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W. Herz, H. Grisebach, G. W. Kirby

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Bioluminescence: Chemical Aspects

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With 9 Figures

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Note on Abbreviations

The abbreviations used in this review are as follows: LH and LH_2 commonly refer to the luciferin molecule under discussion, L refers to dehydroluciferin and L=O to oxyluciferin. E commonly refers to the luciferase molecule under discussion. $FMNH_2$ and FMN are the reduced and oxidized forms of flavin mononucleotide respectively. DPNH and DPN are the reduced and oxidized forms of nicotinamide adenine dinucleotide. ATP and PP_i represent adenosine triphosphate and inorganic pyrophosphate respectively, ANS is anilinonaphthalenesulfonate and RCHO is used for an unspecified long chain aldehyde.

I. Introduction

Bioluminescence may be defined simply as the emission of visible radiation by an enzyme-catalyzed reaction. An analogous phenomenon, chemiluminescence, may be defined as the emission of visible radiation by a nonenzymic (purely chemical) reaction. Generally bioluminescence represents an efficient reaction with quantum yields (number of photons emitted divided by the number of molecules of substrate oxidized) ranging from about 0.1 to nearly 1.0 (*34, 113, 120, 141*). On the other hand chemiluminescent reactions cover a broad spectrum of quantum yields.

In general chemiluminescent systems are often limited either by an emitter molecule with an intrinsically low quantum yield or by quenching effects both of which are selected against in most bioluminescence reactions. Thus the enzymes involved in catalyzing bioluminescent reactions must play an important role in increasing the efficiency of such reactions, probably both by decreasing the rates of excited state quenching and by increasing the efficiency of excited state formation. Bioluminescence takes place in an aqueous environment at near neutral pH, conditions which do not allow chemiluminescence to occur. The environmental requirements for chemiluminescence generally include strong base and an aprotic solvent. Presumably the enzyme provides the appropriate basic and aprotic environment to allow not only for an efficient reaction but for the appropriate transition levels as well. Certainly this concept must apply to enzymic catalysis in general.

The terms luciferin and luciferase have become part of the terminology in the field of bioluminescence primarily due to the classic work of DUBOIS (*28*). In the classic sense luciferase refers to an enzyme that catalyzes the oxidation of the substrate, luciferin, with light emission. During this oxidative reaction a large amount of energy is released (50—72 kcal per mole quanta). This energy is utilized to create an electronically excited state either directly or by energy transfer to some fluorescent species formed, or present, during the reaction. This fluorescent species, or emitter, may be a product of luciferin, luciferin itself, or some

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Table 1. *A List of Known Bioluminescent Reactions Separated According to Type Reaction*

Type Reaction	Examples	Emission Maxima (nm)	
		in vivo	*in vitro*
A. Pyridine-nucleotide linked:			
1. $DPNH + H^+ + FMN \underset{}{\overset{\text{Dehydrogenase}}{\rightleftharpoons}} FMNH_2 + DPN$ $FMNH_2 + RCHO + O_2 \xrightarrow{\text{Luciferase}} \text{Light}$	Bacteria	470—505	490
2. $DPNH + H^+ + L \overset{\text{Dehydrogenase}}{\rightleftharpoons} LH + DPN$ $LH + O_2 \xrightarrow{\text{Luciferase}} \text{Light}$	Fungi	528	—
B. Adenine-nucleotide linked:			
1. $LH + ATP + O_2 \xrightarrow[Mg^{2+}]{\text{Luciferase}} \text{Light}$	Firefly	552—582	562 (ph 7.6)
2. $\text{LH-Sulfate} + DPA \overset{\text{Luciferin Sulfokinase}}{\rightleftharpoons} LH + PAPS$ $LH + O_2 \xrightarrow{\text{Luciferase}} \text{Light}$	*Renilla* (coelenterate)	509	488
	Cavernularia (coelenterate)	509	488
	Yarella (fish)	—	—
C. Enzyme-substrate systems:	*Cypridina* (crustacean)	—	460
	Porichthys (fish)	—	460
	Apogon (fish)	—	460
	Parapriacanthus (fish)	—	460
	Pholas (clam)	—	480
$LH + O_2 \xrightarrow{\text{Luciferase}} \text{Light}$	*Gonyaulax* (protozoan) sol. sys.	470	470
	Odontosyllis (annelid)	—	507
	Latia (limpet)	—	535

Table 1 (continued)

Type Reaction	Examples	Emission Maxima (nm)	
		in vivo	*in vitro*
	Euphausia **(crustacean)**	—	476
	Diaphus **(myctophid fish)**	—	—
	Hoplophorus (decapod)	—	—
D. Peroxidation systems:			
$LH + H_2O_2 \xrightarrow{\text{Luciferase}} \text{Light}$	***Balanoglossus*** **(acorn worm)**	—	—
	Diplocardia **(earthworm)**	507	507
E. Activation of "precharged" systems:			
1. Precharged Particle $\xrightarrow{H^+;\,O_2} \text{Light}$	*Gonyaulax* (protozoan)	470	470
2. Precharged Protein $\xrightarrow{Ca^{2+}} \text{Light}$	***Aequorea*** **(coelenterate)**	509	460
	Halistaura **(coelenterate)**	—	460
	Obelia **(coelenterate)**	509	475
	Clytia **(coelenterate)**	509	—
	Phialidium **(coelenterate)**	509	—
	Ptilosarcus **(coelenterate)**	509	—
	Pelagia **(coelenterate)**	—	—
	Campanularia **(coelenterate)**	—	—
	Mnemiopsis **(coelenterate)**	485	485
F. Unclassified systems:	***Chaetopterus*** **(annelid)**	—	460
	Meganyctiphanes **(crustacean)**	—	476

protein bound chromophore in close association with luciferase, depending upon the system being investigated.

It is important to note that luciferin is a generic term which does not apply to any specific chemical structure. Although luciferin structures may vary widely, as will be shown subsequently in this chapter, we also find in a number of instances that luciferins isolated from widely diverse animals may be identical. Similar observations have been made for the luciferases.

The basic problem in bioluminescence may be stated simply: how is energy, derived from chemical oxidation, efficiently coupled to electronic excitation? One of the very interesting properties of luciferase proteins that catalyze bioluminescent reactions is their ability to manipulate large amounts of energy and to catalyze the creation of an electronically excited state. Sufficient work has been done on the chemistry of certain of these systems to allow us to make a start in the formulation of mechanism and to ponder the answer to the question posed above.

As will be shown subsequently there is a great deal of similarity in the mechanisms of bioluminescent reactions. This might be expected since the requirements for mechanisms which allow for the manipulation of a large amount of energy and release of this energy in a single reaction step may be extremely selective as to both the substrate requirements and the details of the oxidation reaction.

Work done by numerous investigators over the past decade has made this chapter possible. Many of the interesting observations made on bioluminescence will not be touched upon since it is beyond the scope of this chapter to do so. We have concentrated rather on those systems about which some chemical information is available. Additional aspects of bioluminescence have been covered in recent reviews (*18, 19, 42, 45, 55, 68, 96*).

CORMIER and TOTTER (*18, 19*) have developed a useful classification of bioluminescent reactions in which a particular system falls under one of several "type reactions". Table 1 gives an expanded version of their original classification based on recent information.

II. Renilla (Sea Pansy) Bioluminescence

1. General Comments

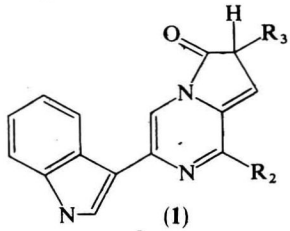
Renilla reniformis (commonly referred to as the sea pansy) is a marine animal (coelenterate) whose bioluminescence is controlled by a nerve network. As the result of a mechanical or electrical stimulus, concentric

waves of greenish luminescence can be seen to move over the animal surface. Light from the living animal is green and exhibits a very narrow, structured emission (*141*) having a maximum at 19,640 cm^{-1} (509 nm). At low enzyme concentration the *in vitro* reaction produces a blue structureless emission (*141*) with a maximum at 20,500 cm^{-1} (488 nm).

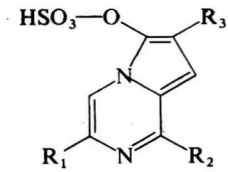
A good deal of information is now available regarding the requirements for this light emission process and some information is now at hand which allows us to speculate on the control mechanisms involved. As described below, studies on *Renilla* will provide an understanding of the bioluminescent reaction in a number of marine organisms since several of these have been shown to have essentially identical biochemical requirements for light emission.

2. Chemical Requirements for Light Emission

The tentative structure of the major portion of *Renilla* luciferin (**1**) has recently been reported (*23, 66*). Some uncertainties in the structure still remain regarding groups R_2 and R_3. Note the indole-pyrazine ring structure and the similarity of this structure to that of *Cypridina* luciferin (**22**). Unlike the situation which obtains in *Cypridina* luciferin (*18*) groups R_2 and R_3 are not amino acid side chains (*78*). Free luciferin is easily autooxidized (*65*), but the living animal stabilizes it and stores luciferin as a sulfonated derivative (*15*) which we term luciferyl sulfate (**2**). The position of the sulfate group has been recently established by infra-red data and chemical synthesis (*66*). For example luciferin exhibits an absorption at 1630 cm^{-1} which is assigned to an amide linkage present in the fused pyrrole ring. This absorption is absent in luciferyl sulfate but new absorption bands appear at 1215 cm^{-1} and 1265 cm^{-1} which are assigned to an acid sulfate linkage equivalent to such linkages found in indolyl-3-sulfate and ascorbic acid sulfate. We have also been able to synthesize luciferyl sulfate from luciferin and sulfamic acid. This methods result in the synthesis of sulfate esters of alcoholic and phenolic hydroxyls. The synthesized product is identical with the natural compound in chromatographic behavior and absorption characteristics. In addition requirements for light production with synthetic luciferyl sulfate are identical with those reported for the natural compound and the kinetics are the same (*11*). Thus the structure of luciferyl sulfate is **2.**



(1)



(2)

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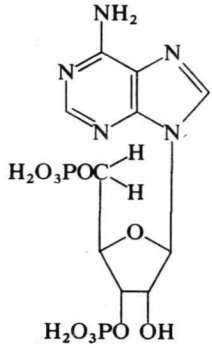
Since luciferyl sulfate does not react with luciferase to produce light the animal must possess a means for effecting its conversion to luciferin. We have isolated an enzyme from *Renilla* that does catalyze the conversion of luciferyl sulfate to luciferin which we have named luciferin sulfokinase (*15*). As shown by CORMIER *et al.* (*15*) luciferin sulfokinase catalyzes the conversion of luciferyl sulfate to luciferin in the presence of 3′,5′-diphosphoadenosine (DPA) (**3**). The activity of partially purified luciferin



(**3**)

sulfokinase is stimulated by the addition of calcium ion but that of the purified enzyme is not (*11*, *86*). As shown in Scheme 1 the other reaction product is 3′-phosphoadenylyl sulfate (PAPS). S^{35} experiments have shown that the reaction is reversible (*15*).

$$\text{Luciferyl sulfate} + \text{DPA} \xrightleftharpoons[\text{Sulfokinase}]{\text{Luciferin}} \text{Luciferin} + \text{PAPS}$$

Scheme 1

Most of the luciferin in *Renilla* exists in the form of luciferyl sulfate except for small amounts which we find bound to protein. Furthermore the conversion of luciferyl sulfate to luciferin by luciferin sulfokinase is a very slow reaction (*15*). In addition it is well known that repeated stimulation of *Renilla* results in a bioluminescence fatigue phenomena (*109*) which requires considerable time (1—2 hours) for complete recovery. These observations suggest that the low rate of synthesis of luciferin by luciferin sulfokinase plays an important role in this recovery process and is thus one of the controlling features of bioluminescence in *Renilla*.

Once luciferin is formed the only requirement for bioluminescence *in vitro* are luciferin, oxygen and luciferase. As shown in Scheme 2 luciferase catalyzes the oxidation of luciferin to produce light, CO_2 and oxyluciferin which represents the product excited state in the reaction (*23*, *141*).

$$\text{Luciferin} + O_2 \xrightarrow{\text{Luciferase}} \text{Oxyluciferin}^* + CO_2$$
$$\downarrow$$
$$\text{Oxyluciferin} + h\nu$$

Scheme 2

Luciferase is an energy conversion enzyme of low molecular weight, approximately 24,000. The evidence suggest that it is a dimer of identical subunits with a molecular weight of 12,000 (*73*). Its Stokes radius is 27 Å, its diffusion coefficient, $D_{20,w}$, is 8×10^{-7} cm^2 sec^{-1} and its extinction coefficient, $\overset{0,1\%}{\underset{280}{}}$, at pH 7.5 is 1.04. Its amino acid composition has also been determined (*73*).

3. Mechanism of the Light Reaction

During the bioluminescent oxidation of luciferin by luciferase (Scheme 2) one mole of CO_2 is produced per mole of luciferin present (*23*). The only other products are light and oxyluciferin (**8**, Scheme 3) whose fluorescence emission spectrum essentially matches that of the bioluminescence emission (Fig. 1). This same compound is released into the surrounding sea water by *Renilla* when they are stimulated to luminesce (*141*). Thus this fluorescence product must represent the *in vitro* emitter.

When light emission proceeded in an $^{18}O_2$ atmosphere there was negligible incorporation of ^{18}O into the CO_2. However when the reaction was carried out in the presence of $H_2{}^{18}O$, both oxygens in the released CO_2 were labelled (*23*). The production of CO_2 and the production of the *in vitro* product (**8**) as well as labelling of the CO_2 by $H_2{}^{18}O$ requires the presence of both luciferase and luciferin (**1**). A mechanism that predicts the ^{18}O labelling data during *Renilla* bioluminescence is shown in Scheme 3. One of the two labelled oxygens found in the CO_2 most probably arises from a non-enzymic exchange of water oxygen with the ketone group of luciferin (**1**) where R_1 is the indolyl moiety. We postulate the formation of a carbanion (**5**) followed by oxygen attack to give a linear hydroperoxide (**6**). Hydroxyl ion then adds at the carbonyl carbon to form **7** which then rearranges to form the electronically excited state product **8** and CO_2. Compound **8** then returns to the ground state with the release of a photon. For convenience this mechanism will be referred to as the "hydroxyl ion mechanism".

When luciferase catalyzes the oxidation of luciferin according to Scheme 3, blue light is observed with a maximum at 20,500 cm^{-1} (488 nm) as shown in Fig. 1. The quantum yield of this reaction with respect to luciferin is low, i.e. about 0.04 (*141*). The color of light produced from