SOURCE OF OXYGEN IN THE CO_2 PRODUCED DURING CHEMILUMINESCENCE OF FIREFLY LUCIFERYL-ADENYLATE AND *RENILLA* LUCIFERIN[1]

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SUMMARY. The chemiluminescent reactions of firefly luciferyl-adenylate and *Renilla* luciferin utilize molecular oxygen and produce CO_2. If the reactions are allowed to proceed in the presence of $^{18}O_2$, no incorporation of 18-oxygen is detected in the CO_2. This is true over a wide range of substrate concentrations (0.03 μmoles - 6 μmoles). If $H_2{}^{18}O$ is added to the reaction medium there is incorporation of 1 oxygen-18 into the CO_2. These results are consistent with the oxygen-18 labelling observed with the bioluminescent reactions.

The bioluminescent reactions catalyzed by luciferases from firefly, *Renilla* and *Cypridina* all utilize molecular oxygen and produce CO_2 as one of the products (1, 2, 3). Experiments have been done with each of these systems in an effort to determine the source of oxygens in the CO_2. We have reported that with firefly bioluminescence and chemiluminescence as well as *Renilla* bioluminescence there is no incorporation of oxygen - 18 into CO_2 when the reactions are allowed to proceed in the presence of $^{18}O_2$ (3, 4, 5). Results obtained with the *Cypridina* luciferase are different in that molecular oxygen is incorporated into the CO_2 (6). Shimomura and Johnson have reported that

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non-enzymatic exchange of CO_2 and H_2O becomes significant when less than 1 μmole of CO_2 is produced in several ml of water (7). They have also examined the effect of various buffers and pH's on the non-enzymatic exchange (8). Since the isotope incorporation data is very important in establishing the mechanism for the creation of the excited state product, we have repeated and extended our studies on the firefly and Renilla systems to eliminate any possible artifacts due to a non-enzymatic exchange.

The present studies show that during the chemiluminescence of Renilla luciferin and firefly luciferyl-adenylate in aprotic solvents, the 18-oxygen labelling patterns are consistent with those previously observed during bioluminescence (3, 4).

Materials and Methods

Crystalline firefly luciferin was synthesized according to the method of Seto et al (9). Luciferyl-adenylate (LH_2-AMP) was synthesized as described by Morton et al (10) and dried at least 1 hour in a dessicator to remove excess acetone. The concentration was determined from the absorbance at 336 nm after chromatography on Sephadex G-25 in 0.01 M Na acetate + 0.04 M NaCl pH 4.5. The molar absorbancy of LH_2-AMP at 336 nm is 15,000 at pH 5 (10). Six μmoles of LH_2-AMP were dissolved in 3 ml of dry DMSO and placed in one side of the reaction vessel. The other side of the vessel contained 0.3 ml of butanol with 0.01 M potassium t-butoxide. When the reaction was run in the presence of $H_2{}^{18}O$, 0.1 ml of $H_2{}^{18}O$ was added to the DMSO. The collection and analysis of the CO_2 was done exactly as described previously (4, 5). The chemiluminescent reaction was over in less than 15 seconds.

Synthetic Renilla luciferin was prepared as described previously (11). Samples were weighed on a Cahn electrobalance (Model G-2). Varying amounts were dissolved in dry 3 ml DMSO. Other conditions are exactly the same as described above. After mixing, the chemiluminescent reaction was complete in 30 seconds.

$H_2{}^{18}O$ and $^{18}O_2$ were obtained from Miles Laboratories, Elkhart, Indiana.

Table 1 Source of oxygens in CO_2 from chemiluminescent oxidation of firefly luciferyl-adenylate.

Conditions	Light Emission	Oxygens Incorporated in CO_2
(1) Blank + $^{18}O_2$ (94.2%)	-	0
(2) Blank + $^{18}O_2$ (94.2%)	-	0
(3) 6 μmoles LH_2-AMP + $^{18}O_2$ (94.2%)	+	< 0.1
(4) 6 μmoles LH_2-AMP + $^{18}O_2$ (94.2%)	+	< 0.1
(5) 6 μmoles LH_2-AMP + $H_2{}^{18}O$ (40%)	+	0.65

Samples 1 and 2 contained 6 μmoles of decomposed luciferyl-adenylate (see text). There was no detectable CO_2 produced.

Samples 3 and 4 contained 6 μmoles of freshly synthesized luciferyl adenylate and $^{18}O_2$.

Sample 5 contained 6 μmoles luciferyl adenylate and $H_2{}^{18}O$. For details see Materials and Methods.

Results and Discussion

Table I shows the results obtained with firefly LH_2-AMP in the chemiluminescent reaction. Samples 1 and 2 were controls in which LH_2-AMP had been allowed to stand at room temperature, during which time it breaks down to luciferin and AMP. This material was then mixed with tertiary butoxide and butanol in the presence of $^{18}O_2$. There was no light emission and no production of CO_2. These experiments rule out the possibility of CO_2 arising from something other than the chemiluminescent reaction.

In samples 3 and 4, 6 μmoles of LH_2-AMP were reacted with tertiary butoxide in the presence of $^{18}O_2$. There was a bright light emission lasting about 15 seconds. There was essentially no incorporation of oxygen-18 into the CO_2. This amount of substrate is 200 times more than was used in previously reported

Table 2 Source of oxygens in CO_2 produced from the chemiluminescent oxidation of Renilla luciferin.

Conditions	Light Emission	Oxygens Incorporated in CO_2
0.05 μmoles luciferin + $^{18}O_2$ (94.2%)	+	< 0.2
3 μmoles luciferin + $^{18}O_2$ (94.2%)	+	< 0.20
3 μmoles luciferin + $^{18}O_2$ (94.2%)	+	< 0.20
3 μmoles luciferin + $H_2{}^{18}O$ (40%)	+	1
2.9 μmoles luciferin + $H_2{}^{18}O$ (40%)	+	1

Experimental details are given in Materials and Methods.

experiments. In the presence of $H_2{}^{18}O$, 0.65 atoms of oxygen were incorporated. Since the quantum yield of the chemiluminescent reaction is about 0.28 this is consistent with the water incorporation arising via a light producing pathway.

The bioluminescent reaction was also repeated with 60 nmoles of luciferin and 130 nmoles of luciferase. There was no incorporation of molecular oxygen into the CO_2.

Table II shows the results obtained with Renilla luciferin during chemiluminescence. There is less than 0.20 oxygen atoms incorporated from molecular oxygen into CO_2. This is true whether 0.05 or 3 μmoles of luciferin are used. Over this same luciferin concentration range, there is incorporation of one oxygen into CO_2 when $H_2{}^{18}O$ is added to the system. This is in contrast to the data obtained with the enzyme catalyzed reaction in which 2 oxygens were incorporated into the CO_2 from $H_2{}^{18}O$. The second oxygen was attributed to exchange of the ketone with water during the long reaction time (40 minutes).

In the chemiluminescent reaction the time was much shorter, 30 seconds, and presumably no exchange occurred with the ketone.

In all of the bioluminescence and chemiluminescence experiments which have been done with the firefly and Renilla systems over a concentration range of 0.03 μmoles to 6 μmoles, we have never observed any significant incorporation of molecular oxygen-18 into CO_2.

The experiments reported here do not support the mechanism earlier proposed (12,13) for the efficient generation of excited state singlet molecules during luminescent reactions. To achieve a clear understanding of the oxidative mechanism involved in these processes, further work is needed. A mechanism is required which provides a theoretical explanation for the generation of the excited state and which also is consistent with the $^{18}O_2 + H_2{}^{18}O$ labelling patterns.

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