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Source of oxygen in the CO_2 produced in the bioluminescent oxidation of firefly luciferin

(luminescence/luciferase/oxygen exchange/reaction mechanism)

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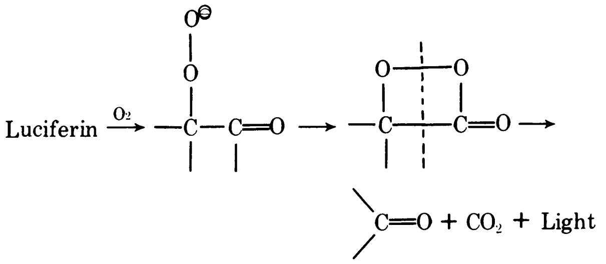
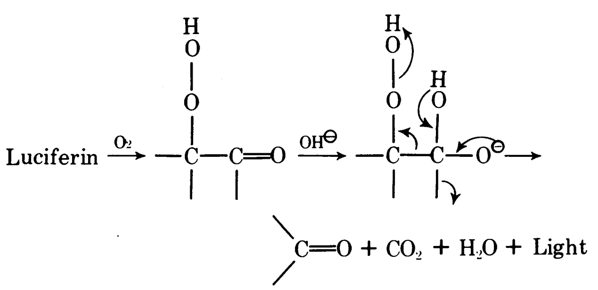
ABSTRACT Incorporation of ^{18}O into the CO_2 produced in the bioluminescent oxidation of firefly luciferin was studied. In $H_2{}^{16}O$ medium with $^{18}O_2$ gas, the product CO_2 contained up to 75% $C^{16}O^{18}O$, showing that one O of the product CO_2 arose from the O_2 that oxidized luciferin. This result is consistent with a dioxetane mechanism. Analysis of the mass spectral data of the CO_2 obtained in high-enrichment $H_2{}^{18}O$ medium with $^{16}O_2$ gas indicated the presence of about 20% contaminating CO_2, which contributes approximately 70% of the total incorporated ^{18}O. Thus the values of incorporated ^{18}O in $H_2{}^{18}O$ medium with $^{16}O_2$ gas have no significance in the present context. Data obtained with luciferases of the American firefly *Photinus* and Japanese firefly *Luciola* were similar.

Bioluminescence and chemiluminescence of the luciferins of the firefly, the ostracod *Cypridina*, and the sea pansy *Renilla* are similar in that all require molecular oxygen and produce CO_2 as a product (1–3). A mechanism that involves a dioxetane intermediate, scheme 1, has been proposed for the luminescence of both firefly luciferin (4–6) and *Cypridina* luciferin (7).



[1]

Here, one O of the product CO_2 originates from molecular oxygen. To test this scheme, DeLuca and Dempsey studied the labeling of the product CO_2 with ^{18}O in the bioluminescence reaction of firefly luciferin (8, 9). Their data indicated that one O of the CO_2 was labeled when the reaction was carried out in $H_2{}^{18}O$ medium with $^{16}O_2$ gas, but not in $H_2{}^{16}O$ medium with $^{18}O_2$ gas. They proposed reaction scheme 2 instead of 1.



[2]

In chemiluminescence of firefly luciferin, the data reported by White *et al.* (10) were consistent with scheme 1 but did not completely rule out scheme 2 (11), in contrast to the data of DeLuca *et al.* (12), which supported scheme 2 but failed to rule out scheme 1. Studies on the bioluminescence of *Cypridina* luciferin by labeling product CO_2 with ^{18}O fully supported scheme 1 (13–15). In regard to *Renilla* luciferin, however, one O of the product CO_2 was reported to arise from solvent H_2O in both bioluminescence (3) and chemiluminescence (12), which would seem odd in view of the structural similarity between *Renilla* luciferin and *Cypridina* luciferin.

The present study unambiguously supports scheme 1, but not scheme 2, for the bioluminescence of firefly luciferin. Moreover, present data cast a serious doubt on the validity of previously reported data (3) on the bioluminescence of *Renilla* luciferin.

MATERIALS AND METHODS

Firefly D-luciferin was synthesized according to Seto *et al.* (16). $H_2{}^{18}O$ and $^{18}O_2$ were purchased from Prochem, Summit, NJ. Dilutions of $H_2{}^{18}O$ to the desired atom % were done at least 1 day prior to use. All buffer solutions were made up on the day of use. Contaminating CO_2 in $^{18}O_2$ and $^{16}O_2$ (air) was separated in advance, by immersing the container in liquid nitrogen at least 1 hr then transferring the gas into another container. Temperature was 0–5° for the purification of luciferase and 23–25° for other experiments, except as noted.

Firefly Luciferase. Purification was by the following procedure, hitherto unpublished. Acetone powder prepared from 10 g of lanterns of freeze-dried *Photinus* fireflies (collected at Princeton, NJ) was mixed with 200 ml of 25 mM Tris·HCl buffer containing 1 mM EDTA (pH 7.9 at 5°) and the pH of the mixture was readjusted to 7.9 with Tris, then centrifuged. The precipitate was mixed with 50 ml of the same buffer and the pH was adjusted to 7.9; then the mixture was centrifuged again. The supernatants were combined and fractionated with $(NH_4)_2SO_4$; the fraction precipitated between 0.32 and 0.58 saturation was saved. This preparation was purified on a column of Sephadex G-150 (Pharmacia) (2.6 × 80 cm) equilibrated with 10 mM Tris·HCl buffer containing 2 mM EDTA and 10% $(NH_4)_2SO_4$ (pH 7.75), and finally recrystallized three times by dialysis as described by Green and McElroy (17).

The luciferase of *Luciola* fireflies (collected in Japan) was extracted by the same procedure as described above. In fractionation with $(NH_4)_2SO_4$, the precipitate that formed between 0.3 and 0.6 saturation was saved. Further purification was achieved by column chromatographies on Sephadex G-150 and Ultrogel AcA 34 (LKB), using the same buffer system as used for *Photinus* luciferase. Due to rapid inactivation of this luciferase in solutions of low ionic strength, crystallization was not achieved.

Luciferase activity was assayed by recording the light emitted when 0.3 ml of 13 mM ATP (pH 7.9) was rapidly injected into a mixture of 2 ml of 20 mM Tris·HCl buffer containing 5 mM $MgCl_2$ (pH 7.9), 0.1 ml of 0.6 mM luciferin solution (pH 7.9), and a few microliters of luciferase solution to be tested, at 24–25°. Light emission reached maximum intensity in less than 1 sec. The maximum intensity for three-

Abbreviation: m/e, mass-to-charge ratio.

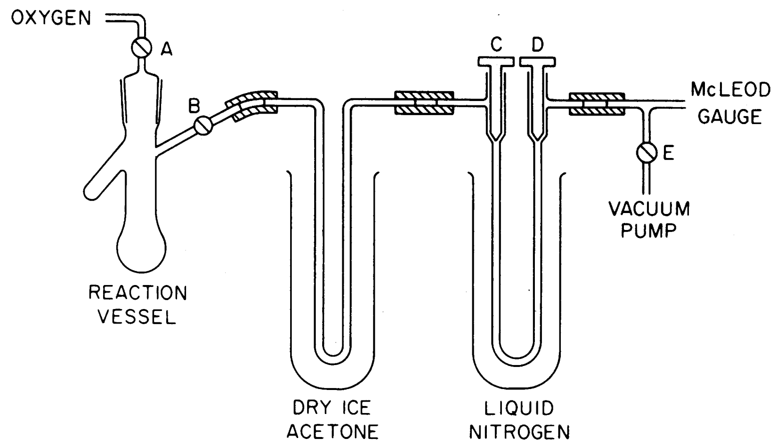


FIG. 1. Apparatus used to collect CO_2 produced in the bioluminescent reaction of firefly luciferin. The inside volume of the reaction vessel was 55 ml, C and D were Teflon plug stopcocks (Ace Glass Inc., Vineland, NJ, catalog no. 8195), and the hatched connections were Tygon vacuum tubing.

times-crystallized *Photinus* luciferase was 7.8×10^{14} photons mg^{-1} sec^{-1}, employing the absorbance $A^{0.1\%}_{1\,cm} = 0.75$ at 278 nm (18). The corresponding value for the purified *Luciola* luciferase, assuming the same absorptivity, was 0.75×10^{14} photons mg^{-1} sec^{-1}. Thus, *Luciola* luciferase was only one tenth as active as *Photinus* luciferase.

Experimental Procedure. The apparatus (Fig. 1) was evacuated to about 1 μm Hg (1 mm Hg = 133 Pa) for at least 2 hr before an experiment, in addition to a more thorough outgassing in advance of the U-tube section equipped with stopcocks C and D.

An amount of *Photinus* luciferase was first added to 1 ml of buffer (made up with $H_2{}^{16}O$ or $H_2{}^{18}O$) and dissolved with the aid of adding 25 mg of $(NH_4)_2SO_4$, then diluted with 4 ml of the buffer alone. *Luciola* luciferase was first precipitated with $(NH_4)_2SO_4$, then dissolved in 5 ml of the buffer. The luciferase solution was placed at the bottom of the reaction vessel, and 0.35 ml of luciferin solution (pH 7.9, made up with $H_2{}^{16}O$), containing 0.3 μmol of luciferin, 10 mg of ATP, and sufficient Tris to adjust the pH, was added into the side arm. Without coolant for the traps, the reaction vessel was slowly evacuated by carefully opening stopcock B (stopcocks A, C, D, and E open, with stopcock of O_2 container, not shown in Fig. 1, closed), resulting in a heavy bubbling that ceased within 2–3 min. The vessel was intermittently evacuated and stirred with a swivel motion of the vessel for the next 20 min, then, with stopcock B closed, oxygen gas was introduced into the reaction vessel (*ca* 20–200 mm Hg), and stopcock A was now closed. After the vessel had been agitated briefly (½ min) to dissolve the oxygen gas in the solutions, the two solutions were mixed vigorously to start the luminescent reaction. The bright light emission ceased in about 20 sec. At just 30 sec after mixing the vessel containing the mixture was placed into a dry ice/acetone bath and kept there for 20 min. Two traps were now immersed in the respective designated coolants (Fig. 1). Stopcock D was closed, then stopcock B was opened. CO_2 in the reaction vessel was collected in the liquid nitrogen trap by a slight opening of stopcock D. Stopcock C was closed at the pressure of 100 μm Hg to minimize contamination of the collected CO_2 with water, then stopcock D was closed at 10 μm Hg. Samples of CO_2 obtained in this manner were analyzed on a Hitachi-Perkin Elmer mass spectrometer model RMU-6D, by Morgan-Schaffer Corp., Montreal. A trace of water vapor in the CO_2 sample did not affect the results.

Determination of CO_2 plus Bicarbonate in Degassed Buffer. Two U-tube traps (Fig. 1) were omitted in this experiment. Five milliliters of test solution and 0.4 ml of 1 M $KHSO_4$ were placed in the bottom and side arm, respectively, of the reaction vessel, followed by degassing for 20 min in the same manner as described under *Experimental Procedure*. The two solutions were mixed and agitated for 30 sec, then the mixture was frozen in a dry ice/acetone bath for 20 min. After stopcock E had been closed, stopcock B was opened and the McLeod gauge was read. The amount of CO_2 was calculated from the pressure and the inner volume involved, ignoring the lower temperature of the lower part of the reaction vessel. Control experiments with known amounts of Na_2CO_3, but without buffer, indicated the experimental measurement by this procedure to be 0–15% too low, although no correction was made to the data obtained.

Calculations. When the ratio of peak heights at mass-to-charge ratio m/e 44, 46, and 48 for CO_2 is X:Y:Z, assuming that each peak is strictly proportional to the number of CO_2 molecules, the atom fraction of ^{18}O in CO_2, *C*, is given by

$$C = \frac{Y + 2Z}{2(X + Y + Z)}. \qquad [3]$$

When Z is negligibly small, and Y/X is defined equal to R,

$$C = \frac{R}{2(R + 1)}. \qquad [4]$$

It should be pointed out here that the calculation used in some previous reports (9, 19), i.e., $C = R/(2 + R)$, results in an error that increases as the value of R increases.

The atoms of incorporated oxygen per mole of CO_2, *N*, is

$$N = \frac{2(100C - 0.20)}{E - 0.20} \qquad [5]$$

in which E is the atom % of ^{18}O in $^{18}O_2$ or $H_2{}^{18}O$ used in the experiment, and 0.20 is the natural abundance of ^{18}O.

RESULTS AND DISCUSSION

In the bioluminescent oxidation of firefly luciferin in $H_2{}^{16}O$ medium with $^{18}O_2$ gas, a large part of CO_2 molecules (up to 75%) obtained from the reaction product contained one atom of ^{18}O (see Table 1, experiments 1, 3, and 5). These data alone would be sufficient to conclude that one O of any CO_2 molecule that was formed in the light-emitting process originated from the O_2 molecule that oxidized luciferin, because (*a*) any side reaction can be practically ruled out by the quantum yield of the reaction, 0.88 (20), and (*b*) the labeling of the product CO_2 with ^{18}O of $^{18}O_2$ gas is only possible through the oxidation of luciferin, and not by any exchange reaction in the condition involved. Previously reported results (8, 9, 12, 19) are contra-

Table 1. Incorporation of ^{18}O into product CO_2 in the bioluminescent oxidation of 0.3 μmol of firefly luciferin

Exp.	Buffer*	Luciferase†	Source of ^{18}O‡	Mass spectral data, %			*N*§
				m/e 44	m/e 46	m/e 48	
1¶	T	*P*, 22 mg	$^{18}O_2$ (99%)	37.2	62.6	0.2	0.63 (0.75, 0.67, 0.66, 0.45)
2	T	*P*, 22 mg	$H_2{}^{18}O$ (17.7%)	89.7	9.6	0.68	0.60
3‖	G	*P*, 21 mg	$^{18}O_2$ (95%)	74.2	25.7	0.1	0.27 (0.27, 0.27)
4	G	*P*, 21 mg	$H_2{}^{18}O$ (29.5%)	80.8	16.8	2.4	0.72
5	T	*L*, 120 mg	$^{18}O_2$ (99%)	37.1	62.7	0.2	0.63
6	T	*L*, 120 mg	$H_2{}^{18}O$ (17.13%)	91.3	8.1	0.60	0.53

* T: 25 mM Tris·HCl containing 5 mM $MgCl_2$, pH 7.8 at 25 °. G: 25 mM glycylglycine–NaOH containing 5 mM $MgCl_2$, pH 7.8.

† *P*, *Photinus* luciferase; *L*, *Luciola* luciferase.

‡ Numbers in parentheses represent atom %. Vaues for $H_2{}^{18}O$ are for the final solutions.

§ Atoms of incorporated oxygen per mole of CO_2, calculated by Eqs. 4 and 5 (experiments 1, 3, and 5) or by Eqs. 3 and 5 (experiments 2, 4, and 6). Individual data, in the case of multiple experiments, are shown in parentheses. The values corrected for the presence of contaminating CO_2 (assuming 20% for experiments 1, 2, 5, and 6, 26% for experiments 3 and 4; see *text*) are 0.79, 0.21, 0.365, 0.21, 0.79, and 0.143, respectively, for experiments 1, 2, 3, 4, 5, and 6.

¶ Average of four experiments carried out under the same conditions.

‖ Average of two experiments carried out under the same conditions.

dictory to the above. The following data and discussion are offered in explanation of the earlier results.

Four possible sources of error in the present experiments are considered, namely, (*i*) exchange of O between the COOH of luciferin and solvent H_2O, (*ii*) exchange of O between the product CO_2 and solvent H_2O, (*iii*) dilution of the product CO_2 by contaminating CO_2, (*iv*) residual $^{16}O_2$ before the introduction of $^{18}O_2$ gas.

In $H_2{}^{16}O$ medium with $^{18}O_2$, the effects of (*ii*), (*iii*), and (*iv*) are to reduce the amount of ^{18}O incorporated in the product CO_2, whereas (*i*) has no effect. In $H_2{}^{18}O$ medium with $^{16}O_2$ gas, (*i*), (*ii*), and (*iii*) all contribute to increase the amount of incorporated ^{18}O, whereas (*iv*) has no effect. Thus, corrections for decrease or increase due to these factors could only strengthen the above conclusion that one O in the CO_2 comes from O_2 gas (scheme 1).

Taking present data into account, the previously reported "exchange of O between solvent H_2O and product CO_2" in the bioluminescent oxidation of *Cypridina* luciferin (14, 15) is now considered to be the combined effect of (*ii*) and (*iii*) to a large extent.

Exchange of O between the Carboxylic Group of Luciferin and Solvent H_2O. Although no data are available with respect to firefly luciferin, oxygen exchange of carboxylic acids at neutral pH and at room temperature is generally slow (21). We assume this effect to be negligible in the present experimental conditions in which the reaction time between luciferin and $H_2{}^{18}O$ is only 30 sec.

Exchange of O between the Product CO_2 and Solvent H_2O. The reversible hydration of CO_2 may result in a considerable exchange of O even in the 30 sec reaction time (21, 22), but because of a large gas phase in the reaction vessel, it would be difficult to estimate the extent of this exchange. The fact of such exchange, however, was clearly, even though qualitatively, demonstrated in the following experiment.

The evacuated reaction vessel containing the frozen spent solution of experiment 1 ($H_2{}^{16}O$ medium) was immersed and stirred around in a water bath at 30°. As soon as the ice completely melted, the solution was again frozen in a dry ice/acetone bath and kept in the same bath for 20 min. CO_2 that had evolved from the melted solution was now collected into the liquid nitrogen trap. The mass spectrum indicated that ^{18}O in the CO_2 of this sample was only 28% of that in the CO_2 of the initially collected sample (compare Table 1).

Residual O_2 in Degassed Solutions. If the degassed solutions of luciferin and luciferase were mixed together prior to the introduction of O_2 gas, there was always some light emission without introduction of O_2, thus indicating the presence of residual O_2. We had hoped to dilute the residual O_2 with a large excess of $^{18}O_2$ in the present experiments, though this would not work if the molecules of residual O_2 were bound at or near the active site of luciferase in a manner that would not allow exchange with other O_2 molecules. The actual amount of residual O_2 was not measured in the present study due to limitations in the large amounts of firefly luciferase required in the main experiments (compare Table 1).

Presence of Contaminating CO_2. The amount of residual CO_2 in degassed solutions was studied by two methods. In the first method, CO_2 plus HCO_3^- (total carbonate) in degassed buffer solutions was directly measured by acidification as described in the *Materials and Methods* section. As shown in Table 2, Tris buffer and glycylglycine buffer both yielded considerable amounts of the total carbonate even immediately after the preparation of these solutions. Total carbonate in the glycylglycine buffer steadily increased on standing. The increase due to added luciferase was quite large.

The amount of total carbonate in the degassed solutions that were used in experiments for the data of Table 1 can be estimated from the data of Table 2 to be approximately 0.8 μmol. This total residual carbonate should contain 0.03 μmol of CO_2 in the absence of a gas phase, or should give 0.26 μmol of CO_2 after complete equilibration with the 10-volume gas phase of the reaction vessel. We suppose that the actual amount of CO_2 which diluted the CO_2 that was produced by the luminescent reaction is in between the two figures of 0.03 μmol and 0.26 μmol.

In the second method the effect of contaminating CO_2 was estimated by analyzing the mass spectral data of ^{18}O-labeled CO_2 obtained in $H_2{}^{18}O$ medium with $^{16}O_2$ gas. When $C^{16}O_2$ is labeled in a large excess of $H_2{}^{18}O$, of which the atom fraction of ^{18}O is A, the ratio of m/e 44 ($C^{16}O_2$), m/e 46 ($C^{16}O^{18}O$), and m/e 48 ($C^{18}O_2$) will be

$$(1 - a)^2 : 2a(1 - a) : a^2$$

in which a = A at the complete equilibration of labeling, and a < A before the equilibrium is reached. The mass spectral data of experiments 2, 4, and 6 all clearly deviate from this ratio. We consider this to be largely due to the influence of contaminating CO_2 which was completely equilibrated with $H_2{}^{18}O$ of the buffer solution.

Table 2. Amount of CO_2 plus bicarbonate (μmol) in 5 ml of buffer solution degassed for 20 min

Buffer	Time after the preparation of buffer			
	<1 hr	6 hr	1 day	1 week
25 mM Tris·HCl containing 5 mM $MgCl_2$, pH 7.8 at 25°	0.13		0.16	0.23
The same buffer as above plus 15 mg *Photinus* luciferase	0.60			
25 mM glycylglycine–NaOH containing 5 mM $MgCl_2$, pH 7.8	0.16	0.42	0.8 (0.6*)	1.6

* Instead of the present method of degassing, the solution was degassed by three cycles of freezing, evacuation, and thawing, with a dry ice/acetone bath used to freeze the solution. Although the freezing procedure appeared more efficent, it rapidly inactivates firefly luciferase.

In the equations below, we take the fraction of contaminating CO_2 in the total CO_2 as b, the atom fraction of ^{18}O in the medium water as A, and the observed ratio of m/e 44, m/e 46, and m/e 48 as X:Y:Z, wherein X + Y + Z = 1. We assume that the contaminating CO_2 is completely equilibrated with $H_2{}^{18}O$ medium of the reaction mixture.

$$X = b(1 - A)^2 + (1 - b)(1 - a)^2 \quad [6]$$

$$Y = 2bA(1 - A) + 2a(1 - b)(1 - a) \quad [7]$$

$$Z = bA^2 + (1 - b)a^2 \quad [8]$$

The first terms on the right side of these equations represent the contribution of contaminating CO_2. By elimination of a and $(1 - a)$ from Eqs. **6**, **7** and **8**,

$$b = \frac{4XZ - Y^2}{4A^2X + 4Z(1 - A)^2 - 4AY(1 - A)}. \quad [9]$$

The values of b calculated by Eq. **9** for experiments 2, 4, and 6 are 0.20, 0.26, and 0.20, respectively. These values correspond to the amounts of contaminant CO_2 of 0.075 μmol, 0.1 μmol and 0.075 μmol (based on 0.3 μmol of CO_2 produced), which amounts are well within the range estimated from the data of Table 2 discussed above. The contributions of ^{18}O in the contaminant CO_2 to the total ^{18}O found in experiments 2, 4, and 6 are calculated as 65%, 71%, and 73%, respectively, by Eqs. **7** and **8**. These figures indicate that the data of experiments 2, 4, and 6 given in Table 1, for experiments in $H_2{}^{18}O$ medium with $^{16}O_2$ gas, are hardly meaningful in the interpretation of the reaction mechanism.

In studies by DeLuca *et al.* (8) and Tsuji *et al.* (19)‡, only 33 nmol/6.5 ml of firefly luciferin (about 1/10 of the present experiment) was used. Therefore, the effect of contaminant CO_2 relative to the CO_2 formed in the luminescence reaction should be far greater than in the present investigation. The situation seems worse in the study of *Renilla* bioluminescence (3), in which the luminescent reaction of 39 nmol/3.7 ml of *Renilla* luciferin took 40 min to complete, thereby adding more exchange of O between medium H_2O and the CO_2 produced.

Use of ^{13}C-Labeled Luciferin. On the basis of the present data, we propose the use of ^{13}C-labeled luciferin (90 atom % or more) labeled at the carbon that yields CO_2 in the luminescence reaction ($^{13}COOH$ in the case of firefly luciferin), instead of a regular luciferin. The use of such a luciferin will make it possible to distinguish readily by mass spectrometry the CO_2 that is formed in the luminescent reaction from contaminating CO_2. This technique should be especially effective when a very small amount of luciferin (0.1 μmol or less) is used, or when the amount of contaminating CO_2 and bicarbonate is not sufficiently small.

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‡ In the same paper, three sets of data were reported on the source of O in CO_2 produced in the *Cypridina* bioluminescent reaction, contradictory to more than 40 data previously reported (13–15). In the procedure used by Tsuji *et al.*, degassing prior to the luminescent reaction possibly did not remove any contaminating CO_2 from the reaction vessel, because one side arm of the vessel was in liquid nitrogen at the time of evacuation. Moreover, the reported values of incorporated O in the bicarbonate of reaction medium seem mysteriously small; the time needed for 50% equilibrium of O between bicarbonate and solvent H_2O at pH 7.8 at room temperature can be estimated to be approximately 1 hr (22). The values reported by Tsuji *et al.* thus correspond to less than 5 min of the equilibration process. Consequently, the analyzed CO_2 arose probably not from the bicarbonate, at least to a large extent. Clarification of these matters is needed in order to evaluate the results they reported.