CHEMISTRY OF BIOLUMINESCENCE

TOSHIO GOTO

Department of Agricultural Chemistry, Nagoya University, Nagoya, Japan

THE STRUCTURE OF THE LUCIFERINS

With their fascinating phenomena we recognize many bioluminescent organisms, such as firefly, luminous bacteria, luminous fungi, and luminous deep-sea fish. Some eighty years ago, Dubois found that the bioluminescence of the luminescent beetle *Pyrophorus* is due to the luciferin–luciferase reaction. Bioluminescence[1–9] was observed when a hot aqueous extract ("luciferin") of the insect was mixed with a cold aqueous extract ("luciferase") after the initial luminescence had disappeared. This observation led to numerous studies on the luciferin–luciferase reaction[9, 10] in many bioluminescent organisms (*Table 1*). It is now known that hot water extracts luciferin and destroys luciferase; the cold-water extract contains luciferase, and also luciferin at first, but the latter changes into an inactive oxidation product on standing in air.

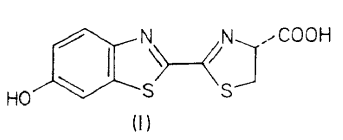
Table 1. Luciferin–luciferase reaction in bioluminescent organisms

Luminous organisms	*Observation of luciferin–luciferase reaction*
Pyrophorus (elaterid beetle)	1885, Dubois
Pholas dactylus (clam)	1887, Dubois
Photinus (American firefly)	1916, Harvey
Luciola (Japanese firefly)	1917, Harvey
Cypridina (ostracod crustacea)	1917, Harvey
Pyrocypris	1921, Harvey
Odontosyllis (marine fireworm)	1931, Harvey
Systellaspis (shrimp)	1931, Harvey
Latia (fresh-water limpet)	1950, Bowden
Achromobacter & *Photobacterium* (luminous bacteria)	1953, Strehler 1953, McElroy
Heterocarpus (shrimp)	1955, Haneda
Gonyaulax (dinoflagellate)	1957, Hastings and Sweeney
Apogon & *Parapriacanthus* (fish)	1958, Haneda and Johnson
Renilla (sea pansy)	1959, Comier
Collybia, *Armillaria* & *Omphalia* (fungi)	1959, Airth and McElroy
Balanoglossus (marine euteropneust)	1963, Dure and Comier
Octochaetus multiporus (earthworm)	1966, Johnson, Shimomura and Haneda
Hoplophorus (shrimp)	1966, Johnson, Stachel, Shimomura and Haneda

The luciferin–luciferase reaction is the emission of light as a result of the enzyme (a luciferase)-catalysed oxidation (generally with oxygen) of a substrate (a luciferin). Thus, "luciferin" is the name given to a substance

having special properties, and is not the name of one compound or of a class of compounds. For example, luciferin from the American firefly *Photinus* is completely different from luciferin from the marine crustacean *Cypridina*. The luciferase of these organisms are also different. It is noteworthy, however, that luminous fish, *Apogon* and *Parapriacanthus*, produce luciferin identical with that of crustacean *Cypridina*[11].

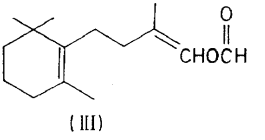
At present there are only three luciferins whose structures have been elucidated; namely firefly luciferin (I)[12], *Cypridina* luciferin (II)[13], and very recently *Latia* luciferin (III)[14] (*Figure 1*). No relationship is apparent from the structures of these luciferins.



(I)

Firefly Luciferin

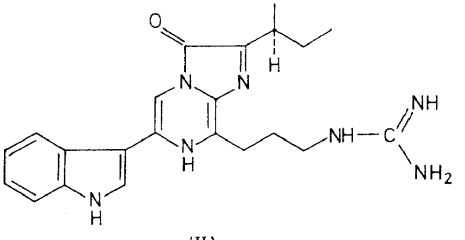
(White, McCapra & Field, 1961)



(III)

Latia Luciferin

(Shimomura & Johnson, 1968)



(II)

Cypridina Luciferin

(Kishi, Goto, Hirata, Shimomura & Johnson, 1966)

Figure 1. Luciferins whose structures have been established

Extensive studies on the bioluminescence mechanisms have been carried out only in the case of firefly[15–18] and luminous bacteria[15, 16]. In the latter case, however, the reaction is complex and it is not certain which substance is to be regarded as the bacterial luciferin. The mechanism of firefly luminescence has been studied extensively by McElroy and his coworkers. The luminescence of firefly requires the presence of luciferin, luciferase, magnesium ions, ATP and oxygen. As can be seen from the *Figure 2*, after luminescence ceased, the reaction product remains bound to the enzyme, and hence the reaction stops even if the luciferin is still present. Because of this product inhibition, the reaction leading to the emission of light depends on the quantity of luciferase. Thus, the kinetics of this reaction are very complex and difficult to analyze. Contrary to the firefly bioluminescence, the kinetics of *Cypridina* bioluminescence are very simple, and Harvey observed[1] as long ago as 1919 that the *in vitro* luminescence of *Cypridina* is a first-order reaction. *Cypridina* bioluminescence system is, therefore, regarded as most suitable for study of bioluminescence mechanisms.

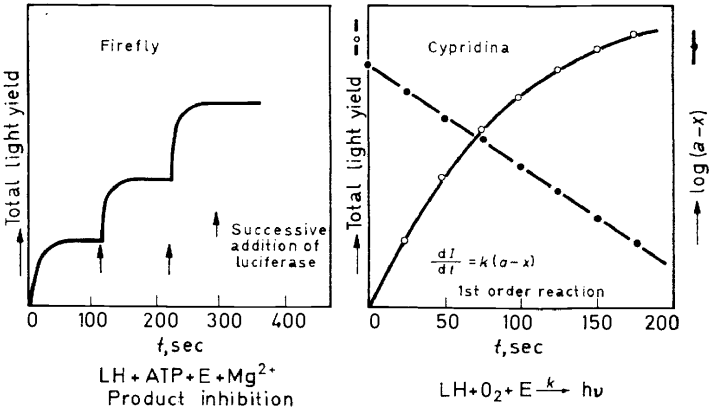
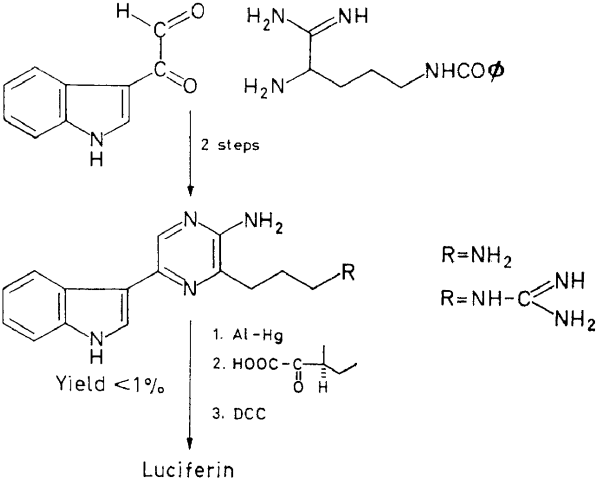


Figure 2. Bioluminescence of firefly and *Cypridina*

Crustacean *Cypridina hilgendorfii* is abundant along the coast of Japan. The organism is about 3 mm long, and emits a strongly luminescent secretion into sea water. The luminescent activity is preserved almost indefinitely in completely dry *Cypridina*, and the luminescence is restored by moisture. We isolated *Cypridina* luciferin in a crystalline state in 1957[19] and elucidated its structure as indicated in *Figure 3* in 1966[13, 20]. As is apparent from this structure, luciferin is composed of tryptamine, arginine and L-isoleucine moieties, and indeed in suitable conditions it gives isoleucine and arginine on hydrolysis[20], and a hydrogenation product of luciferin shows a typical indole absorption[13]. The synthetic route was designed in consideration of this biogenesis[21]. Thus, condensation of 3-indolylglyoxal with the α-amino-amidine gives the 2-aminopyrazine derivative. The direction of condensa-



(Kishi, Goto, Inoue, Sugiura and Kishimoto; 1966)

Figure 3. Synthesis of luciferin (II)

tion was confirmed by comparison of n.m.r. and u.v. spectra with other synthetic analogues having unambiguous structures. Etioluciferamine ($R = NH_2$) thus obtained is converted into etioluciferin [$R = —NHC(= NH)NH_2$] by condensation with *S*-methylisothiourea. This compound is one of the bioluminescent oxidation products of luciferin, thus indicating the destruction of the imidazolone ring during the luminescent reaction. Reduction with aluminium amalgam of the pyrazine ring and condensation with α-keto-β-methylvaleric acid (obtained from L-isoleucine) in the presence of DCC afforded luciferin (*Figure 3*). The yield of this last step is very low and this step makes the application of this route to the synthesis of other analogues difficult[40]. The natural and the synthetic luciferins show the same luciferin–luciferase reaction.

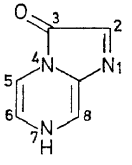
CHEMILUMINESCENCE OF *CYPRIDINA* LUCIFERIN AND RELATED COMPOUNDS

Johnson and coworkers[22] found that *Cypridina* luciferin gives light without enzyme when dissolved in dimethyl sulphoxide, but the quantum yield of this chemiluminescence is extremely low. We found that when diethylene glycol dimethyl ether (diglyme) containing acetate buffer (pH 5·6) is used as a solvent, the light yield of the chemiluminescence is improved nearly 100 times compared with that in DMSO; it being over 10 per cent of the quantum yield of *Cypridina* bioluminescence[23] (*Table 2*). This high chemiluminescence efficiency, and the mild condition used, suggest that both chemi- and bio-luminescence involve similar mechanisms. Thus study of the mechanism of the chemiluminescence should contribute to the elucidation of the bioluminescence mechanism. General discussions of the mechanisms of chemiluminescence have appeared in the literature[24–26].

Table 2. Chemiluminescence of luciferin (II)

Solvent	*Relative quantum yield* (Bioluminescence = 100)
DMSO	< 0·2 (Johnson *et al.* 1965)
DGM	0·05
DGM + H_2O	1·2
DGM + KOH	3·0
DGM + Ac (pH 5·6)	12 1st order reaction on luciferin

Since as mentioned before the imidazolone ring is destroyed during the luminescence of *Cypridina* luciferin the structure essential for the luminescent reaction is considered to be the 3,7-dihydroimidazo[1,2-a]pyrazin-3-one nucleus, and hence we planned to synthesize this unsubstituted compound to see whether it chemiluminesces or not. While our synthesis was in



3, 7-Dihydroimidazo[1,2-a] pyrazin-3-one

progress, McCapra and Chang[27] reported the synthesis of a dimethyl phenyl derivative (V) of the compound (IV) by the route shown in *Figure 4*, and the examination of its chemiluminescence activity in DMSO in the presence of t-butoxide. They isolated the acetamidopyrazine derivative as the main reaction product, and assumed the anion of this compound to be the light emitter by comparison of the luminescence and fluorescence spectra of these compounds. This synthetic method is, however, difficult to apply to the synthesis of the unsubstituted compound (IV).

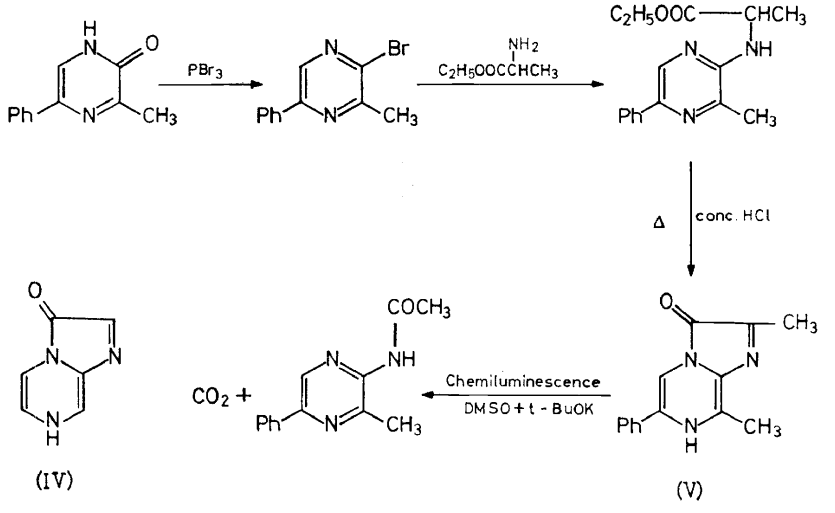


Figure 4. Synthesis of a dimethylphenyl derivative of 3,7-dihydroimidazo[1,2-a]pyrazin-3-one

Our synthesis[23] started with 2-aminopyrazine (*Figure 5*), which was condensed with formaldehyde and sodium cyanide to give 2-pyrazylaminoacetamide (VIII) and 3-aminoimidazo[1,2-a]pyrazine (VII). In this reaction, the intermediate nitrile (VI) could not be isolated. Treatment of the amide (VIII) with sodium methoxide in methanol gave the desired compound (IV). Alternatively, hydrolysis of the amide (VIII) with conc. hydrochloric acid afforded in low yield the corresponding acid (IX) as its hydrochloride, which was converted without purification into its methyl ester (X) by heating with methanol. Reaction of the ester with sodium methoxide in methanol, or of the acid with DCC in ethanol, afforded the same compound (IV). Its 2-methyl-(XI) and 2-methyl-6-phenyl-(XII) derivatives were also synthesized by essentially the same method. As expected, these three compounds emit light in aprotic solvents, such as diglyme DMSO, tetrahydrofuran in the presence of small amounts of base. Methylation or acetylation of the NH group in the 2-methyl derivative causes complete loss of luminescence activity. The following data on chemiluminescence were obtained using the 2-methyl derivative. (a) A first-order (pseudo) dependence of light production on the amount of the methyl derivative (XI) is observed using a constant pressure of oxygen (*Figure 6*).

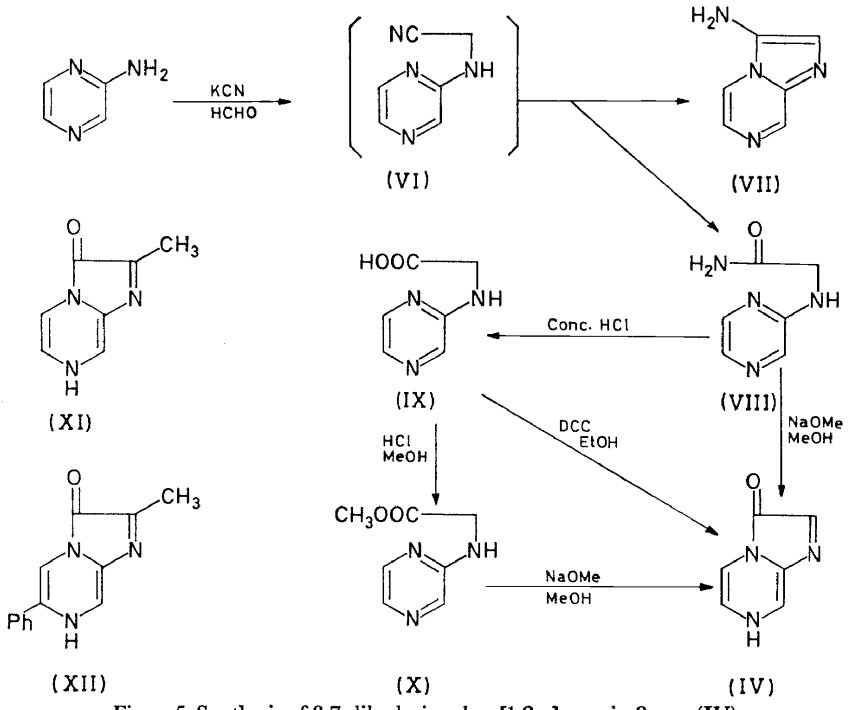
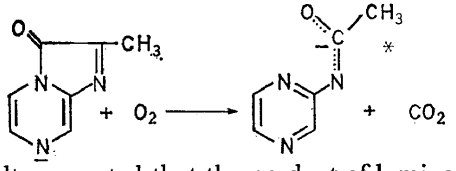


Figure 5. Synthesis of 3,7-dihydroimadazo[1,2-a]pyrazin-3-one (IV)
(T. Goto, S. Inoue, S. Sugiura, 1968)

(b) The total light yield is directly proportional to the amount of the substrate, whereas the rate constant is independent of the concentration (*Figure 7*). (c) The luminescence rate is directly proportional to the oxygen pressure, and *one mole* of oxygen is consumed during the reaction (*Figure 8*). (d) The main product of this luminescence reaction is 2-acetamidopyrazine, whose fluorescence spectrum in diglyme containing potassium t-butoxide is in agreement with the luminescence spectrum (*Figure 9*). From these results the reaction is represented by the following equation:



The above results suggested that the product of luminescence of *Cypridina* luciferin is the acylaminopyrazine derivative (XIII). We had proposed[13] structure (XIIIa) for oxyluciferin, which is considered as a primary product of *Cypridina* bioluminescence, without synthetic proof. Reinvestigation of the structure by physical methods strongly suggested that structure XIII is indeed correct[28]. Since oxyluciferin is hydrolyzed easily with the enzyme solution, the structural change of luciferin in the presence of luciferase can be represented as in the *Figure 10*. In order to ascertain whether oxyluciferin has structure XIII, its synthesis was carried out. Attempted synthesis of

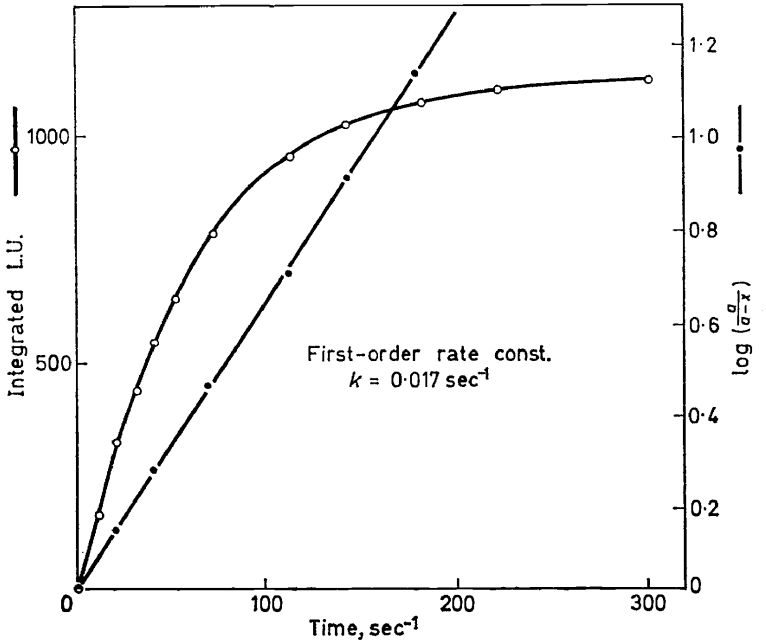


Figure 6. A pseudo first-order dependence of light production on the amount of the methyl derivative (XI) (the experiment was carried out with 5 μg of XI in 5 μl n-BuOH + 2 ml DGM + 10 μl 0·8 M t-BuOK in t-BuOH)

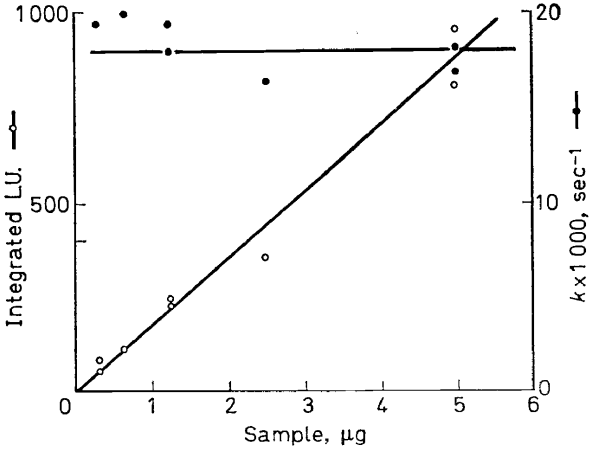


Figure 7. Variation of light yield with the amount of substrate (the experiment was carried out with a solution of the sample XI in 5 μl n-BuOH + 2 ml DGM + 10 μl 0·8 M t-BuOK in t-BuOH at 21·5°; pressure 1 atm)

oxyluciferin by means of direct acylation of etioluciferin (XIV) gave only crude products because the acylation may occur on the guanidino group.

Synthesis of oxyluciferin was achieved by the route shown in *Figure 11.* The amino group in etioluciferamine (R = NH_2) was converted with *N*-bis-

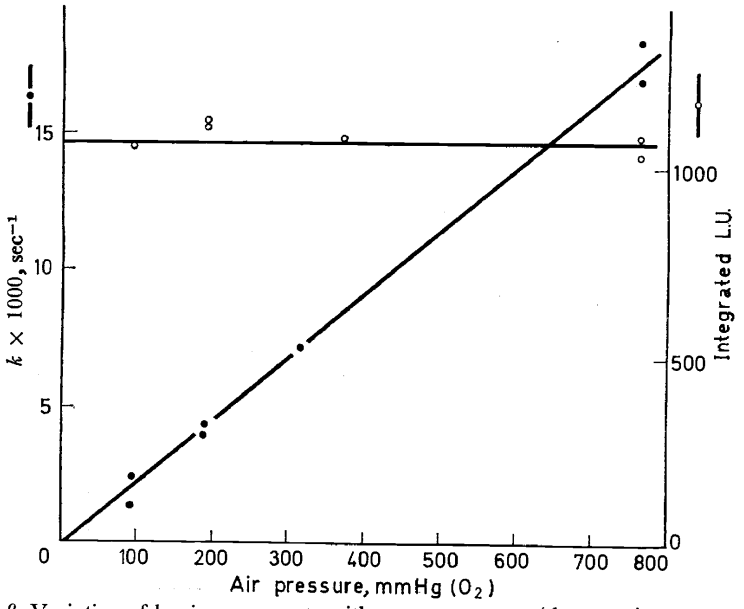


Figure 8. Variation of luminescence rate with oxygen pressure (the experiment was carried out with 5 μg of the sample XI in 5 μl n-BuOH + 2 ml DGM + 10 μl 0·8 M t-BuOK in t-BuOH; temp. 21°)

(dimethylthio)methylene-toluene-*p*-sulphonamide into the isothiourea derivative. This protecting group was later convertible into a guanidino group. This compound was acylated with α-methylbutyric acid anhydride in the presence of pyridine to give the acylamino compound, which on treatment with sodium in liquid ammonia, and of the product with ethanol–ammonia, afforded oxyluciferin identical in all respect to the natural compound.

As mentioned before, the methyl compound gives a chemiluminescence spectrum identical with the fluorescence spectrum of the acetamidopyrazine anion. The chemiluminescence spectrum of *Cypridina* luciferin in diglyme containing acetate buffer (pH 5·6) is, however, similar to the fluorescence spectrum of oxyluciferin in a neutral solvent and different from that in a basic solution, indicating that the emitting species is an un-ionized, rather than an ionized, molecule of oxyluciferin. Now we must answer the question whether the un-ionized excited molecule is produced directly during the reaction, or whether protonation occurs after the ionized-excited molecule is produced[28] (*Figure 12*). Interestingly, the 2-methyl-6-phenyl derivative under suitable conditions undergoes chemiluminescence, the spectrum suggesting that the emission comes from un-ionized and ionized 2-acetamido-5-phenylpyrazine in their excited states. In this case proton-transfer reactions are involved in the molecules in electronically excited states. Since the spent solutions of this luminescence reaction show fluorescence spectra which are the same as that of the neutral molecules of the amide, it is apparent that the anion of the acylaminopyrazine is first formed in excited states and then protonated to produce the excited neutral molecules. Deuterium isotope effects also support this mechanism. Thus, when the ionized form is produced

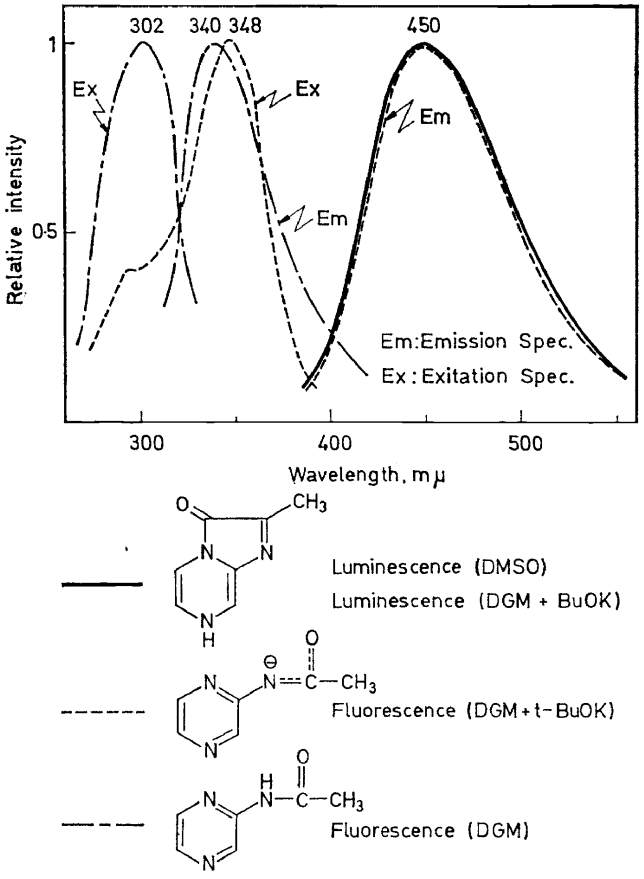


Figure 9. Luminescence spectra of (XI) and fluorescence spectra of 2-acetamidopyrazine in diglyme

first and then protonated to give the neutral molecules in excited states. The luminescence intensity corresponding to the ionized forms becomes stronger in deuterium oxide than in water, since the rates of proton-transfer reactions are usually faster in water than in deuterium oxide[29]. A similar effect to that in deuterium oxide is also observed when the concentration of water in the reaction mixture is decreased.

Now, we turn to a discussion on the structure of the precursor of the emitter. Strongly chemiluminescent substances, such as lophine and indole derivatives, give isolable hydroperoxides, which react rapidly with base to give bright chemiluminescence, indicating that the hydroperoxides are the intermediates of the chemiluminescence of the starting materials[30–34] (*Figure 13*). Comparison of the structures of the hydroperoxides and the emitters led to the assumption that the anions of the hydroperoxides decompose via four-membered cyclic peroxides[17, 18, 27, 35–38]. Interestingly, oxalic esters react with hydrogen peroxide in the presence of a fluorescent substance (fluorescer) to give chemiluminescence, with a spectrum which is

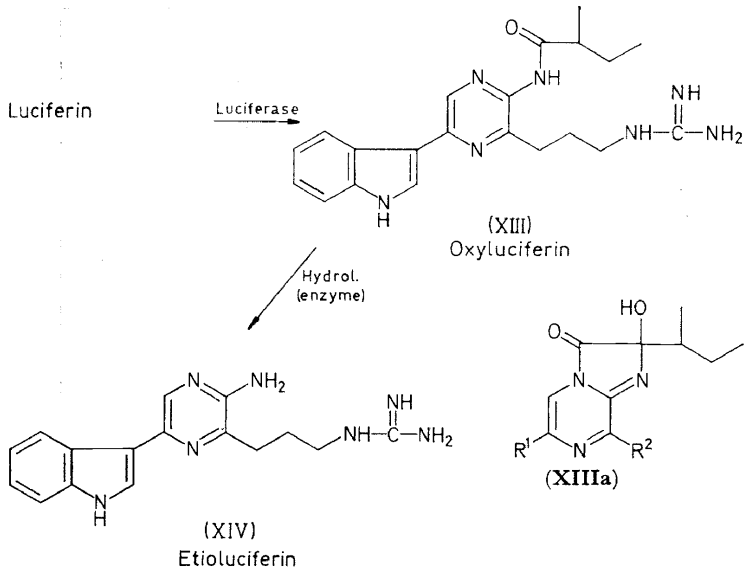


Figure 10. Structural change of luciferin (II) in the presence of the enzyme luciferase

identical with the fluorescence spectrum of the fluorescer; the energy in the excited carbon dioxide molecules is transfered to the fluorescer[37, 38]. In contrast to lophine, the indoles and their hydroperoxides, which require strongly basic conditions, oxalic esters luminesce without addition of bases. This difference may be explained in terms of the dissociation constants of the hydroperoxides; the pK_a of the former hydroperoxides are in a range of 11–12, whereas the latter compounds are peracids and hence have a pK_a between 7 and 8. Dissociation of the hydroperoxides are necessary for luminescence.

In the case of the luciferin analogues two structures are possible for their hydroperoxides, i.e. A and B (*Figure 14*). Although McCapra and Chang suggested[27] structure A as the structure of the hydroperoxides, structure B would be preferred since the substances chemiluminesce under very mild conditions; for example, in diglyme containing acetate buffer (pH 5·6), or even containing acetic acid; in the case of the 2-methyl-6-phenyl derivative, even in aqueous solution at pH 5·6 (acetate buffer) in the presence of hydrogen peroxide and ferric ions.

The next problem is how the hydroperoxides are produced from the luciferin analogues. The proposed reaction mechanisms are summarized in *Figure 15*. Luciferin is very susceptible to oxygen in aqueous solution, even in the absence of enzyme. Thus orange coloured aqueous solution of luciferin turns red in air, and then colourless owing to autoxidation[19]. A corresponding change is observed in the spectrum of the bioluminescence of luciferin in the presence of the enzyme when the concentration of luciferin is high, but the enzyme-catalyzed reaction is extremely fast. The initial red shift points to the formation of a red substance, luciferin-R, which emits light only very slowly in the presence of the enzyme, but the luminescent activity can be restored by chemical reduction with sodium borohydride. The same

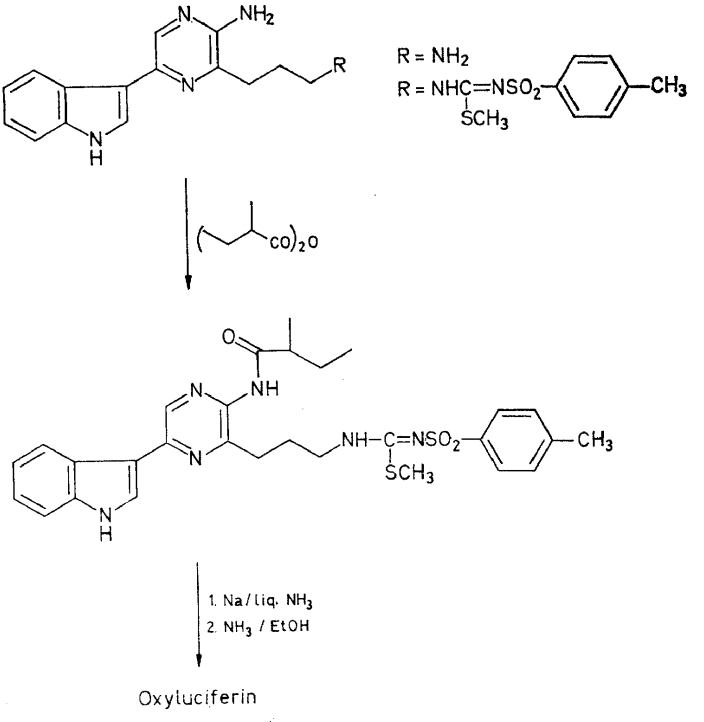
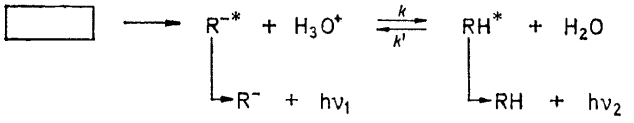
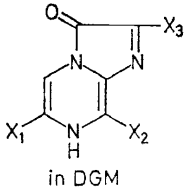


Figure 11. Synthesis of oxyluciferin (XIII)





X_1	X_2	X_3	t-BuOK	AcpH5·6
H	H	CH_3	R^{-*}	
Φ	H	CH_3	R^{-*}	$R^{-*}+RH^*$
Luciferin				RH^*

Figure 12. Structure of the emitter

red substance can be prepared by chemical oxidation using lead dioxide, DPPH radical, ceric ion, cupric ion, etc. Since only one mole of DPPH radical is necessary for this oxidation, it must be a one-electron oxidation to give a radical (L·). However, no e.s.r. signal was detected with a DMSO solution of the red substance obtained by oxidation of luciferin with lead dioxide in vacuum. When air was introduced into the solution, a strong e.s.r. signal appeared (*Figure 16*). Cupric ions oxidize luciferin catalytically in the presence of oxygen. Luciferin-R must therefore be a dimer of the

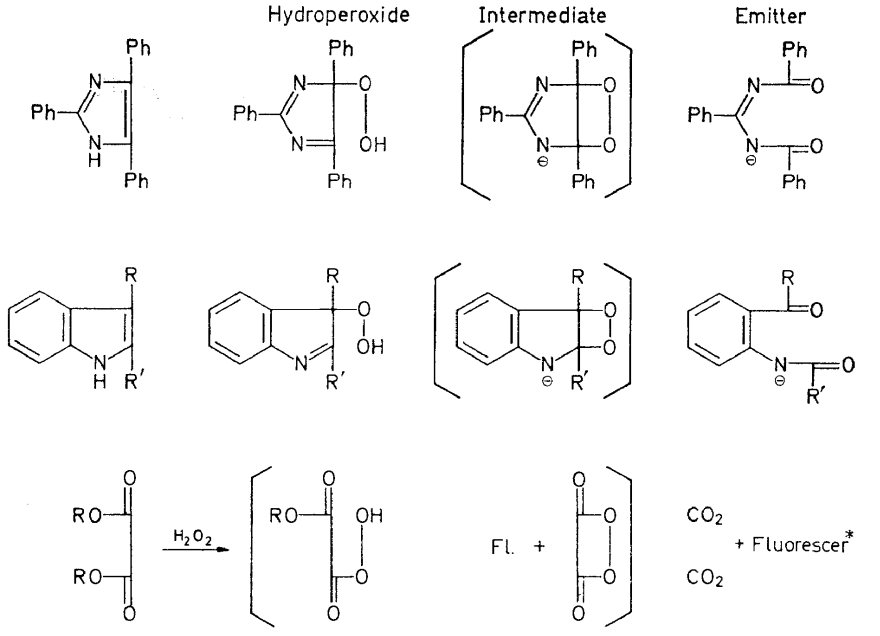


Figure 13. Comparison of the structures of the hydroperoxides and the emitters

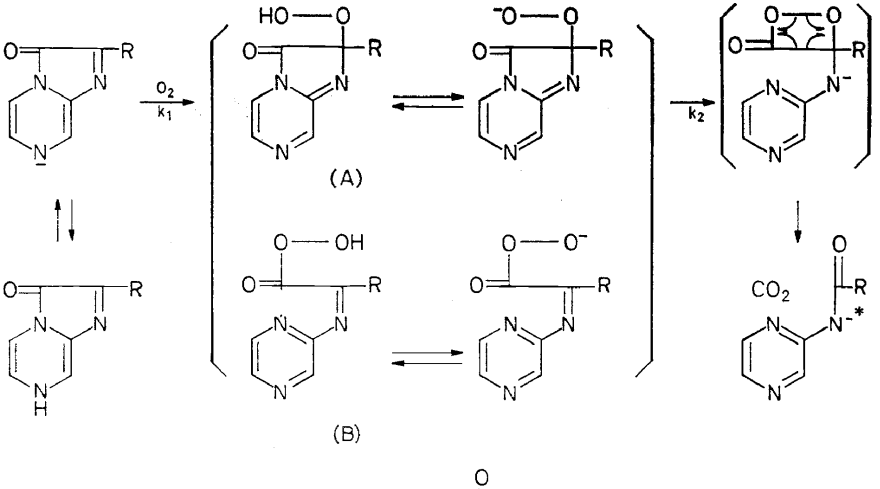


Figure 14. Structures of the hydroperoxides of luciferin analogues

initially produced radical. Ultraviolet spectra of luciferin and luciferin-R are shown in *Figure 17.* Luciferin-R also chemiluminesces in diglyme in the presence of oxygen, although the reaction is very slow. This reaction reveals a one-half order dependence on the amount of luciferin-R (*Figure 18*), suggesting that the latter is indeed a dimer. The dimer, the radical, and the

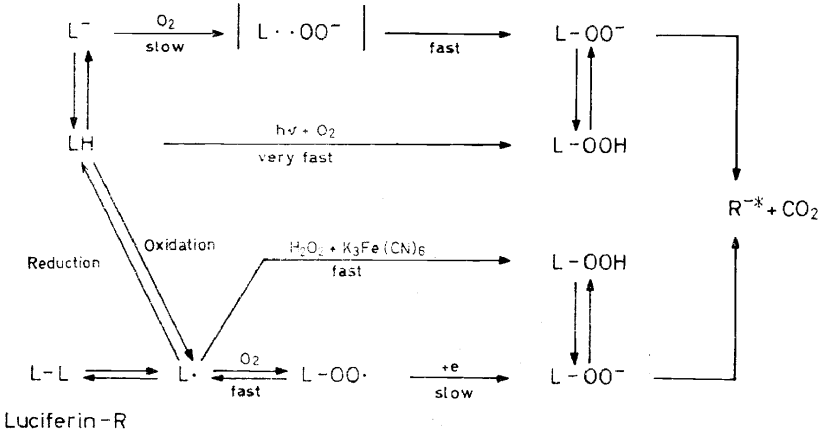


Figure 15. Chemiluminescence mechanism of luciferin (LH)

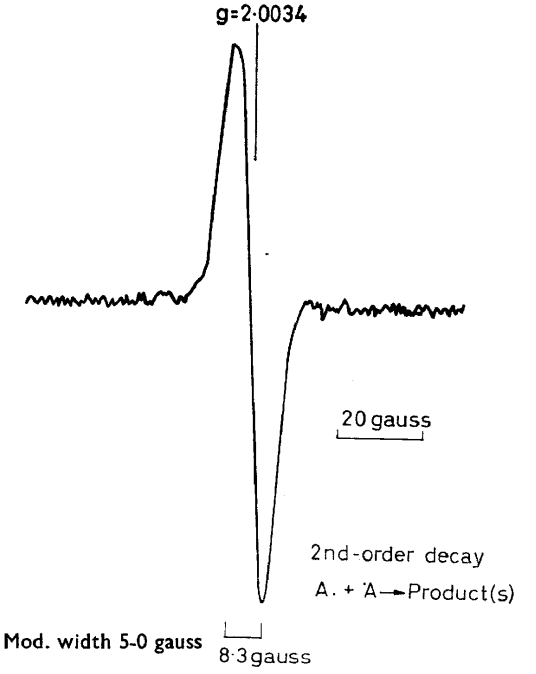


Figure 16. E.s.r. spectrum of luciferin-R in DMSO + air (O_2) at 23·5°

peroxide radical are in equilibrium, and the one-electron reduction step of the peroxide radical into hydroperoxide anion is slow and becomes a rate-determining step. That no acceleration was observed on addition of bases is also understandable from this scheme.

When hydrogen peroxide and ferricyanide are added, however, luciferin-R emits light very fast (*Figure 19*); the reaction rate shows first-order dependence on the concentration of luciferin-R, indicating that the dissociation of the

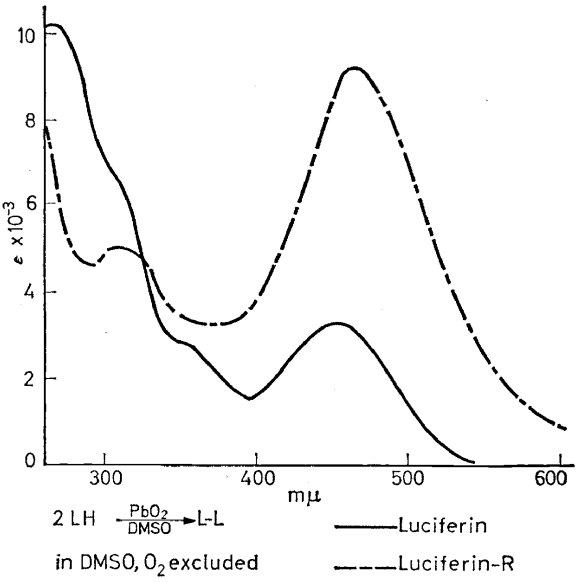


Figure 17. Ultraviolet spectra of luciferin and luciferin-R in DMSO

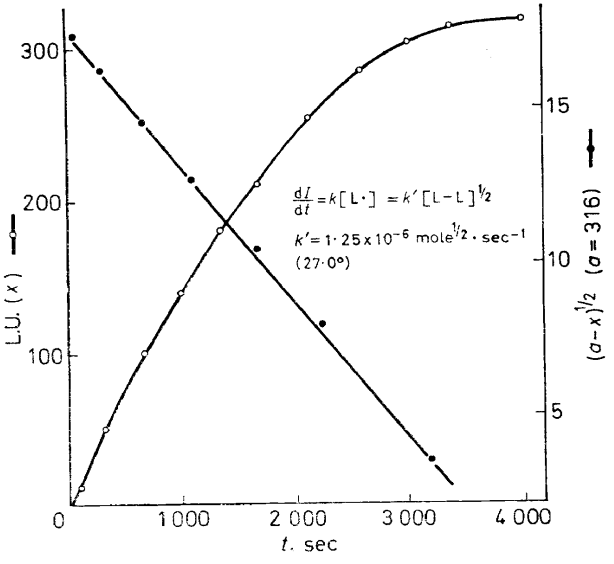


Figure 18. Chemiluminescence of luciferin-R in diglyme solution in the presence of oxygen [the experiment was carried out with a sample of XII (5×10^{-3} M) in a solution of 5 μl BuOH in the presence of DPPH (5×10^{-3} M), 5 μl; 0·6 M acetate buffer (pH 5·6); DGM, 2 ml]

dimer becomes the rate-determining step. Reaction of hydrogen peroxide and ferricyanide would give a hydroperoxy radical, which combines rapidly with a luciferin radical. This is also supported by the observation that this reaction is faster than that of luciferin itself under the same conditions.

$$H_2O_2 + Fe(CN)_6^{3-} \rightarrow HOO\cdot + Fe(CN)_6^{4-} + H^+$$

$$L{-}L + H_2O_2 + K_3Fe(CN)_6 \qquad k = 0{\cdot}017 \text{ sec}^{-1}$$

$$LH + H_2O_2 + K_3Fe(CN)_6 \qquad k = 0{\cdot}004 \text{ sec}^{-1}$$

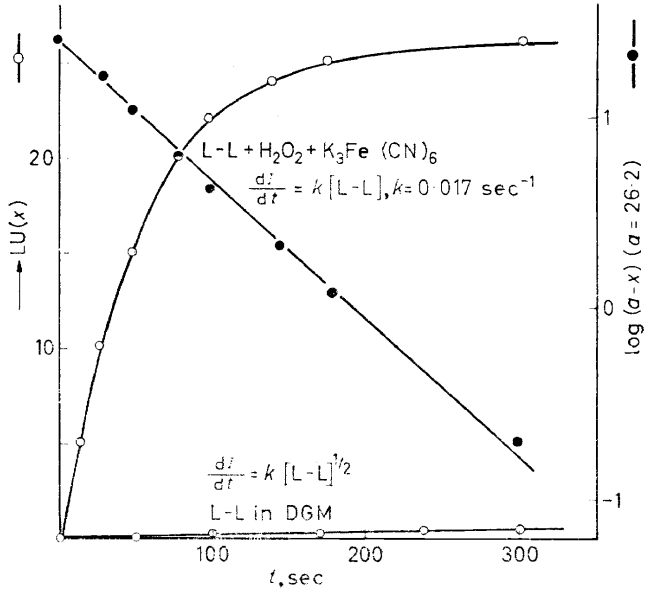


Figure 19. Effect of the addition of hydrogen peroxide and ferricyanide on the chemiluminescence of luciferin-R

L–L + H_2O_2 + $K_3Fe(CN)_6$		L–L in DGM	
L–L 0·42 mg/ml DMSO	5 µl	L–L 0·42 mg/ml DMSO	5 µl
1% $K_3Fe(CN)_6$	2·6 µl	H_2O	10 µl
3% H_2O_2	10 µl	DGM	2 ml
0·6 M Ac buffer pH 5·6	10 µl		
H_2O	30 µl		
DGM	2 ml		

Under certain conditions luciferin itself gives luminescence extremely slowly (*Figure 20*). Under these conditions, u.v. irradiation of the luminescent solution greatly accelerates the luminescence reaction rate as shown in *Figure 20*. The observed half-life period is about 10 sec. This can be explained as follows: the formation of the hydroperoxide is so fast that the subsequent step, the decomposition of hydroperoxide, becomes the rate-determining step, with a half-life of about 10 sec. The rate decomposition of the hydro-

peroxide, which depends on hydrogen ion concentration, may be represented by the equation:

$$\frac{dI}{dt} = k(\text{L—OO}^-) = k\frac{a}{1 + (1/K)[\text{H}^+]}$$

a = Total amount of hydroperoxide

K = Dissociation constant of hydroperoxide

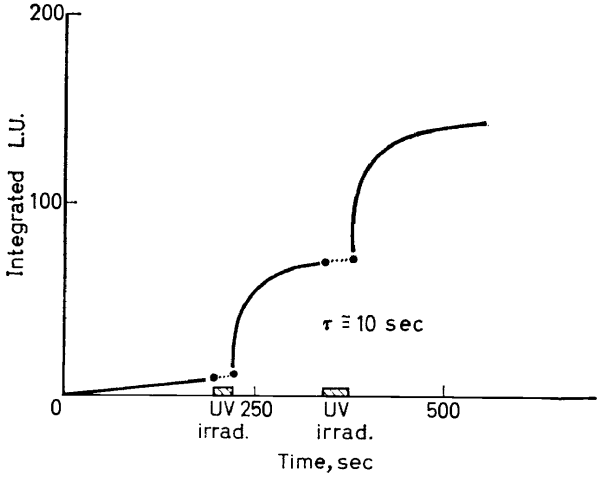


Figure 20. Effect of u.v. irradiation of the luminescent solution on the rate of luminescence reaction [the experiment was carried out with 5 μg of (II) in 5 μl n-BuOH + 2 ml ethyl orthoformate]

Taking into account these results, the mechanism of the usual chemiluminescence is assumed to be that in *Figure 15.* The pK_a of luciferin is about 8·3. Luciferin dissociates first into its anion which is then oxidized slowly with molecular oxygen to give a luciferin radical and a hydroperoxy radical anion, both of which combine rapidly to produce the hydroperoxide anion. The anion decomposes through the cyclic four-membered peroxide into an acylaminopyrazine anion in excited states as has been discussed. The activation energy of this chemiluminescent reaction is 16·9 kcal/mole. Supporting data for this mechanism are given in *Table 3.*

Table 3. Effect of addition of substances on chemiluminescence

Added substance	*Chemiluminescence*	
	Rate	*Light yield*
L–L	—	—
Et_2NCS_2Na	—	decrease
$(NH_2)_2CS$	—	decrease
Na_2 EDTA	retarded	increase
2,6-Di-t-butylphenol	—	—
Peroxide in DGM	—	—
$H_2O_2 + K_3Fe(CN)_6$	—	—

Usually oxidation of organic substances with molecular oxygen, i.e. autooxidation, proceeds by a radical-chain mechanism[39], as shown below.

$$R\cdot + O_2 \rightarrow R{-}OO\cdot$$

$$R{-}OO\cdot + RH \rightarrow R{-}OOH + R\cdot$$

If such a mechanism were operating, addition of luciferin-R must accelerate the reaction rate. However, the luminescence rate of mixtures of luciferin and luciferin-R is almost equal to that calculated by addition of each reaction rate. Addition of radical scavengers, such as di-t-butylphenol, has no effect; compounds containing sulphur also show no effect on the rate but decrease the total light yield. This effect may be explained as due to the inactivation of intermediates by the reagents; the fluorescence intensity of the spent solution of the luminescence reaction diminishes markedly when the sulphur compounds are initially added. Addition of EDTA prevents unnecessary oxidation induced by traces of metallic impurities, and thus increases the light yield and retards the apparent reaction rate. That both the peroxides contained in diglyme and the mixture of hydrogen peroxide and ferricyanide has no effect also provides evidence against chain mechanisms.

Chemiluminescence is regarded as an autooxidation reaction, but with this major difference: common autooxidations proceed by radical-chain mechanisms producing hydroperoxides which decompose further into radicals, and the reaction kinetics are usually autocatalytic; in the case of chemiluminescence, however, hydroperoxides produced decompose rapidly by ionic mechanisms, and hence the reactions are not autocatalytic.

Chemiluminescence is often referred to also as being due to the reverse reaction of photochemical reaction, since the former changes energy produced by chemical reactions into light energy, whereas in the latter the chemical reaction is induced by light. However, these chemical reactions are quite different, because in the photochemical reaction molecules in singlet or triplet excited states participate, whereas in the case of luminescence molecules participating in the reaction are not in such excited states.

BIOLUMINESCENCE OF *CYPRIDINA* LUCIFERIN AND RELATED COMPOUNDS

Let us now turn to the problem of bioluminescence. Kinetics of bioluminescence of luciferin are very similar to those of chemiluminescence. Thus, in the presence of the enzyme, luciferin gives bioluminescence, which is a first-order reaction on the substrate, whereas luciferin-R gives a one-half order kinetics; the rate of the latter reaction being nearly 1000 times slower than the former. This similarity of the kinetics between bio- and chemiluminescence strongly suggests that both reactions have the same reaction mechanism apart from the catalytic action of the enzyme (*Figure 21*).

It is apparent that the enzyme catalyzes the reaction between luciferin and molecular oxygen to produce the radical pair; this step being the rate-determining step. The specificity of the enzyme was tested by using modified luciferins as the substrates (*Table 4*). As shown in *Table 4*, change in the length of the chain between the guanidine and the imidazopyrazine ring produces a dramatic change in the rate of bioluminescence, whilst it has no

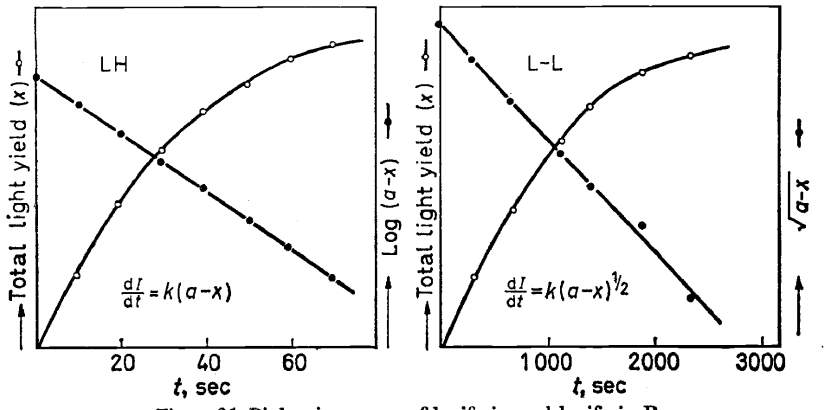
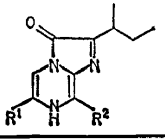


Figure 21. Bioluminescence of luciferin and luciferin-R

LH + Luciferase (× 1) in 0·1 M phosphate buffer (pH 7·0) containing 0·1 M sodium chloride

L − L + Luciferase (× 30) in 0·1 M phosphate buffer (pH 7·0) containing 0·1 M sodium chloride

Table 4. Effect of chain length between the guanidine and the imidazopyrazine ring on the rate of bioluminescence



R_1	R_2	*Relative rate of luminescence* Bioluminescence	Chemiluminescence	*Ratio of light yield* Bio./Chem.
3-Indolyl	$-(CH_2)_2NHC(=NH)NH_2$	1	100	10
3-Indolyl	$-(CH_2)_3NHC(=NH)NH_2$	100	100	10
3-Indolyl	$-(CH_2)_4NHC(=NH)NH_2$	1	100	
3-Indolyl	$-(CH_2)_5NHC(=NH)NH_2$	4	100	10
3-Indolyl	$-(CH_2)_7NHC(=NH)NH_2$	0·6	100	10
3-Indolyl	$-(CH_2)_3NH_2$	0·2	100	0·5
Phenyl	$-(CH_2)_3NHC(=NH)NH_2$	0·4	200	1
—H	$-(CH_2)_3NHC(=NH)NH_2$	0	< 0·01	
3-Indolyl	—H	0·1	30	0·5
Phenyl	—H	0	(25)	

effect on the chemiluminescence rate. Hence it is clear that the guanidino group in luciferin plays an important role in the interaction between luciferin and luciferase. It is interesting that the bioluminescence rate of the five-methylene compound is faster than that of the four-methylene compound. A zig-zag shape of the methylene side chain might be responsible for this anomally. If the guanidino group is removed, this side chain loses most of its activity. The ratio of light yield corresponds to luminescence efficiency. It is noted that in the case of luciferin analogues having indole and guanidino group, bioluminescence efficiency is about 10 times better than chemiluminescence efficiency. When the indolyl group is changed to a phenyl group, the chemiluminescence rate becomes rather faster whilst the bioluminescence rate is markedly slower, but still measurable. The

ratio of light yield becomes almost unity. Thus no enhancement of luminescence efficiency by the enzyme is observed. This indicates that the indolyl group is necessary for the high quantum yield of bioluminescence. Removal of the aromatic group at position-6 makes the chemiluminescence rate nearly 10 000 times slower, whereas the rate is retarded only a few times by removal of the aliphatic side chain at position-8.

The last, but nonetheless important, problem is that of the bioluminescence spectra. In the case of the bioluminescence of *Cypridina* luciferin the luminescence spectrum is not in agreement with the fluorescence spectrum of oxyluciferin (*Table 5*). While the fluorescence of oxyluciferin in organic solvents such as diglyme is strong, in aqueous solutions it is very weak. If we assume that the emitter of *Cypridina* bioluminescence is oxyluciferin, the fluorescence quantum yield of oxyluciferin in aqueous solutions must be high, since the

Table 5. Luminescence spectral data of luciferin and oxyluciferin

Compound		*Solvent*	λ_{max} *Relative intensity*	
Luciferin (II)	[B]	Luciferase Phosphate buffer (pH 7·0)	450 mμ	
	[C]	DGM 0·3% MeOH	428 mμ	
Oxyluciferin (XIII)	[F]	DGM + 0·3% MeOH	417 mμ	100
		DGM + 5% MeOH	439 mμ	
		MeOH	473 mμ	7
		Phosphate buffer (pH 7·0)	480 mμ	0·3

[B] Bioluminescence; [C] Chemiluminescence; [F] Fluorescence

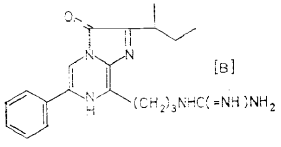
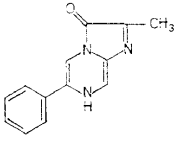
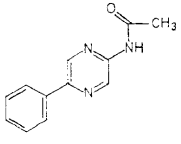
bioluminescence quantum yield is high, and the luminescence spectrum should be identical with the fluorescence spectrum of oxyluciferin. These contradictions may be explained as follows: the presence of a small amount of protic solvent such as methanol influences markedly the emission maximum as shown in *Table 5*, suggesting that in a suitable protic–aprotic environment, which may be expected to be in a cave of the enzyme, oxyluciferin can give fluorescence maximum at 450 mμ with a high fluorescence efficiency. This environment, however, could not be made by addition of a large excess of luciferase to an aqueous solution of oxyluciferin, because oxyluciferin was easily hydrolyzed to etioluciferin, whose fluorescence intensity is extremely high in aqueous solution.

It is also understandable that although luciferin gives strong chemiluminescence in aprotic solvents, in aqueous solution it gives no light, since the fluorescence quantum yield of oxyluciferin is negligible in aqueous solution. This can be verified by the observation that the phenyl analogues can chemiluminesce in aqueous solutions at pH 5·6 in the presence of hydrogen peroxide and ferric ions, since the corresponding acylaminopyrazine shows strong fluorescence in aqueous solutions as well as in aprotic solvents (*Table*

6). Chemi- and bio-luminescence spectra in this case are almost identical with the fluorescence spectra of the acylaminopyrazine.

In conclusion, the *Cypridina* bioluminescence is regarded as one of the best systems for studying not only bioluminescence mechanisms but also enzyme-substrate interactions and kinetics of enzyme reactions, since this system has an extremely sensitive visible indicator of reaction velocity, and since the substrate can be modified easily in numerous ways by synthesis. There are many outstanding investigations which have been carried out with other bioluminescent systems. However, I could not refer to these investigations in this lecture simply because lack of time obliged me to limit my subject to *Cypridina* bioluminescence.

Table 6. Luminescence spectral data of luciferin analogues

Compound	*Solvent*	λ_{max} *Relative intensity*
 [B]	Luciferase Phosphate buffer (pH 7·0)	380 mμ
 [C]	H_2O_2 + Fe^{3+} Acetate buffer (pH 5·6)	380 mμ
 [F]	Phosphate buffer (pH 7·0) DGM	380 mμ 100 365 mμ 70

[B] Bioluminescence; [C] Chemiluminescence; [F] Fluorescence

ACKNOWLEDGEMENTS

Grateful acknowledgement is made to Professor Y. Hirata for the suggestion to initiate this work and for his continued interest. I am indebted to my coworkers, Professor S. Inoue, Drs. Y. Kishi and O. Shimomura for their contributions to the experimental work in various phases. Some experiments were carried out by Professor D. A. Coviello, Miss S. Sugiura, Mr. M. Isobe, Mr. Y. Abe, and Mr. K. Nishikawa, to whom special thanks are due. I wish to thank Mr. S. Miyamoto and Mr. H. Miyamoto for collecting *Cypridina hilgendorfii*.

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[40] *Note added in proof.* Condensation of etioluciferin (XIV) with α-keto-β-methylvaleraldehyde in dilute hydrochloric acid gave (racemic) luciferin (II) in excellent yield —— S. Inoue, S. Sugiura and T. Goto, unpublished results.