Properties and Reaction Mechanism of the Bioluminescence System of the Deep-Sea Shrimp *Oplophorus gracilorostris*†

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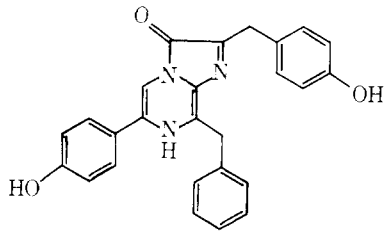
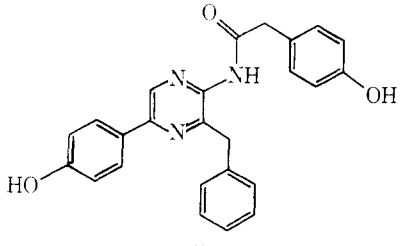
ABSTRACT: The bioluminescent reaction of *Oplophorus* takes place when the oxidation of coelenterazine (the luciferin) with molecular oxygen is catalyzed by *Oplophorus* luciferase, resulting in light of maximum intensity at 462 nm and the products CO_2 and coelenteramide. *Oplophorus* luciferase has now been obtained in a highly purified state. Optimum luminescence occurs at pH 9 in the presence of 0.05–0.1 M NaCl at 40 °C, and, due to the unusual resistance of this enzyme to heat, visible luminescence occurs at temperatures above 50 °C when the highly purified enzyme is used, or at over 70 °C when partially purified enzyme is used. The specific activity of purest preparations is 1.75×10^{15} photons s^{-1} mg^{-1} at 23 °C. At pH 8.7, native luciferase has a molecular weight of approximately 130 000, apparently comprising 4 monomers of 31 000; at lower pHs, the native luciferase tends to polymerize. The quantum yield of coelenterazine is 0.34 at 22 °C with this enzyme. After the luminescent reaction, the spent solution is nonfluorescent, and likewise solutions of luciferase alone. When the bioluminescent reaction was carried out in the presence of $^{18}O_2$, the product CO_2 contained more than 50% $C^{18}O^{16}O$, supporting the dioxetane mechanism, but without ruling out the linear peroxide mechanism.

It has been a widely held belief until several years ago that specific components in luminescent systems of different types of organisms have arisen independently in the course of evolution and are chemically unrelated (cf. Glass, 1961, p 849). Recent investigations, however, have revealed a remarkable, unanticipated similarity of the chemical structure of key compounds in a considerable number of bioluminescence systems among different types of luminous organisms. Thus, identically the same compound "coelenterazine" (I) (Shimomura & Johnson, 1975a) has been found to occur as a luciferin or as a functional group of a photoprotein, and "coelenteramide" (II) has been found as the product of the luminescent reaction of both the luciferin and photoprotein. Both I and II have been found in the hydromedusan jellyfish *Aequorea* (Shimomura et al., 1974; Shimomura & Johnson, 1973a), the anthozoan sea pansy *Renilla* (Hori & Cormier, 1973; Inoue et al., 1977a; Shimomura & Johnson, 1975a), the myctophid fish *Neoscopelus* (Inoue et al., 1977b), and the decapod shrimps *Oplophorus* (*Hoplophorus*) and *Heterocarpus* (Inoue et al., 1976). Possibly the same is true of all coelenterates (Cormier et al., 1973; Shimomura & Johnson, 1975a) and ctenophores as well (Ward & Seliger, 1974; Ward & Cormier, 1975). In addition, disulfated forms of I and also of II occur in the firefly squid *Watasenia* (Goto et al., 1974; Inoue et al., 1975). Such a wide-spread occurrence of coelenterazine and coelenteramide in various luminescent organisms would imply a possibly important, somewhat general biological role of these compounds not necessarily in luminescence alone.



I



II

In regard to the organisms cited above, detailed chemical studies of the bioluminescence systems, including a complete purification of either the luciferase or photoprotein, have been reported only in two instances, i.e., *Aequorea* (Shimomura & Johnson, 1969) and *Renilla* (Matthews et al., 1977). The present study constitutes the third example.

The decapod shrimp *Oplophorus gracilorostris*, which was once thought to be *Heterocarpus sibogae* (Haneda, 1955), possesses secretory luminous glands at the base of the antennae and legs in addition to luminous organs also on the legs. This shrimp ejects a cloud of brightly luminescent secretion from the base of antennae when stimulated. Both fresh and dried material give the traditional luciferin–luciferase reaction (Haneda, 1955). Partial purification of *Oplophorus* luciferin and *Oplophorus* luciferase has been reported with data suggesting that the luciferin, luciferase, and molecular oxygen are the only components necessary for the luminescent reaction (Johnson et al., 1966).

Materials and Methods

Specimens were caught in Suruga Bay, Japan, air dried, then desiccated with $CaCl_2$ until used. Extraction and purification of natural luciferin and of native luciferase were carried out at or near 0 °C. Synthetic luciferin of *Oplophorus*, i.e., coelenterazine (Inoue et al., 1975), was a gift from Dr. S. Inoue. Coelenteramide was prepared as previously described (Shimomura & Johnson, 1973a). Luciferase activity was

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TABLE I: Purification of *Oplophorus* Luciferase Extracted from 200 g of Defatted, Dried Material.[a]

Step	Method	Spec act.[b] (quanta s^{-1})	Total yield of act. (quanta s^{-1})
1	Adsorption on DEAE-cellulose, batch method	0.4×10^{14}	1.8×10^{17}
2[c]	Sephadex G-150 column chromatography, 2.6 × 90 cm	3.3×10^{14}	1.25×10^{17}
3	Sephadex G-150 column chromatography, 2.6 × 90 cm	9×10^{14}	0.84×10^{17}
4	DEAE-cellulose column chromatography, 2 × 20 cm	14.5×10^{14}	0.63×10^{17}
5	Ultrogel AcA 34 column chromatography, 1.6 × 80 cm	17×10^{14}	0.50×10^{17}

[a] Total activity of crude extract: 2×10^{17} quanta s^{-1}. [b] Activity in 1 ml/OD (1 cm) at 280 nm. [c] The product of step 1 was divided into three portions which were chromatographed successively on this column.

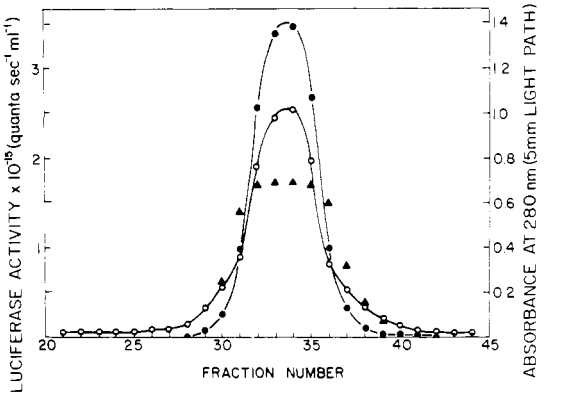


FIGURE 1: Elution curves of *Oplophorus* luciferase from the Ultrogel AcA 34 column (step 5 in Table I), for luciferase activity (●), absorption at 280 nm (○), and specific activity (activity of 1 mL/OD for 1-cm light path; ▲). Fractions of 3.1 mL were collected at an elution speed of 8 mL/h. Fractions 32–35 were pooled and saved as the purified luciferase.

measured by the initial maximum intensity of the light emitted when 5 mL of 15 mM Tris-HCl buffer (pH 8.3) containing 50 mM NaCl and a known amount of natural luciferin or synthetic coelenterazine (0.45 μg, except as noted) was rapidly added to a small volume (5–10 μL) of luciferase sample at 23–25 °C. Luciferin (coelenterazine) was assayed by measuring the total light emitted when a large excess of luciferase in the buffer referred to above was added to a small amount of luciferin sample. Light emission was measured on a photomultiplier–amplifier–recorder assembly that was calibrated with *Cypridina* bioluminescence (emission maximum at 465 nm, quantum yield 0.3; Shimomura & Johnson, 1970).

Natural Luciferin. Luciferin was extracted and partially purified from 500 g of dried shrimp as previously reported (Johnson et al., 1966). The product was further purified by gel filtration on a column of Sephadex LH-20 (Pharmacia) using as the solvent ethanol containing a trace of ammonia, followed by chromatography on a column of DEAE-cellulose (Whatman, DE-32) with NaCl gradient elution in 50% ethanol.

Luciferase. Dried shrimp (200 g) was ground with mortar and pestle, washed with benzene on a Buchner funnel, and then air dried. The dried powder was added to 3 L of water, and the mixture was gently stirred for 2 h and then filtered. The filtrate was diluted with 5 L of water and mixed with 200 g (dry weight) of DEAE-cellulose which had been previously neutralized and equilibrated with 10 mM sodium phosphate (pH 6.8). Luciferase adsorbed on the DEAE-cellulose was eluted with 10 mM sodium phosphate (pH 6.8) containing 0.4 M NaCl, by the batch method. Luciferase was precipitated from this eluate (1.5 L) by saturating with $(NH_4)_2SO_4$ and purified further by four additional steps of column chromatography, as summarized in Table I. The first and the second steps (steps 2 and 3 in Table I) were gel filtration on Sephadex G-150 (Pharmacia), the next (step 4) was ion exchange on DEAE-cellulose with NaCl linear gradient elution, and the last (step 5) was gel filtration on Ultrogel AcA 34 (LKB), all in 10 mM sodium phosphate buffer, pH 6.8 (pH 7.5 for the last column), with 0.2 M NaCl added for the gel filtrations. In the DEAE-cellulose chromatography, the gradient of NaCl started at 0.05 M and ended at 0.5 M. In each chromatography, every fraction was tested for luciferase activity and OD at 280 nm, and the fractions which had the highest value for the ratio of activity/OD and which contained 75–80% of eluted total activity were saved. In purification of this enzyme, gel filtration was generally more effective than ion-exchange chromatography. The elution profile of the last chromatography is shown in Figure 1.

Determination of Molecular Weight of Luciferase. Gel filtration was carried out at 4 °C on a column of Sephadex G-150 (1.6 × 80 cm) equilibrated with 10 mM Tris-HCl containing 0.2 M NaCl (pH 8.7) or with 0.1 M sodium phosphate containing 0.2 M NaCl (pH 6.8). The column was calibrated with egg albumin, aldorase (both from Pharmacia), bovine albumin, and apoferritin (Schwarz/Mann). Sodium dodecyl sulfate acrylamide gel electrophoresis was performed according to Weber & Osborn (1969) on 10% gel columns, using cytochrome *c* (Schwarz/Mann), chymotrypsinogen A (Pharmacia), egg albumin, and bovine albumin as the standards. After electrophoresis, gels were stained with 0.2% Coomassie Brilliant Blue R-250 (Bio-Rad), in 7% acetic acid, in 50% methanol. Mobility (M) was calculated as follows:

$$M = PL_1/BL_2$$

where P is the migration distance of protein, B is the migration distance of Bromophenol Blue, L_1 is the length of gel before staining, and L_2 is the length of gel after staining.

Extinction Coefficient of Luciferase. Approximately 2 mL of luciferase solution was dialyzed against two changes of 2 L of 10 mM ammonium acetate (pH 7.5) for 16 h each time. After recording the absorption spectrum, 1.5 mL of the dialyzed luciferase solution was freeze-dried over P_2O_5 in a vacuum desiccator; then the protein residue was further dried at 120 °C for 2 h in an oven. The dried residue was weighed after cooling the sample in a desiccator.

Incorporation of ^{18}O of $^{18}O_2$ Gas into CO_2 Produced in the Bioluminescent Reaction. The apparatus, technique, and calculation method was essentially the same as reported for the *Cypridina* (Shimomura & Johnson, 1973b, 1975b) and firefly bioluminescence systems (Shimomura et al., 1977). Approximately 20 mg of purified luciferase in 5 mL of 15 mM Tris-HCl buffer (pH 8.3) containing 50 mM NaCl was placed in the bottom of the reaction vessel (total inner volume including side arm = 55 mL), and 120 μg of coelenterazine in 0.4 mL of 50% methanol was added into the side arm. After degassing the reaction vessel for 20 min by intermittent evacuation and agitation, $^{18}O_2$ gas (95 atom %, Bio-Rad Labora-

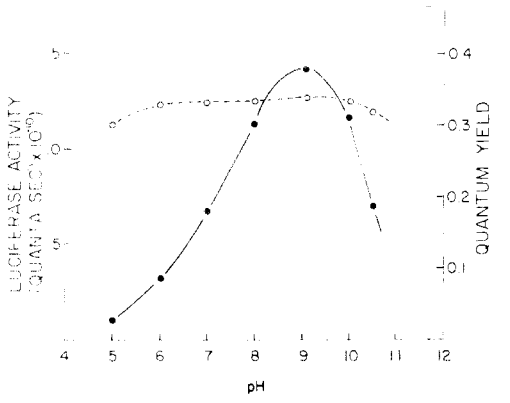


FIGURE 2: Influence of pH on luciferase activity (●) and quantum yield of coelenterazine (○) in the bioluminescence of *Oplophorus*. The luciferase activities were measured with 4.5 μg of coelenterazine plus 0.02 μg of luciferase, and the quantum yields with 0.1 μg of coelenterazine plus 100 μg of luciferase, in 5 mL of buffer solution at 24 °C. The buffer systems used were 10 mM each of sodium acetate–acetic acid (pH 5.0), sodium phosphate (pH 6.0 and 7.0), Tris-HCl (pH 8.0 and 9.1), and $NaHCO_3$–Na_2CO_3 (pH 10 and 10.5), all containing 50 mM NaCl.

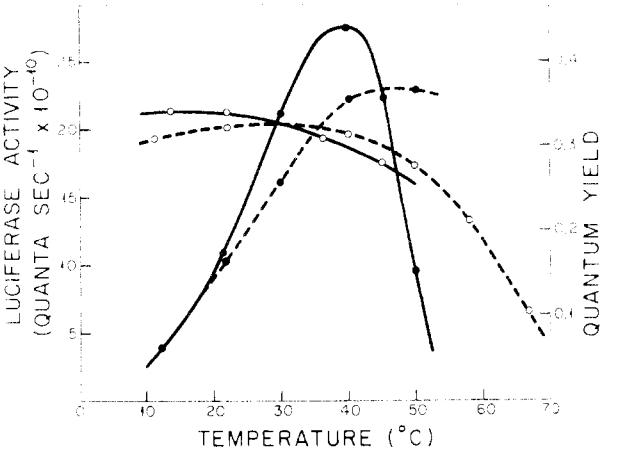


FIGURE 3: The activities of luciferase (●) and quantum yields of coelenterazine (○) in the *Oplophorus* bioluminescent reaction at various temperatures, measured with purified luciferase (solid lines) or with partially purified luciferase of approximately 20% purity (broken lines). The activity was measured with 4.5 μg of coelenterazine and 0.05 μg of luciferase, and the quantum yield was measured with 0.2 μg of coelenterazine and 200 μg of luciferase, in 5 mL of 15 mM Tris-HCl containing 50 mM of NaCl (pH 8.3) at 25 °C. Coelenterazine was first added to the buffer solution at the designated temperature; then the luminescence reaction was started by a rapid injection of 0.1 mL of luciferase solution.

tories; freed of contaminating CO_2 by cooling in advance with liquid nitrogen) was introduced, and the luminescent reaction started by mixing the two solutions at 23 °C. After 1 min of the luminescence reaction, the mixture in the reaction vessel was frozen in a dry ice–acetone bath. CO_2 produced in the reaction was collected in a liquid nitrogen trap, and the CO_2 sample was analyzed on a Hitachi-Perkin-Elmer mass spectrometer Model RMU-6D by Morgan-Schaffer Corp., Montreal.

Under the conditions described above, not all of the coelenterazine was oxidized. In order to determine the amount of residual coelenterazine, the frozen spent solution still in the reaction vessel was thawed after adding 1 mL of methanolic 1 N HCl; then 10 μL of the thawed mixture was used in the assay of coelenterazine.

Results and Discussion

Molecular Properties of Luciferase. Oplophorus luciferase purified by the present procedure was colorless, with an absorption maximum at 280 nm and a trough at 252 nm as commonly found for many simple proteins. The absorption coefficient $E_{1cm}^{1\%}$ was 6.1 at 280 nm and the ratio of OD_{252}/OD_{280} was 0.52.

Sodium dodecyl sulfate acrylamide gel electrophoresis of purified *Oplophorus* luciferase revealed a slightly broadened, single band, which corresponded to a molecular weight of 31 000. A trace of a side band corresponding to a slightly smaller molecular weight was also visible. Thus, the luciferase was apparently highly purified, even if it was not completely pure.

Sephadex G-150 gel filtration of native luciferase gave molecular weights of 130 000 at pH 8.7 and 220 000 at pH 6.8. The accuracy of the latter value would not be high because this value is close to the exclusion limit of Sephadex G-150, although the molecular weight at pH 6.8 was clearly much greater than that at pH 8.7 as judged by the elution volume. These figures indicate that the native, active form of this enzyme is probably a tetramer, having a tendency to further polymerize at lower pHs. We have not succeeded in detecting an active form of a monomer by gel filtration even at pH 10.

Conditions for Optimum Light Emission. Using the highly purified materials now available, we have reconfirmed that the

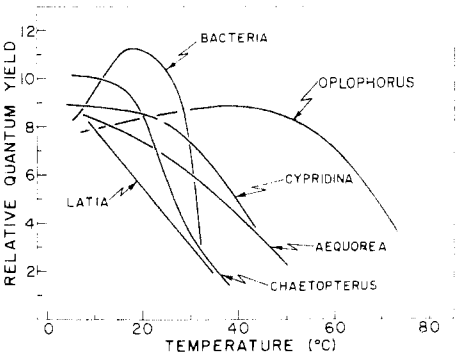


FIGURE 4: Effect of temperature on the relative quantum yield of various bioluminescent systems: *Oplophorus* (0.1 μg of coelenterazine plus 200 μg of luciferase at approximately 20% purity, in 2.5 mL of 10 mM Tris-HCl containing 50 mM NaCl (pH 8.3) at test temperatures), luminous bacteria (Hastings & Gibson, 1963), the sea-firefly *Cypridina* (Shimomura & Johnson, 1970), the jellyfish *Aequorea* (5 μg of aequorin plus 5 mL of 10 mM calcium acetate), the marine worm *Chaetopterus* (Shimomura & Johnson, 1966), and the fresh-water limpet *Latia* (Shimomura et al., 1966).

essential components necessary in the bioluminescence of *Oplophorus* are only luciferase, coelenterazine, and molecular oxygen, in addition to the solvent water. No cofactor is required. Light emission with these components can be significantly affected, however, by pH, salt concentration, and temperature.

The optimum pH for rate of the bioluminescence reaction was found to be around 9 (Figure 2). Below pH 8, the rate progressively decreased, becoming about one-tenth the maximum rate at pH 5, and virtually zero at pH 4. Changing the pH, however, did not significantly affect the quantum yield of coelenterazine. A certain level of ionic strength seems essential for the maximum activity of luciferase. In the case of NaCl, the optimum concentration was found to be between 0.05 and 0.1 M.

The bioluminescence system of *Oplophorus* is an unusually heat-resistant example (Figures 3 and 4). In other known luciferin-luciferase systems, e.g., *Cypridina* (Shimomura &

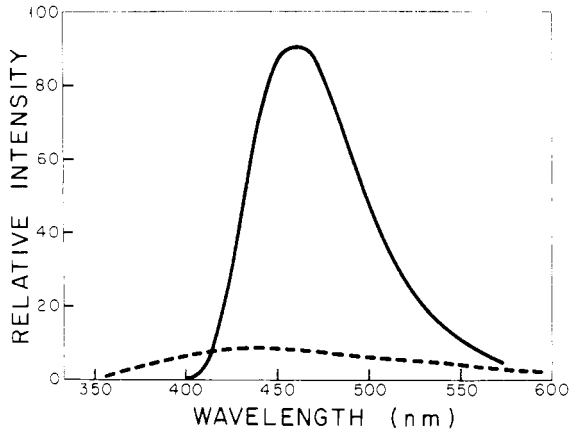


FIGURE 5: Bioluminescence spectrum (solid line) of coelenterazine (5 μg) in the presence of *Oplophorus* luciferase (approximately 2 μg) in 1 mL of 15 mM Tris-HCl (pH 8.3) containing 50 mM NaCl, and fluorescence emission spectrum (broken line) of the same solution after completion of the luminescence reaction, excited at 330 nm, measured on an Aminco-Bowman spectrofluorometer, both at 25 °C. The data have been corrected for variation in sensitivity of the photomultiplier at different wavelengths. An addition of 100 μg of luciferase, or 10 μg of coelenteramide, to the spent solution did not affect the fluorescence spectrum.

Johnson, 1970) and *Latia* (Shimomura et al., 1966), inactivation of the enzyme is pronounced at much lower temperatures, and the quantum yields of the various luciferins usually fall to zero at about 40–50 °C. With pure *Oplophorus* luciferase, the optimum temperature for rate of light emission was found at about 40 °C. The quantum yield of coelenterazine was nearly constant for temperatures below 20 °C, with relatively small decreases at higher temperatures up to 50 °C. At temperatures above 50 °C, the inactivation of luciferase became so rapid that reliable data on quantum yields could not be obtained.

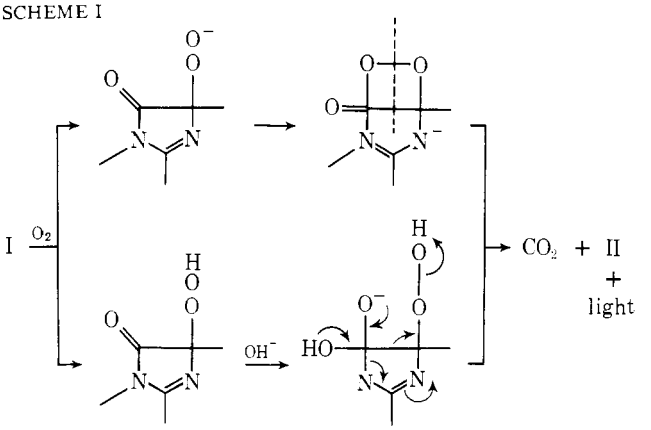
Partially purified luciferase (approximately 20% pure) was more resistant to heat than practically pure luciferase. With the former, the optimum temperature for rate of light emission rose to almost 50 °C. Remarkably, light emission was clearly observable at 70 °C, and momentarily even at 80 °C, when luciferase was injected into the solution of coelenterazine maintained at this temperature. It seems probable that protein impurities in the partially purified luciferase preparation had a protective effect against the inactivation of luciferase. In fact, bovine serum albumin (1 mg/mL) added to highly purified luciferase has shown a detectable stabilizing effect.

The Quantum Yield of Luciferin and the Activity of Luciferase. The quantum yield of coelenterazine in the luciferase-catalyzed bioluminescence reaction in 15 mM Tris-HCl buffer (pH 8.3) containing 0.05 M NaCl at 22 °C was 0.34, which is evidently close to the maximum value (cf. Figure 3). The specific activity of pure luciferase in the presence of a large excess of coelenterazine (4.5 μg/5 mL) in the same buffer at 23 °C was 1.75×10^{15} photons s^{-1} mg^{-1}. This value corresponds to a turnover number of 65 min^{-1} for the native luciferase tetramer, and 16 min^{-1} for the subunit monomer.

Mechanism of the Luminescence Reaction. When synthetic coelenterazine (I) or purified natural *Oplophorus* luciferin was oxidized with molecular oxygen in the presence of purified *Oplophorus* luciferase, the luminescence spectrum showed a maximum at 462 nm (Figure 5). The spent solution was not fluorescent, but it afforded practically pure coelenteramide (II) upon extraction with ether. The failure to demonstrate the light-emitting species by fluorescence of the spent solution can be attributed to the dissociation of coelenteramide from the light-emitting enzyme complex. An analogous situation exists in the bioluminescence system of *Renilla* (Hori et al., 1973) in which the spent solution did not fluoresce and in which the luciferin has recently been determined to be coelenterazine (Inoue et al., 1977a). Coelenteramide (II) does not fluoresce in aqueous solution but is highly fluorescent in organic solvents. This compound is the product of the bioluminescent oxidation of coelenterazine (I) and is obviously the light-emitting compound in both instances, i.e., *Oplophorus* and *Renilla*.

Two kinds of reaction routes are possible for the luminescent oxidation of coelenterazine (I), as shown in Scheme I. The upper route involving a dioxetane intermediate is consistent

SCHEME I



with ^{18}O-labeling studies of the bioluminescent oxidation of *Cypridina* luciferin (Shimomura & Johnson, 1971, 1973b, 1975b) which is structurally similar to coelenterazine (I). Essentially the same mechanism is applicable to the bioluminescent oxidation of firefly luciferin (Shimomura et al., 1977) which bears no structural resemblance to coelenterazine. The lower route, which is essentially the same as the mechanism that has been first claimed for firefly bioluminescence (DeLuca & Dempsey, 1970, 1973; Tsuji et al., 1977) was thought to be applicable to the bioluminescent oxidation of *Renilla* luciferin (DeLuca et al., 1971) which is identical with coelenterazine (Inoue et al., 1977a).

In the present study, incorporation of ^{18}O into the product CO_2 was measured in the bioluminescent oxidation of coelenterazine (I) with $^{18}O_2$ gas catalyzed by *Oplophorus* luciferase in $H_2{}^{16}O$ medium as described in Materials and Methods. Under such conditions, the atoms of ^{18}O incorporated in the product CO_2 must have come from the $^{18}O_2$ gas that oxidized compound I. In two repeated experiments, approximately 0.2 μmol of I (i.e., two-thirds of the added amount) was oxidized each time in 1 min of the reaction period, and the mass spectra of the product CO_2 samples showed that 39% and 52%, respectively, of the total CO_2 molecules were $C^{18}O^{16}O$. These percent figures indicate that the mechanism of *Oplophorus* bioluminescence is consistent with the upper pathway which involves the dioxetane intermediate, without, however, ruling out a partial involvement of the lower route.

The above values, however, should be considerably lower than the true value of incorporated ^{18}O in the CO_2 that has just been formed in the bioluminescence reaction, because of various factors all of which would decrease the apparent value of the incorporated ^{18}O (Shimomura et al., 1977). Such factors would include the presence of contaminating CO_2, the presence of residual $^{16}O_2$, and the exchange of O between the product CO_2 and H_2O of the medium. It should also be noted that the difference between the two values is not an error of measurement, but rather is a result of fluctuation of the factors just

mentioned, particularly of the presence of contaminating CO_2.[1] Thus, in the experiment that yielded the latter value (52%), evacuation, and agitation in the degassing step should have been more efficient than in the experiment for the former value (39%), and the higher value (52%) should be closer to the true value of incorporation than the lower value (39%).

The liberation of CO_2 in the bioluminescent oxidation of I is quantitative (0.2 μmol). Conservatively assuming the actual amount of contaminant CO_2 as 0.06 μmol (cf. Shimomura et al., 1977) and ignoring the other two factors mentioned above, correction of the value of 52% for the contaminating CO_2 gives a value of 67.5% which is close to the value of 66% that is necessary to rule out the lower pathway on the basis of the quantum yield of 0.34. This calculation makes the involvement of the lower route in the bioluminescence of *Oplophorus* highly unlikely.

The results and discussion given above are contrary to the reported mechanism in the bioluminescence of *Renilla* (DeLuca et al., 1971) in which the luciferin is identically the same as compound I in the bioluminescence of *Oplophorus*. The mechanism of *Renilla* bioluminescence calls for reinvestigation not only because of this contradiction, but also for the various reasons pointed out elsewhere (Shimomura et al., 1977).

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[1] Although we presently have no technique for precise control over the effectiveness of degassing, the effect of contaminating CO_2 should be avoidable by the use of ^{13}C-labeled luciferin (labeled at the carbonyl carbon) (Shimomura et al., 1977), provided that such a luciferin and a further supply of luciferase both become available.