

CHAPTER

1

THE FIREFLIES AND LUMINOUS INSECTS

Since ancient times, the light emitted by fireflies and glow-worms has attracted the curiosity of people. Descriptions of the phenomena are frequently found in old poems, songs and folklores of many countries. Old scientific studies of these phenomena are also numerous, particularly after the 17th century. However, the chemical study was not begun until the early 20th century.

Although the class Insecta (in the phylum Arthropoda) contains bioluminescent organisms in four orders: Collembola, Hemiptera, Coleoptera and Diptera, biochemical studies have been carried out only with several types of organisms of the last two orders, Coleoptera and Diptera. In these orders, the adults have two pairs of wings: in Coleoptera, the front wings are modified as elytra (a heavy protective cover); and in Diptera, the hind wings are reduced to knobs. The order Coleoptera includes Lampyridae (fireflies), Phengodidae (railroad worms), and Elateroidae (click beetles such as *Pyrophorus*), and all the luminous species in this order utilize firefly luciferin in their light emission. The order Diptera contains the glow-worms *Arachnocampa*

and *Orfelia*, and their bioluminescence systems are clearly different from that of Coleoptera.

According to Harvey (1952), there are considerable differences in morphology between males and females among the various genera of fireflies (lampyrids). In many of them, both males and females are winged and can fly, but usually the male has a larger light organ than the female. They use light signals to find each other. In some cases, females are luminous but males are not, and some species do not emit light at all. In *Lucidota* and *Pyropyga*, the larvae are luminous but the adult fireflies are not. The eggs of the fireflies are generally luminous, as was discovered as early as 1643 by Thomas Bartholin. The functions of the light emission of the fireflies and the behaviors of synchronous flashing have been reviewed by John B. Buck (Buck and Buck, 1976; Buck, 1978).

In northern and central Europe, the most common lampyrid is the glow-worm *Lampyrus*. The female of this genus is wingless and has a bright light organ, which attracts the flying male that has a less bright light organ. In Italy and southern Europe, common fireflies are the genera *Luciola* and *Phausia*, of which both males and females are winged. In North America, the genera *Photinus* and *Photuris* are the most common. The females of *Photinus* has partially developed wings and does not fly. *Photuris* is carnivorous and eats fireflies of other species.

Lampyrids are abundant in Japan; Ohba (1997, 2004) lists nine genera of lampyrids containing more than 50 species. The most common of them are *Luciola cruciata* (Fig. 1.1) and *Luciola lateralis*. These two species live in water during their larval stage (one or two years), making a distinct difference from all other species of fireflies; the larvae are luminescent. These lampyrids belong to one of the two kinds of luminous organisms that inhabit freshwater; the other is the New Zealand freshwater limpet *Latia*.

The fireflies, railroad worms, and click beetles use the same luciferin in their luminescence reactions. Recent studies on the railroad worms and the click beetles have greatly contributed to the biochemical understanding of the firefly bioluminescence (see Section 1.2). Concerning luminous Diptera, significant progress has been made only recently.

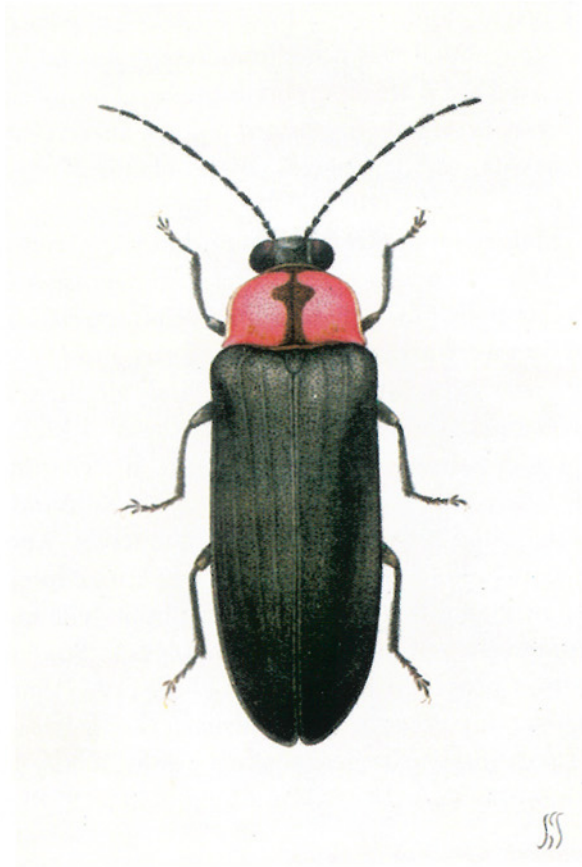


Fig. 1.1 The firefly *Luciola cruciata* (male) drawn by Sakyō Kanda (1874–1939), a pioneer of the study of bioluminescence in Japan, showing his extraordinary artistic talent (reproduced from Kanda, 1935).

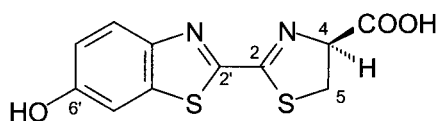
1.1 The Fireflies

1.1.1 An Overview of the Firefly Luminescence Reaction

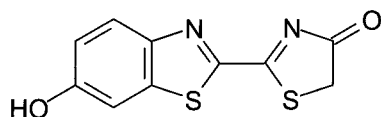
The luciferin-luciferase reaction of fireflies was first demonstrated by Harvey (1917), although the light observed was weak and short-lasting. Thirty years after Harvey's discovery, McElroy (1947) made a crucial breakthrough in the study of firefly bioluminescence. He found that the light-emitting reaction requires ATP as a cofactor. The addition of ATP to the mixtures of luciferin and luciferase

resulted in a bright, long-lasting luminescence. It was not a simple experiment because ATP was not commercially available at the time. McElroy prepared ATP from rabbit muscles. The discovery of the cofactor ATP was extremely important indeed, and it cleared the way for the spectacular, rapid progress in the chemical study of firefly bioluminescence.

In 1949, McElroy and Strehler found that the luminescence reaction requires Mg^{2+} in addition to luciferin, luciferase and ATP. The luciferase was partially purified and various characteristics of the luminescence reaction were investigated by McElroy and Hastings (1955). The luciferase was crystallized by Green and McElroy (1956). The luciferin was purified and crystallized by Bitler and McElroy (1957), which led to the determination of its structure and chemical synthesis (White *et al.*, 1961; 1963). The active luciferin was found to be in the D-form (Fig. 1.2); the L-form is practically inactive. According to a study by Lemberg (1996), L-luciferin is a competitive inhibitor of firefly luciferase, although it can produce some light. The mechanism of the chemiluminescence of luciferin that involves a dioxetanone intermediate was first proposed by Hopkins *et al.* (1967) and McCapra *et al.* (1968). The dioxetanone mechanism in the luciferase-catalyzed luminescence reaction was experimentally confirmed by ^{18}O -labeling studies (Shimomura *et al.*, 1977; Wannlund *et al.*, 1978).



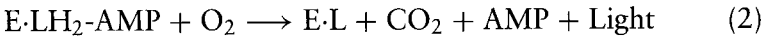
Firefly D-luciferin



Oxyluciferin

Fig. 1.2 Structures of firefly luciferin and oxyluciferin.

The following schemes represent the overall reaction of firefly bioluminescence (McElroy and DeLuca, 1978), where E is luciferase; LH₂ is D-luciferin; PP is pyrophosphate; AMP is adenosine phosphate; LH₂-AMP is D-luciferyl adenylate (an anhydride formed between the carboxyl group of luciferin and the phosphate group of AMP); and L is oxyluciferin.



In the first step, luciferin is converted into luciferyl adenylate by ATP in the presence of Mg²⁺. In the second step, luciferyl adenylate is oxidized by molecular oxygen resulting in the emission of yellow-green light, of which the mechanism is discussed in Sections 1.1.6 and 1.1.7. Both steps, (1) and (2), are catalyzed by luciferase. The reaction of the first step is slower than that of the second step, thus the first step is the rate-limiting step.

1.1.2 Firefly Luciferin and Oxyluciferin

Extraction and purification of luciferin. In the work of purifying and crystallizing firefly luciferin (Bitler and McElroy, 1957), McElroy used a unique method to gather the large quantity of fireflies needed for their research. In the now legendary story, they advertised for the purchase of fireflies at one cent per specimen. Children and youths in the neighborhood responded enthusiastically, collecting a huge number of the bugs for them. In this way, they easily obtained sufficient number of the firefly *Photinus pyralis* for their research.

The live fireflies are dried over calcium chloride in a vacuum desiccator, and then their lanterns are separated by hand. An acetone powder prepared from the dried lanterns is extracted with boiling water. The cooled aqueous extract is extracted with ethyl acetate at pH 3.0, and the ethyl acetate layer is concentrated under reduced pressure. The concentrated luciferin is adsorbed on a column of Celite-Fuller's earth mixture. The column is washed with water-saturated ethyl acetate, and eluted with alkaline water at pH 8.0–8.5. The aqueous eluate of luciferin is adjusted to pH 3.0 with HCl and luciferin is

re-extracted with ethyl acetate. Luciferin is further purified by partition chromatography on a column of Celite using a mixture of butanol-chloroform-water (135:15:50) as the elution solvent, monitoring the absorbance values at 327 nm and 276 nm; the former value parallels the concentration of luciferin, whereas the latter indicates the protein concentration. The purity is judged by the absorbance ratio of 327/276. For crystallization, an aqueous solution of luciferin is extracted with ethyl acetate at pH 3.0. The ethyl acetate layer is washed with water, then evaporated to dryness. The residue of free (acidic) form of luciferin is dissolved in about 3.5 ml of acetone, and 1.5 ml of water is added. The acetone is slowly evaporated by bubbling a stream of nitrogen gas until crystals are formed. From 70 g of acetone powder (about 15,000 fireflies), 9 mg of crystalline luciferin were obtained.

In 1968, Kishi *et al.* reported another method to purify luciferin from the Japanese firefly *Luciola cruciata*. In this method, the first ethyl acetate extract of Bitler and McElroy was chromatographed on a column of cellulose powder using ethyl acetate-ethanol-water (5:2:3) as the eluting solvent, followed by chromatography on a column of DEAE-cellulose (elution with a gradient of NaCl concentration). They obtained 5.5 mg of crystalline luciferin from 233 g of the acetone powder of the abdomens (from 12,000 fireflies). The lower yield compared with that by Bitler and McElroy is probably due to the species used.

Properties of luciferin. The crystals are microscopic needles, which melt with decomposition at 205–210°C (Bitler and McElroy, 1957). It is a quite stable luciferin compared with some other luciferins, such as *Cypridina* luciferin and the luciferins of krill and dinoflagellates. It is not significantly affected by 10 mM H₂SO₄ and 10 mM NaOH at room temperature in air. The absorption spectral data of luciferin are shown in Fig. 1.3 (McElroy and Seliger, 1961). The molar absorption coefficient of the 328 nm peak in acidic solutions and that of the 384 nm peak in basic solutions are both 18,200 (Morton *et al.*, 1969). Luciferin is fluorescent, showing an emission maximum at 537 nm in both acidic and basic conditions, although the intensity of the fluorescence is lower in acidic solution than in basic solution (fluorescence quantum yields: 0.62 in basic condition, and 0.25 in acidic condition; Morton *et al.*, 1969). The chemical synthesis

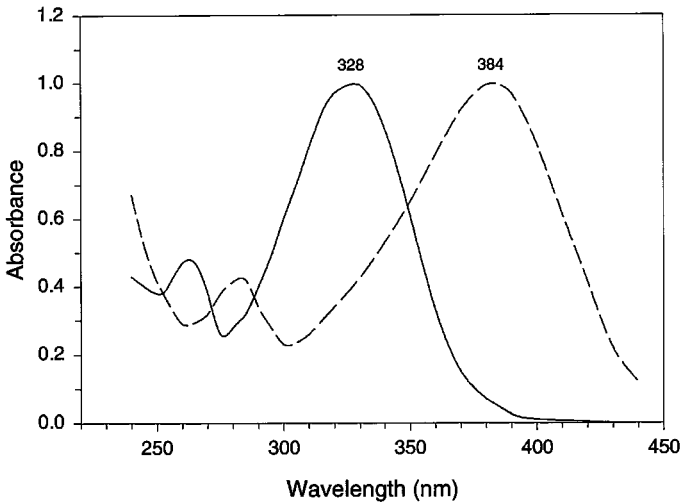


Fig. 1.3 Absorption spectra of firefly luciferin at pH 7.0 or below (solid line, λ_{\max} 327–328 nm) and at pH higher than 9.0 (dashed line, λ_{\max} 381–384 nm). Reproduced from McElroy and Seliger, 1961, with permission from the Johns Hopkins University Press.

of luciferin was accomplished by White *et al.* (1961; 1963); certain details and the improvements of the synthetic method are discussed by Bowie (1978) and Branchini (2000).

Oxyluciferin. Firefly oxyluciferin is an extremely unstable compound; it has never been isolated in a completely pure form (White and Roswell, 1991). A group in Nagoya synthesized the compound and its properties were investigated (Suzuki *et al.*, 1969; Suzuki and Goto, 1971). The fluorescence of oxyluciferin in DMSO in vacuum in the presence of potassium *t*-butoxide is yellow-green (λ_{\max} 557 nm), the same emission maximum as the chemiluminescence of luciferin in DMSO in the presence of potassium *t*-butoxide, suggesting that oxyluciferin is the light emitter in the chemiluminescence of luciferin. In the bioluminescence reaction, the absorption peak of synthetic oxyluciferin at pH 7 (382 nm; Suzuki *et al.*, 1969) closely coincides with the absorption peak of the luciferase-oxyluciferin complex in the spent luminescence solution (Fig. 1.4; Gates and DeLuca, 1975), suggesting that oxyluciferin is the light-emitter as in the case of chemiluminescence. However, the fluorescence emission maximum of the spent

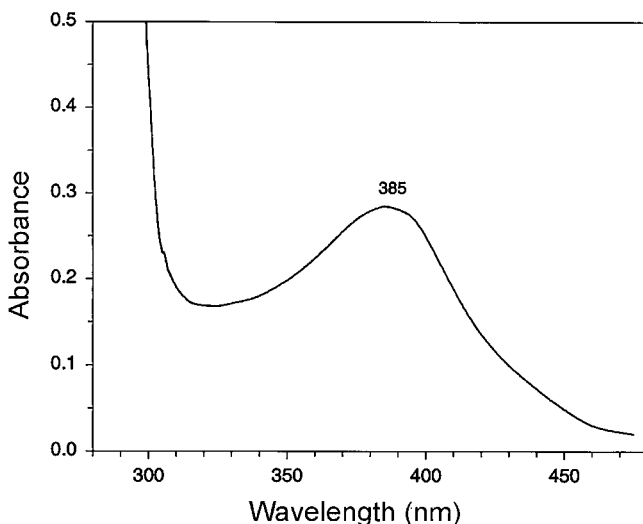


Fig. 1.4 Absorption spectrum of a spent luminescence solution of firefly luciferin containing luciferase-oxyluciferin after dialysis in 0.1 M potassium phosphate, pH 7.8. Replotted from the data of Gates and DeLuca, 1975, with permission from Elsevier.

luminescence solution is found at 523 nm, clearly different from the bioluminescence maximum at 562 nm (Fig. 1.5). Gates and DeLuca attributed this difference to the environmental change of the oxyluciferin molecule that is caused by the conformational change of luciferase after the emission of light.

1.1.3 Firefly Luciferase

Firefly luciferase has been purified, crystallized and partially characterized by Green and McElroy (1956). The acetone powder prepared from the dried lanterns of *Photinus pyralis* is extracted with water containing 1 mM EDTA, at pH 7.8. The luciferase extracted is purified by calcium phosphate gel adsorption, ammonium sulfate fractionation, and then crystallized by dialysis against a low ionic strength buffer. Although the ammonium sulfate precipitated fractions are stable in the frozen state, the crystalline luciferase is inactivated by freezing and thawing. For the storage of luciferase, the crystals are dissolved and precipitated with 2.4 M $(\text{NH}_4)_2\text{SO}_4$ in the presence of 1 mM EDTA

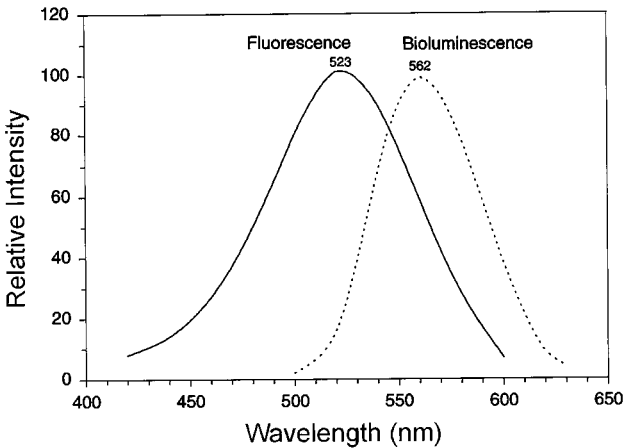


Fig. 1.5 Fluorescence emission spectrum of the luciferase-oxyluciferin complex in the same solution as in Fig. 1.4 (solid line), compared with the luminescence spectrum of firefly luciferin measured in glycyglycine buffer, pH 7.6 (dotted line). The former curve from Gates and DeLuca, 1975; the latter from Selinger and McElroy, 1960, both with permission from Elsevier.

at a pH between 7.5 and 8.0, then stored at 4°C. For use, the suspension is centrifuged, and the precipitate is dissolved in 1 mM EDTA at pH 7.9. This solution is stable for days at 4°C at a protein concentration of about 10 mg per ml. The molecular weight of luciferase was estimated at 100,000 (however, see next page), the isoelectric point is found at pH 6.2–6.3, and the absorbance of a 1 mg/ml solution at 278 nm is 0.75. A purification method of luciferase involving high-performance liquid chromatography (HPLC) was reported by Branchini and Rollins (1989).

Using the American firefly *Photinus pyralis*, the cloning of luciferase was achieved first by *in vitro* translation of RNA by Wood *et al.* (1984), followed by the expression of the cDNA in *Escherichia coli* by De Wet *et al.* (1985, 1986). The purification and cDNA cloning of firefly luciferase are also reported for several other species of fireflies: Japanese fireflies *Luciola cruciata* (Tatsumi *et al.*, 1989), *Luciola lateralis* (Tatsumi *et al.*, 1992) and *Pyrocoelia miyako* and *Hotaria parvula* (Ohmiya *et al.*, 1995); European firefly *Luciola mingrellica* (Devine *et al.*, 1993); and American firefly *Photuris pennsylvania* (Ye *et al.*, 1997; Leach *et al.*, 1997).

The role of the sulfhydryl groups of luciferase in the firefly bioluminescence reaction has been a target of intensive investigation since the early 1960s. DeLuca *et al.* (1964) found that luciferase contains 6–8 sulfhydryl groups per 100 kDa of protein. The catalytic activity of luciferase is completely lost by treatment with *p*-mercuribenzoate. However, in the presence of excess amounts of luciferin, Mg^{2+} and ATP for forming the luciferin-luciferase-ATP complex, two sulfhydryl groups are protected from *p*-mercuribenzoate and the catalytic activity is maintained, implying the necessity of these sulfhydryl groups for the activity. Despite these results, Alter and DeLuca (1986) concluded that the sulfhydryl groups of luciferase are not essential for the activity of luciferase. They also reported that the treatment of luciferase with methyl methanethiosulfonate produces an enzyme that causes the emission of red light, differing from the native luciferase that results in yellow-green light. It seems possible that the modified enzyme contains a distorted active site. Later, Ohmiya and Tsuji (1997) confirmed that the sulfhydryl groups are nonessential based on the results of the replacement of the cysteine residues by the site-directed mutagenesis.

The apparent molecular weights of both natural *P. pyralis* luciferase and an active luciferase obtained from *P. pyralis* by the *in vitro* RNA translation were 62,000 by SDS-PAGE (Wood *et al.*, 1984), in contrast to the value of 100,000 that had been widely referred to in the field for almost 30 years. Luciferases from other species of firefly probably have similar molecular weights. Presently, the molecular masses of firefly luciferases are considered to be 60–62 kDa.

Conti *et al.* (1996) solved the crystal structure of the *P. pyralis* luciferase at 2.0 Å resolution. The protein is folded into two compact domains, a large N-terminal portion and a small C-terminal portion. The former portion consists of a β -barrel and two β -sheets. The sheets are flanked by α -helices to form an $\alpha\beta\alpha\beta\alpha$ five-layered structure. The C-terminal portion of the molecule forms a distinct domain, which is separated from the N-terminal domain by a wide cleft. It is suggested that the two domains will close up in the course of the luminescence reaction.

1.1.4 Assays of Luciferase Activity, ATP and Luciferin

Various assay mixtures of different compositions have been used to measure the activity of luciferase and the amount of ATP (Leach, 1981). A typical mixture for luciferase assay contains 10–25 mM Tris-HCl or glycylglycine buffer, pH 7.5–7.8, 5 mM MgCl₂, 1–5 mM ATP, and 0.1 mM luciferin. Because luciferase at very low concentrations is rapidly inactivated, 0.5–1 mM EDTA and 0.1% BSA are included in some formulae to prevent the inactivation. Usually ATP is injected into the rest of the mixture to start luminescence, producing a sharp flash of light that diminishes rapidly (DeLuca and McElroy, 1978). The peak of the flash occurs about 0.3–0.5 second after the injection of ATP (Fig. 1.6), and the light intensity of the peak is proportional to the amount of luciferase in a wide range of luciferase concentration. If the measurement of flash height is difficult to carry out for some reasons (such as a slow response of recorder), the light intensity at 5 or 10 seconds after the ATP injection is measured instead of the flash height. Although the measured light intensity in this case

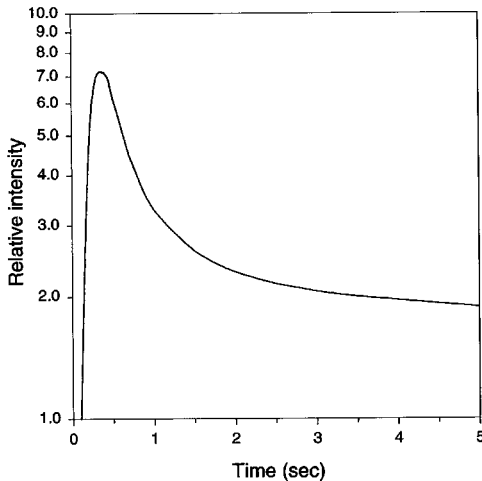


Fig. 1.6 The time course of luminescence reaction initiated by the injection of ATP. The light intensity first rises rapidly, reaching a maximum in 0.3–0.5 sec, followed by relatively rapid decrease for the first few seconds and then a much slower decay that lasts for several minutes or more. From McElroy and Seliger, 1961, with permission from the Johns Hopkins University Press.

is lower than the intensity of a flash, it is still proportional to the amount of luciferase as long as the same method and the same conditions are used. Luciferin and ATP can be assayed with appropriate modifications of the method.

1.1.5 General Characteristics of the Bioluminescence of Fireflies

The color of the luminescence of common fireflies varies slightly depending on the species, with their *in vivo* emission peaks in a range from 552 nm to 582 nm (Seliger and McElroy, 1964). The color of the *in vitro* luminescence using purified luciferin and *Photinus pyralis* luciferase (plus ATP and Mg^{2+}) under neutral or slightly alkaline conditions is yellow-green (λ_{max} 560 nm; Fig. 1.7), with a quantum yield of 0.88 ± 0.25 (Seliger and McElroy, 1959; 1960); in spite of the large error range, the quantum yield is clearly greater than those of *Cypridina* luciferin and coelenterazine (both about 0.3). The quantum yield and the color of luminescence are affected by the pH of the reaction medium (Fig. 1.8). Under acidic conditions, red luminescence (λ_{max} 615 nm) with a decreased light intensity is emitted. A similar red shift of luminescence is also observed by raising the reaction

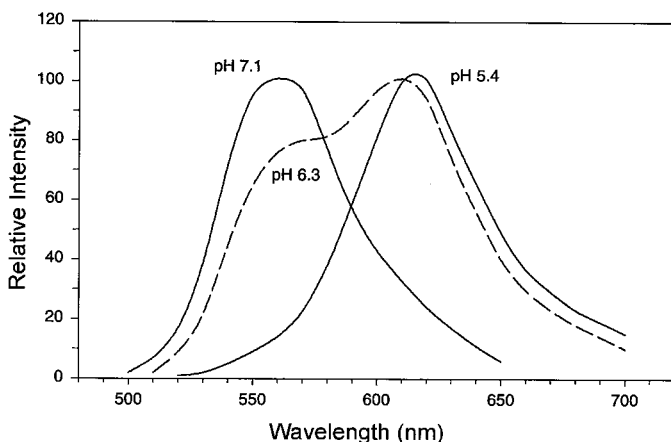


Fig. 1.7 Spectral change of the *in vitro* firefly bioluminescence by pH, with *Photinus pyralis* luciferase in glycylglycine buffer. The normally yellow-green luminescence (λ_{max} 560 nm) is changed into red (λ_{max} 615 nm) in acidic medium, accompanied by a reduction in the quantum yield. From McElroy and Seliger, 1961, with permission from Elsevier.

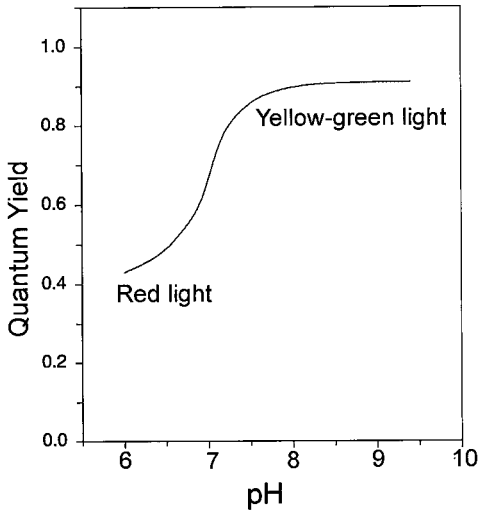


Fig. 1.8 Quantum yield of firefly bioluminescence as a function of pH. From McElroy and Seliger, 1961, with permission from Elsevier.

temperature, by carrying out the reaction in a glycylglycine buffer (pH 7.6) containing 0.2 M urea, and by adding a small concentration of Zn^{2+} , Cd^{2+} , or Hg^{2+} (Seliger and McElroy, 1964).

The optimum pH for the luminescence reaction is about 7.8, and the luminescence intensity is strongly affected by the buffer salt used

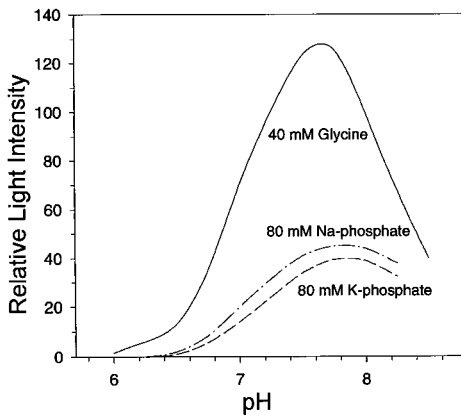


Fig. 1.9 Effects of pH and buffer on the activity of luciferase measured at the same luciferase concentration. The optimum pH with glycine buffer is approximately 7.8. From Green and McElroy, 1956, with permission from Elsevier.

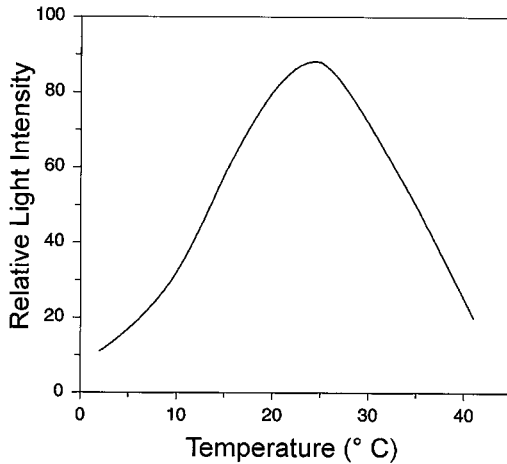


Fig. 1.10 Effect of temperature on the activity of luciferase. From McElroy and Seliger, 1961, with permission from Elsevier.

(Fig. 1.9; Green and McElroy, 1956). The optimum temperature for luminescence is 23–25°C (Fig. 1.10; McElroy and Strehler, 1949; Green and McElroy, 1956). In the presence of a low concentration of ATP, high concentrations of Mg^{2+} are inhibitory, while in the presence of a low concentration of Mg^{2+} , high concentrations of ATP are inhibitory (Fig. 1.11; Green and McElroy, 1956).

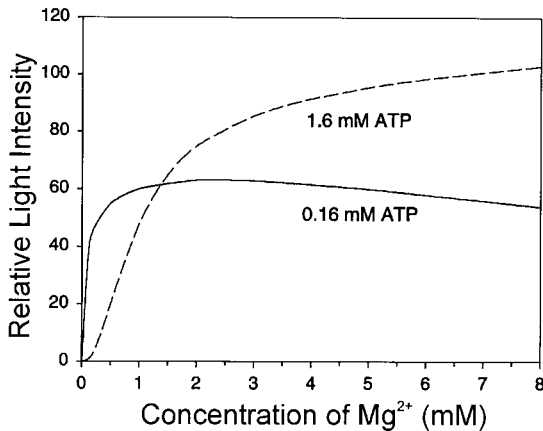
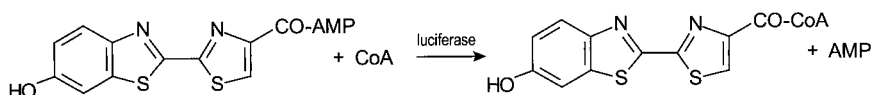


Fig. 1.11 Effect of Mg^{2+} concentration on the activity of luciferase in the presence of 0.16 mM and 1.6 mM ATP. From Green and McElroy, 1956, with permission from Elsevier.

The sharp flash in the firefly bioluminescence reaction (Fig. 1.6) is due to the formation of a strongly inhibitory byproduct in the reaction. The inhibitor formed is dehydroluciferyl adenylate, having the structure shown below at left. In the presence of coenzyme A (CoA), however, this inhibitory adenylate is converted into dehydroluciferyl-CoA, a compound only weakly inhibitory to luminescence. Thus, an addition of CoA in the reaction medium results in a long-lasting, high level of luminescence (Airth *et al.*, 1958; McElroy and Seliger, 1966; Ford *et al.*, 1995; Fontes *et al.*, 1997, 1998).



1.1.6 Mechanisms of the Firefly Bioluminescence

Seliger and McElroy (1962) discovered that the esters of firefly luciferin emit chemiluminescence. They reported that luciferyl adenylate (Rhodes and McElroy, 1958) emitted a red light (λ_{\max} 625.5 nm) in dimethyl sulfoxide upon the addition of a base. The emission spectrum was dependent upon pH, producing a yellow-green light in the presence of a large excess of base. The observation of yellow-green light was also reported later by other authors (White *et al.*, 1969, 1971; however, see Section 1.1.7). The product of the luminescent oxidation of luciferin is oxyluciferin (the structure shown in Fig. 1.12), an extremely unstable compound. Hopkins *et al.* (1967) found that 5,5-dimethyloxyluciferin, an oxyluciferin analogue having no H atoms at position 5, shows a red fluorescence in the presence of a base, coinciding with the red chemiluminescence spectrum of luciferyl adenylate. Considering these findings, the bioluminescence reaction of firefly was postulated as shown in Fig. 1.12 (Hopkins *et al.*, 1967; McCapra *et al.*, 1968; White *et al.*, 1969, 1971, 1975; Shimomura *et al.*, 1977; Koo *et al.*, 1978).

In the postulated bioluminescence mechanism, firefly luciferin is adenylated in the presence of luciferase, ATP and Mg^{2+} . Luciferyl adenylate in the active site of luciferase is quickly oxygenated at its tertiary carbon (position 4), forming a hydroperoxide intermediate (A).

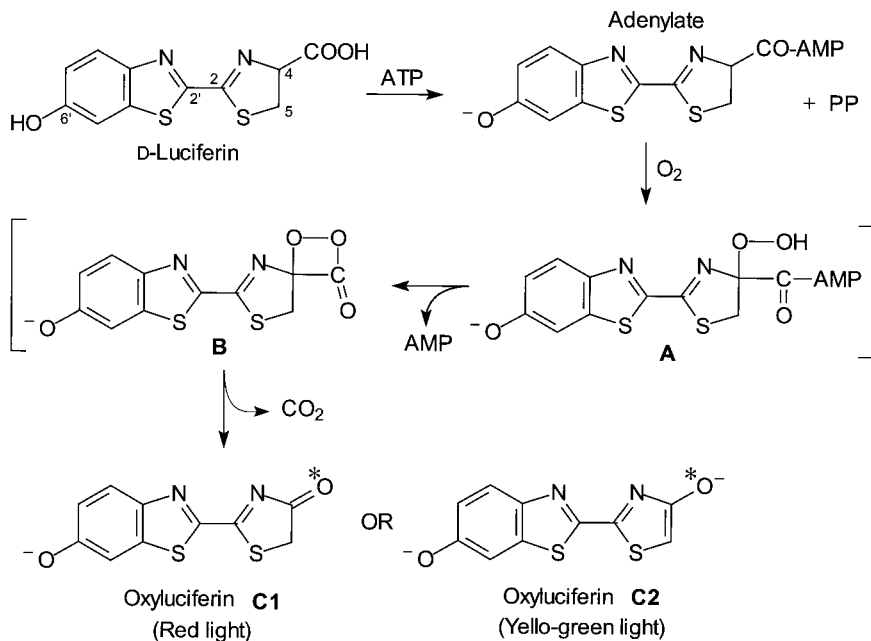


Fig. 1.12 Mechanism of the bioluminescence reaction of firefly luciferin catalyzed by firefly luciferase. Luciferin is probably in the dianion form when bound to luciferase. Luciferase-bound luciferin is converted into an adenylate in the presence of ATP and Mg^{2+} , splitting off pyrophosphate (PP). The adenylate is oxygenated in the presence of oxygen (air) forming a peroxide intermediate **A**, which forms a dioxetanone intermediate **B** by splitting off AMP. The decomposition of intermediate **B** produces the excited state of oxyluciferin monoanion (**C1**) or dianion (**C2**). When the energy levels of the excited states fall to the ground states, **C1** and **C2** emit red light (λ_{max} 615 nm) and yellow-green light (λ_{max} 560 nm), respectively.

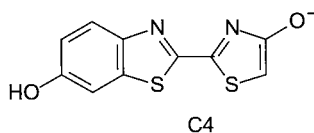
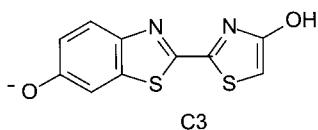
The hydroperoxide forms a very unstable 4-membered dioxetanone ring (**B**), splitting off AMP. The dioxetanone decomposes by a concerted cleavage, yielding the keto-form oxyluciferin (**C1**) and CO_2 , accompanied by emission of light. It is possible that the decomposition of dioxetanone results in light emission by the chemically initiated electron-exchange luminescence (CIEEL) mechanism (McCapra, 1977; Koo *et al.*, 1978). The formation of the dioxetanone intermediate was confirmed by ^{18}O -labeling experiments, by showing that one of the O atoms of the product CO_2 was derived from molecular oxygen (Shimomura *et al.*, 1977; also see Section 1.1.8).

Because luciferyl adenylate emitted a red chemiluminescence in the presence of base, coinciding with the red fluorescence of 5,5-dimethyloxyluciferin, the keto-form monoanion **C1** in its excited state is considered to be the emitter of the red light. Thus, the emitter of the yellow-green light is probably the enol-form dianion **C2** in its excited state, provided that the enolization takes place within the life-time of the excited state. Although the evidence had not been conclusive, especially on the chemical structures of the light emitters that emit two different colors, the mechanism shown in Fig. 1.12 was widely believed and cited until about 1990.

1.1.7 Light Emitters in the Firefly Luminescence System

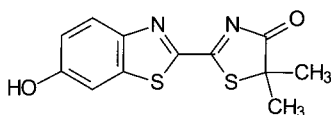
Bioluminescence of firefly luciferin can produce a wide range of colors when catalyzed by different luciferases obtained from various species of fireflies, with their emission maxima ranging from 535 nm (yellow-green) to 638 nm (red). Apparently, each spectrum is emitted from a single emitting species; they are not the composites of the yellow-green peak and the red peak (Seliger and McElroy, 1964).

White and Roswell (1991) investigated the fluorescence emission properties of the 5-methyl and *O*-methyl derivatives of oxyluciferin, and concluded that the structures **C3** and **C4** (shown below) should also be the candidates for the emitter of the yellow-green light, in addition to **C2**. Moreover, they have stated that the chemiluminescence of the esters of firefly luciferin produces only red light and does not produce yellow-green light, even in the presence of a high concentration of strong base. They wrote: "It is not clear how the earlier work produced contrary observation." Thus, it became difficult to understand why the chemiluminescence of luciferyl adenylate emits only red light, differing from the luciferase-catalyzed reaction that normally produces yellow-green light.



McCapra *et al.* (1994) and McCapra (1997) suggested that the color variation could be caused by the conformational difference of the oxyluciferin molecule, when the plane of thiazolinone is rotated at various angles against the plane of benzothiazole on the axis of the 2-2' bond; the red light would be emitted at 90° angle, reflecting its minimum structural energy.

However, Branchini *et al.* (2002) reported a surprising discovery that the adenylate of D-5,5-dimethyluciferin emits light in two different colors in the bioluminescence reaction catalyzed by two different luciferases, one from *Photinus pyralis* and the other from a green-emitting click beetle *Pyrophorus plagiophthalmus*. In the presence of Mg²⁺ and at pH 8.6, a yellow-green light (λ_{\max} 560 nm) was produced with *P. pyralis* luciferase and a red light (λ_{\max} 624 nm) was emitted with *P. plagiophthalmus* luciferase. In both cases, the reaction product was 5,5-dimethyloxyluciferin (shown below) that has no H atom on its C5; it cannot take the tautomeric enolized form, such as in C2, C3 or C4, that had been proposed to be the emitter of yellow-green light.

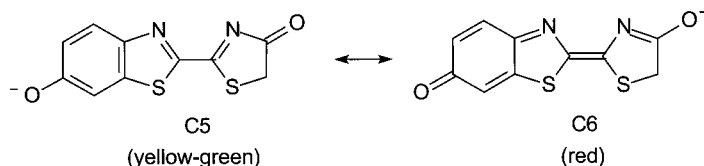


This finding by Branchini *et al.* (2002) clearly indicates that 5,5-dimethyloxyluciferin is able to emit the two different colors. This conclusion, however, does not rule out the involvement of the enolized oxyluciferin in the bioluminescence reaction of firefly.

Orlova *et al.* (2003) theoretically studied the mechanism of the firefly bioluminescence reaction on the basis of the hybrid density functional theory. According to their conclusion, changes in the color of light emission by rotating the two rings on the 2-2' axis is unlikely, whereas the participation of the enol-forms of oxyluciferin in bioluminescence is plausible but not essential to explain the multicolor emission. They predicted that the color of the bioluminescence depends on the polarization of the oxyluciferin molecule (at its OH and O⁻ termini) in the microenvironment of the luciferase active site; the

smaller the H–O polarization, the greater the blue shift of the absorption (and excitation). By this mechanism, the range of colors observed in the bioluminescence could be obtained with various forms of oxyluciferin. The most likely light emitter is keto-*s-trans* monoanion, but the enol-*s-trans* monoanion and keto-*s-cis* monoanion structures may also be involved. Their conclusion is in agreement with that of Branchini *et al.* (2002), in that the involvement of keto-enol tautomerism is not essential to explain the two different colors.

According to Branchini *et al.* (2004), luciferase modulates the emission color by controlling the resonance-based charge delocalization of the anionic keto-form of oxyluciferin in the excited state. They proposed the structure C5 as the yellow-green light emitter, and the structure C6 as the red light emitter.



It should be pointed out that the structure C5 (yellow-green emitter) is identical to the structure C1 that was previously assigned to the red light emitter.

1.1.8 A Note on the Dioxetanone Pathway and the ¹⁸O-incorporation Experiment

In the luminescence reaction of firefly luciferin (Fig. 1.12), one oxygen atom of the product CO₂ is derived from the molecular oxygen while the other originates from the carboxyl group of luciferin. In the chemiluminescence reaction of an analogue of firefly luciferin in DMSO in the presence of a base, the analysis of the product CO₂ has supported the dioxetanone pathway (White *et al.*, 1975).

Contrary to the dioxetanone pathway, DeLuca and Dempsey (1970) proposed a mechanism of the bioluminescence reaction that involves a multiple linear bond cleavage of luciferin peroxide

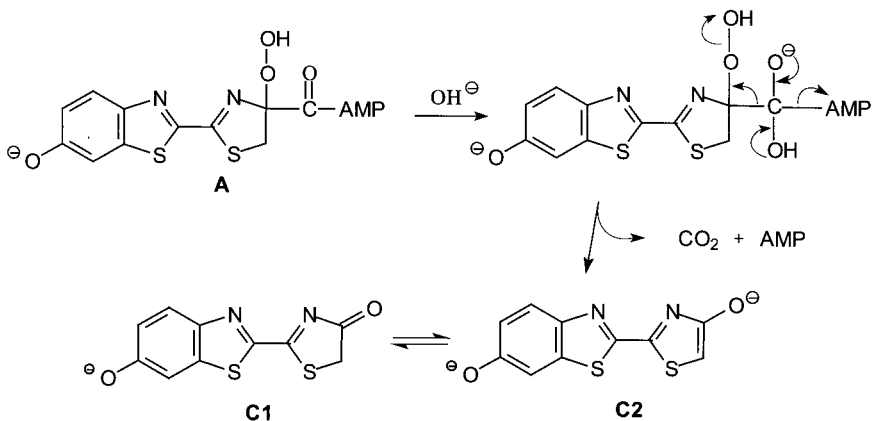


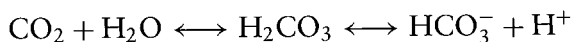
Fig. 1.13 A mechanism of the decomposition of luciferin-4-peroxide in the firefly bioluminescence reaction proposed by DeLuca and Dempsey (1970), which involves a multiple linear bond cleavage.

(Fig. 1.13). They carried out the luminescence reaction in H_2^{18}O solvent under $^{16}\text{O}_2$ atmosphere, and also in H_2^{16}O solvent under $^{18}\text{O}_2$ atmosphere, and analyzed the product CO_2 by mass spectrometry. Under the former condition, they found that one atom of ^{18}O was incorporated into the CO_2 , whereas under the latter condition, no ^{18}O was found in the CO_2 . Thus, they concluded that one O atom of the product CO_2 came from the solvent water, as the basis of their linear bond cleavage hypothesis. Although organic chemists are reluctant to accept this linear mechanism (e.g. White *et al.*, 1971), three follow-up papers involving four laboratories have confirmed and verified the DeLuca and Dempsey 1970 report, supporting the linear bond cleavage hypothesis (DeLuca and Dempsey, 1973; DeLuca *et al.*, 1976; Tsuji *et al.*, 1977). Moreover, the same mechanism has also been applied to the bioluminescence and chemiluminescence of two kinds of imidazopyrazinone-type luciferins, *Renilla* luciferin (coelenterazine) and *Cypridina* luciferin (DeLuca *et al.*, 1971, 1976; Tsuji *et al.*, 1977), despite the fact that the bioluminescence reaction of *Cypridina* had previously been shown to involve the dioxetanone mechanism (Shimomura and Johnson, 1971).

However, the linear bond cleavage hypothesis of the firefly bioluminescence was made invalid in 1977. It was clearly shown by Shimomura *et al.* (1977) that one O atom of the CO₂ produced is derived from molecular oxygen, not from the solvent water, using the same ¹⁸O-labeling technique as used by DeLuca and Dempsey. The result was verified by Wannlund *et al.* (1978). Thus it was confirmed that the firefly bioluminescence reaction involves the dioxetanone pathway. Incidentally, there is currently no known bioluminescence system that involves a splitting of CO₂ by the linear bond cleavage mechanism.

It seems important to identify the factors that have led DeLuca, Dempsey, and others into a misjudgment. The following explanation is included here for future experimentalists (see also Section C6 in Appendix).

When gaseous CO₂ is equilibrated with aqueous buffer solution in a closed vessel, a large portion of the CO₂ is dissolved in the aqueous phase, mostly in the form of bicarbonate, maintaining the equilibrium of the following three phases:



Thus, the O atom of CO₂ is exchangeable with the O atom of H₂O. When the luminescence reaction is carried out in a H₂¹⁸O medium under an atmosphere of ¹⁶O₂, the C¹⁶O₂ formed by the dioxetanone mechanism is spontaneously converted into C¹⁶O¹⁸O. If the reaction is carried out in a H₂¹⁶O medium under an atmosphere of ¹⁸O₂, the C¹⁶O¹⁸O formed is spontaneously converted into C¹⁶O₂. Thus, the result of ¹⁸O-incorporation experiment can be obscured by the exchange of O atom between CO₂ and H₂O. In addition to this exchange, the presence of contaminating CO₂ can also obscure the result. The occurrence of CO₂ is ubiquitous and clean air normally contains approximately 0.03% (v/v) of CO₂. In our experiments, carefully prepared fresh buffer solutions contained 0.02–0.03 μmol/ml of CO₂ plus HCO₃⁻ even after vacuum degassing, and the amount was much greater when luciferase had been included (Shimomura *et al.*, 1977). Thus, the CO₂ produced from a small amount of luciferin (for example, 0.033 μmol in 3.5 ml: DeLuca and Dempsey, 1970) will be

obscured by the contaminating CO_2 even without considering the effect of the O atom exchange.

In the ^{18}O -incorporation experiment of *Cypridina* bioluminescence, the effects of the O atom exchange and contaminating CO_2 are clearly seen in the relationship between the amount of luciferin luminesced and the amount of ^{18}O atoms incorporated into the product CO_2 (Fig. 1.14; Shimomura and Johnson, 1973a). The experiments were done in glycylglycine buffer, pH 7.8, the same buffer as chosen by DeLuca and Dempsey (1970). The total volume of the reaction mixture was 4 ml, with 40 ml of gas phase (see the reaction vessel in Fig. A.5 in the Appendix). The data of the luminescence reaction with $^{18}\text{O}_2$ gas in the H_2^{16}O medium indicates that at least 1 μmol of

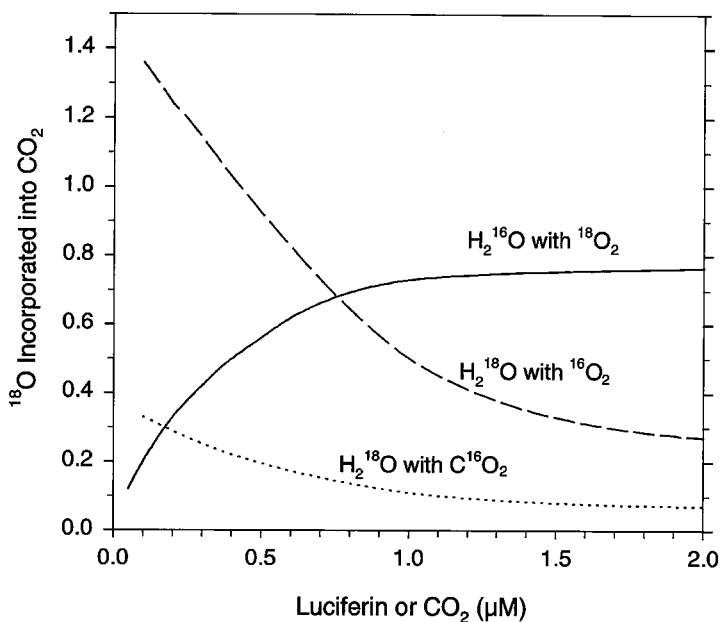
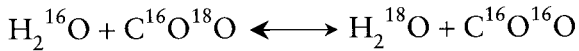


Fig. 1.14 Relationship between the incorporation of ^{18}O into product CO_2 and the amount of luciferin used, in the bioluminescence reaction of *Cypridina* luciferin catalyzed by *Cypridina* luciferase. The reactions were carried out in H_2^{16}O medium with $^{18}\text{O}_2$ gas (solid line); in H_2^{18}O medium with $^{16}\text{O}_2$ gas (dashed line); and the control experiment of the latter using C^{16}O_2 gas instead of luciferin and luciferase (dotted line), all in 20 mM glycylglycine buffer, pH 7.8, containing 40 mM NaCl. From Shimomura and Johnson, 1973a. Reproduced with permission from Elsevier.

luciferin has to be used to obtain a highly reliable conclusion (the theoretical maximum number of ^{18}O atoms to be incorporated is 0.85–0.90; see Section 3.1.8). In the luminescence reaction with $^{16}\text{O}_2$ gas in a H_2^{18}O medium, the effect of oxygen atom exchange is even greater; the number of ^{18}O atoms incorporated is about 0.5 when 1 μmol of luciferin is used, and the value reaches 1.0 when 0.4 μmol of luciferin is used (theoretically, there should be no incorporation of ^{18}O in the dioxetanone mechanism). The ^{18}O -incorporation was much less in the control experiment that contained C^{16}O_2 instead of luciferin. These results would be reasonable when the following matters are considered: (1) the O atom exchange occurs only in the solution, not in the gas phase; and (2) in the following equilibrium reaction, the rate going to the left is twice that going to the right (Cohn and Urey, 1938).



Based on the data in Fig. 1.14, it is apparent that the O atom exchange and contaminating CO_2 can easily give misleading result and erroneous conclusion when a small amount of luciferin is used. The level of oxygen atom exchange is also affected by the pH and type of buffer used (Shimomura and Johnson, 1975a). The glycylglycine buffer is less suitable than Tris or phosphate buffer, due to the rapid increase in the amount of dissolved CO_2 plus HCO_3^- (Shimomura *et al.*, 1977). See further details in Appendix C6.

1.2 Phengodidae and Elateroidae

The bioluminescence systems of Phengodidae (railroad worms) and Elateroidae (click beetles) are basically identical to that of Lampyridae (fireflies), requiring firefly luciferin, ATP, Mg^{2+} and a luciferase for light emission. However, there seem to be some differences. Viviani and Bechara (1995) reported that the spectra of the luminescence reactions measured with the luciferases of Brazilian fireflies (6 species) shift from the yellow-green range to the red range with lowering of the pH of the medium, like in the case of the *Photinus pyralis* luciferase (see Section 1.1.5), whereas the spectra

measured with the luciferases of Elateroidae (5 species) and Phengodidae (3 species) showed no change with lowering of the pH of the medium.

1.2.1 *Phengodidae*

The railroad worm *Phrixothrix* is well known for displaying two different colors of luminescence from a single organism. This genus is widely distributed in Central and South America. The larva of *Phrixothrix* (and also the adult female) emits a greenish yellow light (λ_{\max} 535–565 nm) from 11 pairs of luminous organs on the posterior lateral margins of the second to the ninth segment, and a red light (λ_{\max} 600–620 nm) from the luminous area on the head (Viviani and Bechara, 1993). The adult male is a typical beetle and does not show a noticeable luminescence.

Viviani and Bechara (1993, 1997) investigated various railroad worm species of 8 genera of phengodidae collected in southeastern and west central Brazil, near the Parque Nacional das Emas. The bioluminescence systems of these phengodids were essentially the same as that of the fireflies, involving the same luciferin (firefly luciferin), ATP and Mg^{2+} . Their emission maxima of luminescence from the lateral and head organs are in the ranges of 535–592 nm and 562–638 nm, respectively. The color differences are probably due to the presence of luciferase isoenzymes (M_r about 60,000) according to the authors.

Gruber *et al.* (1997) reported the purification and cloning of the luciferases of a *Phengodes* species. Viviani *et al.* (1999) cloned the luciferases from the lateral light organs of *Phrixothrix vivianii* (emission λ_{\max} 542 nm) and the head light organs of *Phrixothrix hirtus* (emission λ_{\max} 628 nm).

1.2.2 *Elateridae*

The elaterid *Pyrophorus* is of special importance in the history of bioluminescence, because it was used by Dubois in his first demonstration of the luciferin-luciferase reaction in 1885. The Jamaican click beetle (*Pyrophorus noctilucus*) is commonly found in the West Indies. The beetle possesses two kinds of luminous organs. A

pair of oval-shaped light organs is located on the head. These organs look like eyes, and emit very strong greenish luminescence. The second kind of light organ is ventrally located on the first abdominal segment, and it emits orange light only when flying or when the elytra are expanded. The bioluminescence system is chemically the same as those of fireflies and railroad worms.

Colepicolo-Neto *et al.* (1986) investigated the adults of 12 species of elaterids collected in Brazil. They found that the luminescence emission maxima of the abdominal light organs are shifted toward red (20–40 nm) relative to those of the head organs (λ_{\max} 525–560 nm). Thin-layer chromatography under various conditions revealed that the luciferins extracted from both types of light organs are identical to firefly luciferin.

Wood *et al.* (1989) generated 11 cDNA clones from mRNA isolated from the abdominal light organs of a Jamaican click beetle *Pyrophorus plagiophthalmus*. When expressed in *E. coli*, these clones produced four types of luciferase distinguishable by the colors of bioluminescence they produce: green (λ_{\max} 546 nm), yellow-green (λ_{\max} 560 nm), yellow (λ_{\max} 578 nm), and orange (λ_{\max} 593 nm). Molecular cloning of the luciferase of a Brazilian click beetle *Pyrearinus termitilluminans* was also reported (Viviani *et al.*, 1999a).

1.3 Diptera

The order Diptera (flies) contains the glow-worms *Arachnocampa* and *Orfelia*. The bioluminescence systems of dipterans do not utilize firefly luciferin in their light-emitting reactions, differing from the bioluminescence systems of coleopterans. In dipterans, it is extremely intriguing that the bioluminescence system of *Arachnocampa* appears different from that of *Orfelia*: the former luminescence is activated by ATP, whereas the latter luminescence is stimulated by DTT but not by ATP.

1.3.1 The Glow-worm *Arachnocampa*

The glow-worm *Arachnocampa* is distributed in New Zealand and Australia. The larvae emit blue light continuously from their light

organs located at the posterior extremity. They are found most often on the roofs of caves. The glow-worms usually stay on the horizontal network of mucous tubes suspended from rocks, and they hang down long sticky threads of “fishing lines” from the tubes to catch small insects. The spectacular view of glow-worms at the Waitomo Cave in New Zealand attracts hundreds of tourists everyday.

Earlier studies indicated that the bioluminescence emission maximum of the New Zealand glow-worm *A. luminosa* is 487–488 nm, and that the bioluminescence reaction probably requires ATP as a cofactor (Shimomura *et al.*, 1966), similar to the firefly luminescence reaction. According to Lee (1976), the luminescence emission spectrum of the Australian glow-worm *A. richardsae* (λ_{\max} 488 nm) was not significantly influenced by pH in a wide range, i.e. 5.9–8.5, and firefly luciferin does not cross-react with the spent luminescence mixture, indicating differences from the firefly luminescence system. However, the luminescence was quenched by EDTA, but not by EGTA that does not chelate Mg^{2+} , suggesting that Mg^{2+} is probably required for the luminescence reaction, like the firefly luminescence.

The luciferin-luciferase reaction of *Arachnocampa* was first demonstrated by Wood (1993), by mixing a cold-water extract and a cooled hot-water extract. The cold-water extract was prepared with 27 mM Tricine, pH 7.4, containing 7 mM MgSO_4 , 0.2 mM EDTA, 10% glycerol and 1% Triton X-100, and incubated with 1 mM ATP on ice for 18 hr. The hot-water extract was prepared by heating the cold water extract before the addition of ATP at 98°C for 5 min. The luminescence reaction was performed in the presence of 1 mM ATP.

Extraction and purification of luciferin and luciferase (Viviani *et al.*, 2002a) To isolate luciferin, the lanterns of the Australian *A. flava* were homogenized in hot 0.1 M citrate buffer, pH 5, and the mixture was heated to 95°C for 5 min. The mixture was acidified to pH 2.5–3.0 with HCl, and luciferin was extracted with ethyl acetate. Upon thin-layer chromatography (ethanol-ethyl acetate-water, 5:3:2 or 3:5:2), the active fraction of luciferin was fluorescent in purple (emission λ_{\max} 415 nm when excited at 290 nm). To isolate the luciferase, the cold-water extract prepared according to Wood (1993; see above) was chromatographed on a column of Sephacryl S-300. On the same

column, the molecular mass of luciferase was estimated at approximately 36 kDa.

Luminescence reaction (Viviani *et al.*, 2002a) The luciferin-luciferase luminescence reaction was carried out in 0.1 M Tris-HCl, pH 8.0, containing 2 mM ATP and 4 mM Mg^{2+} . Mixing luciferase with luciferin and ATP resulted in an emission of light with rapid onset and a kinetically complex decay. Further additions of fresh luciferase, after the luminescence has decayed to about 10% of its maximum value, resulted in additional luminescence responses similar to the initial one (Fig. 1.15). According to the authors, the repetitive light emission occurred in consequence of the inhibition of luciferase by a reaction product, as seen in the case of the firefly system (McElroy *et al.*, 1953). The luminescence spectrum showed a peak at 487 nm (Fig. 1.16).

1.3.2 The American Glow-worm *Orfelia*

The American glow-worm *O. fultoni* is found in the Appalachian Mountains. The larvae of *Orfelia* live on damp stream banks, and they

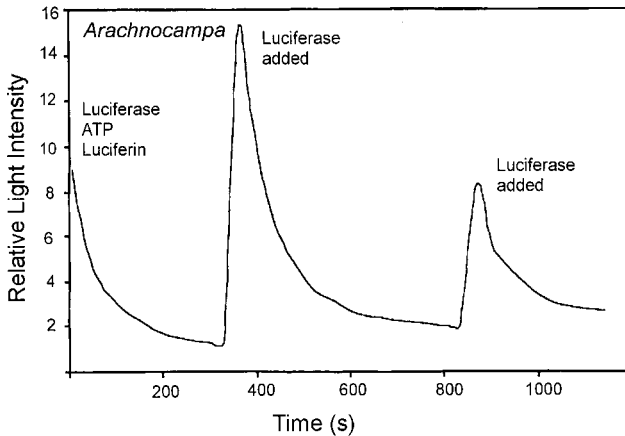


Fig. 1.15 Effect of successive additions of 10 μ l of *Arachnocampa* luciferase (cold-water extract) to the assay mixture (90 μ l) containing 2 mM ATP, 4 mM $MgSO_4$ and 5 μ l of luciferin solution (hot-water extract made with 10 mM DTT). From Viviani *et al.*, 2002a, with permission from the American Society for Photobiology.

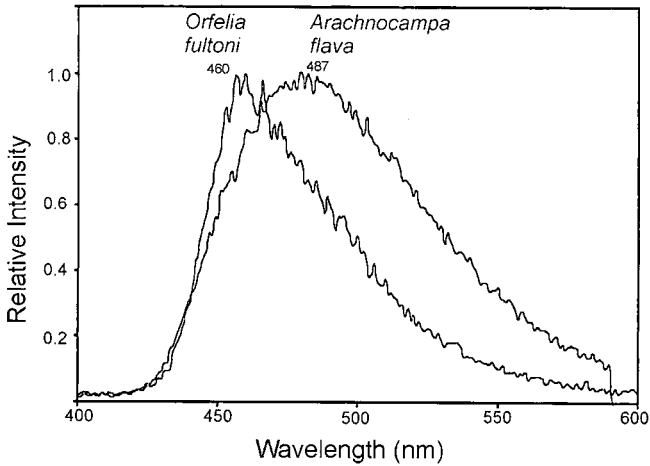


Fig. 1.16 The *in vitro* bioluminescence spectra of *O. fultoni* and *A. flava*. From Viviani *et al.*, 2002a, with permission from the American Society for Photobiology.

are luminous just like *Arachnocampa*. Viviani *et al.* (2002a) demonstrated a luciferin-luciferase reaction with a cold-water extract and a hot-water extract. The cold-water extract was prepared with 0.1 M phosphate, pH 7.0, containing 1 mM EDTA and 1% Triton X-100.

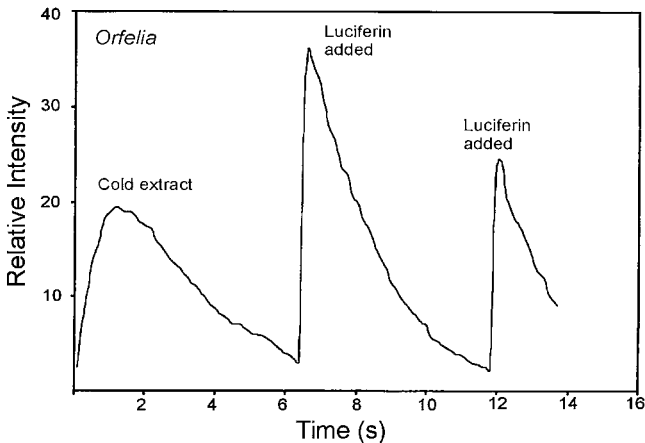


Fig. 1.17 Effect of successive additions of 10 μ l of *Orfelia* luciferin solution (hot-water extract made with 10 mM DTT) to the assay mixture (90 μ l) containing cold-water extract and 10 mM DTT after the luminescence has decayed. From Viviani *et al.*, 2002a, with permission from the American Society for Photobiology.

The hot-water extract was prepared by heating the cold-water extract at 95°C for 5–10 min in the presence of 10 mM DTT under argon gas. The luminescence reaction was performed in 0.1 M Tris-HCl, pH 8.0. The reaction was strongly stimulated by DTT and ascorbic acid, but not by ATP, indicating that the *Orfelia* luminescence system is different from the luminescence systems of the fireflies and *Arachnocampa* that require ATP for light emission. After the luminescence of a cold-water extract in the pH 8.0 buffer containing DTT had decayed to about 10% of the peak intensity, an addition of a hot-water extract caused an immediate increase in light emission (Fig. 1.17), suggesting that the decay of luminescence is caused by the depletion of luciferin. The molecular mass of the luciferase was estimated at about 140 kDa by gel filtration. The luminescence of *O. fultoni* is the bluest of all luminous insects (λ_{\max} 460 nm; Fig. 1.16).