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The Chemi- and Bioluminescence of Firefly Luciferin: An Efficient Chemical Production of Electronically Excited States

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The chemi- and bioluminescence of firefly luciferin results in the emission of either red light or yellow-green light, depending on the amount of base present. The red emission stems from the formation of a dioxetane intermediate, with subsequent cleavage to yield the light emitter **22** in an electronically excited state. Yellow-green light emission stems from a second emitter **23**, derived from **22** during the lifetime of the excited state by a proton loss. These assignments were made by a combination of approaches involving the use of luciferin analogs, model light emitters, and a careful comparison of the light emitted in chemiluminescence with that emitted in bioluminescence and with the fluorescence of suitable model compounds.

INTRODUCTION

Chemiluminescence, the production of light by chemical reactions,

$$A + B \rightarrow C^* \rightarrow \text{light},$$

has been the subject of curiosity since antiquity and of serious investigation since the days of Robert Boyle (*1*, *2*). In chemiluminescence, light emission stems from electronically excited states which are produced adiabatically. Black body irradiation is

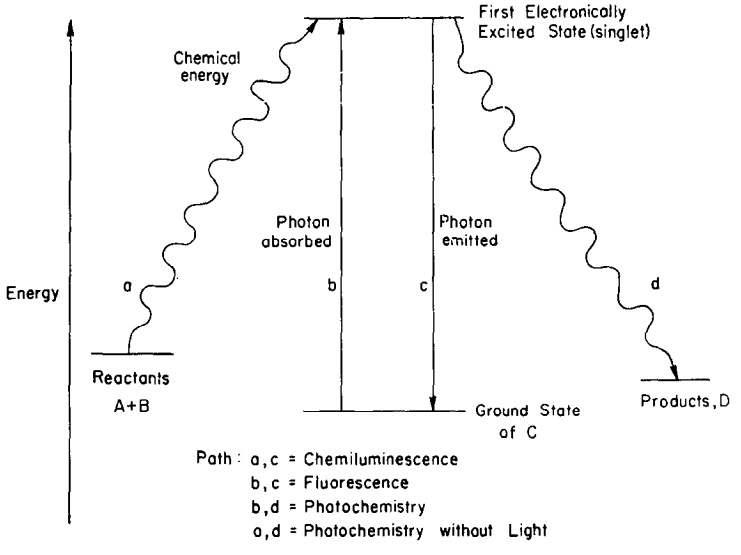

FIG. 1. The relationship between various processes involving light.

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not involved; in fact, black body emission from bodies at room temperature is invisible to humans. Chemiluminescence bears a close relationship to fluorescence and to photochemistry, phenomena which also involve excited states, as illustrated in Fig. 1 (*3*).

Chemical Production of Excited States

The process whereby chemical energy is converted into electronic energy (Fig. 1, process a) is therefore the unique aspect of chemiluminescence (*4*). This process can be visualized as a crossing between ground state and excited state energy curves or energy surfaces that intersect (Fig. 2) (*5*). The overall efficiency of chemiluminescence

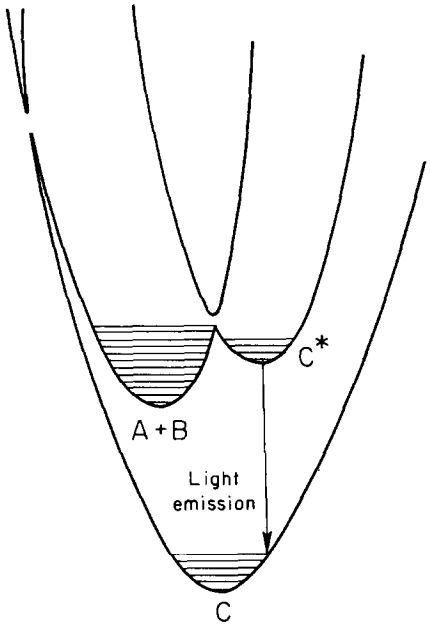


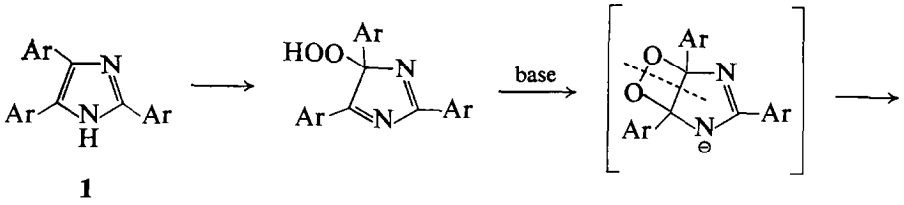
FIG. 2. The production of excited states of C by the chemical reaction A + B.

is thus a product of a factor indicating what fraction of the chemical reaction (for example, an oxidation) proceeds with the "right" chemistry, a factor for the efficiency of crossover to the excited state, and a factor for the efficiency of fluorescence of the excited molecule. The overall efficiency of light production, or quantum yield (Q), is the ratio of the number of photons emitted by the reaction to the number of molecules that react, and it is a resultant of the three factors outlined above. A value near 1, the expected upper limit for Q, has been reported for the bioluminescent reaction of firefly luciferin (*6*); most "bright" chemiluminescent reactions, however, fall in the range 0.01–0.25 (*2*). In marked contrast, values as low as 10^{-15} have been measured for simple reactions such as the neutralization of hydronium ion (*7*).

The fluorescence quantum yield of the emitter molecule in chemiluminescence can be controlled; in general, electron-releasing substituents increase the yield (*2a*). Furthermore, changes in reaction conditions can determine what fraction of the reactants undergo the "correct" chemistry. The third factor in determining the quantum yield of chemiluminescence—the proportion of molecules that cross over to the excited

state energy surface—has been relatively ill defined. Only recently have satisfactory explanations of this process been advanced.

At the present time, most chemiluminescent reactions for which reaction mechanisms have been advanced belong to one of two categories, the allocation depending on whether (1) dioxetanes (1,2-dioxacyclobutanes), or (2) radical ions are intermediates. The chemiluminescence of the lophines (arylimidazoles, **1**) is an example of the first



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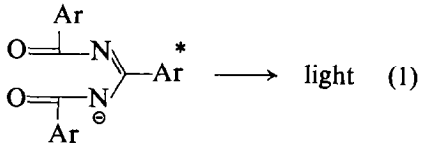
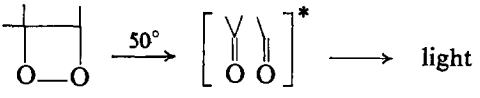


light (1)

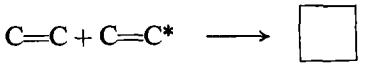
2

category (*8*). Diaroylamidines (**2**) are the chief products of the reaction and, in the more efficient cases, are the light emitters in the reaction. This assignment follows from the observation of a match between the wavelength maxima of chemiluminescence in a series of the arylimidazoles and the wavelength maxima of fluorescence of the corresponding diaroylamidines (**2**). Dioxetanes have also been proposed as key intermediates in the chemiluminescence of indoles (*9*), acridine compounds (*10*), peroxalates (*11*), and aminoethylenes (*12*), *e.g.*

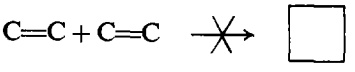
Recently, several alkyl-substituted dioxetanes [Eq. (3)] have been prepared and shown to be isolable compounds (*13*). These simple dioxetanes also dissociate to give



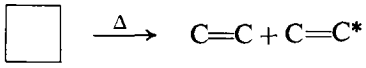
excited states of their carbonyl products (*13*, *14*). An explanation for the production of excited states from the dioxetanes has appeared recently (*15*). An intuitive argument can be advanced based on an analogy with carbocyclic compounds. Cyclobutanes can



be readily made photochemically, but experience has shown that the cyclization in simple cases does not occur with ground state ethylenes. Looking at the reaction in the

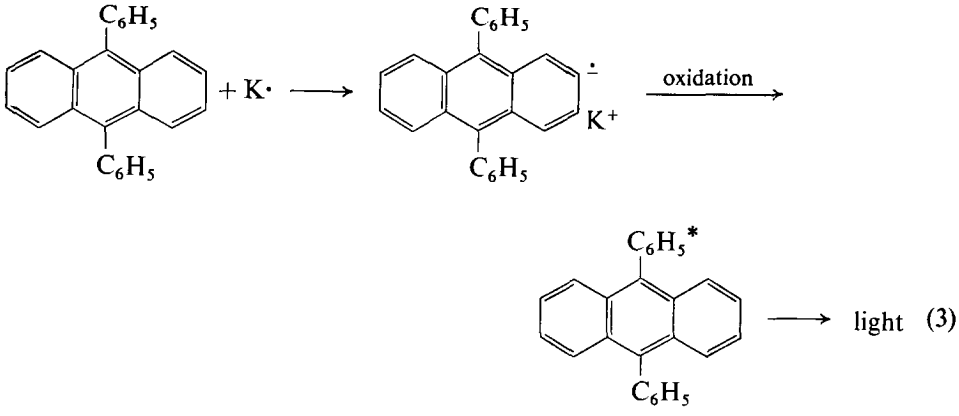


opposite sense would suggest that, provided sufficient energy were available, cleavage of a cyclobutane should lead to an ethylene molecule in the excited state. In the case



of the dioxetanes [Eq. (2)] the presence of the weak O–O bond, the high bond strength of the carbonyl group, and the strain energy in the reactant insure that sufficient energy is available for the process. These intuitive views have been placed on a firmer basis by the orbital symmetry considerations of Woodward and Hoffman (*15*, *16*). The case of the dioxetanes has been treated explicitly by Kearns (*17*).

Concerning the second type of chemiluminescence, light emission from radical ions was first reported by Chandross (*18*). Subsequently a number of studies have been



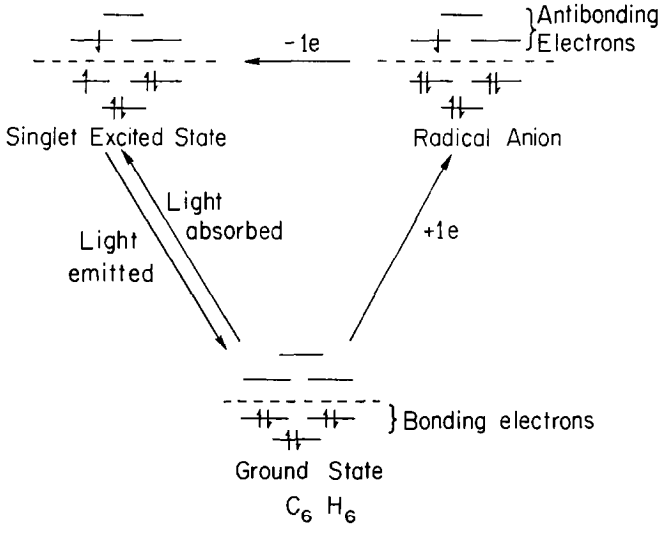


FIG. 3. Relationship between the electron distribution in the ground state of benzene, an excited singlet state, and the radical anion.

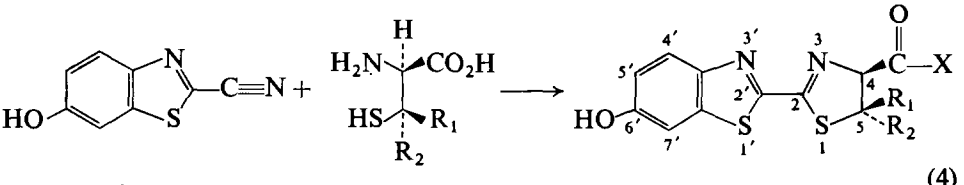
carried out on the related phenomenon of electroluminescence involving radical ions (*19*). The process whereby excited states are formed from radical ions is illustrated in Fig. 3 with benzene as the model (*5*, *20*). As shown, the oxidation of the radical anion is believed to lead to the loss of a bonding electron to yield a state identical to that obtained by the irradiation of benzene. The efficiency is relatively low, and in most of the oxidative events, an antibonding electron is removed, a process which leads directly to the ground state ("chemidarkness!").

In the present paper we present evidence to show that the chemi- and bioluminescence of firefly luciferin belongs to the first category of chemiluminescent reactions—that is, that dioxetanes are reaction intermediates in the production of light.

RESULTS

Syntheses and Preparations

Firefly luciferin (**6a**) was prepared as previously described (*21*). The final step in the synthesis, the condensation of 6-hydroxy-2-cyanobenzothiazole (**4**) with D-cysteine (**5**),



4 **5** (4)

a, X = OH c, X = OC_6H_5 **6**, $R_1 = R_2 = H$

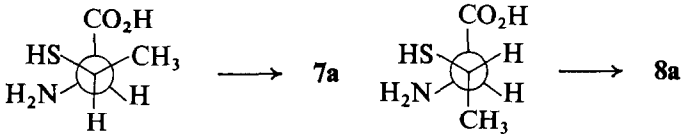
b, X = OCH_3 d, X = AMP **7**, $R_1 = CH_3$, $R_2 = H$

8, $R_1 = H$, $R_2 = CH_3$

9, $R_1 = R_2 = CH_3$

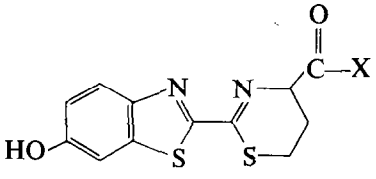
was also used in the synthesis of various analogs (*22*), starting with both modified benzothiazoles and cysteine analogs [Eq. (4)]. The method of Seto et al. (*23*) was used in the preparation of most of the benzothiazoles.

Cis- and *trans*-DL-2-(6′-hydroxy-2′-benzothiazolyl)-Δ^2-thiazoline-5-methyl-4-carboxylic acids (5-monomethyl luciferins) (**7a** and **8a**, respectively) were prepared by reacting 2-cyano-6-hydroxybenzothiazole (**4**) with the corresponding β-methylcysteines (prepared *in situ* from the *S*-benzyl compounds). The α-amino-β-benzylthioacids were prepared and separated to the two diastereomeric racemates according to the method of Carter, Stevens, and Ney (*24*) by fractional crystallization of the β-phenyl ethylamine salts of the *N*-benzoyl derivatives. The *erythro* form reacted to give the *cis*-5-monomethyl luciferin (**7a**) whereas the *threo* gave *trans*-5-monomethyl luciferin (**8a**) [Eq. (4)]: The structures were confirmed by the nmr spectra of the luciferins. The *cis* isomer **7a** has a higher coupling constant for protons 4 and 5 ($J_{4,5} = 9$ Hz) than the *trans* isomer **8a** ($J_{4,5} = 6$ Hz). According to the Karplus equation (*25*) the dihedral angle was calculated to be 0° for **7a** and 142° for **8a** and models of **7** and **8** are consistent with these data. The proton in position 4 absorbs at τ 4.76 in the case of **7a** and at τ 5.00 in **8a**. In the *trans* isomer **8a**, this proton is shielded by the C–CH_3 σ bond and appears at a higher field.

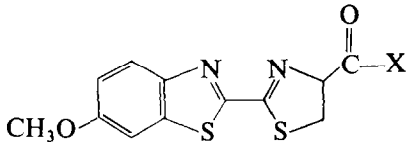


erythro *threo*

DL-2-(6′-Hydroxy-2′-benzothiazolyl)-Δ^2-dihydro-1,3-thiazine-4-carboxylic acid (homoluciferin) (**10a**) was prepared by the reaction of DL-homocysteine with **4**. Homo-



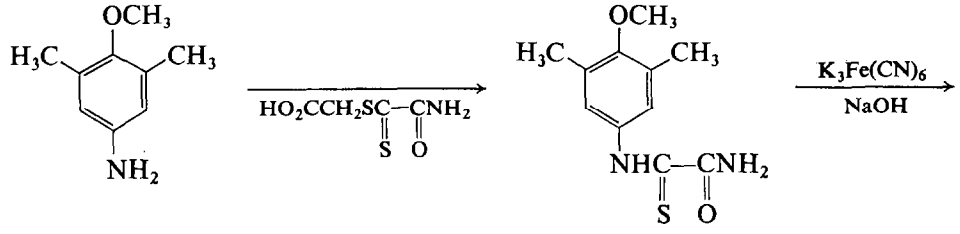
10a, c



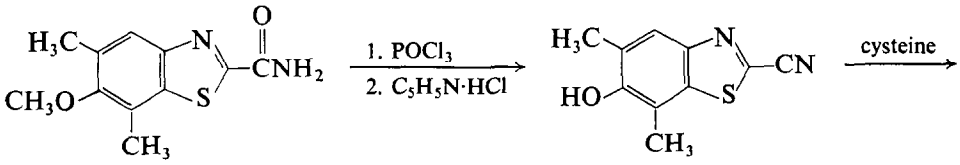
11c

luciferin proved much more sensitive to hydrolysis than luciferin (**6a**) and generation of the free acid in aqueous solutions is difficult. The nmr spectrum of **10a** in DMSO-d_6 shows a quartet for the C-4 proton at τ 5.4 ppm, a signal representing the x part of an ABX spectrum for the protons on C-4 and C-5 (*26*).

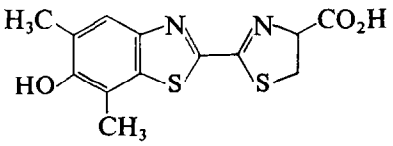
2-(6′-Hydroxy-5′,7′-dimethyl-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (**16**) was synthesized starting with 2,6-dimethylanisole. Nitration and catalytic hydrogenation led to 4-amino-2,6-dimethylanisole (**12**) which was subjected to Seto



12 **13**



14 **15**



(5)

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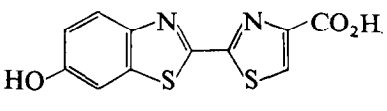
et al.'s (*23*) synthesis. With carbamoylthiocarbonylthioacetic acid in aqueous ethanol, it gave 3,5-dimethyl-4-methoxythiooxanilamide (**13**) which in turn was oxidized to 2-carbamoyl-5,7-dimethyl-6-methoxybenzothiazole (**14**). The amide was converted to the corresponding nitrile, which was demethylated with pyridine hydrochloride to yield the hydroxynitrile (**15**). A reaction of **15** with cysteine led to 5′,7′-dimethyl-luciferin **16**.

The reaction of 2-cyano-6-hydroxybenzothiazole (**4**) with ethyl-2-mercaptoisobutyrate in ethanol with triethylamine yielded 2-(6′-hydroxy-2′-benzothiazolyl)-Δ^2-thiazoline-5,5-dimethyl-4-one (**18**), the product in the chemiluminescent reaction of activated derivatives of dimethylluciferin **9**.

The synthesis of the phenyl esters **6c**, **7c**, **8c**, and **9c** was achieved by the use of phenol and trifluoroacetic anhydride (*21*). The same reagent mixture was used for the synthesis of the phenyl ester of 2-(6′-methoxy-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (**11**) and (with methanol) the methyl ester of **6a**.

Dehydroluciferin (**17**) was prepared by a modification of the earlier synthesis (*21*). Nitrile **4** was converted to the corresponding thioamide, which was condensed with



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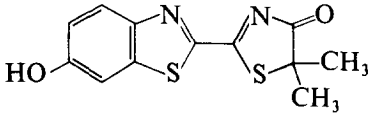

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ethyl bromopyruvate to give the ethyl ester of dehydroluciferin; basic hydrolysis yielded dehydroluciferin (**17**).

Firefly luciferin deteriorates on standing and older samples were purified by aqueous Sephadex (G-25 fine) adsorption chromatography. The isomeric purity of D-luciferin was determined by the quantum yield of bioluminescence and by ORD. The adenylates of the different luciferin analogs were synthesized by condensing the free acids with adenosine monophosphate using dicyclohexylcarbodiimide (*28*). D-Luciferyl adenylate was prepared in dimethylsulfoxide and separated on Sephadex. The enzyme luciferase was isolated and purified from dried tails of *Photinus pyralis*.

Chemiluminescent Reactions

The esters and mixed anhydrides of firefly luciferin yield a strong red light on reaction with a base and oxygen.

$$R{-}\overset{\overset{O}{\|}}{C}{-}X \xrightarrow[O_2]{\text{base}} \text{Red light.}$$

Since the chemiluminescent reaction products were unstable in aqueous systems (*29*) and since a relatively high fluorescence quantum yield for **6a** was observed in anhydrous basic dimethylsulfoxide, the chemiluminescent properties were usually measured in this solvent. Various base catalysts were used to initiate the reactions in air-saturated solvent. Chemiluminescence and fluorescence data are summarized in Table 1.

The phenyl ester of luciferin (**6c**) yielded a red chemiluminescent emission ($\tilde{\nu}_{max}$ 15 850 cm^{-1}) when treated with small amounts of base. Additional base led to simultaneous red and yellow-green chemiluminescence, and high base concentrations led exclusively to a yellow-green emission ($\tilde{\nu}_{max}$ 18 000 cm^{-1}, Fig. 4B, Table 1). The phenyl

TABLE 1

CHEMILUMINESCENCE AND FLUORESCENCE EMISSION CHARACTERISTICS[a]

Compound	Conditions[c]	Maxima (cm^{-1})[b]		
		Chemiluminescence emission	Fluorescence of spent reaction mixture	Fluorescence
6d	0.005 *M* base	15 850 (1750)	18 000 (2300)	
6d	0.05 *M* imidazolate	15 400 (1850)		
9d	Base	15 850 (1750)	15 850 (1750)	
18	Base			15 850 (1750)
6c, 7c, 8c	0.005 *M* base	15 850 (1750)	18 000 (2300)	
	0.05 *M* base	15 850; 18 000	18 000 (2300)	
	0.5 *M* base	18 000 (2150)	18 000 (2300)	
10c	Base	15 900 (1750)		
11c	0.5 *M* base	21 400 (2300)	16 900 (3075)	
	0.005 *M* base	16 900 (3000)	16 900 (3075)	16 800 (3025)[d]
6b	0.005 *M* base	15 850 (1750)	18 000 (2300)	

[a] All concentrations 10^{-5} *M* in dry dimethylsulfoxide.

[b] Full band width between half-maximum intensity points of the spectrum (FWHM) is reported in parentheses. The accuracy was estimated to be $\bar{\nu}_{max} \pm 1\%$ and FWHM $\pm$ 10%.

[c] Base concentration refers to solutions of potassium *tert*-butoxide in dimethylsulfoxide. Imidazolate anion was used as base in aqueous solutions. Where base concentration is not specified, the emission wave number is independent of base concentration in dimethylsulfoxide.

[d] These values are for a product, not obtained pure, from the reaction of 2-cyano-6-methoxybenzothiazole with butyl 2-mercaptoglycolate (*30*).

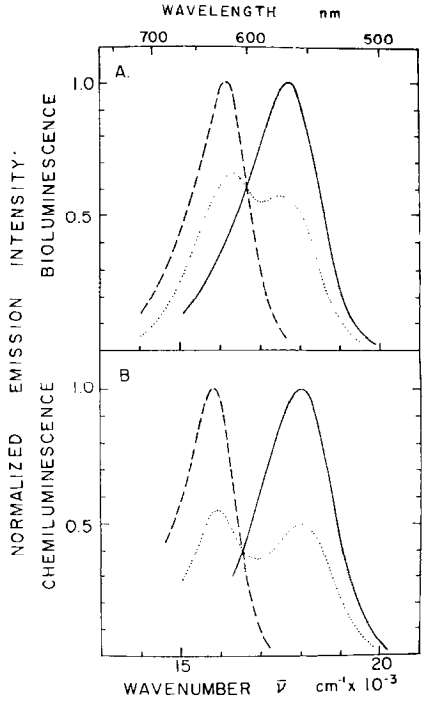


FIG. 4. Light emission in luciferin oxidation. A. *P. pyralis in vitro* bioluminescence at pH 8.0 (——), pH 6.8 (· · · · ·), and pH 6.0 (– – – –) in 0.05 *M* Tris using ATP (2×10^{-3} *M*); $MgSO_4$ (5×10^{-3} *M*); luciferin (1×10^{-6} *M*); enzyme (4×10^{-8} *M*) and coenzyme A (10^{-3} mg/ml).

B. Chemiluminescence of luciferin (phenyl ester, **6c**) (10^{-5} *M*) in dimethylsulfoxide at potassium *tert*-butoxide concentrations of 0.5 *M* (——), 0.05 *M* (· · · · ·), and 0.005 *M* (– – – –).

esters of both 5-monomethylluciferins (**7c** and **8c**) showed the same base-dependent colors of the chemiluminescent emission. However, the phenyl ester of homoluciferin (**10c**) and also compound **9d** yielded a red emission ($\tilde{\nu}_{max}$ 15 850 cm^{-1}) independent of base concentration. Treatment of **11c** with small amounts of base led to a blue emission while higher base concentrations yielded a low-intensity orange chemiluminescent emission.

Fluorescence

The fluorescence emission of the spent red-emitting (low base) chemiluminescent reaction mixtures of luciferin analogs which possess enolizable hydrogens at position 5 (**6b**, **7c**, and **8c**) were found to give a short-lived red fluorescence ($\tilde{\nu}_{max}$ 15 850 cm^{-1}) identical to the red chemiluminescence (Fig. 5). In these cases the red fluorescence

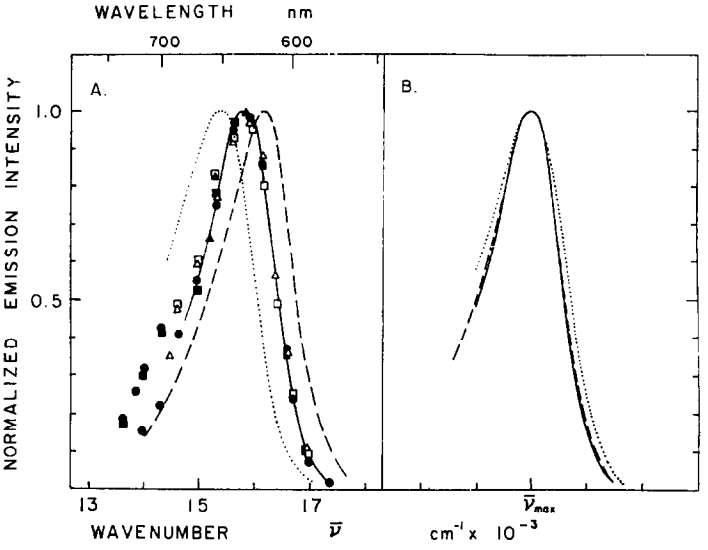


FIG. 5. Red light emission. A. Chemiluminescence of 5,5-dimethyl luciferyl adenylate (**9d**) (—△—) and luciferyl adenylate (**6d**) (—□—) in dimethylsulfoxide plus imidazolate (10^{-2} *M*). Fluorescence of the resulting reaction mixes (—▲—) and (—■—) and fluorescence of the 5,5-dimethyl keto anion (**18**) (—●—) in DMSO. Aqueous chemiluminescence of luciferyl adenylate (· · · · ·) at pH 9.0 (0.1 *M* imidazole). Bioluminescence of luciferin at pH 6.0 (– – – –).

B. Superposition of aqueous luciferyl adenylate chemiluminescence (· · · · ·), dimethyl ketone anion fluorescence in dimethylsulfoxide (——), and pH 6.0 bioluminescence (——) plotted with common peak wave number ($\tilde{\nu}_{max}$) positions.

shifted within minutes to a yellow-green fluorescence ($\tilde{\nu}_{max}$ 18 000 cm^{-1}) which was independent of base concentration. The fluorescence emission of the spent chemiluminescent reaction mixture of compounds **9d** and **10d** remained red ($\tilde{\nu}_{max}$ 15 850 cm^{-1}) independent of base concentration. The fluorescence of the spent chemiluminescent reaction mixture of **11c** was orange ($\tilde{\nu}_{max}$ 16 900 cm^{-1}) independent of base concentration (*30*).

The fluorescence emission spectrum of basic dimethylsulfoxide solutions of ketone **18** matched the chemiluminescence of compound **9d**, and it matched the fluorescence of the spent chemiluminescence reaction mix as well (Fig. 5). In neutral dimethylsulfoxide solutions, compound **18** itself is only weakly fluorescent. Its absorption spectrum ($\tilde{\nu}_{max}$ 25 600 cm^{-1}) shifts to the red ($\tilde{\nu}_{max}$ 17 300 cm^{-1}) accompanied with

the appearance of a red fluorescence ($\bar{\nu}_{max}$ 15 850 cm^{-1}) upon addition of 2% triethylamine (Fig. 6). The spent chemiluminescent reaction mix of 5,5-dimethylluciferin (**9d**) was shown to contain compound **18**. The adenylate of dehydroluciferin (**17**) did not yield light with potassium *tert*-butoxide in DMSO, or with potassium imidazolate in water.

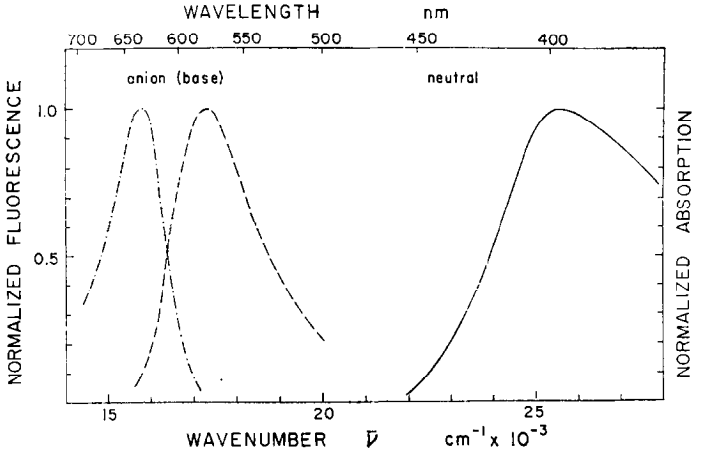


FIG. 6. Red-emitting product anion spectral characteristics. Normalized absorption of 5,5-dimethyl ketone anion (**18**) in neutral dimethylsulfoxide (——). Absorption (— — —) and fluorescence (—·—) of 6′-phenolate anion of **18** formed upon addition of 2% triethylamine.

Bioluminescence Reactions

Bioluminescence of firefly luciferin requires enzyme (luciferase) catalysis of: (1) formation of the mixed anhydride, D-luciferyl adenylate (**6d**), from D-luciferin (LH_2), ATP, and Mg^{2+} followed by, (2) formation of a light-emitting excited state from

$$LH_2 + ATP \xrightarrow[E]{Mg^{2+}} LH_2AMP + POP$$

the oxidation of the D-luciferyl adenylate with oxygen (*31*).

$$LH_2AMP + O_2 \xrightarrow[E]{} \text{light.} \qquad (6)$$

By using synthetic adenylates (LH_2AMP, e.g.) (*32*) considerable simplification occurs, since only luciferase and oxygen are now required for light production. The effect of pH on the bioluminescence (*33*), both with luciferin (**6a**) and with various analogs, was determined; values are listed in Table 2

The 6-methoxy-, homo-, and 5′,5′-dimethyl analogs of luciferin (**11**, **10**, and **9**) were inactive in light emission with the enzyme at both pH 6 and 8 using either the luciferin and ATP-Mg^{2+} approach or the synthetic adenylate derivatives. Both *cis*- and *trans*-5′-monomethyl DL-luciferins (**7**, **8**) strongly inhibit light production by D-luciferin (**6**) and the enzyme. Both are relatively inactive in bioluminescence as the adenylates. A very slow rate of light production at pH 8.0 results from either isomer used with ATP-Mg^{2+} and the enzyme.

The fluorescence emission spectrum of an anaerobic reaction mix of D-luciferyl adenylate (**6d**) with excess enzyme ($\bar{\nu}_{max}$ 18 150 cm^{-1}, FWHM 2650 cm^{-1}) is given in Fig. 8 where it is compared with the fluorescence emission of D-luciferyl adenylate

TABLE 2

pH EFFECTS ON THE BIOLUMINESCENCE EMISSION SPECTRA[a]

Compound	pH (aqueous)	Maxima ($\bar{\nu}_{max}$ cm^{-1})	Bandwidths (FWHM cm^{-1})
D-Luciferin (**6a**)	8.0	17 700	2200[b]
	6.0	16 150	1700
7a, **8a**[c,d]	8.0	(17 700)[c]	(2200)[c]
16a	9.0	15 450	1850
	8.0	15 400	1750
	6.3	15 300	1650

[a] Reaction conditions: 0.05 *M* phosphate buffer, ATP 2×10^{-3} *M*, $MgSO_4$ 5×10^{-3} *M*, luciferase 10^{-7} *M*, coenzyme A 0.002 mg/ml, luciferin or derivative 5×10^{-6} *M*.

[b] The previously reported values of 2400 (*33*) and 2050 (*34*) were drawn from the literature.

[c] The rate of emission (intensity) is <0.01 that for D-luciferin. The quantum yield is therefore difficult to determine. It is on the order of 0.01–0.001. In this range the possibility of artifacts is very real.

[d] Synthesized and used as racemic mixtures.

($\bar{\nu}_{1max}$ 18 100 cm^{-1}, FWHM 3000 cm^{-1} without enzyme). Upon addition of a very slight excess of oxygen to this anaerobic preparation, the fluorescence emission spectrum was again measured ($\bar{\nu}_{max}$ 18 100 cm^{-1}, FWHM 3300 cm^{-1}; $\bar{\nu}_{2max}$ 22 000 cm^{-1}, FWHM ~3300 cm^{-1}) as is shown in Fig. 8. This spectrum is compared with the bioluminescence emission at pH 8.0. These results are summarized in Table 3.

TABLE 3

THE FLUORESCENCE OF SELECTED REACTION COMPONENTS[a]

Compound	Maxima ($\bar{\nu}_{max}$ cm^{-1})	Bandwidths (FWHM cm^{-1})
Luciferyl adenylate (**6d**)	18 100	3000
6d + luciferase (anaerobic)	18 150	2650
6d + luciferase + O_2	18 100[b]	(3300)[c]
	22 000[b]	(3300)[c]

[a] Reaction conditions: pH 8.0, 0.05 *M* phosphate buffer; 0.0001 *M* EDTA; luciferyl adenylate 1×10^{-6} *M*; luciferase 4×10^{-6} *M*.

[b] Compound emission (two peaks).

[c] Estimated from the components of the compound emission curve.

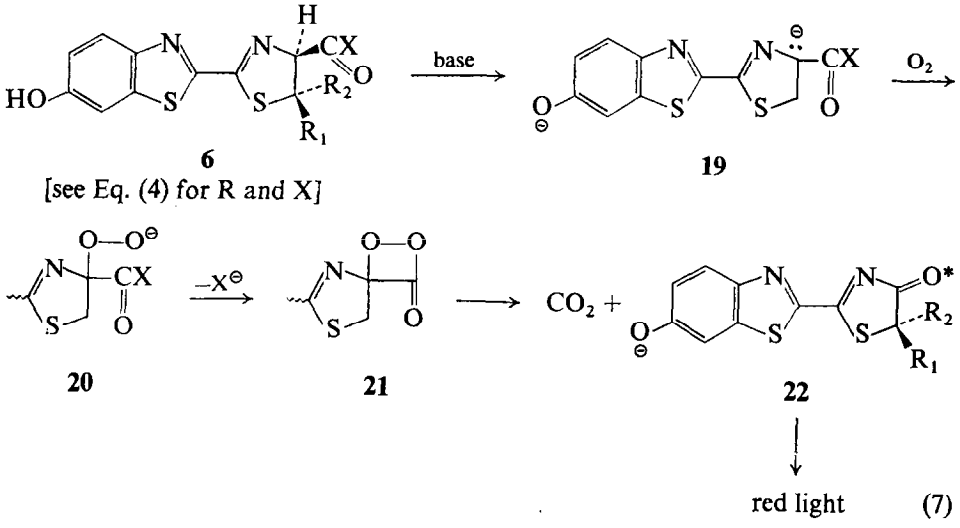
Throughout this paper, spectroscopic data, especially emission spectra, were converted to wave number plots so that comparisons between spectral shapes for curves with different peak positions were possible. It is only valid to compare the shapes of spectra with different wavelength maxima when plotted against wave number, not wavelength, because intervals of wavelength are not equivalent in energy (*34*).

DISCUSSION

The complete identification of a product excited state in a luminescent chemical reaction requires isolation and identification of the presumed product, proof of its structure, and implication of the suspected excited state through the exact matching of its fluorescence emission with the luminescent reaction emission. In the present study the instability of the reaction products in the basic oxygen-containing media necessary for light emission rendered isolation of the presumed product impossible, except in one case. However, analysis by fluorescence of the reaction mixes immediately following light emission revealed complete consumption of the reactant involved and the presence of a single product. Synthesis of the presumed product was possible in only a few instances. Thus very careful measurements of emission spectra were necessary for unambiguous product identification.

Red Chemiluminescence

The fact that dioxetanes are involved in many examples of chemiluminescence (Introduction section), and various observations made with the enzyme-catalyzed emission (*vide infra*), led to the following working hypothesis for the chemi- and



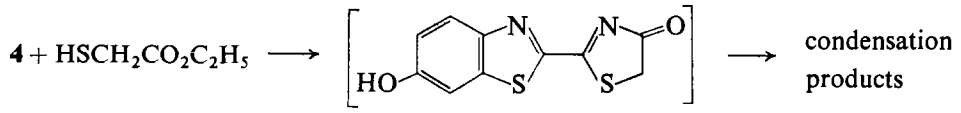
6 [see Eq. (4) for R and X] **19** **20** **21** **22** (7)

bioluminescence of firefly luciferin (*35*). We found, in fact, that when luciferyl adenylate (**6**, R = H, X = AMP) in dimethylsulfoxide was treated with potassium *tert*-butoxide, a strong red emission resulted (*36*). Similarly, the phenyl and methyl esters of luciferin yielded red light under these conditions, as did various analogs of luciferin. The emission maxima are reported in Table 1.

Normally, fireflies emit in the yellow-green region of the spectrum. However, it had long been noted that when fireflies are heated, the emission becomes reddish, as it does also when fireflies frozen in liquid air are allowed to thaw (*37*). Furthermore, the *in vitro* enzymatic reaction itself leads purely to red emission at acid pH values (Results section) (*33*). The red emission noted above was thus thought to have some significance.

Considerable effort has gone into attempts to isolate or identify products of the reaction [Eqs. (6) and (7)]. An examination of spent reaction mixtures (enzyme catalyzed) by fluorescence techniques showed only weak absorption, at a position (18 100

and 22 000 cm^{-1}) different from that of the normal emission (17 700 cm^{-1}; Tables 2 and 3) (*34*, *38*). The isolation attempts led to a mixture of pigments from which no single component could be fully characterized (*29*). A similar mixture was obtained in attempts to synthesize the postulated product.



Only a few syntheses of thiazolinones have been reported, and the properties listed for the products indicate that they were also probably polymeric (*39*). The first true synthesis, that of 2-phenyl-Δ^2-thiazoline-4-one, was reported recently by Jansen and Crossland (*40*). They showed that the compound existed predominantly as the enol in neutral dimethylsulfoxide solutions. Self-condensation of thiazolinones is of the aldolic type (*40*) and renders the isolation of the monomer very difficult. We therefore shifted our attention to a model compound, 5,5-dimethylluciferyl adenylate (**9d**), in which condensation at the active methylene group is blocked. Esters of the dimethylluciferin on air oxidation in basic solution led to the same red light emission as from the parent luciferin (Table 1, Fig. 5). Furthermore, we were able to synthesize the postulated emitter in this case.

$$\mathbf{4} + HSC(CH_3)_2CO_2C_2H_5 \rightarrow \mathbf{18}$$

Evidence that it is the actual light emitter stems from an exact correspondence between its fluorescence spectrum (in the ionized state, **22**; Figs. 5 and 6), and the spectrum of the chemiluminescence light emission [Eq. (7); Fig. 1a, b, and c). The neutral form of this compound gives a blue fluorescence in DMSO ($\bar{\nu}_{max}$ 23 000 cm^{-1}), but addition of triethylamine (2%) to the solvent shifts the absorption peak from the deep blue ($\bar{\nu}_{max}$ 25 600 cm^{-1}) to green ($\bar{\nu}_{max}$ 17 300 cm^{-1}), and the fluorescence to red ($\bar{\nu}_{max}$ 15 850 cm^{-1}; Fig. 6). Note that the bandwidth of this red emission (1700 cm^{-1}) is remarkably narrow. The red-shift measured when the reaction is performed in an aqueous medium (Fig. 5A) is probably due to solvent-environment effects of the type measured with luciferin fluorescence in various solvents and aqueous environments (Fig. 7) (*35*, *41*) (see below).

Considerable additional evidence supports the sequence of reactions illustrated in Eq. (7). Earlier work with luciferin itself (*41*) showed that efficient fluorescence required the ionization of the phenolic group at C-6′. Consistent with this view is the observation that esters of the 6′-methoxy analog (**11**) of luciferin yield only a weak, blue light on reaction with low concentrations of base and oxygen, while at higher base concentrations an orange emission appears (Fig. 7).

Facile removal of the C-4 proton is suggested by the ease of racemization of LH_2AMP; the hydrolysis of LH_2AMP which occurs rapidly at pH values above neutrality is always accompanied by racemization. The 4-proton is labile even in luciferin itself, and deuterium can be readily introduced at this position in basic deuterium oxide (*22*b). An indication of the base strength necessary for rapid carbanion formation is available from the success of the anion of imidazole (imidazolate) as a catalyst in DMSO or water. The pK_a of imidazole is estimated to be 14 (*42*). Weaker organic base catalysts (pyridine, triethylamine) do not catalyze light emission.

The formation of hydroperoxides [Eq. (7)] from attack of oxygen on carbanions has ample analogy in the literature (*43*). Analogy also is available for the key intermediacy of the dioxetane (Introductory section). The ring closure step [Eq. (7)] should be

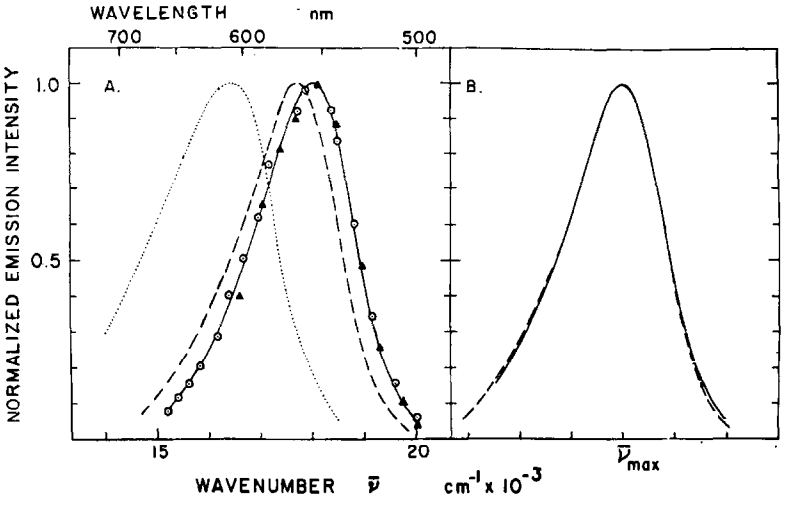


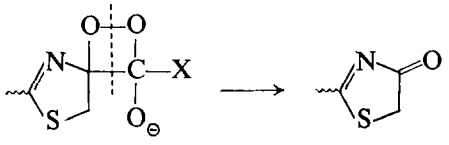
FIG. 7. Yellow-green light emission. A. Chemiluminescence of luciferin, phenyl ester (**6c**) (—⊙—) and fluorescence of the resulting reaction mix (—▲—) in dimethylsulfoxide with high base concentrations (0.5 *M* potassium *tert*-butoxide). Orange chemiluminescence of methyl luciferin phenyl ester (**11c**) with high base (· · · · ·). Bioluminescence of luciferin at pH 8.0 (——).

B. Superposition of chemiluminescence (——) in dimethylsulfoxide at high base concentrations and bioluminescence at pH 8.0 (——) plotted with common peak wave number ($\bar{\nu}_{max}$) positions.

facilitated by "good leaving groups" (X), that is, by conjugate bases of strong acids. The light yields decrease in the order:

$$X = AMP \sim OC_6H_5 > OCH_3 > OH.$$

With the less efficient derivatives, decomposition may occur before the ketodioxetane (**21**) is formed:



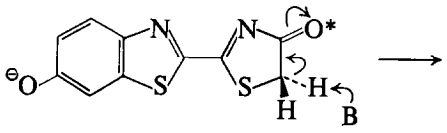
or possibly other dark reactions intervene.

A red emission from the phenyl ester of 5,5-dimethylluciferin has also been reported by another group of workers (*44*); the mechanism they proposed for the reaction is essentially the same as that outlined in Eq. (7).

Yellow-Green Chemiluminescence

Esters or anhydrides of luciferin yield red light on treatment with potassium *tert*-butoxide in air-saturated DMSO, but an excess of base shifts the emission to the yellow-green range, which matches light emission in the enzymatic reaction (Fig. 7) (*45*). Similar behavior was found for various analogs of luciferin. However, no such wavelength shift was found for esters of 5,5-dimethylluciferin (**9d**) or homoluciferin (**10d**).

Thus, it seemed likely that proton abstraction at C-5 was involved during the lifetime



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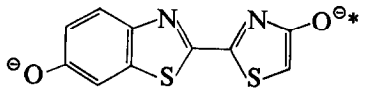
(8)



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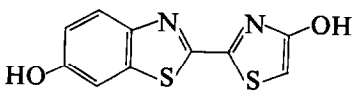
of the excited singlet state of ketone **22** (*46*). By gradually increasing the concentration of base, a shift in emission from red to purely the yellow-green (as in the enzymatic reaction, Fig. 4A) can be measured (Fig. 4B). This shift is not a gradual shift induced by changing environment; instead, it is a result of the change in the ratio of red- to yellow-green-emitting species which has a direct analog in the bioluminescence. That proton abstraction is involved is further supported by the narrow range of base concentration (100X; Fig. 4) necessary for a complete (~99%) shift in emitting species. Thus the apparent equilibrium inferred by the spectra (Fig. 4) is, in fact, a *rate* phenomenon.

The ionization of excited states is well documented, and Forster in particular has furnished a number of examples of this process (*46*). Specifically, this type of ionization involving the phenolic group at C-6′ has been shown to occur with luciferin (*34*). The photoionizations discussed above involve a proton loss from oxygen and nitrogen; photoionization involving the C–H bonds would be expected to be slower (*47*). The failure of homoluciferin to chemiluminesce in the yellow-green region suggests that the ionization for protons activated only by a carbonyl group is too slow to compete with light emission, and that the rate is enhanced in cases where resonance stabilization results from the ionization [Eq. (8)] (*48*); stabilization of the transition state by the *d* orbitals of sulfur might also be a factor.

Confirmation of the postulate that the dianion (**23**) is the light emitter in the yellow-green chemiluminescence comes from fluorescence measurements on spent reaction mixtures, which match the chemiluminescence measurements (Fig. 7). Also, a synthesis has been reported recently for the expected product from the luminescence of luciferin (*49*). It apparently exists largely in the enolic form (**24**) in neutral media, and it yields a yellow-green fluorescence in basic media (*49*).



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Emission Bandwidth Differences

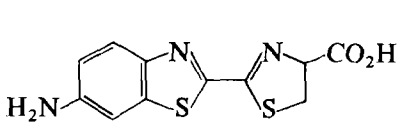
In addition to obvious color differences in the red and green emissions in chemiluminescence, there is a distinctive narrowness to the red emission (FWHM ~1700 cm^{-1}) as compared to the yellow-green (FWHM 2150 cm^{-1}). Red and yellow-green *in vitro* bioluminescence emissions have effectively the same FWHM values as listed

above (Tables 1 and 2). The narrowness of the red emission (FWHM 1700 cm^{-1}) may result from the rigidness of the emitter (**22**) (a result of the partial double bond character of bond 2,2′ between the ring systems). An unusually narrow emission has also been noted for several porphyrins (*50*a, b). Even the green emission (FWHM 2150 cm^{-1}) is narrow compared to the FWHM's of the luciferin (3300 cm^{-1}) and luciferyl adenylate (3000 cm^{-1}) fluorescence emissions (*50*c), and the methoxy luciferin phenyl ester (**11**c) chemiluminescence (FWHM 3000 cm^{-1}, Fig. 7).

Bioluminescence

Light production by the firefly involves two steps catalyzed by the enzyme luciferase: the generation of the mixed anhydride of luciferin and AMP and the oxidation of this species [Eq. (6)] (*31*). The *in vitro* reaction system, using synthetic D-luciferin and ATP-Mg^{2+} or synthetic D-luciferin adenylate (*32*, *53*), emits the same yellow-green light (Fig. 4A) at pH 7.8–8.0 with a quantum yield nearly unity (*6*). At pH values below this (down to 5.8), or in the presence of divalent heavy metal ions (Hg^{2+}, Cd^{2+}, Zn^{2+}), or at elevated temperatures (20–45°C), the color of light emission shifts toward the red, due to the emission from a distinct excited state product which becomes the only product emitter (Fig. 4A) at pH 6.0 or at high (2×10^{-2} *M*) concentrations of heavy metal ions (*51*). At intermediate pH values or heavy metal ion concentrations the emission spectrum is a mix of the yellow-green and red emissions (Fig. 4A). The pH *optimum* for red emission is 6.8 (though at this pH there is still considerable yellow-green emission) where the quantum yield for red emission is about 0.3 (*34*).

Photinus pyralis firefly luciferase is specific for the D-isomer of luciferin and the D-enantiomorph of luciferyl adenylate (*52*). Very few of the analogs of luciferin are active in light emission. 6-Aminoluciferin (**25**) gives only a red emission (*24*, *34*) wheres



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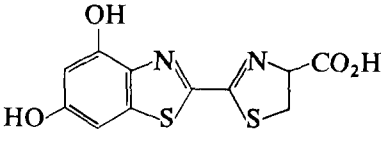

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the 4-hydroxy derivative (**26**) gives both red and yellow-green emissions with a different pH optimum compared to luciferin (*34*).

Product Identification

As outlined in an earlier section, attempts to isolate or detect the product of the enzymatic oxidation of luciferin have failed. Furthermore, neither the use of special techniques for monitoring fluorescence during bioluminescence emission (*38*) nor use of stoichiometric amounts of oxygen (Fig. 8) has allowed detection of a fluorescence emission spectrum matching the bioluminescence emission [the weak fluorescence of the bioluminescence product reaction mixture observed (Fig. 8) is apparently due to some degradation or condensation product of the emitter **23**]. As a result, identification of the product excited states must follow from their emission spectra alone.

Various firefly species (all using D-luciferin as the substrate) emit light over a wide range of wavelengths from green to orange (*51*, *54*); the shapes of the emission spectra are essentially identical, however (*54*). Presumably, the same excited state is formed in each case, but specific weak interactions of the solvent and of enzyme functional groups

(hydrogen bonding, etc.) lead to shifts of the emission maxima. Identification of the product excited state in the present case can thus be made by a comparison of the shape of the bioluminescence emission with the shapes of the fluorescence and chemiluminescence emissions of model systems. The comparison should include the effects of pH and other variables.

We find that the red bioluminescence emission spectrum is matched exactly in shape by the red chemiluminescence of luciferyl esters and also by the fluorescence of the ketophenolate product (see Fig. 5A and B). Also the yellow-green bioluminescence spectrum is exactly matched in shape by the yellow-green chemiluminescence and by

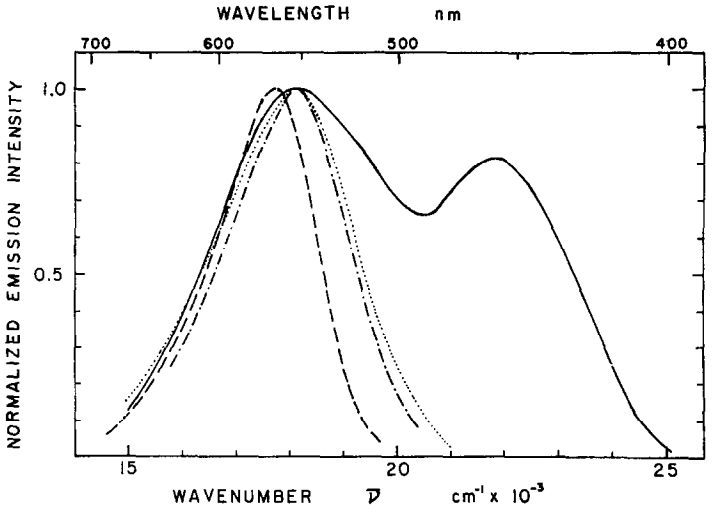


FIG. 8. Fluorescence of selected *P. pyralis in vitro* bioluminescence reaction components at pH 8.0. D-Luciferyl adenylate (**6d**) (· · · · ·), 2×10^{-6} *M*; anaerobic reaction mix of D-luciferyl adenylate (2×10^{-6} *M*) plus excess enzyme (5×10^{-6} *M*) (—·—); spent reaction mix after addition of oxygen (2×10^{-6} *M*) (——), and *P. pyralis in vitro* bioluminescence (——).

the fluorescence of the enolate (dianion) (Fig. 4A and B). We therefore attribute the yellow-green bioluminescence to this enolate dianion (enzyme-bound excited state, E·**23**), and the red emission to its pH-dependent related keto form (E·**22**).

Consistent with these assignments are the findings that (1) the C-5 monomethyl luciferin derivatives (**7** and **8**) are active in the enzyme-catalyzed yellow-green light production, (2) the 6′-methoxy analog of luciferin (**11**) is inactive in bioluminescence, and (3) synthetic **24** spent reaction mixtures, and the dimethyl analog (**18**) have fluorescence spectra which match the bioluminescence emissions (solely red in the case of **18**).

Environmental Perturbation of Emission Spectra

Morton et al. (*41*) used the 6′-phenolate fluorescence emission of luciferin as a model in studying the effect of changing the environment surrounding the product on the character of its emission. It was found that gradual shifts in the peak position ($\bar{\nu}_{max}$) without significantly effecting the FWHM could be produced in two ways: (1) less polar solvents led to a blue shift typical of $\pi \rightarrow \pi^*$ transitions in which the dipole moment increases upon excitation (*55*), and (2) a red shift was found when bidentate complexes, analogous to those formed by α-α′-dipyridyl (*56*), were formed with

Cd^{2+} and Zn^{2+}. This effect is *unrelated* to the red shift in the bioluminescence emission of *P. pyralis* in the presence of these ions (*41*). The range over which effects 1 and 2 shifted the peak position of luciferin fluorescence with little change in FWHM was $>4000\ cm^{-1}$.

Similarly, the solvent-environmental effects probably account for part of the differences in peak positions found in the present work (red chemiluminescence at $15\,400\ cm^{-1}$ in water, $15\,850\ cm^{-1}$ in DMSO, and red bioluminescence at $16\,150\ cm^{-1}$; yellow-green chemiluminescence in DMSO at $18\,000\ cm^{-1}$ and yellow-green bioluminescence at $17\,700\ cm^{-1}$; Tables 1 and 2). In this connection, the emission from different firefly species, *in vivo* and *in vitro* with D-luciferin at pH 8, spans a wide range in peak positions, which blanket the above values (the shapes and FWHM of which are similar) (*54*). Species variation in the enzyme is clearly involved here. Since the range of species emissions spans both sides of the dimethylsulfoxide chemiluminescence, more than just a solvent-type environmental interaction must be involved —more specific interactions must play a part.

Reaction Pathway

A reaction pathway for the enzymatic light emission similar to that proposed for the chemiluminescence [Eqs. (7) and (8)] is consistent with the experimental findings. Presumably, groups on the enzyme (B_1, B_2, and B_3—or some combination) are acting as bases leading to proton abstraction at the 6′ hydroxyl group, the C-4 C–H and the C-5 C–H, respectively.

The following observations support this mechanism:

Ionization at C-6′. The fact that enzyme-bound LH_2AMP (anaerobic conditions) fluoresces strongly in the green region ($\tilde{\nu}_{max}$ 18 150, Fig. 8) indicates that the phenolic OH at C-6′ is ionized (*34*). Un-ionized luciferin (and its derivatives), and the 6′ methoxy analogs fluoresce weakly in the blue region of the spectrum. [The fact that the bandwidth of the enzyme-bound luciferyl adenylate is different (FWHM $2600\ cm^{-1}$) from that of luciferin itself (FWHM $3200\ cm^{-1}$) suggests specific interactions between the enzyme and luciferin.]

Anion formation at C-4. D-Luciferyl adenylate is readily racemized, and the rate of bioluminescence emission is less if the LH_2AMP is deuterated at C-4. Bioluminescence initiated with luciferyl adenylate follows complex kinetics. There is an initial rapid rise in the intensity followed by a slow decay. The rate of the initial rise is a function of pH, the rise time doubling over the pH range 7.4–6.4. Using C-4 deuterated DL-luciferyl adenylate increases the time before maximum intensity by a factor of 1.6 at pH 7.4.

Carbon dioxide production. Plant et al. (*29*) have shown a quantitative formation of carbon dioxide from carboxyl-labeled (^{14}C)-luciferin in the enzymatic reaction.

Anion formation of the excited ketone. That a base-catalyzed step is necessary for yellow-light emission is obvious from the pH-color dependence. (The action of Zn^{2+}, Cd^{2+}, and Hg^{2+} in shifting the normal yellow-green emission to red is viewed as a blockage of the proton abstraction at C-5, probably by the derivatization of the basic group on the enzyme by the metals.)

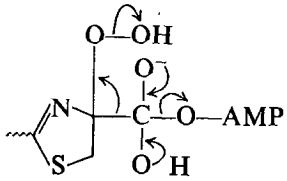
Some idea of the timing involved stems from the observation that no rate differences have been observed in red vs. green emission intensities when a reaction is initiated with ATP-Mg^{2+} and D-LH_2 or with D-LH_2AMP in an *aerobic* system at pH 6.8 (where both emissions occur with the same quantum yield). In contrast, such a measurement has shown a slower rate for the rise in intensity of yellow-green emission relative to the red (0.3 vs. 0.8 sec to reach maxima) when oxygen is injected into an anaerobic solution

of D-LH_2AMP with an excess of enzyme. Since the red vs. yellow-green rate difference shows up in the rise time rather than in the decay of the intensity, there may be a difference in the rate of O_2 attack governed by whether the enzyme is "primed" for yellow-green emission or for red emission.

That the enzyme can efficiently (*57*) remove the proton at C-4 (to yield **19**, *e.g.*) is a catalytic feat, considering the weak protein side-chain base catalysts available (sulfhydryl anion, amino or imidazole group, etc.). Proton abstraction from C-5 in yellow-green bioluminescence is no less impressive since it must complete with red light emission. In dimethylsulfoxide, conjugate bases of weak acids (pK_a of *tert*-butanol = 27) (*58*) are necessary for the success of this reaction. The catalytic proximity effect resulting from enzymatic binding is considerable but other factors are almost certainly at play as well. Both sulfhydryl and imidazole groups have been suggested as being implicated in the enzyme catalysis (*31*). Sulfhydryls, though present (*59*), may not be at the catalytic (light-emitting) site (*60*); histidine may also be involved (*61*).

Recent experiments utilizing oxygen-18 labeling have been reported to be inconsistent with the mechanism proposed above (*62*). In enzyme-catalyzed runs with ^{18}O-labeled oxygen gas, no ^{18}O was found in the carbon dioxide, and in runs with ^{18}O-labeled water, one oxygen atom of ^{18}O was found in the carbon dioxide. The introduction of this oxygen atom was proposed to have mechanistic significance (**27**), but it seems to us that at least a part of the ^{18}O exchange may have occurred at the adenylate stage of the reaction (LH_2AMP, **6d**), or with the carbon dioxide (*64*).

The reaction mechanism proposed by DeLuca and Dempsey (reproduced in **27**) also



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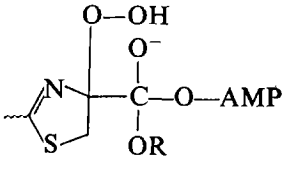
requires comment. Mechanisms of this general type, multiple linear bond cleavages, have been proposed for a few chemiluminescent reactions (*63*); however, the theoretical basis for such reactions leading to electronically excited states is on less firm ground than the dioxetane or radical ion hypotheses discussed in the Introduction section. With specific reference to *27* no strong analogy from the field of organic reaction mechanisms can be cited.

Furthermore, as we have shown, a strong similarity exists between the chemiluminescence of luciferin and the bioluminescence. In chemiluminescence, *t*-butoxide ion (RO^-) is an effective base, yet its adduct related to **27** [Eq. (28)] cannot undergo



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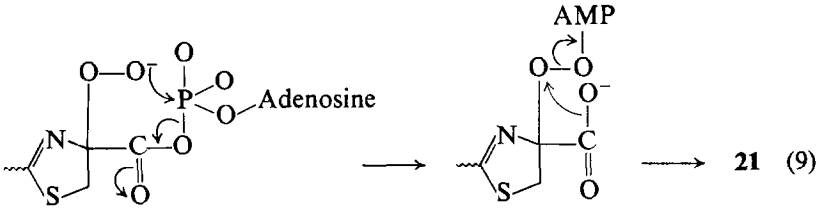
such a cleavage. Lastly, the pK_a of hydroperoxides is in the range of ~12 (*65*) whereas that of *t*-butanol is 26.9 in DMSO (*58*). Thus the hydroperoxide group would be expected to be largely ionized under our reaction conditions [as illustrated in Eq. (7)].

In any case, in the event that a more carefully controlled experiment verifies the lack of carbon dioxide incorporation of ^{18}O from the oxygen gas, a modification of the dioxetane mechanism could readily account for the result, as illustrated in Eq. (9).



(9)

"Photobiology Without Light"

Photochemistry, detailing various reactions of excited states (Fig. 1, process b, d), is of immense importance in chemistry and biology. As indicated in Fig. 1, identical excited states are obtained from the chemical production (process a) as from light absorption (process b); process a, d, "photochemistry without light," should therefore be a realizable process. In fact, a number of examples are now known involving the singlet state of oxygen (*66*) and the excited states of various organic compounds (*14*a, b, *67*). Extrapolating further, "photobiology without light" should also be a distinct possibility, and several promising areas for such a process were pointed out recently (*14*c). Since then, a number of other possible applications have been cited. Buu-Hoï and Sung have proposed that carcinogenic aromatic hydrocarbons may be active via their excited states arrived at chemically (*68*), and Pettus and Moore have considered chemically produced excited states in the biogenesis of the dictyopterenes (*69*). Also, the role of singlet oxygen (generated chemically in some cases) has been reviewed (*70*). Organisms living in the darkness of caves would appear to be fruitful subjects in a search for chemically produced excited states in cellular processes. In summary, it is clear that chemically produced excited states are of central importance in bioluminescence; it seems moderately certain that they will also be found to be important in other areas of biology.

Note Added in Proof: Lamola (*Biochem. Biophys. Res. Commun.* **43**, 893 (1971)) has recently reported the formation of pyrimidine dimers in DNA using chemically produced excited states.

EXPERIMENTAL

Melting points were taken with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analysis was performed by Galbraith Laboratories (Knoxville, Tenn.). Infrared spectra were determined on a Perkin–Elmer Model 337 instrument, ultraviolet spectra were determined on a Cary Model 14 spectrophotometer, and optical rotatory spectra were measured on a Cary Model 16 spectrophotopolarimeter. Proton magnetic resonance (pmr) spectra were determined on Varian Associates A-60 and HR-100 instruments. Chemical shifts are reported in τ units relative to tetramethylsilane (TMS).

Materials

Potassium *tert*-butoxide (MSA Research Corp.) was used as received. Dimethylsulfoxide (Matheson Coleman and Bell) was stirred overnight over crushed potassium

hydroxide, decanted, and distilled *vacuo* from potassium *tert*-butoxide. Other solvents, except where stated, were reagent grade and used as received. Adenosine-5′-triphosphate (ATP), adenosine monophosphate (AMP), coenzyme A (CoA), and EDTA were the best grade obtainable from the Sigma Chemical Company. Dicyclohexylcarbodiimide (Aldrich Chemical Co.) and triethylamine (Eastman) were found to contain no significant fluorescent contaminants and were used as received. The following compounds were prepared following literature procedures: 2-cyano-6-hydroxybenzothiazole (**4**) (*23*), 2-cyano-6-methoxybenzothiazole (*23*), DL-2-(6′-hydroxy-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (**6a**) (firefly luciferin) (*21*), DL-2-(6′-methoxy-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (**11a**) (*22*b), DL-2-(6′-hydroxy-2′-benzothiazolyl)-Δ^2-thiazoline-5,5-dimethyl-4-carboxylic acid (**9a**) (*22*b), ethyl-2-mercaptoisobutyrate (*71*), DL-threo-α-amino-β-benzylthiobutyric acid (*24*), and DL-*erythro*-α-amino-β-benzylthiobutyric acid (*24*).

DL-Trans-*2-(6′-hydroxy-2′-benzothiazolyl)-Δ^2-thiazoline-5-methyl-4-carboxylic acid* (**8a**). DL-*Threo*-α-amino-β-benzylthiobutyric acid (80 mg, 0.35 mmole) was dissolved in freshly distilled liquid ammonia (50 ml) and reduced with small pieces of sodium until the blue color persisted for 10 min. The excess sodium was destroyed with ammonium chloride. The ammonia was evaporated with a stream of nitrogen, the dry residue was dissolved (all operations under nitrogen) in 10 ml of oxygen-free water (nitrogen bubbling was sufficient) and the pH was adjusted to 7.5 with 0.1 *N* hydrochloric acid. Methanol (5 ml, oxygen free) was added followed by a solution of 2-cyano-6-hydroxybenzothiazole (**4**) (54 mg, 0.32 mmole) in methanol (5 ml). The color of the solution changed immediately to orange, and the stirred mixture was allowed to react in the dark for 90 min. The reaction was followed by ultraviolet absorption, the initial absorption at 324 nm changed to 332 nm at the end of reaction (aliquots were acidified before determining the spectra). The solution was diluted with water (20 ml) and the pH adjusted to 8. It was then extracted with ethyl acetate to remove neutral materials. The aqueous layer was acidified slowly (nitrogen bubbling) to pH 1 with 10% hydrochloric acid. The product was extracted with ethyl acetate which was present during the acidification. The ethyl acetate solution was dried and evaporated to dryness to give 76 mg (0.26 mmole, 87%) of crude product. The product was recrystallized twice from acetone–cyclohexane to give pure material, mp 190–192°C dec. It showed one spot on tlc (cellulose with ethanol:water:ammonia, 8:1:1). ir (KBr) 3400–2800 (broad) and 1750 cm^{-1}; uv (95% ethanol) 332 ($\log\epsilon$ 4.25) and 269 nm (3.83); nmr (perdeuteroacetone) τ 2.09 (1 H, d, $J = 9$ Hz), 2.56 (1 H, d, $J = 2$ Hz), 2.89 (1 H, pair of doublets, $J = 9$ Hz, $J = 2$ Hz), 5.00 (1 H, d, $J = 6$ Hz), 6.39 (1 H, m) and 8.47 (3 H, d, $J = 7$ Hz).

Anal. Calcd for $C_{12}H_{10}N_2O_3S_2$: C, 48.99; H, 3.43; N, 9.52; S, 21.76. Found: C, 48.84; H, 3.41; N, 9.33; S, 21.60.

DL-Cis-*2-(6′-hydroxy-2′-benzothiazolyl)-Δ^2-thiazoline-5-methyl-4-carboxylic acid* (**7a**). DL-*Erythro*-α-amino-β-benzylthiobutyric acid (80 mg, 0.35 mmole) was reduced with sodium in liquid ammonia and reacted with 2-cyano-6-hydroxybenzothiazole (**4**) (54 mg, 0.3 mmole) in the same manner as described for the synthesis of the *trans* isomer. The crude reaction product (71 mg, 81%) was recrystallized twice from acetone–cyclohexane, mp 189–190°C dec. The pure product showed one spot on tlc (cellulose with ethanol:water:ammonia, 8:1:1). ir (KBr) 3400–2800 (broad) and 1750 cm^{-1}; uv (95% ethanol) 332 ($\log\epsilon$ 4.22) and 269 nm (3.81); nmr (perdeuteroacetone) τ 2.06 (1 H, d, $J = 9$ Hz), 2.54 (1 H, d, $J = 2$ Hz), 2.88 (1 H, pair of doublets, $J = 9$ Hz, $J = 2$ Hz), 4.76 (1 H, d, $J = 9$ Hz), 6.39 (1 H, m), and 8.64 (3 H, d, $J = 7$ Hz).

Anal. Calcd for $C_{12}H_{10}N_2O_3S_2$: C, 48.99; H, 3.43; N, 9.52; S, 21.76. Found: C, 48.90; H, 3.65; N, 9.67; S, 21.55.

DL-*2-(6′-Hydroxy-2′-benzothiazolyl)-Δ²-dihydro-1,3-thiazine-4-carboxylic acid* (**10a**). DL-Homocystine (120 mg, 0.44 mmole) was dissolved in freshly distilled liquid ammonia (50 ml) and reduced with small pieces of sodium until the blue color persisted for 10 min. Excess sodium was destroyed with ammonium chloride. The ammonia was removed with a stream of nitrogen, and the dry residue was dissolved in oxygen-free water (nitrogen bubbling) (5 ml). The pH was adjusted to 7.5 with 0.1 *N* hydrochloric acid. A solution of 2-cyano-6-hydroxybenzothiazole (**4**) (60 mg, 0.35 mmole) in methanol (5 ml) was added, and the reaction mixture was stirred at room temperature and under nitrogen. Aliquots were removed during the reaction, acidified, and their ultraviolet spectrum was determined. The absorption at 324 nm changed completely to 330 nm after 2 hr. The reaction solution was slowly acidified to pH 4 and extracted immediately with ethyl acetate which was present during acidification. The organic solution was dried and concentrated *in vacuo* until the appearance of several crystals. The product was obtained upon cooling (41 mg, 0.14 mmole, 40%). It was recrystallized from acetone–cyclohexane to give pure material, mp 164–165°C dec. The product showed one spot on tlc (cellulose with ethanol:water:ammonia, 8:1:1). ir (KBr) 3400–2800 (broad) and 1750 cm^{-1}; uv (95% ethanol) 330 ($\log\epsilon$ 4.26) and 269 nm (3.86); nmr (perdeuterodimethylsulfoxide) τ 2.11 (1 H, d, $J = 9$ Hz), 2.63 (1 H, d, $J = 2$ Hz), 2.98 (1 H, pair of doublets, $J = 9$ Hz, $J = 2$ Hz), 5.40 (1 H, q), 6.83 (2 H, m), and 7.88 (2 H, m).

Anal. Calcd for $C_{12}H_{10}N_2O_3S_2$: C, 48.99; H, 3.43; N, 9.52; S, 21.76. Found: C, 49.05; H, 3.38; N, 9.46; S, 21.50.

Phenyl DL-*2-(6′-hydroxy-2′-benzothiazolyl)-Δ²-thiazoline-4-carboxylate* (**6c**). DL-2-(6′-Hydroxy-2′-benzothiazolyl)-Δ²-thiazoline-4-carboxylic acid (**6a**) (65 mg, 0.25 mmole) was mixed at room temperature with phenol (240 mg, 2.5 mmoles), and trifluoroacetic anhydride (3 ml) was added with cooling and under nitrogen. The reaction solution was stirred for 1 hr, and the excess of phenol and trifluoroacetic anhydride was removed under vacuum. The dry residue was dissolved in ethyl acetate (50 ml) and washed twice with water. The organic layer was then dried and evaporated to dryness to give a crude product which was recrystallized twice from acetone–cyclohexane (60 mg, 0.18 mmole, 72%), mp 189–190°C dec. ir (KBr) 3600–3300 (broad), 3050, 2920, and 1750 cm^{-1}; uv (95% ethanol) 334 ($\log\epsilon$ 4.05) and 269 nm (3.50).

Anal. Calcd for $C_{17}H_{12}N_2O_3S_2$: C, 57.30; H, 3.37; N, 7.86; S, 17.97. Found: C, 56.98; H, 3.18; N, 7.80; S, 17.64.

Phenyl DL-*2-(6′-hydroxy-2′-benzothiazolyl)-Δ²-dihydro-1,3-thiazine-4-carboxylate* (**10c**). DL-2-(6′-Hydroxy-2′-benzothiazolyl)-Δ²-dihydro-1,3-thiazine-4-carboxylic acid (**10a**) was esterified with phenol in trifluoroacetic anhydride as described in the preceding experiment. The phenyl ester was recrystallized twice from acetone–cyclohexane to give pure material in 58% yield, mp 180–182°C dec. ir (KBr) 3350, 3050, 2920, and 1750 cm^{-1}; uv (95% ethanol), 328 ($\log\epsilon$ 4.18) and 269 nm (3.86).

Anal. Calcd for $C_{18}H_{14}N_2O_3S_2$: C, 58.38; H, 3.81. Found: C, 58.53; H, 4.04.

Phenyl DL-*2-(6′-methoxy-2′-benzothiazolyl)-Δ²-thiazoline-4-carboxylate* (**11c**). Trifluoroacetic anhydride (4 ml) was added carefully with cooling to DL-2-(6′-methoxy-2′-benzothiazolyl)-Δ²-thiazoline-4-carboxylic acid (**11a**) (0.4 mg, 1.36 mmoles) followed by phenol (0.61 g, 6.5 mmoles). The resulting solution was stirred at room temperature for 30 min and evaporated to dryness under vacuum. The dry residue was dissolved in ethyl acetate which was washed twice with 5% aqueous sodium bicarbonate and twice with water. The organic solution was dried and evaporated to dryness. The product was recrystallized twice from ethyl acetate–cyclohexane to give 0.39 g (1.05 mmoles, 77%) of the phenyl ester, mp 210–212°C dec. ir (KBr) 2920 and 1725 cm^{-1}.

Anal. Calcd for $C_{18}H_{14}N_2O_3S_2$: C, 58.38; H, 3.81; N, 7.56; S, 17.29. Found: C, 58.24; H, 3.62; N, 7.29; S, 17.07.

Methyl DL-*2-(6'-hydroxy-2'-benzothiazolyl)-Δ^2-thiazoline-4-carboxylate* (**6b**). DL-2-(6'-Hydroxy-2'-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid (**6a**) (0.1 g, 0.42 mmole) was stirred at 0°C with 3 ml trifluoroacetic anhydride, and methanol (1 ml) was added dropwise at this temperature. The reaction mixture was stirred for an additional hour and ethyl acetate (50 ml) was added. The organic solution was washed twice with 5% aqueous sodium bicarbonate and twice with water. The ethyl acetate solution was dried and evaporated until crystallization began; cooling gave yellow needles which were recrystallized from acetone–cyclohexane (0.106 g, 0.36 mmole, 86%), mp 182–184°C. ir (KBr) 3330 and 1690 cm^{-1}; uv (95% ethanol) 328 (log ϵ 4.28) and 269 nm (3.88); nmr (perdeuteroacetone) τ 2.04 (1 H, d, $J = 9$ Hz), 2.50 (1 H, d, $J = 2$ Hz), 2.81 (1 H, pair of doublets, $J = 9$ Hz, $J = 2$ Hz), 4.54 (1 H, t, $J = 9$ Hz), 6.18 (2 H, d, $J = 9$ Hz), and 6.64 (3 H, s).

Anal. Calcd for $C_{12}H_{10}N_2O_3S_2$: C, 48.99; H, 3.43; N, 9.52; S, 21.76. Found: C, 48.64; H, 3.50; N, 9.39; S, 21.98.

4-Nitro-2,6-dimethylanisole. The compound was prepared according to Rowe, Bannister, and Storey (*72*). Nitric acid (70%, 52 ml) was added gradually with cooling to a solution of 2,6-dimethylanisole (25 g, 0.18 mole) in glacial acetic acid (38 ml). The resulting solution was warmed slowly to 60–70°C, and then cooled, whereupon the reaction mixture solidified. It was diluted with 600 ml of ice and water and filtered. Recrystallization from ethanol give 21 g (0.11 mole, 61%) of the nitro derivative, mp 92–93°C [Ref. (*72*) 92°C].

4-Amino-2,6-dimethylanisole (**12**). 4-Nitro-2,6-dimethylanisole (18 g, 0.1 mole) was catalytically hydrogenated under pressure (60 psi) in ethanol (150 ml) using 10% Pd–C (1 g) as catalyst. The theoretical amount of hydrogen was absorbed within 2 hr. The solution was warmed and filtered from the catalyst. The solvent was evaporated and the residue recrystallized from ethanol to give long colorless needles of the amine (14.5 g, 0.096 mole, 96%) mp 64–66°C [Ref. (*72*) 66°C].

3,5-Dimethyl-4-methoxythiooxanilamide (**13**). A solution of carbamoylthiocarbonylthioacetic acid (*23*) was prepared from trichloroacetamide (14 g) and chloroacetic acid (12 g) in 50% aqueous ethanol (250 ml). This solution was added immediately to 4-amino-2,6-dimethylanisole (9.5 g, 62.9 mmoles) in 50% aqueous ethanol. The resulting solution was cooled in a refrigerator for 3 days whereupon yellow needles precipitated. The product was filtered and recrystallized from ethanol (2.55 g, 10.8 mmoles, 11%), mp 123–124°C. ir (KBr) 3370, 3270, 2940, and 1700 cm^{-1}.

Anal. Calcd for $C_{11}H_{14}N_2O_2S$: C, 55.46; H, 5.92; N, 11.76; S, 13.43. Found: C, 55.29; H, 5.95; N, 11.86; S, 13.27.

2-Carbamoyl-5,7-dimethyl-6-methoxybenzothiazole (**14**). A solution of 3,5-dimethyl-4-methoxythiooxanilamide (980 mg, 4.1 mmoles) in 10% aqueous sodium hydroxide (110 ml) was added dropwise to a stirred solution of potassium ferricyanide (17 g) in water (40 ml). A colorless precipitate appeared immediately and stirring was continued for an additional 30 min. The precipitate was collected and washed thoroughly with water to give 732 mg (3.1 mmoles, 75%) of material which was used without further purification. The melting point was 158–160°C and the typical amide absorption in the infrared (KBr) appeared at 3300, 3200, and 1680 cm^{-1}.

2-Cyano-5,7-dimethyl-6-methoxybenzothiazole. A solution of 2-carbamoyl-5,7-dimethyl-6-methoxybenzothiazole (0.61 g, 2.6 mmoles) in pyridine (30 ml) with phosphorous oxychloride (10 ml) was refluxed for 0.5 hr. The solvent was partially evaporated and ethyl acetate (100 ml) was added. The solution was well stirred with

water and ice (100 ml), and the aqueous layer was extracted with two additional portions of ethyl acetate (50 ml each). The ethyl acetate solution was treated with Norit A, dried, and evaporated to dryness. Recrystallization from benzene–petrol ether gave white needles (479 mg, 2.2 mmoles, 85%), mp 147–148°C. ir (KBr) 3080, 2970, 2960, 2890, 2230, and 1600 cm^{-1}; uv (95% ethanol) 306 ($\log \epsilon$ 4.14) and 250 nm (3.91).

Anal. Calcd for $C_{11}H_{10}N_2OS$: C, 60.55; H, 4.59; N, 12.84; S, 14.66. Found: C, 60.35; H, 4.43; N, 12.76; S, 14.39.

2-Cyano-5,7-dimethyl-6-hydroxybenzothiazole (**15**). 2-Cyano-5,7-dimethyl-6-methoxybenzothiazole (230 mg, 1.06 mmoles) and pyridine hydrochloride (600 mg) were heated at 150–160°C for 3 hr and cooled to room temperature. Ethyl acetate (50 ml) and water (20 ml) were added while the flask was cooled in an ice bath. The aqueous layer was extracted twice with ethyl acetate (25 ml each time), and the combined organic solution was treated with Norit A, dried, and evaporated to dryness. The residue was recrystallized from benzene–petrol ether to give white needles (140 mg, 0.69 mmole, 65%), mp 196–198°C dec. ir (KBr) 3450, 2230, and 1605 cm^{-1}; uv (95% ethanol) 330 ($\log \epsilon$ 4.14) and 250 nm (3.83).

Anal. Calcd for $C_{10}H_8N_2OS$: C, 58.85; H, 3.92; N, 13.72; S, 15.68. Found: C, 58.99; H, 3.96; N, 13.65; S, 15.44.

DL-*2-(6′-Hydroxy-5′,7′-dimethyl-2′-benzothiazolyl)-Δ^2-thiazoline-4-carboxylic acid* (**16**). DL-Cystine (36 mg, 0.15 mmole) was dissolved in liquid ammonia (20 ml) and reduced with sodium metal. Small pieces of sodium were added to the stirred solution until the blue color persisted for 10 min. The excess sodium was destroyed with ammonium chloride, and the ammonia was removed with a stream of nitrogen. The dry residue was dissolved in 10 ml of oxygen-free water (nitrogen bubbling), and the pH was adjusted to 7.5 with 1 *N* hydrochloric acid. Methanol (10 ml) was added followed by a solution of 2-cyano-5,7-dimethyl-6-hydroxybenzothiazole (50 mg, 0.24 mmole) in methanol (10 ml). The addition was followed by stirring the resulting solution for 1 hr (all operations under nitrogen) during which time the ultraviolet absorption shifted from 330 to 334 nm. The solution was diluted with water (20 ml), and the methanol was removed under vacuum. The aqueous solution (total vol 50 ml) was stirred with ethyl acetate (50 ml) while acidified slowly to pH 1 with concentrated hydrochloric acid. The ethyl acetate solution was dried and evaporated until several crystals appeared; cooling gave yellow needles which were recrystallized from acetone–cyclohexane to give 65 mg (0.21 mmole, 88%) of the dimethyl luciferin, mp 163–164°C dec. ir (KBr) 3505, 3400 (broad), and 1740 cm^{-1}; uv (95% ethanol) 334 ($\log \epsilon$ 4.29) and 266 nm (3.87); nmr (perdeuteroacetone) τ 2.34 (1 H, s), 4.58, (1 H, t, $J = 9$ Hz), 6.26 (2 H, d, $J = 9$ Hz), 7.56 (3 H, s), and 7.62 (3 H, d, $J = 1$ Hz).

Anal. Calcd for $C_{13}H_{12}N_2O_3S_2$: C, 50.66; H, 3.92; N, 9.09; S, 20.78. Found: C, 50.35; H, 3.87; N, 8.92; S, 20.91.

6-Hydroxybenzothiazole-2-thiocarboxamide. 2-Cyano-6-hydroxybenzothiazole (**4**) (50 mg, 0.28 mmole) was dissolved in pyridine (3 ml), and two drops of triethylamine and hydrogen sulfide was bubbled through the solution for 2 hr (*73*). The solvent was removed *in vacuo* and the residue was triturated with benzene and collected. The crude product was recrystallized twice from aqueous methanol to give the pure thioamide (52 mg, 0.25 mmole, 90%) mp 243–244°C. ir (KBr) 3430, 3350, and 1620 cm^{-1}. uv (95% ethanol) 360 ($\log \epsilon$ 4.30), 319 (sh) (4.03), and 271 nm (3.93).

Anal. Calcd for $C_8H_6N_2OS_2$: C, 45.71; H, 2.86; N, 13.33; S, 30.48. Found: C, 45.81; H, 2.92; N, 13.10; S, 30.51.

Ethyl and methyl 2-(6′-hydroxy-2′-benzothiazolyl)-thiazole-4-carboxylate (ethyl and methyl esters of dehydroluciferin). Ethyl bromopyruvate (0.42 g, 1.3 mmoles) was added

to a suspension of 6-hydroxybenzothiazole-2-thiocarboxamide (0.1 g, 0.48 mmole) in methyl alcohol (10 ml) (*74*). The reaction mixture was stirred at room temperature for 15 hr, and then heated on a steam bath for 2 hr. A yellow-colored product (0.11 g, 0.36 mmole, 75%) precipitated upon cooling. The crude product was recrystallized from methanol to give yellow crystals mp 256°C (sinters at 235°C). ir (KBr) 3300 and 1730 cm^{-1}; uv (95% ethanol) 353 (log ϵ 4.33) and 276 nm (3.77).

Elementary analysis showed partial ester interchange.

Anal. Calcd for $C_{12}H_8N_2O_3S_2$ (methyl ester): C, 49.33; H, 2.76; N, 9.59. Found: C, 50.21; H, 2.97; N, 9.31.

2-(6′-Hydroxy-2′-benzothiazolyl)-thiazole-4-carboxylic acid (dehydroluciferin) (**17**). The ester of 2-(6′-hydroxy-2′-benzothiazolyl)-thiazole-4-carboxylate (0.1 g, 0.34 mmole) was dissolved in 10% aqueous sodium hydroxide (10 ml), and methanol (5 ml) was added to the solution. The fluorescent solution was refluxed for 3 hr and filtered while hot. Slow acidification with 10% hydrochloric acid yielded a yellow crystalline solid (0.085 g, 0.30 mmole, 88%). The product was purified by dissolving it in methanol containing aqueous sodium hydroxide and slowly acidifying with dilute hydrochloric acid; mp 320–322° [Ref. (*21*) 315–321°C] and mixed mp 316–320°C. uv (95% ethanol) 351 (log ϵ 4.37), 275 (3.89), and 271 nm (3.87).

D-*Luciferin* (**6a**). The isomeric purity of D-luciferin was determined by maximum quantum yield of bioluminescence (*6*, *34*) and by ORD. Impure luciferin samples were rendered pure by aqueous Sephadex (G-25 fine) adsorption chromatography (*41*). Aqueous solutions were kept cold, dark, and near neutral pH.

Optical rotatory dispersion (ORD) spectra of luciferin were measured at concentrations where the optical density at λ_{max} was less than 0.1. D-Luciferin shows a negative Cotton effect, crossing 0° at 330 nm. The molar rotation [*M*] at the negative maximum (312 nm) is measured to be −5600°. At 270 nm the [*M*] is +7500°. The ORD of L-luciferin measured to be almost a mirror image of the D-luciferin ORD ($[M]_{312}$ +5100°; $[M]_{270}$ −12,200°; λ_0 320 nm). The measurements were made with aqueous solutions.

4-Deuterioluciferin. This was prepared by dissolving D-luciferin (3 mg, 1.1 mmoles) in 20 ml D_2O (Nuclear-Chicago), 0.01 *M* in NaOH, and saturated with nitrogen. Within 5 min the mixture is racemic (determined by the light assay for D-luciferin). The solution is then acidified to pH 3.0, extracted with ethyl acetate, and dried after removal of solvent to yield 3 mg of product. Previous results involving the nmr spectrum of luciferin in basic D_2O (*22*b) showed that the C-4 proton was the first exchanged in the molecule (of those attached to carbon).

Firefly luciferyl adenylate (**6d**). Luciferin (9 mg, 3.2 mmoles) and adenylic acid (13 mg, 5.4 mmoles) were dissolved in 1.0 ml of pyridine acidified with 0.1 ml of 0.5 *N* HCl. To this solution was added *N*,*N*-dicyclohexylcarbodiimide (250 mg, 71 mmoles) dissolved in 1.0 ml pyridine. The mixture was allowed to react 30 min at 0°C. Cold acetone (10 ml, −20°C) was then added and the fine precipitate was immediately filtered and washed with cold acetone (5 ml). The precipitate, a mixture of the urea, anhydride (~30%), and adenylic acid, was used without further purification for chemiluminescence studies in nonaqueous systems. For subsequent work in aqueous systems, the precipitate was extracted with an acetate buffer (0.05 *M*, pH 4.8, 0–4°C). This solution was chromatographed on Sephadex G-25 (fine). Although LH_2AMP hydrolyzes rapidly, the effluent contained less than 5% luciferin.

While this method gives good yields of the mixed anhydride, its synthesis is accompanied by racemization. Practically pure D-luciferyl adenylate was prepared using distilled anhydrous DMSO instead of acidified pyridine. A slight separation of the D- and L-enantiomorphs is obtained in the Sephadex G-25 (fine) chromatography (0.04

M NaCl, 0.01 *M* acetate, pH 4.5). Early fractions contain the highest D/L-enantiomorph ratio.

The criteria for purity used for luciferyl adenylate included (1) absorption and fluorescence spectra (*34*, *41*), (2) yield of photons/mole when the LH_2AMP was added to luciferase (no ATP-Mg^{2+}) relative to D-luciferin (with ATP-Mg^{2+}, see below), which assays D-luciferyl adenylate content, and (3) analysis by spectral characteristics and tlc of the hydrolysate (DL-luciferin, see below). ORD proved to be difficult to use since DL-luciferyl adenylate gave a complex ORD spectrum, possibly due to formation of intramolecular complexes (*41*).

Luciferyl adenylate (**6d**) hydrolysis rates were measured as a function of pH (1) by following the change in optical density (at 330 nm) with time or (2) when pure D- or L-enantiomorphs were used, by following the change in photons emitted per mole with luciferase in bioluminescence (relative to D-luciferin + ATP-Mg^{2+}, see below). The fluorescent product of D- and L- or DL- hydrolysis is shown always to be DL-luciferin by the combination of (1) identification and quantitation by chromatography and spectral characteristics and (2) assay of D-isomer content by relative photons emitted per mole in bioluminescence and also by the ORD of the product. Luciferyl adenylate hydrolysis is always accompanied by racemization. The rate of hydrolyses is apparently first order and directly proportional to pH (6–9) with $t_{1/2}$ ~10 min at pH 8.2. In the presence of 10^{-2} *M* Mg^{2+} at pH 8.2, $t_{1/2}$ is reduced to 5 min.

Adenylates of luciferin analogs. These were prepared as the dried acetone precipitate and were used without further purification.

2-(6′-Hydroxy-2′-benzothiazolyl)-Δ^2-thiazoline-5,5-dimethyl-4-one (**18**). Ethyl-2-mercaptoisobutyrate (163 mg, 1.15 mmoles) was dissolved in ethanol (13 ml), and the solution was flushed with nitrogen. 2-Cyano-6-hydroxybenzothiazole (**4**) (176 mg, 1.01 mmoles) was added followed by triethylamine (0.16 ml). The reaction mixture was refluxed for 4 hr under nitrogen and was followed by tlc (silica gel with chloroform:ethanol, 20:1). The red fluorescent solution was evaporated to dryness under vacuum. The red residue was heated under vacuum (80°C, 20 μ) for 2 hr. It was then sublimed (125°C, 15 μ), and fractions (10–15 mg) were collected and analyzed by tlc. Fractions 1–7 contained mixtures of 2-cyano-6-hydroxybenzothiazole and the expected product. The temperature was raised to 200°C, at which point the sublimate and residue showed only one spot on tlc. The sublimate and residue (175 mg, 0.66 mmole, 66%) were crystallized from ethanol to yield pure **18**, mp 280–283°C. ir (KBr) 3175, 2920, and 1765 cm^{-1}; uv (95% ethanol) 390 (logϵ 4.17), 307 (3.81), 277 (3.87), and 272 nm (3.88); nmr (perdeuterodimethylsulfoxide) τ 1.84 (1 H, d, $J = 9$ Hz), 2.39 (1 H, d, $J = 2$ Hz), 2.80 (1 H, pair of doublets, $J = 9$ Hz, $J = 2$ Hz), and 8.33 (6 H, s).

Anal. Calcd for $C_{12}H_{10}N_2O_2S_2$: C, 51.81; H, 3.62; N, 10.07. Found: C, 52.01; H, 3.67; N, 10.20.

2-(6′-Methoxy-2′-benzothiazolyl)-Δ^2-thiazoline-4-one. *n*-Butyl thioglycolate (53 mg, 0.3 mmole) was dissolved in glyme (5 ml), and the solution was flushed with nitrogen. 2-Cyano-6-methoxybenzothiazole (50 mg, 0.3 mmole) was added followed by 0.1 ml triethylamine. The reaction mixture was heated (reflux) for 2 hr under nitrogen. The solution was then evaporated to dryness, and the residue was sublimed at 147°C (12 μ) and then at 212°C (13 μ). The latter sublimate showed only one spot on thin layer chromatography.

Anal. Calcd for $C_{11}H_8N_2O_2S_2$: C, 50.0; H, 2.5; N, 10.6. Found: C, 51.6; H, 2.8; N, 10.2.

Physical data suggested that the compound was present in dilute solutions, but that it polymerized in concentrated solutions. However, see Ref. (*49*).

2-(6′-Hydroxy-2′-benzothiazolyl)-Δ^2-thiazolin-4-one (ground state of **22**). An attempt to synthesize this compound was made as follows: 2-cyano-6-hydroxybenzothiazole (**4**) (15 mg, 0.1 mmole) was dissolved in 5 ml of distilled dimethylsulfoxide. Triethylamine (0.3 ml) was added, and the solution was thoroughly deaerated with nitrogen, placed in a closed flask, then placed under vacuum. Previously deaerated butyl thioglycolate (0.1 ml, 5 X excess) was introduced by injection through a rubber septum. The mixture was allowed to react at room temperature while the appearance of product was followed by tlc and by fluorescence (samples were withdrawn using a syringe). Although starting material was consumed (tlc) within 20–30 min, the fluorescence emission was never red. Rather, the fluorescence emission was yellow-green ($\bar{\nu}_{max}$ ~18 000 cm^{-1}). It is possible that our failure to isolate a product (attempted sublimation resulted only in a polymeric material) was due to the sensitivity of the expected compound to oxygen and to polymerization [see, however, Ref. (*49*)].

Luciferase (enzyme). This enzyme was isolated and purified from dried tails (light organs) of *Photinus pyralis*. Preparations were crystalline according to Green and McElroy (*75*). These proved of too high a fluorescence content for fluorescence studies involving the enzyme. In these cases further purification on DEAE-Sephadex (A-50) using a 0.03–0.3 *M* phosphate gradient (pH 7.9) achieved the necessary low fluorescence levels. Anaerobic preparations were achieved by repeated cycles of evacuation and nitrogen saturation in an all-glass vacuum-tight mixing system (*30*). Since luciferase is very sensitive to surface denaturation, anaerobisis was achieved by 500 cc/min flushing of nitrogen over the surface of a concentrated (10–30 mg/ml) solution of the enzyme at 0°C for 1 hr before use. The concentrated enzyme solution is obtained from dissolving an ammonium sulfate precipitate in 0.2 *M* ammonium sulfate, 0.1 *M* phosphate, pH 7.9, 10^{-3} *M* EDTA, 10^{-4} *M* Cleland's reagent. Calculations of concentrations were based on a mol wt of 50 000 (*76*) and the optical density at 278 nm (*75*).

Spectral Measurements

Fluorescence and bioluminescence emission spectra were obtained using either a 1 m (f/3 grating), 0.5 m (f/4.5 grating), or a 0.25 m (f/8.0) grating spectrometer designed by W. Fastie using either an RCA 7326 or an EMI 9558 photomultiplier (S-20 response). These instruments were calibrated for relative photon spectral sensitivity (efficiency) using an NBS color temperature lamp (*77*).

All spectral data are plotted as a function of wave number (cm^{-1}). A wavelength scale (nm) is given at the top of Figs. 4–8 for orientation. The ordinate for emission spectra has the unit photons per unit wave number ($dN/d\bar{\nu}$), and the curves are corrected for variations in the spectrometer's relative photon spectral sensitivity (*34*). They are then normalized to unity at the wave number of peak intensity. The $dN/d\bar{\nu}$ vs. $\bar{\nu}$ plots can be used for the determination of energy differences between absorption and emission shifts and can be used to draw comparisons between either absorption spectra or emission spectra bands which peak at different wave numbers. 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 (*34*).

Fluorescence measurements were taken with awareness of the possible ambiguities introduced by minute amounts of contaminants. A considerable effort was made to insure the purity of our preparations. All emission and fluorescence spectra were measured at or near room temperature (24°C) in solutions of absorbance less than 0.05 at the peak wavelength of emission. No deviations from Beer–Lambert behavior in concentration-dependent self-quenching of fluorescence was observed.

Chemiluminescence reactions were carried out in 6 × 75-mm round glass tubes. This geometry was found not to contribute to emission spectra artifacts (by refraction or scattered light). Reactions in air-saturated solvent were initiated by rapid injection of a small amount of base catalyst dissolved in the solvent. Imidazole anion in DMSO was produced by dissolving imidazole, to strength, in a solution of DMSO over crushed KOH pellets. In water, imidazole was dissolved (usually 1.0 *M*) and adjusted to a given pH level—usually buffered—and the concentration of imidazole anion concentration was calculated from its p*K* of 14. Methoxide was produced from methanolic KOH. Corrections for intensity changes (generally an exponential decrease) were made by first recording several spectra in rapid succession then correcting for the observed intensity decrease with time before correcting for the spectral efficiency of the spectrometer.

Photometric determinations were made by measuring the output of an RCA 1P 21 or EMI 9558 photomultiplier-photometer exposed to the reacting solution (enzyme + substrates or chemiluminescence). Since the quantum yield of bioluminescence relative to D-luciferin is 0.88 ± .25 (*6*) and the emission spectrum and quantum yield at pH 7.8–8.0 using *P. pyralis* luciferase is a constant, measurements of the total intensity output from a known quantity of D-luciferin provides a calibration (secondary) of a photometer for this reaction as well as providing a method for determining the optical purity (D-isomer content) of D-luciferyl adenylate. Thus photons sec^{-1} were recorded for the intensity measured under these conditions (*P. pyralis* enzyme, pH 7.8–8.0).

The quantum yield of luciferin (ester) chemiluminescence (0.3 ± 50%) was determined relative to yellow-green bioluminescence (0.325, pH 6.8) (*34*) by comparing the photons/mole emitted by chemiluminescence relative to bioluminescence. This ratio was corrected for the difference in the position of the $\tilde{\nu}_{max}$ in the two emissions.

Biochemical Studies

Luciferase was assayed by "flash height" in the D-luciferin calibrated photometer (see above) using fresh solutions of ATP (~2×10^{-3} *M*), $MgSO_4$ (~5×10^{-3} *M*), and purified D-luciferin (~5×10^{-6} *M*). The ATP-Mg^{2+} solution was usually injected into the enzyme-luciferase solution. The concentrations of these components was such that the response to enzyme concentration was linear. The specific activity, measured in photons sec^{-1} mg^{-1}, was compared with that of crystalline enzyme.

Simultaneous measurements of red and yellow-green emissions in the bioluminescent reaction were made at pH 6.8 where the red and yellow-green emission quantum yields are nearly equivalent (*34*). The measurement was made using two photomultiplier-photometer detectors viewing the same reaction mix. One photomultiplier was masked with a blue-green (536 nm) interference filter, which passes emission for only the yellow-green emission. The other photomultiplier was masked with a red (645 nm) transmitting filter. The two photometer outputs were recorded simultaneously on a two-channel high speed recorder.

D-Luciferin and D-luciferyl adenylate isomeric purities were determined by comparing the photons $mole^{-1}$ emitted with excess enzyme (plus ATP-Mg^{2+} with D-luciferin) to that with purified D-luciferin.

Since bioluminescence emission spectra could not be determined accurately in some cases with luciferin analogs, bioluminescence quantum yields are not quoted for these compounds.

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REFERENCES

1. E. N. HARVEY, "A History of Luminescence." American Philosophical Society, Philadelphia, 1957.
2. Surveys of the field of chemiluminescence appear in several reviews: (a) E. H. WHITE, "Light and Life" (W. D. McElroy and B. Glass, Eds.). Johns Hopkins Press, Baltimore, 1961; (b) F. MCCAPRA, *Quart. Rev. Chem. Soc.* **20**, 485 (1966); (c) K. D. GUNDERMANN, "Chemilumineszenz Organischer Verbindungen." Springer-Verlag, Berlin, 1968. Also: K. D. GUNDERMANN, *Angew. Chem. Int. Ed. Engl.* **4**, 566 (1965); (d) J. W. HAAS, *J. Chem. Educ.* **44**, 396 (1967).
3. Light emission in chemiluminescence normally occurs from the lowest excited singlet state. Reports of emission from the lowest triplet state (phosphorescence) have appeared, but need verification. The oxidation of the radical anion of *N*-phenylcarbazole leads to light emission, which was suggested to be, in part, the phosphorescence of *N*-phenylcarbazole [E. A. CHANDROSS AND F. I. SONNTAG, *J. Amer. Chem. Soc.* **88**, 1089 (1966); *J. Amer. Chem. Soc.* **86**, 3179 (1964)]. However, the reducing agents used to prepare radical anions (Na, etc.) are known to cleave *N*-aryl bonds (The Emde Reaction. See H. GILMAN, "Organic Chemistry," 2nd ed., Vol. 2, p. 1173. Wiley, New York, 1943), and the fluorescence of carbazole anion is similar to the "phosphorescence" reported. Also, A. ZWEIG, D. L. MARICLE, J. S. BRINEN, AND A. H. MAURER [*J. Amer. Chem. Soc.* **89**, 473 (1967)] reported the phosphorescence of phenanthrene in electrochemiluminescence; highly efficient scavenging of oxygen was proposed to account for the phosphorescence in this emission from liquid solutions at room temperature. A notable exception concerning phosphorescence is the work of Vassil'ev on autoxidation chemiluminescence, involving low concentrations of oxygen and ultimate weak emission from various carbonyl compounds formed as products [R. F. VASSIL'EV, *Progr. React. Kinet.*, **4**, 305 (1967) and references cited therein].
4. Early discussions of this problem are given by M. G. EVANS, E. EYRING, AND J. F. KINCAID *J. Chem. Phys.* **6**, 349 (1938), by F. H. JOHNSON, H. EYRING, AND M. J. POLISSAR, "The Kinetic Basis of Biology." Wiley, New York, 1954, and by J. MAYER, "The Luminescence of Biological Systems" (F. H. Johnson, Ed.), p. 248. American Association for the Advancement of Science, Washington, D.C., 1955.
5. More detailed considerations are given in: (a) R. A. MARCUS, *J. Chem. Phys.* **43**, 2654 (1965); (b) D. M. HERCULES, *Accounts Chem. Res.* **2**, 301 (1969).
6. H. H. SELIGER AND W. D. MCELROY, *Arch. Biochem. Biophys.* **88**, 136 (1960).
7. R. AUDUBERT, *Trans. Faraday Soc.* **35**, 197 (1939); V. Y. SHLYAPINTOK, R. F. VASIL'EV, O. N. KARPUKHIN, L. M. POSTNIKOV, AND L. A. KIBALKO, *J. Chim. Phys. Physicochim. Biol.* **57**, 1113 (1960).
8. E. H. WHITE AND M. J. C. HARDING, *Photochem. Photobiol.* **4**, 1129 (1965).
9. F. MCCAPRA AND Y. C. CHANG, *Chem. Commun.*, 522 (1966).
10. F. MCCAPRA, D. G. RICHARDSON, AND Y. C. CHANG, *Photochem. Photobiol.* **4**, 1111 (1965). See also Ref. (*11*).
11. M. M. RAUHUT, *Accounts Chem. Res.* **2**, 80 (1969).
12. W. H. URRY AND J. SHEETO, *Photochem. Photobiol.* **4**, 1067 (1965); W. CARPENTER AND E. M. BENS, *Tetrahedron* **26**, 59 (1970).
13. K. R. KOPECKY AND C. MUMFORD, *Can. J. Chem.* **47**, 709 (1969).
14. (a) E. H. WHITE, J. WIECKO, AND D. R. ROSWELL, *J. Amer. Chem. Soc.* **91**, 5194 (1969); (b) E. H. WHITE, J. WIECKO, AND C. C. WEI, *J. Amer. Chem. Soc.* **92**, 2167 (1970); (c) E. H. WHITE AND C. C. WEI, *Biochem. Biophys. Res. Commun.* **39**, 1219 (1970).
15. F. MCCAPRA, *Chem. Commun.*, 155 (1968).
16. R. HOFFMANN AND R. B. WOODWARD, *Accounts Chem. Res.* **1**, 17 (1968); R. B. WOODWARD AND R. HOFFMANN, *Angew. Chem. Inter. Ed. Engl.* **8**, 781 (1969); R. B. WOODWARD AND R. HOFFMAN, "The Conservation of Orbital Symmetry." Academic Press, New York, 1970.
17. D. R. KEARNS, *J. Amer. Chem. Soc.* **91**, 6554 (1969).
18. E. A. CHANDROSS AND F. I. SONNTAG, *J. Amer. Chem. Soc.* **86**, 3179 (1964).
19. G. J. HOIJTINK [mentioned in the article by E. A. CHANDROSS AND F. I. SONNTAG, *J. Amer. Chem. Soc.* **86**, 3179 (1964)]; D. M. HERCULES, *Science* **145**, 808 (1964); articles listed in Ref. (*2c*).
20. A more detailed account is given in E. A. CHANDROSS AND F. I. SONNTAG, *J. Amer. Chem. Soc.* **88**, 1089 (1966); and in Ref. (*5*).

21. E. H. WHITE, F. MCCAPRA, AND G. F. FIELD, *J. Amer. Chem. Soc.* **85**, 337 (1963).
22. (a) E. H. WHITE, H. WORTHER, H. H. SELIGER, AND W. D. MCELROY, *J. Amer. Chem. Soc.* **88**, 2015 (1966); (b) E. H. WHITE, H. WORTHER, G. F. FIELD, AND W. D. MCELROY, *J. Org. Chem.* **30**, 2344 (1965); (c) E. H. WHITE AND H. WORTHER, *J. Org. Chem.* **31**, 1484 (1966).
23. S. SETO, K. OGURA, AND Y. NISHIYAMA, *Bull. Chem. Soc. Jap.* **36**, 331 (1963).
24. H. E. CARTER, C. M. STEVENS, AND L. F. NEY, *J. Biol. Chem.* **139**, 247 (1941).
25. M. J. KARPLUS, *J. Amer. Chem. Soc.* **85**, 2870 (1963).
26. K. B. WIBERG AND B. J. NIST, "Interpretation of NMR Spectra." Benjamin, New York, 1962.
27. J. M. TEDDER, *Chem. Rev.* **55**, 787 (1955).
28. P. BERG, *J. Biol. Chem.* **233**, 608 (1958).
29. P. J. PLANT, E. H. WHITE, AND W. D. MCELROY, *Biochem. Biophys. Res. Commun.* **31**, 98 (1968).
30. T. A. HOPKINS, Ph.D. thesis, Johns Hopkins University, 1968.
31. W. D. MCELROY, H. H. SELIGER, AND E. H. WHITE, *Photochem. Photobiol.* **10**, 153 (1969).
32. (a) W. D. MCELROY AND A. GREEN, *Arch. Biochem. Biophys.* **64**, 257 (1956); (b) W. C. RHODES AND W. D. MCELROY, *J. Biol. Chem.* **233**, 1528 (1958).
33. H. H. SELIGER AND W. D. MCELROY, *Radiat. Res.*, Suppl. 2, 528 (1960).
34. H. H. SELIGER AND R. A. MORTON, "Photophysiology," (A. C. Giese, Ed.), Vol. 4, pp. 315–353. Academic Press, New York, 1968.
35. T. A. HOPKINS, H. H. SELIGER, E. H. WHITE, AND M. W. CASS, *J. Amer. Chem. Soc.* **89**, 7148 (1967).
36. A *weak* emission of luciferyl adenylate (6d) in dimethylsulfoxide was previously observed [H. H. SELIGER AND W. D. MCELROY, *Science* **138**, 683 (1963)].
37. E. N. HARVEY, "Bioluminescence," p. 428. Academic Press, New York, 1952.
38. H. H. SELIGER AND W. D. MCELROY, "Bioluminescence in Progress" (F. H. Johnson and Y. Haneda, Eds.), pp. 405–426. Princeton Univ. Press, Princeton, N.J., 1966.
39. H. BEYER AND W. LASSIG, *Chem. Ber.* **84**, 467 (1951).
40. (a) K. A. JANSEN AND I. CROSSLAND, *Acta Chem. Scand.* **17**, 144 (1963); (b) K. A. JANSEN, S. GRONOWITZ, B. MATHIASSON, R. DAHLBOM, AND B. HOLMBERG, *Acta Chem. Scand.* **19**, 1215 (1965).
41. R. A. MORTON, T. A. HOPKINS, AND H. H. SELIGER, *Biochemistry* **8**, 1598 (1969).
42. O. H. MÜLLER AND J. P. BAUMBERGER, *Trans. Electrochem. Soc.* **71**, 169 (1937); P. ZUMAN, *Chem. Listy* **47**, 1234 (1953).
43. (a) G. A. RUSSELL AND A. G. BEMIS, *J. Amer. Chem. Soc.* **88**, 5491 (1966); (b) G. BELLUCCI, B. MACCHIA, AND F. MACCHIA, *Tetrahedron Lett.* **38**, 3239 (1969).
44. F. MCCAPRA, Y. C. CHANG, AND V. P. FRANCOIS, *Chem. Commun.*, 22 (1968).
45. E. H. WHITE, E. RAPAPORT, T. A. HOPKINS, AND H. H. SELIGER, *J. Amer. Chem. Soc.* **91**, 2178 (1969).
46. TH. FORSTER, *Z. Electrochem.* **61**, 340 (1957).
47. Related photoenolizations are listed by R. O. KAN, "Organic Photochemistry," pp. 29–32. McGraw-Hill, New York, 1966.
48. D. J. CRAM, "Fundamentals of Carbanion Chemistry." Academic Press, New York, 1965.
49. N. SUZUKI, M. SATO, K. NISHIKAWA, AND T. GOTO, *Tetrahedron Lett.* **53**, 4683 (1969).
50. (a) R. S. BECKER AND J. B. ALLISON, *J. Chem. Phys.* **32**, 1410 (1960); (b) R. S. BECKER AND J. B. ALLISON, *J. Phys. Chem.* **67**, 2662 (1963); (c) G. E. BLANK, J. PLETCHER, AND M. SAX, *Biochem. Biophys. Res. Commun.* **42**, 583 (1971).
51. H. H. SELIGER AND W. D. MCELROY, *Proc. Nat. Acad. Sci. U.S.A.* **52**, 75 (1964).
52. H. H. SELIGER, W. D. MCELROY, E. H. WHITE, AND G. F. FIELD, *Proc. Nat. Acad. Sci. U.S.A.* **47**, 1129 (1961).
53. R. PHILLIPS, *Chem. Rev.* **60**, 501 (1966).
54. H. H. SELIGER, J. BUCK, W. G. FASTIE, AND W. D. MCELROY, *J. Gen. Physiol.* **48**, 95 (1964).
55. E. LIPPERT, *Z. Electrochem.* **61**, 962 (1957).
56. (a) K. SONE, P. KRUMHOLZ, AND H. STAMMREICH, *J. Amer. Chem. Soc.* **77**, 777 (1955); (b) H. L. SCHLAFER, *Z. Phys. Chem. Frankfurt* **8**, 373 (1956).
57. *In vivo* flash rise times <20 msec have been measured: (a) J. B. BUCK AND J. F. CASE, *Biol. Bull.* **125**, 251 (1961); (b) F. E. HANSON, J. MILLER, AND G. T. REYNOLDS, *Biol. Bull.* **137**, 447 (1969).
58. A pK_a of 26.9 was reported for methanol in DMSO (E. C. STEINER, Petroleum Chemistry Section, 153rd National Meeting of the American Chemical Society, Miami, Fla., April, 1967). *Tert*-butoxide is considered to be a stronger base than methoxide [C. D. RITCHIE AND R. E. VSCHOLD, *J. Amer. Chem. Soc.* **89**, 2960 (1967)].
59. M. DELUCA, G. W. WERTZ, AND W. D. MCELROY, *Biochemistry* 3, 935 (1964).
60. R. LEE AND W. D. MCELROY, *Biochemistry* **8**, 130 (1969).
61. J. TRAVIS AND W. D. MCELROY, *Biochemistry* **5**, 2170 (1966).

62. M. DeLuca and M. E. Dempsey, *Biochem. Biophys. Res. Commun.* **40**, 117 (1970).
63. M. M. Rauhut, D. Sheehan, R. A. Clark, and A. M. Semsel, *Photochem. Photobiol.* **4**, 1097 (1965).
64. DeLuca and Dempsey report a reaction time of 15 sec or less plus a short time interval to reattach the flask to the vacuum line and to freeze the contents (reaction temp. not specified, but probably ~25°C). At pH 7.8, the large majority of carbon dioxide molecules are attacked by water rather than by hydroxide ion [T. H. Maren, *Physiol. Rev.* **47**, 595 (1967)]. The rate constants for hydration and dehydration of carbon dioxide and carbonic acid at 25°C are 0.037 sec^{-1} (Maren) and 13.7 sec^{-1} [B. H. Gibbons and J. T. Edsall, *J. Biol. Chem.* **238**, 3501 (1963)], respectively. These values lead to a half-life of hydration of 18.6 sec. Thus, some exchange of the oxygens of carbon dioxide with water should have occurred during the experiment of DeLuca and Dempsey. The authors reported that a blank run on carbon dioxide showed a zero incorporation of oxygen-18 from water (^{18}O-labeled). These results are difficult to interpret; possibly the rate of solution of the gaseous carbon dioxide was the controlling factor in the blank runs.
65. A. G. Davies, "Organic Peroxides," p. 34. Butterworths, London, 1961. The pK_a of methanol in methanol is 18.3, in comparison [C. D. Ritchie, G. A. Skinner, and V. G. Badding, *J. Amer. Chem. Soc.* **89**, 2063 (1967)].
66. C. S. Foote and S. Wexler, *J. Amer. Chem. Soc.* **86**, 3879, 3880 (1964); C. S. Foote, *Accounts Chem. Res.* **1**, 104 (1968).
67. H. Güsten and E. F. Ullmann, *Chem. Commun.*, 28 (1970).
68. N. P. Buu-Hoï and S. S. Sung, *Naturwissenschaften* **57**, 135 (1970).
69. J. A. Pettrus, Jr., and R. E. Moore, *Chem. Commun.*, 1093 (1970).
70. T. Wilson and J. W. Hastings, "Photophysiology" (A. C. Giese, Ed.), Vol. 5. Academic Press, New York, 1970; M. A. Anbar, *J. Amer. Chem. Soc.* **88**, 5924 (1966); T. Matsuura, H. Matsushima, and R. Nakashima, *Tetrahedron* **26**, 435 (1970); J. E. Baldwin, H. H. Basson, and H. Kraust, Jr., *Chem. Commun.*, 984 (1968).
71. R. M. Acheson, J. Barltrop, M. Hichens, and R. E. Hichens, *J. Chem. Soc.* 650 (1961).
72. F. M. Rowe, S. H. Bannister, and R. C. Storey, *Chem. Ind. London*, 80T (1931).
73. A. E. S. Fairfull, J. L. Lowe, and D. A. Peak, *J. Chem. Soc.*, 742 (1952).
74. J. M. Sprague and A. H. Land, "Heterocyclic Compounds" (R. C. Elderfield, Ed.), Vol. 5. Wiley, New York, 1957.
75. A. A. Green and W. D. McElroy, *Biochim. Biophys. Acta* **20**, 170 (1956).
76. J. Denberg and W. D. McElroy, *Biochemistry* **9**, 4619 (1970).
77. J. Lee and H. H. Seliger, *Photochem. Photobiol.* **4**, 1015 (1965).