH_2. In all of the molecules we have studied, the only form having a high fluorescence yield is one with an ionized 6′-hydroxyl group (phenolate) or a 6′ group capable of

electron pair donation to the chromophore as in 6′-aminoluciferin. A high fluorescence yield is required for the emittor because of the high bioluminescence quantum yield (Seliger and McElroy, 1960a); therefore we suggest that it have the equivalent of such a group.

The pH optimum for the *in vitro* bioluminescent reaction is below that of the phenolate pK_a of LH_2 and so either excited-state ionization of the product or ionization of LH_2 or LH_2AMP prior to the formation of the excited product must take place. From the fluorescence yields in Table I, we would suggest that excited-state ionization is probably not efficient enough, although on the enzyme molecule the rate of proton loss might be increased. If excited-state ionization does not occur, a separate step involving the ionization of the 6′-phenol group or an equivalent ionization would be required during catalysis.

Species Differences in the Color of Bioluminescence Emission. At the present time the *in vitro* emission spectra of approximately 45 species of fireflies have been accurately measured (Seliger *et al.*, 1964; Biggley *et al.*, 1967). These emission spectra are smooth, unstructured single bands with peak intensities (on a $dN/d\lambda$ plot) varying from 546 to 594 mμ, a difference in energy of approximately 1600 cm^{-1}. For several of these species it has also been demonstrated that the luciferin molecule is the same. When *Photinus pyralis* luciferin (III) is reacted with luciferase extracted from another species, the resulting *in vitro* spectral distribution is essentially the same as the *in vivo* spectral distribution of the other species (Seliger and McElroy, 1964). This implies that the luciferase "determines" the color of light emission. The luciferase of different species must produce different enzyme–product excited states.

The results of this paper offer three possible molecular interpretations for this phenomenon. Since the product emits while bound to the enzyme, its emission will be influenced by the environment of the site where it is bound, which may be a "nonaqueous" or "hydrophobic" site, approximated by nonpolar organic solvent environments. This is the case in the binding of fluorescent molecules to other proteins (McClure and Edelman, 1966). The fluorescent emission shifts that we have observed in various solvents (3200 cm^{-1}) can account qualitatively for the differences in bioluminescent emissions observed for different species (1600 cm^{-1}). A second mechanism to explain this phenomenon invokes another possible influence of the enzyme binding site on the bound emitter which is of a more specific nature than the "environmental" effect. In the instance of the luciferin chromophore product molecule there is a shift in the fluorescence emission induced by heavy metals, presumably through formation of a specific complex. An equivalent effect could be produced at the enzyme binding site if a charged group or groups were appropriately oriented relative to the bound product excited-state molecule. This mechanism could be responsible for a shift of 1600 cm^{-1} with no decrease in fluorescence yield. This is in contrast to the first mechanism which allows a greater shift but necessitates a decrease in the fluorescence yield at higher energies of emission.

There is a third mechanism which can perturb the product excited state, the association between the benzothiazole and adenine rings. It is possible that bioluminescence is from an excited product–adenylate complex each being separately bound to the enzyme, but similar to the structure which probably exists in aqueous solution. The various luciferase molecules might allow different degrees of interaction between the rings. One difficulty with this hypothesis is that a decrease in fluorescence yield accompanies these intramolecular associations. A second difficulty is that the range of variation of $\bar{\nu}_M$ observed for this effect is small (about 400 cm^{-1}).

The Red Bioluminescence of P. pyralis. As the pH of the *in vitro* reaction of *P. pyralis* is decreased below 7.8 (Seliger and McElroy, 1960a), as Zn, Cd, or Hg divalent cations are introduced (Seliger and McElroy, 1964), and as the temperature is raised (Seliger and McElroy, 1966), the intensity of the normal yellow-green emission band decreases and a new red emission band appears (Figure 4b). In the limit only the red emission band is present. These data represent a transition from one excited molecular species to another. Light emission is from one of two excited states, such as observed in the emission from two ionic forms of a molecule, and not a gradual shift of peak position such as might occur when solvent–chromophore interactions perturb an excited state. There is, however, a significant change in the band width. Except for this last fact, the observed shift in luciferin fluorescence in the presence of Zn^{2+} and Cd^{2+} is a tempting parallel, especially considering the specificity of the effect. We hesitate to attribute the bioluminescence red shift to complexing of the sort we have observed with luciferin, since in this latter case the band width remains constant. Recent results, in fact, suggest that the two bioluminescence emittors are different tautomers (Hopkins *et al.*, 1967), that is, the red emission from the keto form I, the yellow-green from one of the enol forms IIa, b, or c (for more recent results on this point see White *et al.*, 1969).

Nature of the Active Site. We suggest that the enzyme, in addition to acting as a catalyst for the bioluminescence, (a) assures the formation of the product phenolate ion (possibly not separable from catalysis), (b) affords an environment for the product molecule which determines the color of the excited-state product emission through either a general and/or specific interaction with the chromophore, and (c) may function to establish a pH-dependent tautomeric equilibrium between a keto (red emitting) and enol (yellow-green emitting) form of the product molecule.

Studies of the properties of fluorescent molecules, such as LAMP, bound to the enzyme should eventually provide useful information about the active site of firefly luciferase. For instance, previous experiments indicated that enzyme-bound LAMP is essentially nonfluorescent (Rhodes and McElroy, 1958). This suggests that the binding site is essentially an aqueous environment. This conclusion depends, however, upon the assumption that (HO)-LAMP is the species bound, and this has not yet been investigated. Studies of the fluorescence of other analogs of the active substrate

(D-LH_2AMP) and the proposed product molecules (keto and enol forms) bound to the enzyme might be more conclusive.

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