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STUDIES IN BIOLUMINESCENCE

IV. PROPERTIES OF LUCIFERIN FROM *PHOLAS DACTYLUS*

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SUMMARY

Light could be obtained by the addition of Fe^{2+} to purified luciferin from *Pholas dactylus* in the absence of luciferase. The total light emitted was proportional to the concentration of luciferin used. The characteristics of this nonenzymic emission correspond to those of the fast reaction previously described. It may have a physiological importance since iron is present in the luciferin. The injection of Fe^{2+} alone was not sufficient; the presence of a complexing agent such as phosphate or CN^- or EDTA was also necessary. Light emission could also be obtained by the addition of H_2O_2, in the presence of Fe^{2+}, to luciferin. It has been demonstrated that, for a given amount of luciferin, the total light emitted by the action of varying ratios of Fe^{2+} and luciferase is constant.

INTRODUCTION

We have previously shown[1] that Fe^{2+} has a marked effect on the emission of light by the Pholade luciferin–luciferase system. The present study describes the light emission stimulated by the addition of Fe^{2+} to luciferin in the absence of luciferase. Whereas an addition of Fe^{2+} to luciferase alone has no effect, a similar treatment of a mixture of luciferase and luciferin results in a change in the kinetics of light emission. Somewhat surprisingly it was also noted that Fe^{2+} stimulates light emission when added to pure luciferin in phosphate buffer in the absence of the enzyme luciferase. The characteristics of this emission correspond to the fast reaction previously described rather than to the slow emission. In view of these results, an estimation of the iron in highly purified luciferin was made using the bathophenanthroline technique (this is applicable only to noncovalently bound metal). Assuming a molecular weight of 47500 (which would be in excellent agreement with that determined by filtration methods), a value was obtained corresponding to 1 atom of iron per molecule of luciferin.

RESULTS AND DISCUSSION

Properties of the luminescence system of luciferin and Fe^{2+}

The incubation mixture contained 0.1 M potassium phosphate (pH 7.0), 0.5 M NaCl, 0.1 mM Fe^{2+} *plus* luciferin. Both the maximum of emission and the total light

emitted are proportional to the concentration of luciferin, and the reaction is first order for at least 5 min (Figs. 1a and 1b) with a rate constant which is independent of the concentration of luciferin. It is to be noted that 0.1 μg of luciferin can easily be measured in this reaction, and that in fact 0.001 μg could be detected.

Effect of the concentration of Fe^{2+}

Both I_{max} and L increase with increasing concentrations of Fe^{2+} up to a limit of 0.1 mM (Table I). The kinetics remain first order independent of the Fe^{2+} concen-

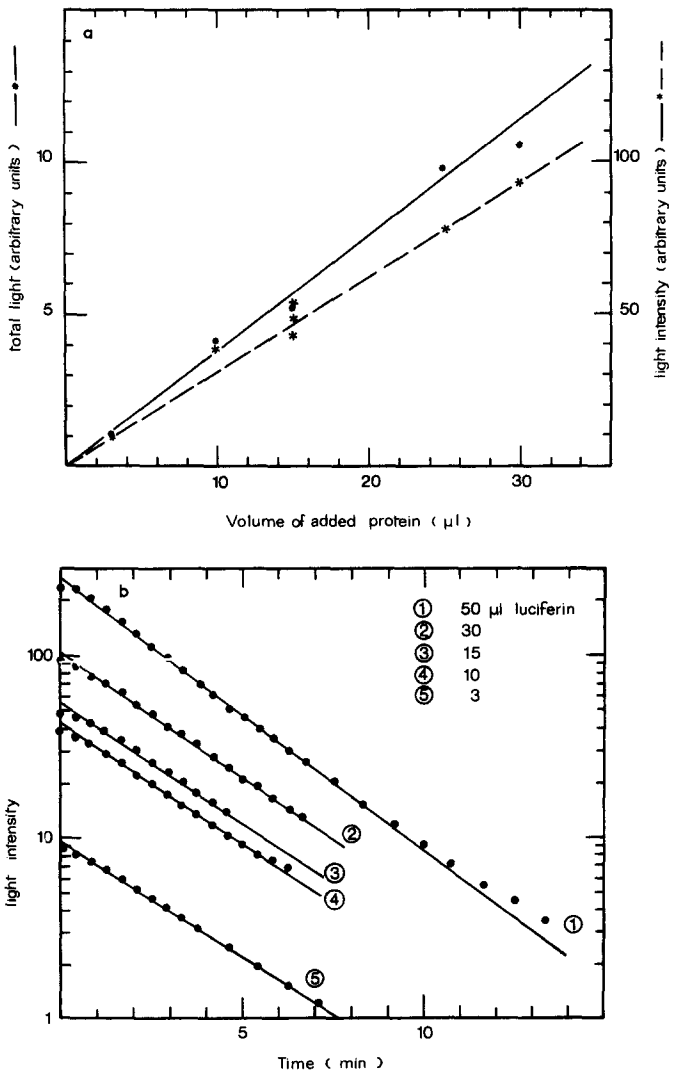

Fig. 1. a. Effect of luciferin concentration on I_{max} and L. Purified luciferin is used. The reaction mixture contains luciferin (stock solution at 0.6 mg/ml), phosphate buffer (pH 7.0; 1 M) 0.3 ml, NaCl (2 M) 0.75 ml and water 1.4 ml. The reaction was initiated by injection of 0.5 ml of a solution of $FeSO_4$ (final concn. 0.1 mM). b. Semi-logarithmic representation of the kinetics. Zero time is at I_{max}.

tration, and except at saturation or at extremely low levels of Fe^{2+} the rate constant is also independent (100–1 μM) (see Fig. 2). The emission of light can be used to detect 0.1 μM Fe^{2+}. The emission can be repeatedly stimulated by the addition of 10 μM Fe^{2+} several times.

TABLE I

EFFECT OF Fe^{2+} CONCENTRATION

Effect of $FeSO_4$ concentration on total light. Details of the experiment are given in Fig. 2.

Fe^{2+} concn. (μM)	*L (arbitrary units)*
0.1	27
0.5	71
1	354
5	4 783
10	11 360
50	25 530
100	55 000
330	62 000
600	65 760

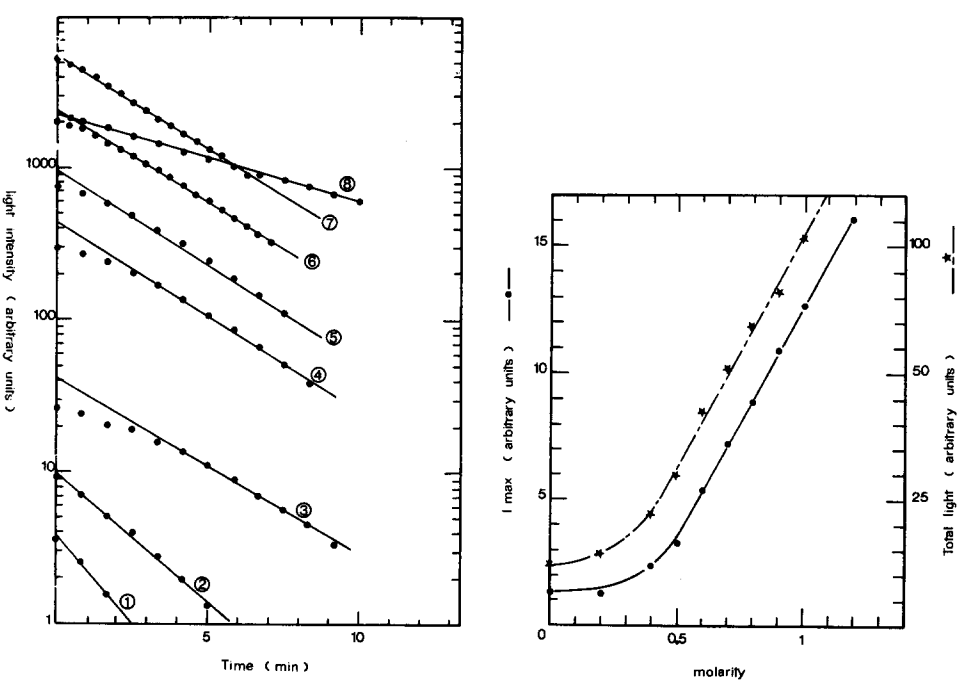

Fig. 2. Effect of $FeSO_4$ concentration. The components in the cuvette are as in Fig. 1, using 30 μl of the purified luciferin solution (final concn. 6 μg/ml). The zero time is at I_{max}. The final sulphate concentrations were: 1, 0.1 μM; 2, 0.5 μM; 3, 1 μM; 4, 5 μM; 5, 10 μM; 6, 50 μM; 7, 330 μM; 8, 600 μM.

Fig. 3. Effect of ionic strength on I_{max} and L. The components are as in Fig. 1 with a final concentration of purified luciferin of 5 μg/ml and $FeSO_4$ 0.1 mM. NaCl varies from 0 to 1.2 M final concn.

Effect of ionic strength

Both I_{max} and L increase with increases in the NaCl concentration up to at least 1.4 M, the kinetics remaining first order (except in extremely dilute solutions) (Fig. 3). In addition, the rate constant also increases, from a value of 0.055 sec^{-1} in 0.2 M NaCl to 0.115 sec^{-1} in 1.4 M NaCl.

Effect of pH

In phosphate buffer, I_{max} shows a maximum at pH 8, whereas the total light emitted (L) shows a maximum at pH 7 (Table II). The rate constant shows a large variation between pH 6 and 7.5 (Fig. 4), increasing with increases in pH.

TABLE II

EFFECT OF pH ON L

The experiment is described in Fig. 4.

pH	*L*	I_{max}
6.0	54 807	$2.18 \cdot 10^{-4}$
6.5	61 896	$4.4 \cdot 10^{-4}$
7.0	72 925	$1.1 \cdot 10^{-3}$
7.5	56 590	$1.13 \cdot 10^{-3}$
8.0	55 200	$1.83 \cdot 10^{-3}$
8.6	39 110	$1.72 \cdot 10^{-3}$

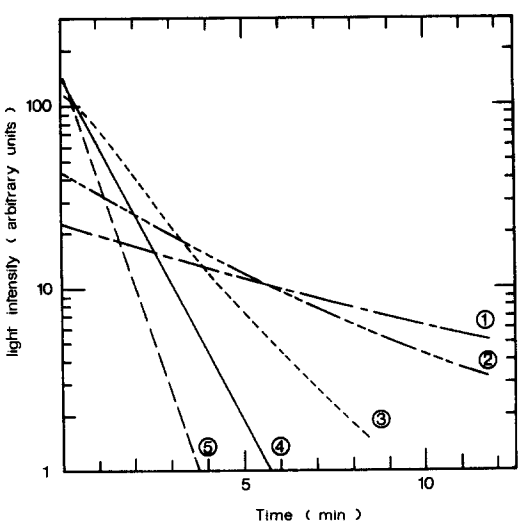

Fig. 4. Variation of the kinetics with pH. In the cuvette: purified luciferin (0.6 mg/ml) 30 μl, phosphate buffer (1 M) 0.3 ml, NaCl (5 M) 1.2 ml, water 0.5 ml. In the syringe: $FeSO_4$ (1 mM) 0.3 ml, NaCl (5 M) 0.6 ml, water 0.1 ml. Zero time is at I_{max}. The pH's of the phosphate buffers were: 1, pH 6.0; 2, pH 6.5; 3, pH 7.0; 4, pH 7.5; 5, pH 8.0.

Effect of the nature of the buffer used

In the work so far described, a phosphate buffer was used. On replacing this by other buffers such as Tris, acetate or citrate, the light emission of luciferin stimu-

lated by Fe^{2+} was greatly reduced. Hence, phosphate plays an essential role in the reaction. Indeed, emission is dependent on the order of addition of reagents. Thus when luciferin is injected into a mixture of Fe^{2+} and phosphate buffer, no light is produced. On the other hand, if phosphate is added to a mixture of Fe^{2+} and luciferin in 0.5 M NaCl (nonluminous) a flash is observed, the characteristics of which are strictly comparable to those obtained by adding Fe^{2+} to luciferin in phosphate buffer. The I_{max} is proportional to the quantity of phosphate injected up to at least 0.5 M phosphate, with an increase in the rate constant simultaneously.

It thus appears that phosphate ions are necessary but that the active component is not ferrous phosphate, and it must be concluded that the interaction of Fe^{2+} and luciferin is extremely rapid, since a flash is obtained even when Fe^{2+} is injected into a solution of luciferin containing a very large excess of phosphate.

Effect of other complexing agents in the absence of phosphate

EDTA. It was found that phosphate ions could be replaced by reagents such as EDTA and CN^- which also complex with iron. Either EDTA was injected into a solution of luciferin and Fe^{2+} in NaCl at pH 7.0, or alternatively the Fe^{2+} was injected into a solution of luciferin, EDTA and NaCl at pH 7. Using the second approach with the injection of 0.1 mM Fe^{2+}, a maximum of emission was obtained with about 0.1 mM EDTA (Table III), though the intensity was some 10 times less than the I_{max} induced by a similar injection of phosphate and the total light emitted some 4 times less. The kinetics of emission were essentially first order though the rate constant was smaller than that observed with phosphate ions. Under conditions of EDTA deficiency the kinetics become more complicated.

CN^-. In a similar manner (injection of Fe^{2+} into a nonbuffered solution of luciferin and CN^- in NaCl at approx. pH 7), the effect of CN^- on light emission was studied. For a given concentration of Fe^{2+}, the I_{max} varies with CN^- concentration and the optimal concentration is a function of the quantity of Fe^{2+}. Under optimal conditions (Fe^{2+} concentration 10 μM and CN^- 27 μM), I_{max} is twice as large as that obtained in the system containing 0.1 M phosphate and 0.1 mM Fe^{2+}, while L is

TABLE III

EFFECT OF EDTA CONCENTRATION

In the cuvette: purified luciferin (stock solution 0.6 mg/ml) 25 μl, EDTA 0.3 ml, NaCl (5 M) 0.3 ml, water 1.6 ml. In the syringe: $FeSO_4$ (1 mM) 0.3 ml, water 0.7 ml. The control is as described in Fig. 2.

EDTA concn. (*mM*)	I_{max} (*arbitrary units*)
0.024	$1.05 \cdot 10^{-5}$
0.081	$2.9 \cdot 10^{-5}$
0.27	$3 \cdot 10^{-5}$
0.9	$1.3 \cdot 10^{-5}$
3	$3 \cdot 10^{-6}$
10	$4.5 \cdot 10^{-7}$
Control with phosphate (0.1 M)	$3 \cdot 10^{-4}$

TABLE IV

EFFECT OF CN^- CONCENTRATION

Effect of CN^- concentration on I_{max}. The components of the reaction are as in Table III.

CN^- concn. (μM)	I_{max}			
Fe^{2+} concn.	0.1 mM	10 μM	1 μM	0
3300	0.5			
1000	7.2–9			120
300	320			75
90	850	4000		50
27	750	6000	210	17
8.1	30	300–550	950	48
2.4		45	15	16–22–34
Control with phosphate (0.1 M)	3200			

reduced by a third (Table IV). The kinetics are first order. Under limiting conditions of CN^-, the rate constant is reduced. However in an excess of CN^-, it is independent of the CN^- concentration and is larger than that obtained using phosphate.

It was also noted that in the absence of Fe^{2+}, light emission was obtained by a simple injection of CN^- into solutions of luciferin in aqueous NaCl. The intensity of this emission increases with the concentration of CN^- employed and can be up to 1/50 of that obtained in the presence of Fe^{2+}.

Certain differences may be noted between the reactions induced by CN^- or phosphate. Thus, for a given quantity of Fe^{2+}, the optimal concentration is very different, that of CN^- (which has a much smaller dissociation constant than ferrous phosphate) being close to the concentration of Fe^{2+} used. In addition, the optimal quantity of Fe^{2+} is some 10 times less in CN^- than in phosphate. The light emission obtained from simple mixtures of luciferin and CN^- is not obtained in the case of mixtures of luciferin and phosphate. Also the rate constant is independent of CN^- concentration (above a certain value), in contrast with the dependence on phosphate concentration. In the case of variation of the rate constant as a function of the Fe^{2+} concentration, no change occurs in phosphate, while in CN^- there is a dependence.

Action of H_2O_2

The addition of H_2O_2 to luciferin and Fe^{2+} in the absence of phosphate, EDTA or CN^- stimulates light emission. For a given quantity of Fe^{2+}, I_{max} increases with increasing amounts of H_2O_2, and similarly with a constant H_2O_2 concentration there is a correlation between I_{max} and the quantity of Fe^{2+}. The total light emitted increases up to a certain concentration of H_2O_2 (0.33 mM) but then diminishes. The kinetics are first order and the rate constant varies with H_2O_2 concentration. Both I_{max} and L are comparable to the values obtained in the system Fe^{2+}–phosphate–luciferin (Table V).

In the absence of Fe^{2+}, the addition of H_2O_2 to luciferin induces a smaller light emission, about 1/50 of that observed in the presence of Fe^{2+}.

TABLE V

EFFECT OF H_2O_2 CONCENTRATION ON I_{max} AND L

In the cuvette: purified luciferin (stock solution 0.6 mg/ml) 25 μl, NaCl (5 M) 0.3 ml, $FeSO_4$ 0.3 ml, water 0.3 ml. In the syringe: H_2O_2 0.3 ml, water 0.7 ml. The H_2O_2 solution was previously tested with $KMnO_4$.

H_2O_2 concn. (mM)	I_{max}			L		
Fe^{2+} concn.	0.1 mM	0.01 mM	0	0.1 mM	0.01 mM	0
Control with phosphate (0.1 M)	350			29 000		
0.03	145	250		10 313	31 768	
0.1	540	420	7.5	41 585	33 972	754
0.33	1050	550		37 252	22 672	

TABLE VI

CONSTANCY OF L EMITTED

Into a cuvette containing purified luciferin (30 μl of stock solution), NaCl (2 M) 0.75 ml, phosphate buffer (1 M) 0.30 ml and water 1.45 ml, two injections were made: first, $FeSO_4$ 0.3 ml and water 0.2 ml were added, followed by luciferase in 0.5 ml phosphate buffer after light emission had ceased.

Fe^{2+} concn. (mM)	L after Fe^{2+} injection ($L1$)	L after luciferase injection ($L2$)	$L1 + L2$
0		9482	
0.01	690	7093	7783
0.03	1780	5979	7759
0.06	2592	5237	7829
0.1	4695	2973	7668

Comparison of the luciferin–Fe^{2+} and luciferin–luciferase reactions

It appears that for the production of light, the enzyme luciferase can be replaced by addition of Fe^{2+} and a complexing agent. The two reactions are similar, and in both cases, the luciferin appears to behave in the same manner. Thus, if Fe^{2+} is added to a solution of luciferin and the reaction allowed to go to completion and then luciferase is added, there is again emission of light. If the concentration of Fe^{2+} is varied (with constant luciferase), the sum of the light emitted in the two consecutive reactions is constant for a given quantity of luciferin (Table VI). The luciferin thus behaves as a photoprotein, the initiation of light emission being stimulated either enzymically by luciferase or chemically by Fe^{2+} and phosphate (or another complexing agent).

CONCLUSION

The term 'photoprotein' has been proposed by SHIMOMURA AND JOHNSON[2] to describe the effective intermediate in light emission from certain bioluminescent systems such as those from Aequorea, Halistaura[3,5], Chaetopterus[2,6] and *Meganyctiphanes norvegica*[7]. The common characteristic of such photoproteins lies in a quantum

yield equal to or less than one, that is, such that no turnover occurs and the protein is consumed in the course of light emission. It is clear that the Pholade system presents certain analogies, though in this case the substrate luciferin (a protein) is consumed and is not subject to turnover, while the enzyme luciferase (also a protein) plays a limited role. A considerable resemblance can be seen between the Pholade and Chaetopterus systems if the Pholade luciferin is regarded as a photoprotein: both can function in the presence of O_2, Fe^{2+} and H_2O_2. However, the molecular weights of the two proteins are quite different and differences also are present in the spectra of fluorescence.

It is clear that identification of Pholade luciferin as a photoprotein raises the question of the role of the enzyme luciferase. A number of speculations involving a controlled transfer of Fe^{2+} or the formation of stable complexes may be evoked. However, a definition of Pholade luciferase will be deferred to future reports as will studies of a possible regenerating enzyme for the reduction of oxidised luciferin. It is of interest that chemiluminescence resulting from the action of H_2O_2 and Fe^{2+} on flavin mononucleotide, for example, is dependent on the presence of a chelating agent such as EDTA[8,9].

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