EXCHANGE OF OXYGEN BETWEEN SOLVENT H_2O AND THE CO_2 PRODUCED IN CYPRIDINA BIOLUMINESCENCE[1]

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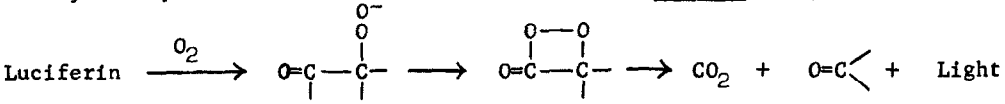
SUMMARY. Bioluminescent oxidation of Cypridina luciferin yields CO_2 besides oxyluciferin and light. The exchange of oxygen between the CO_2 and H_2O of the solvent becomes significant when less than approximately 1 μmol of luciferin is reacted in 4 ml of buffer solution, and the exchanged oxygen in CO_2 markedly increases by decreasing the amount of luciferin. Such an exchange is to be expected in any such system which produces CO_2 in aqueous solution, and must be taken into account in interpreting the results of experiments.

INTRODUCTION

The luminescent oxidation of luciferins of Cypridina, the firefly and Renilla, each catalyzed by the appropriate luciferase in the presence of O_2, all produce CO_2 as one of the products (1,2,3), and information concerning the origin of oxygen in this CO_2 is an important factor in elucidating the mechanism of these reactions.

In the firefly and Renilla reactions, respectively, it has been reported that one or two oxygens in CO_2 arise from H_2O of the reaction medium (3,4), whereas, in Cypridina, we have reported that one of oxygens in CO_2 comes from gaseous O_2 (5). Thus, the following mechanism, which had been suggested for the luminescence of both Cypridina and the firefly (2,6-10) was concluded to be valid for the Cypridina reaction (5), whereas a different mechanism became necessary to explain the results for the firefly and Renilla (3,4).



The structure of Renilla luciferin reported previously (3) has been recently modified, and as a consequence the part of the structure which is directly involved in the reaction now seems to be exactly same as the corres-

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ponding part of Cypridina luciferin (11). A question naturally arises, therefore, as to why the luminescence reactions of Cypridina and Renilla, which have functionally the same structure of luciferins, should have different mechanisms.

The present study was intended to clarify the question just stated, and also to answer some of the comments previously expressed (10) regarding the reported result of ^{18}O incorporation in the firefly reaction (4). We focused our study on the amount of luciferin used in the reaction, because only 0.03 - 0.04 μ mol of luciferin was used in the firefly and Renilla reactions (3,4) in contrast to the much larger amount, viz, 3.5 μ mol of luciferin used in the Cypridina reaction (5).

MATERIALS AND METHODS

Cypridina luciferin dihydrobromide was dissolved in 50% methanol (3-10 mg/ml). Electrophoretically pure Cypridina luciferase (12) was suspended in saturated $(NH_4)_2SO_4$ solution (3-10 mg/ml). Throughout this study, 0.02 M glycylglycine buffer, pH 7.8, containing 0.04 M NaCl was used. The buffer was freshly prepared before use with $H_2{}^{18}O$ (enrichment 4.4%) or with regular H_2O, and the total CO_2 (including carbonate) in the buffer prepared with regular H_2O was determined to be less than 20 nmol/ml.[2] Oxygen gases, $^{18}O_2$ (enrichment 93.3%) and $^{16}O_2$ (air), were shaken with 10% NaOH before use.

The apparatus (Fig. 1) had been continuously evacuated at approximately 1 μ Hg for at least 6 hours prior to experiments. An amount of the luciferase preparation and 4 ml of the buffer were placed at the bottom of the reaction vessel, and the luciferin solution plus 0.2 ml of water into the side arm. Usually, the molar ratio of luciferin·2HBr to luciferase was 1 : 0.01-0.015.

The reaction vessel was evacuated, without coolants for the traps, while

[2] For the determination of the total CO_2, 100 ml of the buffer plus 0.2 ml of H_2SO_4 were bubbled with CO_2-free argon, and CO_2 thus taken up in the argon was then absorbed in 5 ml of 0.002 N NaOH. This NaOH solution was titrated with 0.01 N HCl using phenolphthalein as the indicator.

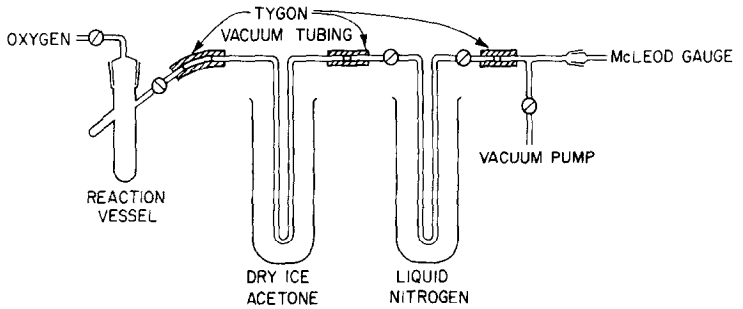

Fig. 1. Apparatus used for the bioluminescent oxidation of Cypridina luciferin and for the collection of the resulting CO_2. The inside volume of the reaction vessel was 44 ml. The mechanical (2-stage) vacuum pump employed was equipped with a dry ice - acetone trap (not shown). For the two stopcocks of the liquid nitrogen trap, Teflon plug stopcocks (Ace, Cat. No. 8195) were used in preference to ground glass stopcocks. Total CO_2 in the reacting solutions after the degassing, and also total CO_2, which became desorbed from glass walls, Tygon tubing, etc. and trapped in the liquid nitrogen trap, were estimated to be both less than 5 nmol.

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stirring with a swivel motion. After bubbling ceased in a minute or so, the vessel was further stirred for 10 minutes with intermittent evacuation.

Two solutions in the reaction vessel were mixed together at 20°C, then $^{18}O_2$ was introduced to the vessel with vigorous stirring. In experiments with $^{16}O_2$ and $H_2{}^{18}O$, however, the sequence of mixing solutions and introducing O_2 was reversed to minimize possible exchange of oxygen between luciferin and $H_2{}^{18}O$. Although the resulting light emission ceased usually in 20 sec., the stirring was further continued with the intention of enlarging the suspected effect of oxygen exchange between CO_2 and H_2O. After 45 sec. of stirring, the solution in the reaction vessel was quickly frozen in a dry-ice acetone bath.

A short piece of glass capillary filled with 1-2 mg of P_2O_5 was inserted into the second trap, and this trap as well as the first trap were now placed in the respective coolants. Finally, CO_2 in the reaction vessel was collected in the second trap under a vacuum of 5-7 μ Hg.

The CO_2 was analyzed on a Hitachi-Perkin-Elmer mass spectrometer Model RMU-6D, at Morgan-Schaffer Corporation, Montreal. From the ratio of

(m/e 46)/(m/e 44), R, the atom per cent of ^{18}O in CO_2, A, was calculated as

$$A = \frac{R}{2(R + 1)} \times 100$$

Thus, the number N of ^{18}O incorporated into CO_2, is given by

$$N = \frac{2(A - 0.20)}{B - 0.20}$$

where B is the atom per cent of ^{18}O in $^{18}O_2$ or $H_2{}^{18}O$, and 0.20 is the natural abundance (%) of ^{18}O.

RESULTS AND DISCUSSIONS

The experimental results are shown in Fig. 2. When luciferin in $H_2{}^{16}O$ was oxidized by $^{18}O_2$ in the presence of luciferase, thus resulting in the formation of CO_2, the number N of ^{18}O found in the CO_2 was close to 0.8 for more than 1 μmol of luciferin, whereas the number decreased to 0.13 for 0.053 μmol of luciferin. Because the quantum yield of the reaction is not appreciably affected by this range of concentration of luciferin (13), the decrease in the incorporation of ^{18}O should be attributed to the exchange of oxygen between $C^{16}O^{18}O$ and $H_2{}^{16}O$, rather than to an alteration in the mechanism of exidation. The incomplete incorporation of ^{18}O even at the highest concentration of luciferin tested may indicate either a simple exchange, the same as above, or a possible difference in pathway of oxidation as previously suggested (5).

An increase in the amount of luciferase did not significantly influence the results; thus we believe that the exchange of oxygen between CO_2 and H_2O is not catalyzed by luciferase and is not an aspect limited to the Cypridina system.

When experiments were carried out with $H_2{}^{18}O$ and $^{16}O_2$, the ^{18}O taken into CO_2 at low concentrations of luciferin was much larger than the decrease in the incorporation of ^{18}O at the corresponding concentrations of luciferin when $H_2{}^{16}O$ and $^{18}O_2$ were used. This is easily understandable from the

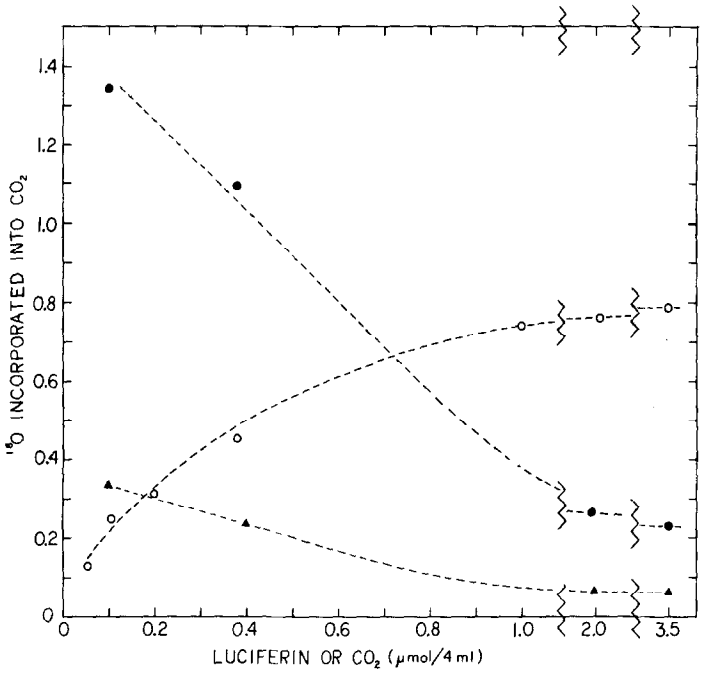

Fig. 2. The number N (see equation in text) of ^{18}O found in CO_2 produced by the bioluminescent oxidation of Cypridina luciferin with gaseous $^{18}O_2$ in $H_2{}^{16}O$ (○), and with gaseous $^{16}O_2$ in $H_2{}^{18}O$ (●). Control experiment with $C^{16}O_2$ instead of luciferin plus luciferase (▲). Lines were drawn by inspection. Reproducibility of the experiments was ± 3% and ± 15%, respectively, at the highest and the lowest concentration of luciferin.

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following reaction scheme, inasmuch as the rate going to the left side is twice that of the rate going to the right side (14).

$$H_2{}^{16}O + C^{16}O^{18}O \rightleftharpoons H_2{}^{18}O + C^{16}O^{16}O$$

The control experiments with CO_2 instead of luciferin, were not quite adequate in purpose, because all the CO_2 was in the gas phase, which was 10 times the volume of the solution, at the start. The results, however, still demonstrated a considerable exchange of oxygen when small amounts of CO_2 were used.

Although the exchange of oxygen between CO_2 and H_2O found in the present study can be suppressed to some extent, if wanted, by shortening time and lowering temperature of the reaction, still a considerable exchange is to be expected at the concentrations of luciferin or CO_2 less than 0.5 μmol/4 ml. In previously reported experiments in the firefly system, however, only 33

nmol of firefly luciferin per 6.5 ml of buffer were used (4), which is less than half the minimum concentration tested in the present study. It is evident that such a small amount of luciferin is not adequate to obtain a really reliable conclusion by the procedure employed.

In experiments on the Renilla system, the luminescence reaction of 39 nmol of Renilla luciferin (in 3.7 ml of buffer) took 40 min. for completion (3). Although the phosphate buffer, pH 7.2, employed may give somewhat less exchange than the buffer presently employed, as judged from our preliminary experiment, such a slow release of CO_2 from such a small amount of luciferin would result in an almost complete equilibrium of oxygen between CO_2 and H_2O.

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