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Identification of the Product Excited States During the Chemiluminescent and Bioluminescent Oxidation of *Renilla* (Sea Pansy) Luciferin and Certain of Its Analogs†

Kazuo Hori, John E. Wampler, John C. Matthews, and Milton J. Cormier*

ABSTRACT: We have synthesized a *Renilla* luciferin [3,7-dihydro-2-benzyl-6-(*p*-hydroxyphenyl)-8-benzylimidazo[1,2-*a*]-pyrazin-3-one] which has an activity equal to that of native luciferin in producing light with *Renilla* luciferase. It will react with luciferase to produce the same color of light with the same quantum yield as that produced by native luciferin. Both the synthetic and the native compounds produce a bluish chemiluminescence when dissolved in dimethylformamide. Oxygen is required for this chemiluminescence, and carbon dioxide and oxyluciferin are the products. The structure of the latter compound has been confirmed by synthesis. Detailed examination of the chemiluminescence and fluorescence emission data of synthetic luciferin, synthetic oxyluciferin and a number of synthetic analogs of each has provided evidence that the monoanion of oxyluciferin represents the electronic excited state responsible for the emission during chemiluminescence. We also find that just as in chemiluminescence the bioluminescent oxidation of luciferin by luciferase and oxygen leads to the formation of carbon dioxide and oxyluciferin. The data also suggest that bioluminescence emission arises from the electronic excited state of the oxyluciferin monoanion. There is sufficient evidence to suggest that *Renilla* luciferin or a structure very similar to it is involved in the bioluminescence of all coelenterates that have been carefully examined including the jellyfish *Aequorea*. The data also suggest that the products of those bioluminescent reactions are analogous to if not identical with those reported here.

The structure and chemical synthesis of a biologically active form of *Renilla* (Sea Pansy) luciferin [3,7-dihydro-2-methyl-6-(*p*-hydrophenyl)-8-benzylimidazo[1,2-*a*]pyrazin-3-one] has recently been reported (Hori and Cormier, 1973a). This compound (I in Figure 1) was found to be 10% as active as native luciferin in producing light with *Renilla* luciferase. Furthermore, the spectral characteristics of I were found to be identical with those of native luciferin as judged by their absorption, fluorescence, and bioluminescence emission characteristics. According to mass spectral data, native luciferin (II in Figure 1) differs from the synthetic compound (I) by the replacement of methyl with a rather bulky group designated R (Hori and Cormier, 1973b). From the mass data we deduced that replacement of the methyl group of I by benzyl would result in a compound similar to but not identical with native luciferin. That is, native luciferin contains a group of 107 mass units attached to the benzyl moiety. However, the synthetic benzyl compound (III in Figure 1) is reported here to be fully active in the bioluminescence assay. Like I it is also active in producing light with luciferases isolated from a variety of bioluminescent coelenterates (Hori and Cormier, 1973b).

We recently observed that *Renilla* luciferin will produce a brilliant bluish luminescence when dissolved in aprotic solvents such as dimethylformamide. Using the biologically active synthetic luciferins we have studied this chemiluminescence and provided evidence for the nature of the products formed and for the identification of the electronic excited state responsible for this emission. Using this information we also provide evidence that the same species is responsible for emission during bioluminescence using *Renilla* luciferase. A preliminary report of some of these data has been published (Hori *et al.*, 1973).

Materials and Methods

Materials. All reagents used for synthetic and purification procedures were the best commercial grades available.

Luciferase Preparation. We have noted recently that the amount of enzymatically active luciferase can vary from preparation to preparation depending on the isolation techniques employed. We have thus developed a new isolation procedure, superior to the one reported previously (Karkhanis and Cormier, 1971), and which results in a higher specific activity luciferase. The specific activity of the current preparations are about 50×10^{12} $h\nu$ sec^{-1} mg^{-1}. The enzyme appears homogeneous by various criteria including chromatographic, disc gel electrophoretic, and sedimentation equilibrium measure-

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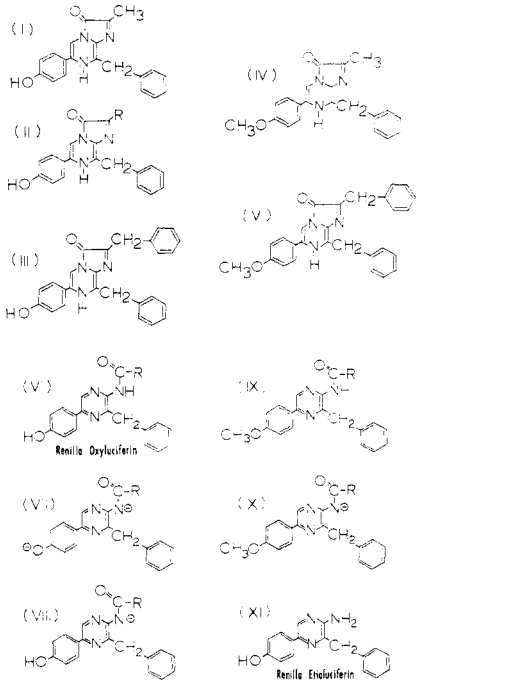

FIGURE 1: Structure of native and synthetic *Renilla* luciferins, *Renilla* oxyluciferin, and certain of their analogs and derivatives. The R group for synthetic oxyluciferin (VI) and its methyl ether (IX) is either methyl or benzyl and these are indicated in the text where appropriate.

ments. The details of this new procedure will be reported elsewhere.

Quantum Yield Measurements. All bioluminescent reactions were carried out in potassium phosphate buffer (pH 7.2, 0.1 M) containing 1 mM EDTA. One-milliliter volumes of buffer plus luciferase were injected into 10–50 μl of methanolic luciferin solutions. For bioluminescence quantum yields the molar ratio of luciferase to luciferin was 5.

For chemiluminescence quantum yields various amounts of methanolic solution of luciferin were placed in assay tubes and dried under a stream of hydrogen. DMF (1.5 ml) was then injected into such assay tubes to initiate chemiluminescence.

During measurements of both bioluminescence and chemiluminescence quantum yields the luminol chemiluminescence reaction was used as an absolute standard (Lee *et al.*, 1966).

Fluorescence quantum yields were determined with DMF solutions of the component to be measured. When a basic environment was desired *tert*-butoxide (saturated solution in *tert*-butyl alcohol) was added to DMF (5% v/v). Quinine bisulfate dissolved in 1 N H_2SO_4 was used as a standard as previously described (Wampler *et al.*, 1971). A fluorimeter system previously described (Wampler *et al.*, 1971; Wampler and DeSa, 1971) employing an on-line computer was used for all the measurements. This same instrument was used in all fluorescence measurements described in the text. Values reported for λ_F were derived from wavelength plots.

Absorption Measurements. Millimolar extinction coefficients for native and synthetic luciferins were determined by taking carefully weighed samples of the dried material, dissolving in deoxygenated methanol, and measuring the absorbance at appropriate wavelengths on a Cary Model 14 spectrophotometer.

High-precision measurements on small changes in absorbancy which occurred during the luciferase-catalyzed oxidation of luciferin (see Figure 5) were determined on an on-line computer spectrophotometer system recently described (DeSa and Wampler, 1973).

Bioluminescence and Chemiluminescence Emission. These data were collected using an absolute spectrophotofluorimeter system previously described (Wampler *et al.*, 1971; Wampler and DeSa, 1971). The values reported for λ_C and λ_B were derived from wavelength plots.

Synthesis of Luciferin, Oxyluciferin, and Their Derivatives. The synthesis of luciferin (I in Figure 1) and its methyl ether (IV) was accomplished as previously described (Hori and Cormier, 1973a). The structures of I, IV, VI (where R is methyl), and IX (where R is methyl) are in agreement with physical data which include mass spectra, uv, ir, and nuclear magnetic resonance (nmr) (Hori *et al.*, 1973). Necessary physical data on newly synthesized compounds are reported below.

The methyl ether of luciferin (V) was synthesized by a previously described method (Hori and Cormier, 1973a) with the following modifications. In the last step methylglyoxal was replaced with the acetal of benzylglyoxal. The acetal of benzylglyoxal was synthesized as earlier described (Dakin and Dudley, 1914) using benzylmagnesium chloride and the piperidide of diethoxyacetic acid (Wohl and Lange, 1908) in ether. The acetal of benzylglyoxal was added to *Renilla* etioluciferin, XI (Kishi *et al.*, 1972), dissolved in ethanol–6 N HCl (2:1). The mixture was heated in a sealed tube at 110° for 2 hr under an argon atmosphere to yield V in 56% yield. V was converted to luciferin (III), as earlier described (Hori and Cormier, 1973a), as brownish-yellow needles: mp 141–144° dec: λ_{max} (methanol) 433 nm (ϵ_{mM} 9.7), 344 (5.2), and 262 (23.6); nmr (DMSO-d_6) δ 3.58 (2 H, s), 4.02 (2 H, s), 6.94 (2 H, AB, J = 9 Hz), 7.09 (5 H, complex), 7.37 (5 H, s), 8.00 (2 H, AB, J = 9 Hz), 8.81 (1 H, s); ms *m/e* 407 (M^+), 377, and 316.

The methyl ether of oxyluciferin (IX, R = methyl) was synthesized by the following procedure. The methyl ether of *Renilla* etioluciferin (XI) (58 mg) was added to 82 mg of acetic anhydride dissolved in 2 ml of pyridine. The mixture was heated for 5 hr at 110° in a sealed tube under an argon atmosphere. The product was dissolved in 80% methanol, evaporated to dryness under a stream of argon, and redissolved in 80% methanol. It was purified by a procedure previously described for the purification of luciferyl sulfate (Hori *et al.*, 1972). The fractions so obtained were concentrated approximately fivefold and allowed to stand overnight at −15°. The product (IX) crystallized as colorless needles (83% yield). The physical data on this compound have been reported (Hori *et al.*, 1973a). The methyl ether of oxyluciferin (IX; R = benzyl) was prepared by the above procedure by substituting phenylacetic anhydride for acetic anhydride as white needles: mp 199–200°: λ_{max} (methanol) 330 nm (ϵ_{mM} 17.2), 293 (17.1), 274 (16.2); nmr (DMSO-d_6) δ 3.72 (2 H, s), 3.84 (3 H, s), 4.09 (2 H, s), 7.05 (2 H, Ab, J = 9 Hz), 7.13 (5 H, complex), 7.36 (5 H, s), 8.02 (2 H, AB, J = 9 Hz), 8.86 (1 H, s); ms *m/e* 409 (M^+), 318, and 291.

Oxyluciferin (VI, R = methyl) was prepared and purified as described above for the synthesis of the methyl ether of oxyluciferin (IX) with two exceptions. Firstly, synthetic *Renilla* etioluciferin (IX) was used as starting material instead of its methyl ether. Secondly, the acetylation step was followed by dissolving the residue in 70% methanol made 0.2 N with NaOH, heating at 60° for 5 min, neutralizing with CO_2 gas, and evaporating to dryness under a stream of argon. As outlined above the product crystallized overnight at −15° as colorless needles (58% yield). The physical data on this com-

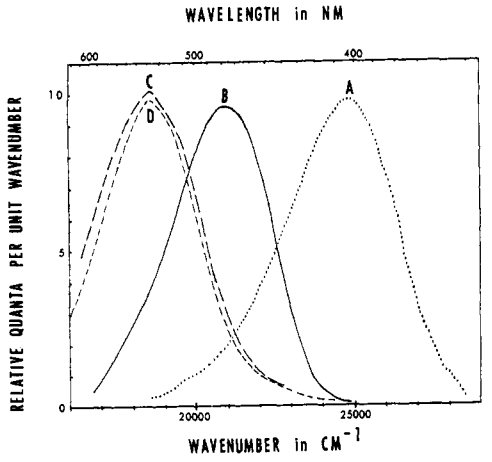

FIGURE 2: Chemiluminescence emission of native and synthetic luciferin and fluorescence emission of their products. Approximately 5–10 nmol of luciferin or oxyluciferin were used in all cases in a total volume of 1.5 ml of DMF. Curve A, fluorescence of the spent chemiluminescence reaction mixture of luciferin (I, II, or III) or oxyluciferin (VI) in DMF. Curve B, chemiluminescence of luciferin (I, II, or III) in DMF. Curve C, chemiluminescence of luciferin (I, II, or III) in DMF plus *tert*-butoxide. Curve D, fluorescence of the spent chemiluminescence reaction mixture of luciferin (I, II, or III) or oxyluciferin (VI) in DMF plus *tert*-butoxide.

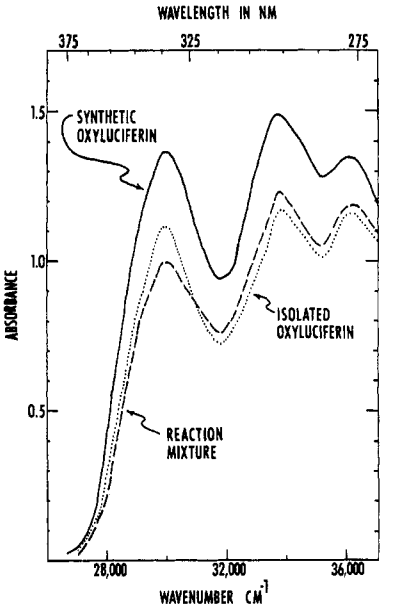

FIGURE 3: Absorption of the product of the chemiluminescence of luciferin (I, II, or III) and a comparison to that of synthetic oxyluciferin (VI). See text for details.

pound have been reported (Hori *et al.*, 1973). Oxyluciferin (VI, R = benzyl) was prepared by the above procedure by substituting phenylacetic anhydride for acetic anhydride. The product crystallized overnight at −15° as colorless white needles (64% yield): mp. 256–257°; λ_{max} (methanol) 332 nm (ϵ_{mM} 20.9), 293 (21.2), 275 (19.6); nmr (DMSO-d_6) δ 3.62 (2 H, s), 4.03 (2 H, s), 6.93 (2 H, AB, J = 9 Hz), 7.08 (5 H, complex), 7.32 (5 H, s), 7.92 (2 H, AB, J = 9 Hz), 8.86 (1 H, s); ms m/e 395 (M^+), 304, and 277.

The absence of two protons in the nmr spectra of oxyluciferin (VI) is to be expected based on its structure and the predicted lability of two protons. This is confirmed for this compound by dissolving in deuterated methanol, taking the solution to dryness and subsequent determination of its mass spectra. A molecular ion was observed that was 2 mass units heavier than that observed with the parent compound.

Results

Properties of Synthetic Renilla Luciferins. The synthesis and properties of a *Renilla* luciferin (I) have previously been reported (Hori and Cormier, 1973a). It will react with *Renilla* luciferase to produce the same color of light as that produced by native luciferin (II). According to initial rate measurements (flash height) I is only 10% as active as II. Furthermore, the bioluminescence quantum yield, Q_B, relative to I is 0.5% compared to a Q_B of 4% measured for the native compound (II). On the other hand, many of the properties of the benzyl derivative (III) are essentially identical with those observed for the native compound (II). Contrasted with the 10% activity level of I, the benzyl derivative (III) is 100% active in the bioluminescence assay. Furthermore, its Q_B is 5% and the kinetics of the reaction are identical when the luciferase-catalyzed light reaction is initiated with either II or III. The color of the light is identical when either I, II, or III is used as substrate (λ_B 490 nm). A comparison of bioluminescence emissions using I and II has been reported (Hori and Cormier, 1973a).

The absorption and fluorescence characteristics of II and III are very similar. Each has an identical fluorescence emission (λ_F 538 nm). In methanol the millimolar extinction coefficients at 262 and 433 nm were found to be 22.8 and 9.0, respectively, for II and 23.6 and 9.7, respectively, for III.

Chemiluminescence of Synthetic and Native Renilla Luciferins. When luciferin (I, II, or III) is added to an aprotic solvent such as dimethylformamide (DMF) a bluish chemiluminescence is observed (λ_C 480 nm) as shown in Figure 2 (curve B). This luminescence is dependent upon dissolved oxygen since flushing the solution with argon extinguishes the light and subsequent flushing with oxygen again initiates light emission. Carbon dioxide was found to be a product of the light reaction by techniques previously reported (DeLuca *et al.*, 1971).

Current theories of the oxidative mechanisms involved in chemiluminescence and bioluminescence (Cormier *et al.*, 1973a) suggested that in addition to CO_2 the other product of the luminescent oxidation of *Renilla* luciferin should be *Renilla* oxyluciferin (VI in Figure 1). This compound was synthesized as described under Methods where R was made either methyl or benzyl. In either case DMF solutions of synthetic oxyluciferin have an absorption illustrated in Figure 3. The millimolar extinction coefficients at 277, 296, and 333 nm in DMF are 13.44, 14.64, and 13.56, respectively. Note in Figure 3 (dashed line) that the spent chemiluminescent reaction mixture has an absorption similar to that of synthetic oxyluciferin. Furthermore, when the product of the luminescent reaction is isolated by techniques previously described for the isolation of luciferyl sulfate (Hori *et al.*, 1972) and redissolved in DMF its absorption is indistinguishable from that of synthetic oxyluciferin (Figure 3). In addition, the mass spectral pattern of the isolated product is identical with that reported under Methods for synthetic oxyluciferin (VI). The fluorescence of synthetic oxyluciferin and of the spent chemiluminescence reaction mixture is identical. As shown by curve A (Figure 2) they exhibit a bluish fluorescence in DMF (λ_F 402 nm). The absorption and fluorescence properties of the chemiluminescence product produced from native luciferin are also identical with those observed with synthetic

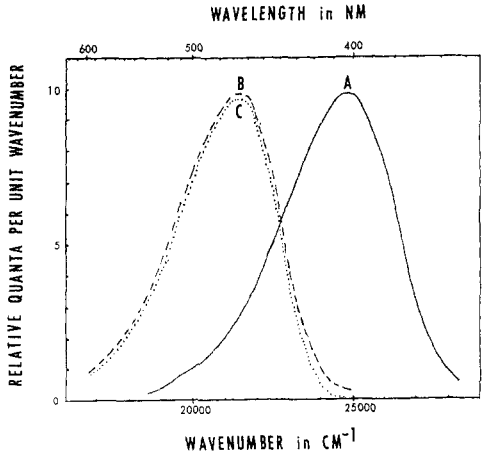

FIGURE 4: Chemiluminescence emission of the methyl ether of luciferin (IV or V) and fluorescence emission of their products. Conditions were the same as in Figure 2. Curve A, fluorescence of the methyl ether of oxyluciferin (IX) in DMF. Curve B, chemiluminescence of the methyl ether of luciferin (IV or V) in DMF with or without *tert*-butoxide. Curve C, fluorescence of the methyl ether of oxyluciferin (IX) in DMF plus *tert*-butoxide.

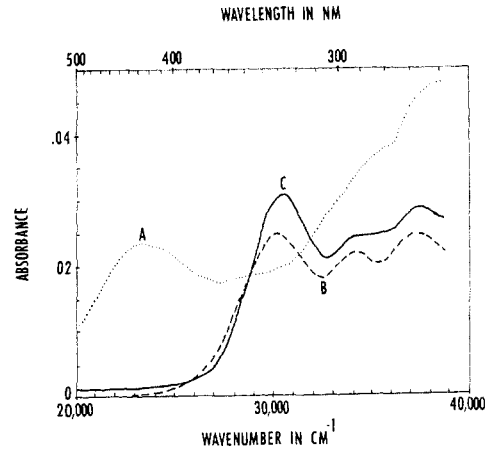

FIGURE 5: Absorption spectrum of the bioluminescence product and its comparison to the absorption of synthetic oxyluciferin and luciferin. Curve A, absorption of luciferin (III) in postassium phosphate buffer (0.1 M, pH 7.2) containing 1 mM EDTA. This same buffer was used for all experiments described here. Curve B, difference spectrum of the spent bioluminescence reaction and luciferase. Equimolar amounts of luciferase and luciferin were used. Curve C, absorption of synthetic oxyluciferin (VI, R = benzyl).

oxyluciferin (VI). Based on the molar extinction coefficients for synthetic oxyluciferin we estimate the yield of oxyluciferin (VI) during chemiluminescence to be at least 80%.

It is evident from Figure 2 that the chemiluminescence emission is not derived from the neutral species of oxyluciferin (VI). That is the chemiluminescence is considerably red shifted over that of the fluorescence of oxyluciferin in DMF (curves A and B, Figure 2). Upon the addition of a strong base the chemiluminescence shifts to longer wavelengths (curve C, Figure 2). Under these same conditions the fluorescence of oxyluciferin (VI) or of the spent chemiluminescence reaction mixture are a perfect match for the chemiluminescence (curve D, Figure 2). In strong base we suggest that the electronic species responsible for the emission is the dianion of oxyluciferin (VII in Figure 1). If this were so the monoanion of oxyluciferin (VIII in Figure 1) would be the logical choice of emitter during chemiluminescence in DMF without base. Evidence for this is presented in Figure 4. We synthesized the methyl ether of oxyluciferin (IX in Figure 1) as well as the methyl ether of luciferin (IV and V in Figure 1) for these experiments as described under Methods. Note in Figure 4 the wavelength distribution of light emission during the chemiluminescence of IV or V in DMF with or without added base (curve B). Note also that the fluorescence of IX in DMF plus base is a perfect match for the chemiluminescence emission (curve C, Figure 4). The fluorescence of IX in DMF without added base is clearly different (curve A, Figure 4). Thus during the chemiluminescence of IV and V we suggest that the electronic species responsible for the emission is the monoanion X (Figure 1). These results also suggest that during the chemiluminescence of I, II, and III in DMF the corresponding oxyluciferin monoanion VIII (Figure 1) is responsible for the emission.

Quantum yield data have provided useful information in the interpretation of mechanisms involved in these luminescent reactions. For native and synthetic luciferins (I–V, Figure 1) the chemiluminescence quantum yield Q_c, in DMF with or without base, is approximately 0.1%. The fluorescence quantum yields, Q_F, of oxyluciferin (VI) and its methyl ether (IX) in DMF are 23 and 38%, respectively. The Q_F for VI and IX in DMF + *tert*-butoxide are 8.5 and 6%, respectively. Thus although the Q_F for the neutral species of VI and IX are considerably higher than the Q_F of the ionized species there is no hint of a contribution by the neutral species during the chemiluminescence of luciferin. These results suggest that the excited state monoanion species is the oxidation product of chemiluminescence and that the fluorescence decay rate of this species is faster than the rate of protonation in DMF. These findings are also consistent with studies on model compounds (Goto, 1968; McCapra and Chang, 1967).

Bioluminescence Studies Using Synthetic Luciferin. A series of experiments were performed in an effort to ascertain whether or not the product excited state produced in bioluminescence is the same as that found for the chemiluminescence of luciferin. For these studies we used the benzyl derivative of *Renilla* luciferin (III, Figure 1) since it is equally as active as native luciferin (II, Figure 1) in producing light with luciferase.

Figure 5 illustrates some absorption studies performed on a spectrophotometer system which incorporates an on-line minicomputer (DeSa and Wampler, 1973). Curve A represents the absorption of luciferin. When this same amount of luciferin is added to luciferase a brilliant bluish bioluminescence occurs which results in the disappearance of the 433-nm absorption band of luciferin. A new absorption band, at 333 nm, appears during this time. At the end of the bioluminescence reaction the absorption of the product is illustrated by curve B (Fig. 5). Curve B represents the difference spectrum of the spent bioluminescence reaction mixture minus luciferase and with a small component of unreacted luciferin subtracted out. Curve B is to be compared with curve C which represents the absorption of synthetic oxyluciferin (VI) under these conditions. Note that the absorption of the bioluminescence reaction product matches the absorption of synthetic oxyluciferin (VI).

The spent bioluminescence reaction mixture does not fluoresce. This observation is consistent with the fact that synthetic oxyluciferin (VI) does not fluoresce in aqueous solutions. Thus the spent mixture was taken to dryness, resuspended in methanol, and centrifuged at 12,000*g* for 10 min to remove denatured protein. The resulting clear supernatant exhibited a brilliant bluish fluorescence. The appearance of

this fluorescence is dependent upon the presence of both luciferin and luciferase in the original reaction mixture. The fluorescent material was further purified on LH-20 as previously described for the isolation of luciferyl sulfate (Hori *et al.*, 1972). The fluorescence of this isolated bioluminescence product was compared to that of synthetic oxyluciferin (VI; R = benzyl) in methanol. The two emissions were essentially identical with emission peaks in each case at 430 nm.

Discussion

Based on absorption, fluorescence, and quantum yield data the products of both the chemiluminescent and bioluminescent oxidation of luciferin (III, Figure 1) are identical with synthetic oxyluciferin (VI, Figure 1). As reported here CO_2 is also a product of the chemiluminescent reaction and it has previously been reported that 1 mol of CO_2/mol of luciferin is produced during the bioluminescent oxidation of *Renilla* luciferin (DeLuca *et al.*, 1971). Analyses of the chemiluminescence and fluorescence emission data (Figures 2 and 4) suggest that the electronic species responsible for the emission during the chemiluminescent oxidation of luciferin is the oxyluciferin monoanion (VIII, Figure 1). We suggest that this oxyluciferin monoanion is also responsible for the emission during bioluminescence. This suggestion is based on the observation that the bioluminescence emission is similar in shape and peak position to the chemiluminescence emission of luciferin in DMF (curve B, Figure 2). The chemiluminescence illustrated by curve B (Figure 2) exhibits an emission peak near 480 nm whereas bioluminescence peaks at 490 nm (Hori and Cormier, 1973a). Thus it seems reasonable that bioluminescence is not derived from the un-ionized product excited state since its fluorescence emission in DMF is 402 nm (curve A, Figure 2). Similarly, bioluminescence appears not to be derived from the dianion product excited state since its fluorescence emission in DMF plus *tert*-butoxide is 535 nm (curve C, Figure 2). If we assume that both DMF and enzyme provide similar environments for oxyluciferin then we can view the pathway to light emission during both chemiluminescence and bioluminescence as shown in Figure 6. The monoanion of oxyluciferin is formed as a product in each case with emission occurring prior to protonation as suggested from our quantum yield studies and our chemiluminescence emission data.

We report a product yield during chemiluminescence of at least 80%. It should be pointed out that we have not detected fluorescent or absorbing products other than oxyluciferin (VI) in the spent reaction mixtures after chemiluminescence. This is also true after bioluminescence with the exception that small amounts of the product of luciferin autoxidation (XI, Figure 1) are formed whose absorption and fluorescence properties are distinct from those of oxyluciferin (Hori and Cormier, 1973b; Shimomura and Johnson, 1972). We will refer to this product as *Renilla* etioluciferin by analogy to *Cypridina* etioluciferin (Cormier *et al.*, 1973a).

The possibility that products other than CO_2 and oxyluciferin (VI) are formed during chemiluminescence and bioluminescence appears to be eliminated by a consideration of the quantum yield data and the absorption spectra of the spent reactions. Whereas the Q_C for luciferin in DMF is about 0.1% the Q_B for luciferin is about 5%. Furthermore, the Q_F for the monoanion of the methyl ether of oxyluciferin (X) in DMF plus *tert*-butoxide is about 6%, and it is inferred that this same value holds true for the oxyluciferin monoanion (VIII) in DMF. It is clear from these quantum yield values that the

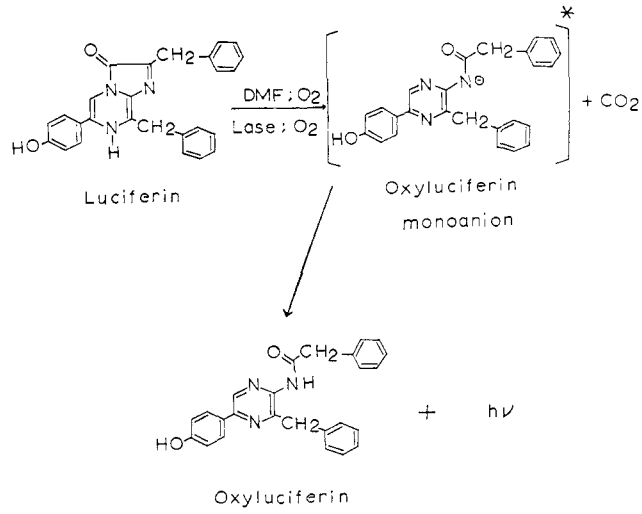

FIGURE 6: Pathway to light emission during both the chemiluminescent and bioluminescent oxidation of synthetic luciferin.

excitation yield for excited state singlet production of the oxyluciferin monoanion (VIII) during *Renilla* bioluminescence could be very high, *i.e.*, in the vicinity of 80% or higher providing that the Q_F for VIII is about the same in either the enzyme or DMF environment. Thus significant dark pathways are eliminated.

We recently presented evidence (Cormier *et al.*, 1973b) that the chemical requirements for bioluminescence among a number of bioluminescent coelenterates that were examined are very similar or identical with those already described for *Renilla* by this laboratory (Cormier *et al.*, 1973a). Components required for luminescence in *Renilla* were also found in a number of bioluminescent coelenterates examined such as *Aequorea*, *Obelia*, *Cavernularia*, *Ptilosarcus*, *Stylatula*, *Acanthoptilum*, *Parazoanthus*, and *Mnemiopsis*. Depending on the organism these included one or more of the following: luciferyl sulfate, luciferase, and luciferin sulfokinase. These isolated components were found to be indistinguishable from those found in *Renilla* as evidenced by their reactivity in the *Renilla* bioluminescent system, by the spectral characteristics of the isolated luciferyl sulfates, by the molecular weights of the luciferases, and by the colors of the bioluminescence produced *in vitro*. More recently, we have also noted that synthetic *Renilla* luciferin (I and III) will produce light typical of the *Renilla in vitro* reaction when added to luciferases isolated from various coelenterates (Hori and Cormier, 1973b).

From the above mentioned observation on coelenterate bioluminescence one can draw the tentative conclusion that the structure of the luciferins of these various bioluminescent systems must be very similar to that of synthetic *Renilla* luciferin (III, Figure 1). Additional evidence for this conclusion can be gleaned from the work of Shimomura and Johnson (1969, 1970, 1972) on the calcium-triggered photoprotein aequorin isolated from the jellyfish *Aequorea*. They reported that treatment of aequorin with urea and a sulfhydryl compound such as cysteine leads to a product which they referred to as AF-350. They deduced the structure of AF-350 to be XI (Figure 1). Note that this structure is identical with that of *Renilla* etioluciferin mentioned earlier and that it forms an integral part of the structure of *Renilla* luciferin. Based on energetics, the native chromophore in aequorin would be expected to contain a fused imidazole ring like that found in *Renilla* luciferin (II). The lack of an oxygen requirement during aequorin luminescence (Shimomura and Johnson, 1969)

could be explained by the formation of a luciferin hydroperoxide intermediate (Cormier *et al.*, 1973a) which is somehow stabilized by aequorin in the absence of calcium ions. Further evidence for the existence of a chromophore in aequorin similar to that of *Renilla* luciferin is obtained by examination of the reported spectral properties of aequorin before and after triggering with calcium ion (Shimomura and Johnson, 1969). Before light emission aequorin has a visible absorption similar to the long wavelength absorption of *Renilla* luciferin. After the addition of calcium ion to aequorin and subsequent light emission the long wavelength absorption of aequorin disappears and a new absorption band appears at about 333 nm. As outlined in the text, this is precisely what happens during the bioluminescent oxidation of synthetic *Renilla* luciferin (III). We know that the 333-nm band is due to oxyluciferin (VI) in the case of *Renilla* and we suggest the formation of a similar product in the case of aequorin luminescence. Hydrolysis of the protein bound oxyluciferin would lead to the formation of *Renilla* etioluciferin (XI). In addition, the product of the aequorin reaction, referred to by Shimomura and Johnson (1970) as the "blue fluorescent protein," exhibits a blue fluorescence similar to that observed by us for the chemiluminescence of *Renilla* luciferin in DMF.

More recently, Ward and Seliger (1973) reported a photoinactivation spectrum of the calcium-triggered photoprotein isolated from the ctenophore *Mnemiopsis*. This photoinactivation spectrum is similar to the absorption of *Renilla* luciferin (Hori and Cormier, 1973b). Again as in the case of the bioluminescent oxidation of *Renilla* luciferin, the luminescent reaction of *Mnemiopsis* photoprotein is accompanied by a loss of absorption near 430 nm and an appearance of a new absorption band near 330 nm (W. W. Ward, personal communication). The appearance of this 330-nm band suggests the formation of a product similar to oxyluciferin (VI).

Taken together, these data suggest that a molecule similar, if not identical, to that of oxyluciferin (VI) represents the product of the bioluminescence reaction in all the coelenterates that we have examined including *Aequorea* (Cormier *et al.*, 1973b). Since the *in vitro* emissions are all in the region 469–490 nm (Cormier *et al.*, 1973a; Shimomura and Johnson, 1970), these data also suggest that the electronic excited state of an oxyluciferin monoanion-like species is responsible for these emissions.

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