THE DECARBOXYLATION OF LUCIFERIN IN FIREFLY BIOLUMINESCENCE

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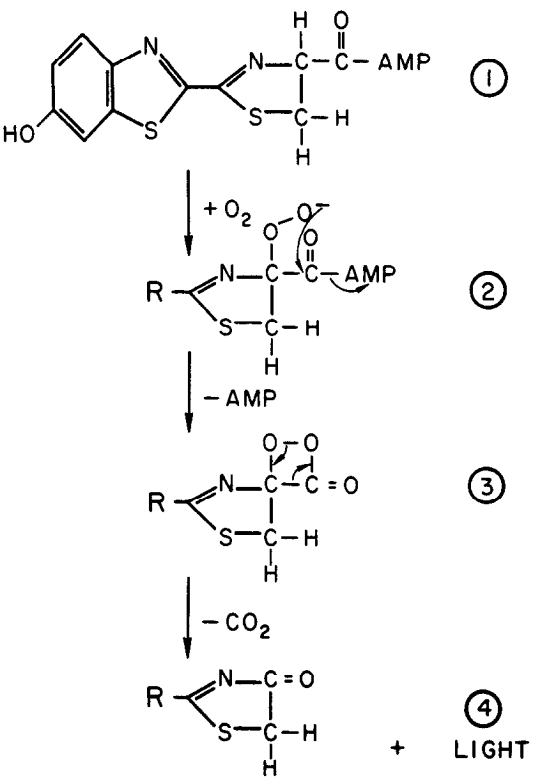
Light emission in the firefly, Photinus pyralis, has been shown to involve the enzyme catalyzed oxidation of D-luciferyl adenylate (LH_2AMP) in which one mole of molecular oxygen is used per mole of substrate (Rhodes and McElroy, 1958; McElroy and Seliger, 1962). The adenylate is formed by a prior reaction of luciferin and ATP as shown in equation (1)

$$LH_2 + ATP + E \overset{Mg^{2+}}{\rightleftarrows} E \cdot LH_2AMP + PP \qquad (1)$$

Evidence has been presented recently concerning the mechanism of the chemiluminescence and the red bioluminescence of LH_2AMP (Hopkins, Seliger, White and Cass, 1967; McCapra, Chang and Francois, 1968). We now report experiments which support this mechanism for the enzyme catalyzed reaction leading to yellow-green emission.

Results and Discussion

The reaction scheme suggested by Hopkins et al. and McCapra et al., 1968 for the chemiluminescent reaction and the red bioluminescence (Fig. 1), indicates that CO_2 liberation should accompany light emission. The reaction scheme was analogous to that for chemiluminescence of lophine (White and Harding, 1964), acridinium carboxylic acids (McCapra and Richardson,1966) and a Cypridina luciferin analog (McCapra and Chang, 1967). A loss of adenylic acid prior to light emission is also indicated. It had been shown previously that adenylic acid liberation accompanies light emission but it could not be determined whether this occurred prior to, during or after light emission (McElroy and Green, 1956).



Support for the mechanism shown in Figure 1, which involves a decarboxylation has come from the bioluminescent reaction of carboxyl labelled C^{14}-luciferin. The more readily available DL-racemic mixture was used here rather than the active D-isomer. Since L-luciferin is not oxidized in the enzymatic reaction (White et al., 1961) only 50% of the initial radioactivity should be found as $C^{14}O_2$. The total absence of light emission from an L-LH_2, ATP, enzyme and Mg^{2+} mixture suggests that racemication under these conditions is negligible. The percentage recovery of counts from the bioluminescence is shown in Table 1.

When luciferase was absent from the reaction mixture no CO_2 liberation was observed. The recovered luciferin (presumably the L form) was inactive for light production when added to a reaction mixture containing enzyme, Mg^{2+} and ATP. The only radioactivity detected on paper chromatographs was in the

Table 1

	Total C.P.M.	Relative counts percent 100 = 1.7 μC.
DL-LH_2 starting material	70,000	100
$C^{14}O_2$ as Na_2CO_3	29,260	41.8
EtOAc extracts of final reaction mixture	30,400	43.4 as LH_2 40.0 as L 3.4
Remnants after EtOAc extraction	4,970	7.1
Total recovery	64,600	92.3

regions associated with luciferin or dehydroluciferin. Under the conditions used the quantum yield of the bioluminescence reaction should be high (approximately 0.9) (Seliger and McElroy, 1960). The fact that over 80% of the C^{14} in the carboxyl group of D-luciferin appeared as CO_2 is strong support for the proposed mechanism presented in Figure 1.

The proposed scheme suggests that the initial product of the light reaction should be the thiazolinone (compound 4). We have attempted to isolate the immediate product(s) of this reaction. Paper chromatographs of the methanol extracts of the bioluminescence reaction mixture showed four spots. Two of these corresponded in Rf to luciferin and dehydroluciferin, and together with a trace of a blue fluorescent spot at Rf 0.88 were present with similar relative intensities in the blank solutions. Dehydroluciferin is known to be formed readily by non-enzymatic oxidation of basic LH_2 solutions (White et al., 1961). The fourth spot, a pink streak Rf 0-0.1, resulted only from the light emitting reaction. Chromatography on LH20 Sephadex or silica gel showed the pink material to be a composite of three distinct compounds designated here A, B, and C. Since the enzymatic reaction is strongly inhibited by a product, only very small amounts (c. 1 mg.) of these materials were available. These highly colored compounds were very unstable in acidic

or basic solutions but the materials had common degradation products and the conversion of A to B and C and of B to C under mild conditions was demonstrated. A summary of ultraviolet spectra is given in Table 2.

Table 2

Compound	Color	λmax in MeOH	λmax with added base
A	pink	rapidly gives a mixture of B and C	
B	orange	278, 366, 486	320, 390(s), 430(s), 475
C	blue	304, 392, 650	276, 365, 470, 700

If the thiazolinone (4) is the immediate product, then it must be very unstable and capable of reacting with itself or other chemicals in the environment. Results which suggest this possibility were obtained when we attempted to synthesize compound 4 by the reaction of 2-cyano-6-hydroxybenzothiazole with ethyl α-mercaptoacetate. Under the conditions used the product is not the expected thiazolinone but a mixture identical with compounds A, B and C obtained from the enzymatic reaction. Comparison was made by ultraviolet spectra at various pH values, behavior on Sephadex and silica gel, Rf of paper chromatographs and identical interconversions. The weak fluorescence of both synthetic and extracted A, B and C, and the lability in basic solution precluded a comparison of fluorescence excitation or emission spectra. Since analogous syntheses of this type give the expected products,* it is reasonable to assume that compound 4 is formed as a direct product of the bioluminescence reaction and is subsequently converted into A, B and C. Fluorescent intermediates were detected in the reaction mixture** but it has not been possible to isolate or identify these transient species. Compounds B and C have also been detected in spent LH_2AMP chemiluminescence reaction mixtures that have been rapidly diluted with anhydrous ethanol and chromatographed.

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*For example the 5,5-dimethyl analog (Hopkins et al., 1967)

**We wish to thank Dr. H. H. Seliger for recording emission spectra of these reaction mixtures.

Further evidence that supports this scheme for bioluminescence is the fact that the oxidative reaction proceeds at a slower rate if the hydrogen atom at C_4 in luciferin is replaced by deuterium (White and DeLuca, unpublished). Little can be said at this stage about the nature of the excited state responsible for the emitted yellow-green light; it should be remembered that the structures shown in Figure 1 refer to enzyme bound species.

Experimental

Luciferase was a five times crystallized preparation (Green and McElroy, 1956). 2-cyano-6-hydroxybenzothiazole was prepared from p-anisidine (Seto et al., 1963). D-luciferin and DL-C^{14}-luciferin were prepared by the condensation of the cyano compound and D-cysteine and DL-1-C^{14}-cysteine respectively (White et al., 1964). Chromatographs of these materials showed only one fluorescent spot; with C^{14}-luciferin > 98% of the radioactivity was concentrated in this spot.

Paper chromatographs were developed using a 70:30 mixture of 95% ethanol and 1 M ammonium acetate solution at pH 7.5. Spots were detected by fluorescence under a u.v. lamp; radioactive compounds were estimated by a strip counte

Methods

The products of the bioluminescent reaction were isolated as follows: A solution of D-luciferin (30 mg) in phosphate buffer (15 ml, 10^{-3} M, pH 7.8) and a mixture of ATP (10^{-3} M) and $MgSO_4$ (10^{-3} M) in phosphate buffer (100 ml) were added in portions to a solution of luciferase (29.4 mg) in buffer (1.50 ml) and the resulting mixture was shaken in air until no further emitted light could be detected. The pH was maintained at 7.6-7.8, the luciferin solution was kept under nitrogen prior to addition, and similar solutions lacking enzyme or ATP served as blanks. The pH of the mixture was adjusted to 7.0, the solution was evaporated to dryness at a temperature $\geq 35^{\circ}$, the residue was extracted with methanol and chromatographed on LH20 Sephadex, silica gel or paper. Similar results were obtained using glycylglycine or tris buffer solutions.

The above mixture yielded strongly colored compounds which were also obtained from the reaction of ethyl α-mercaptoacetate (0.2 ml, 1 mmole) and 2-cyano-6-hydroxybenzothiazole (176 mg, 1 mmole) in 50% aqueous methanol at pH 7.6 under nitrogen.

Carbon dioxide evolution from the bioluminescent reaction was estimated as follows: A solution of ATP and $MgSO_4$ (0.1 M, 0.3 ml) was injected into a closed tube containing luciferase (6 mg), C^{14}-luciferin (8.4 ug, 0.17 uC) and glycylglycine buffer (0.2 ml, 0.1 M) at pH 7.6. The mixture was shaken gently until light emission ceased. The pH was adjusted to 6.2 and a slow stream of nitrogen was bubbled through the solution and into a sodium hydroxide solution (1 ml, 1M). Aliquots of the latter solution were plated and counted directly, a small correction was made for absorption by the sodium hydroxide. The spent reaction mixture was acidified to pH 3, extracted with ethyl acetate and the extracts were chromatographed on paper. Luciferin recovered in this way was tested for light production with luciferase ATP and Mg^{2+}.

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References

Green, A. A. and McElroy, W. D. (1956) Biochim. Biophys. Acta 20, 170
Hopkins, T. A., Seliger, H. H., White, E. H. and Cass, M. W. (1967), 89, 7148
McCapra, F. and Chang, Y. C. (1967) Chem. Comm., 1011
McCapra, F., Chang, Y. C. and Francois, V. P. (1968) Chem. Comm., 22
McCapra, F. and Richardson, D. G. (1964) Tetrahedron Letters, 3167
McElroy, W. D. and Green, A. A. (1956) Arch. Biochem. Biophys. 64, 257
McElroy, W. D. and Seliger, H. H. (1962) Federation Proc. 21, 1006
Rhodes, W. C. and McElroy, W. D. (1958) J. Biol. Chem. 233, 1528
Seliger, H. H. and McElroy, W. D. (1960) Arch. Biochem. Biophys. 88, 136
Seto, S., Ogura, K. and Nishiyama, Y. (1963) Bull. Chim. Soc. Japan 36, 332
White, E. H., McCapra, F., Field, F. G. and McElroy, W. D. (1961) J. Am. Chem. Soc. 83, 2402