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STUDIES IN BIOLUMINESCENCE

III. THE *PHOLAS DACTYLUS* SYSTEM

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SUMMARY

Two components can be obtained from the luminescent mollusc *Pholas dactylus* which, when mixed aerobically, produce light. Purification of the substrate luciferin is described. This component is a protein (mol. wt. 50000); absorption and fluorescence spectra are given.

The kinetics of the reaction leading to light emission were studied. A biphasic reaction could be induced by incubation of luciferin–luciferase in the absence of O_2 or by incubation at pH 4.8 at 0°. Injection of reduced flavin mononucleotide resulted in a flash if injected during the reaction but not after. Among metal ions, only Fe^{2+} showed a strong stimulation.

INTRODUCTION

The boring mollusc *Pholas dactylus* was studied by Aristotle, and bioluminescent aspects of the organism were described by PLINY[1]: 'they shine in men's mouths as they be chawing of them...'. Subsequently, DUBOIS[2] studied the physiology and biochemistry of the system in the period 1885–1925. Since then, little work has been described, though MCELROY AND SELIGER[3] have indicated that the λ_{max} of emission is about 480 nm. In addition, the ultra structure and morphology of the luminescent organs of *P. dactylus* have recently been described by BASSOT[4]. Light emission is continuous after mechanical stimulation of the clam and is extracellular. It appears that two types of cells are involved: one may be responsible for the excretion of the enzyme luciferase and the other for the excretion of the substrate luciferin. The early preparation *in vitro* described by DUBOIS[5] followed the simple techniques available at that time. In view of the lack of a systematic study since the work of DUBOIS, we now present a reexamination of this bioluminescent system. The Pholades were obtained from the Brest coast in France at low tide.

MATERIALS AND METHODS

Before extraction, the siphons of the Pholades were dissected to yield the specific luminescent regions. An acetone powder was then prepared and kept as stock.

Apparatus for measurement of light emission

The apparatus used gives a direct measurement of both the intensity of light emitted at any given moment and also the total light emitted.

The photomultiplier used was a 56 TUVP (Radiotechnique) with a photocathode type S-20. The tube itself was selected for a very feeble dark current. If necessary, the tube can be cooled with dry ice to reduce the background further. The power supply for the multiplier was an Oltronix, type A 2.5 K-10 R. The usual tension during measurements was 1500 V. The current from the photomultiplier was measured by a picoamperometer (Lemouzy) model PA 15 which is capable of measuring currents of 10^{-4}–10^{-15} A. The same apparatus can also be used for integration of the current by means of a condenser. Output was recorded on a galvanometric recorder (Sefram Graphispot) or with a potentiometric recorder (Sargent) type SRLG which records simultaneously the signal and integration of the signal.

The reaction mixtures are contained in cuvettes (32 mm × 45 mm × 6 mm) of which one face is silvered. Between the cuvette and the phototube, there is a system of vertical slits and to this system can be added a continuous interferential filter which can be displaced horizontally in such a manner as to analyse the spectral distribution of the light. Both temperature and atmosphere inside the compartment can be controlled, and in addition, reagent can be introduced into the cuvette by means of a hypodermic needle passing through the top of the apparatus. Final volumes were always 3.0 ml except where otherwise specified.

Acetone powder from P. dactylus

About 100 pholades were dissected at as low a temperature as possible to remove the luminescent parts of the siphon and the poli triangles. The material thus obtained (45 g) was mixed at high speed in a Waring blendor in 800 ml of acetone previously cooled to −20° and then filtered, the residue being washed twice with acetone. The material was then dried *in vacuo* in a dessicator to give about 10 g of powder.

Preparation of crude extracts

Luciferin. Suspensions of the acetone powder in water at 0° (5 mg/ml, 10-ml fractions) were dispersed in a Polytron grinder (Kinematica GMBH-Lucerne) for a short time avoiding excessive heating. The suspension was then centrifuged for 10 min at 15000 rev./min at 0°. The concentration of protein in the supernatant, as measured by the Folin reaction, was 1 mg/ml. This supernatant was then immediately placed in a bath at 65° for 3 min and then in ice, followed by centrifugation at 0°. The supernatant contains about 0.8 mg/ml of protein and about 25–40 % of the original luciferin activity was obtained. Acrylamide-gel electrophoresis showed a somewhat limited number of bands even for this crude preparation due to the use of the luminescent organs as starting material.

Luciferase. The enzyme was also prepared from the acetone powder using somewhat more concentrated solutions for the initial dispersion in 0.01 M phosphate buffer (pH 7). Acetone powder (2 g) was dispersed in 15 ml of buffer using the Polytron grinder, and the suspension centrifuged for 10 min at 15000 rev./min. The precipitate was resuspended in 10 ml of buffer and redispersed. The total supernatant was then combined (25 ml) and adjusted to 0.05 M with respect to NaCl and to pH 4.8 with 0.05 M acetate buffer. After several minutes, the solution was centrifuged and

the supernatant adjusted to 0.1 M phosphate buffer (pH 7.0). A considerable amount of protein was thus removed by precipitation under these conditions. The clear solution was then dialysed overnight against 0.01 M phosphate buffer (pH 7.0) to give a crude solution of luciferase. No luciferin activity is present at this stage since there is no light emission.

Purification of luciferin

Purification was followed by electrophoresis on acrylamide gel after each step as well as by estimation of light emission. The usual mixture for following light output contained 15 μl of luciferin solution, 0.75 ml of 2 M NaCl, 0.3 ml of 1 M phosphate buffer (pH 7.0), 1.45 ml of water and 0.5 ml of crude luciferase (diluted 5 times), the reaction being initiated by injection of the luciferase solution. The protein concentration was followed either by the method of Lowry (Folin reagent) or by following the absorbance at 260 and 280 nm (technique of Warburg and Christian). The initial step followed closely those described for the crude extract of luciferin except that somewhat more concentrated solutions were used (1 g of acetone powder in 30 ml of water) which reduces the recovery somewhat, since the luciferin is consumed more rapidly by the luciferase under these conditions. This extract of luciferin was then adjusted to 0.05 M NaCl and to pH 4.8 by the addition of acetate buffer (0.05 M). The solution was then centrifuged and neutralised with phosphate buffer of pH 7.0 (0.1 M).

$(NH_4)_2SO_4$ precipitation

The usual technique of precipitation by $(NH_4)_2SO_4$ was applied. The luciferin activity was precipitated mainly at 50–80 % saturation of $(NH_4)_2SO_4$. Electrophoresis on polyacrylamide gel showed that at this stage the preparation contained a principal band due to the luciferin.

Filtration on Sephadex G-100

Residual traces of luciferase were removed by this step. About 10 mg of protein in 3 ml of phosphate buffer (0.01 M, pH 7.0) were placed on a column of Sephadex G-100 (50 cm × 2.5 cm, exclusion volume 45 ml). The column was eluted with the same buffer and 2-ml fractions collected. Active fractions were combined and concentrated. Electrophoresis on polyacrylamide gel showed in general only traces of contaminants apart from the major band corresponding to luciferin. Further purification, if necessary, is achieved by chromatography on DEAE-cellulose using for the elution a gradient of NaCl (from 0 to 1 M) in phosphate buffer of pH 7.0 (see Table I).

Partial purification of luciferase

Since the I_{max} of light emission is not strictly proportional to the concentration of luciferase, we have not attempted to define a unit of activity for the enzyme. Partial purification was achieved by precipitation with $(NH_4)_2SO_4$, the enzyme being precipitated in the region of 40–60 % saturation. This material was then dialysed overnight against 0.01 M phosphate buffer (pH 7.0) and further purified by chromatography on DEAE-cellulose using for the elution a gradient of NaCl (from 0 to 1 M) in sodium phosphate buffer of pH 7.0 (see Table I).

TABLE I

SEPARATION OF LUCIFERIN AND LUCIFERASE ON DEAE-CELLULOSE

DEAE-cellulose chromatography was made with crude extracts which were equilibrated with 0.05 M buffers. The column was 15 cm × 1.8 cm. The same column was used twice, for luciferin and for luciferase. Elution was with a linear gradient between 40 ml of 0.05 M buffer and 40 ml of 0.05 M buffer + 1 M NaCl. Fractions of about 2 ml were collected, dialysed against 0.05 M buffer + 0.5 M NaCl and then tested with 0.5 ml of crude luciferin or luciferase extracts.

Buffer	*Molarity of NaCl for elution*	
	Luciferase	*Luciferin*
Acetate (pH 5)	0.28	0.35
Phosphate (pH 7)	0.25	0.28
Carbonate (pH 9)	0.16	0.27

RESULTS AND DISCUSSION

Preliminary separation of the system into enzyme and substrate was achieved as described by DUBOIS[2] (heating the crude extract at 65° for 3 min to destroy the luciferase leaving luciferin activity). First experiments with these crude preparations showed (Fig. 1) that the luciferin thus obtained is indeed a substrate which is consumed during the reaction, in which it is clear that the quantity of light emitted is independent of the concentration of luciferase and proportional to that of luciferin. In this work, the luciferase was simply a crude extract which had been incubated until complete consumption of the luciferin present was accomplished. Luciferin and luciferase are used in the historical sense as introduced by DUBOIS. However, it may well be that in a formal sense the terms are not strictly applicable in this system.

Purification and properties of luciferin and luciferase

When classical techniques for the separation of small molecules were applied to the purification of luciferin, it became evident that the compound has an elevated molecular weight. Thus attempts to separate luciferin and luciferase on Sephadex

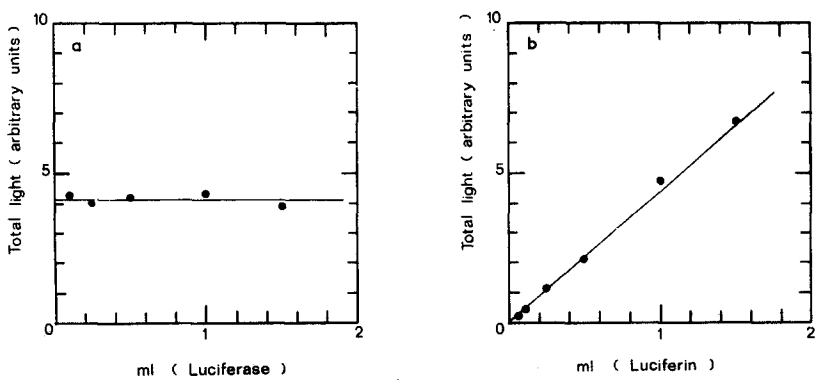

Fig. 1. a. Total light emitted by a given amount (0.5 ml) of crude luciferin with varying quantities of crude luciferase. b. Total light emitted by a given amount (0.5 ml) of crude luciferase with varying quantities of crude luciferin. The time-course of the reaction is from several minutes to several hours.

G-25 or G-50 failed since both molecules were excluded. With filtration on Sephadex G-100, the luciferin (located by reaction with extraneous luciferase) was retained and appeared to have a molecular weight on the order of 50000 (Fig. 2) when compared with bovine serum albumin and trypsin. The luciferase was excluded on this same

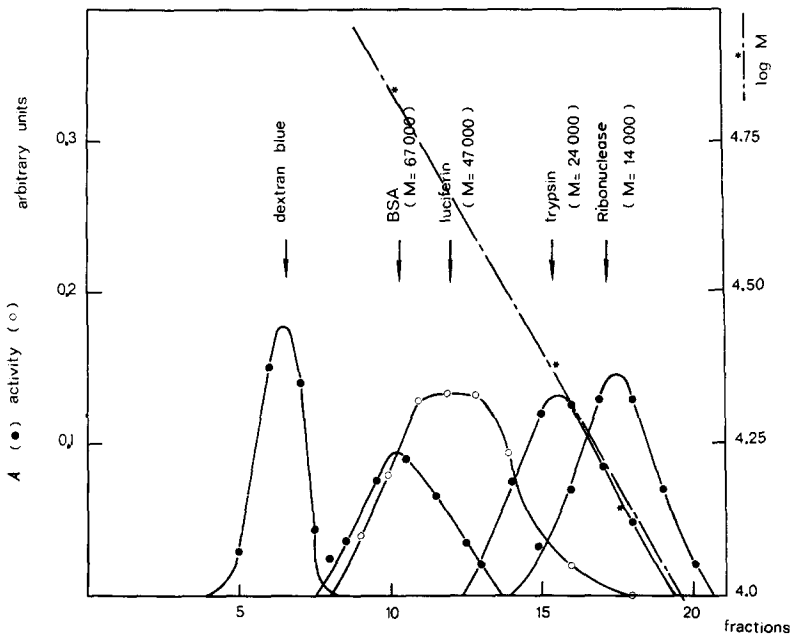

Fig. 2. Filtration on a column (17 cm × 1.2 cm) of Sephadex G-100. The fractions (0.9 ml) were tested for luciferin activity by adding 0.5 ml of a crude luciferase preparation and by comparing the absorbance at 280 nm to that of other proteins. BSA, bovine serum albumin; M, molecular weight.

column, and thus partial separation of luciferin and luciferase from mixtures of the two could be achieved as shown by the presence of three peaks of activity (one emitting light on addition of luciferin; one, spontaneously luminous, *i.e.* the presence of a mixture of luciferin and luciferase due to the partial overlap of the peaks of luciferase and luciferin and the third one emitting light on addition of luciferase). In addition, it was not possible to separate completely these two activities by chromatography on DEAE-cellulose columns (Table I).

Partial separation could also be achieved by precipitation with $(NH_4)_2SO_4$, since the luciferase precipitates in the region of 40–60 % saturation and the luciferin at 50–80 % saturation. However, this approach was not preparative. In accord with the above results, the luciferin does not dialyse as indicated by measurement of the activity retained in the dialysis tube or by dialysis against a solution of luciferase in the apparatus for measuring light emission. Activity within the membrane in comparison with a control remained constant up to 30 h dialysis at 4°.

Attempts to extract luciferin activity from the acetone powder by direct extraction with organic solvents such as methanol, butanol, ethyl acetate were unsuccessful, though extraction with dioxan or dimethyl sulphoxide gave very low yields of light-emitting material. This material could not be identified as luciferin.

To obtain purified luciferin, it was thus necessary to destroy the luciferase activity. Although luciferase activity could be destroyed by heating at 65° for several minutes, it soon became evident that the fraction called luciferin was also not too stable at this temperature. The change in activity with time at a given temperature is shown in Fig. 3, where it can be seen that after 3 min at 65° only 40 % of the original

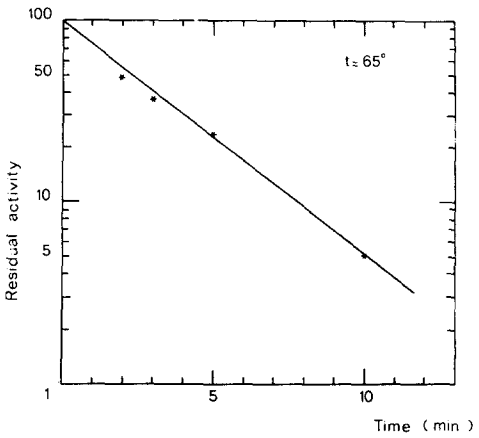

Fig. 3. Luciferin activity after preheating to 65°. Activity was tested at 25° by addition of crude luciferase.

TABLE II

PURIFICATION OF LUCIFERIN

Fraction	*Vol. (ml)*	*Protein (mg/ml)*	*Total protein (mg)*	*Activity per ml (arbitrary units)*	*Total activity*	*Specific activity*
Crude extract	30	4.42	132.6	13.67	410	3.1
Hot extract	30	3.82	114.6	2.71	81.2	0.71
After acid precipitation	30	1.23	36.9	1.73	51.9	1.41
$(NH_4)_2SO_4$ 20–40 % satn.	0.5			0.57	0.28	
$(NH_4)_2SO_4$ 40–60 % satn.	1	2.78	2.78	5.22	5.22	1.88
$(NH_4)_2SO_4$ 60–80 % satn.	2	3.58	7.16	19.54	39.1	5.45
Pooled fractions after Sephadex G-100 column	18	0.235	4.230	1.96	35.2	8.35

activity remains. Under these same conditions the luciferase is completely destroyed. Luciferin activity is also destroyed by proteolytic enzymes, *e.g.* treatment with pepsin for 5 min at 37° and pH 3.0 or incubation at room temperature with pronase, *i.e.* luciferin is a protein. The purification achieved at each step is given in Table II. The luciferin obtained gave a single band on acrylamide-gel electrophoresis. Activity coincident with this band was revealed by incubation with luciferase *in situ* and passage of the gel in front of a photomultiplier using small slit widths. Absorption spectra, fluorescence excitation and emission spectra of luciferin are given in Fig. 4.

The preparation of luciferase showed λ_{max} in ultraviolet absorption of 278 nm and fluorescence spectra gave an excitation maximum at 290 nm with an emission λ_{max} 338 nm. Electrophoresis on polyacrylamide gel showed that the preparation was not 100% pure; among the several bands of protein one corresponded with the enzymic activity as revealed *in situ* by incubation with luciferin. A second extremely

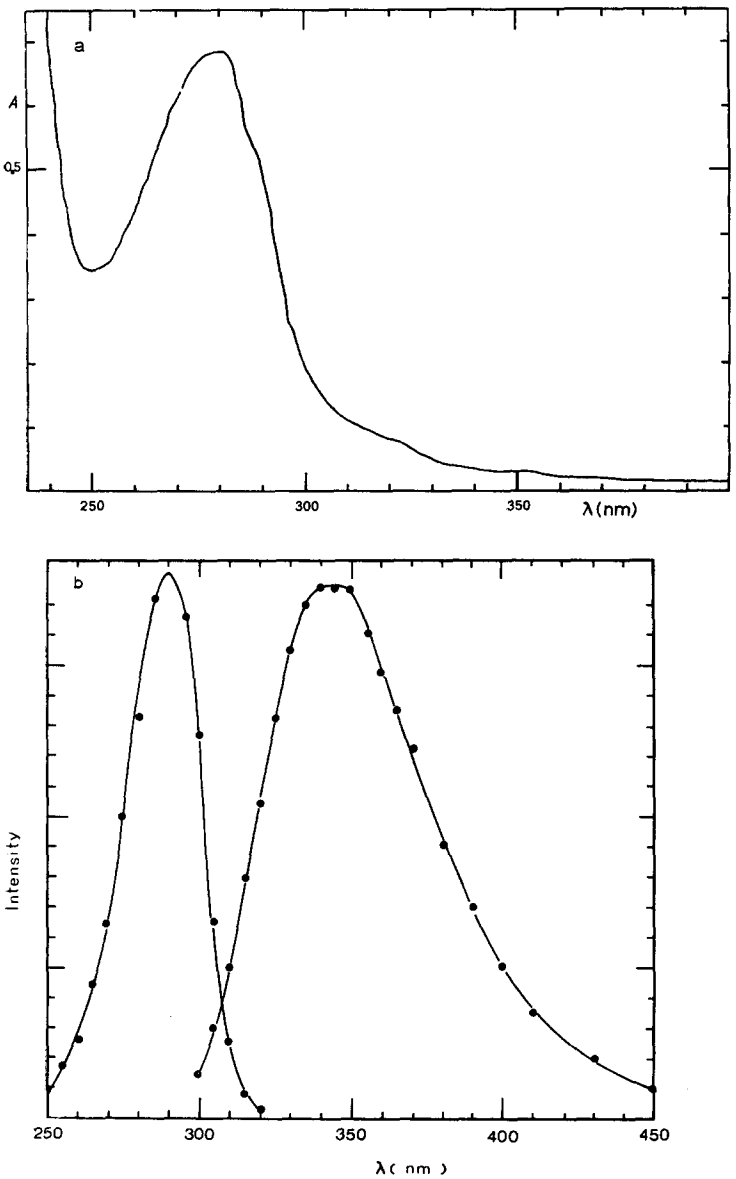

Fig. 4. a. Absorption spectrum of purified luciferin at pH 7.0. Measurements were made with a Cary 14 spectrophotometer. b. Fluorescence activation and emission spectra of the same preparation. Spectra were obtained with a double monochromator Zeiss spectrophotofluorimeter. The spectra are uncorrected for phototube sensitivity and monochromator efficiency.

slight activity could also be observed but did not correspond to a protein band and was presumably due to chemiluminescence.

The position of the bands corresponding to luciferin and luciferase remained unchanged both in intensity and position after bioluminescent reaction. In view of this, the isolated luciferin probably represents a mixture of active and inactive molecules.

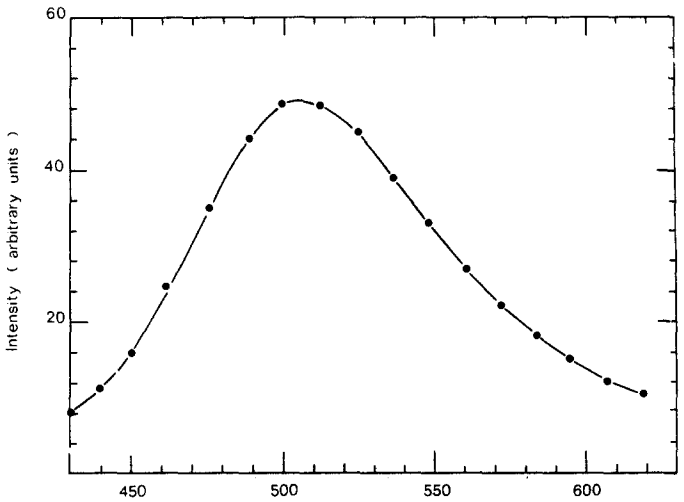

Fig. 5. Bioluminescent emission spectrum of the luciferin–luciferase system. The measurements were made in the light-measuring apparatus, using a continuous interferential filter and slits, during the slow reaction. The results are not corrected for phototube sensitivity and filter transmission. The bandwidth is about 20 nm.

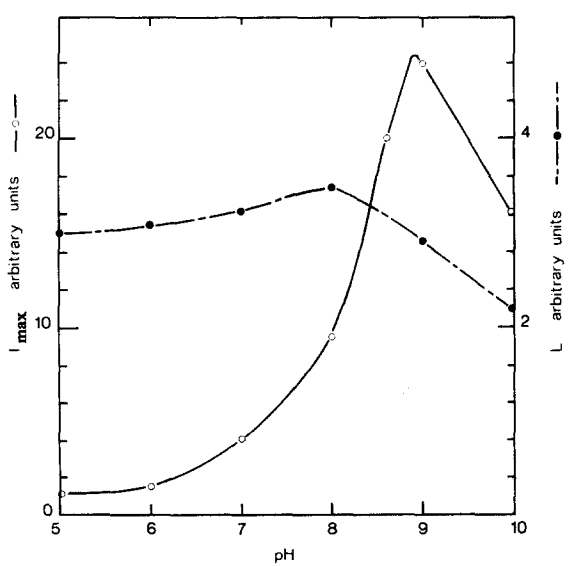

Fig. 6. Variation of I_{max} and L with pH. Incubations were at pH 5 in acetate buffer, at pH 6 in acetate and phosphate buffers, at pH 7 and 8 in phosphate buffer, at pH 8.6 in phosphate and carbonate buffers, at pH 9 and 10 in carbonate buffer. The buffers were 0.05 M.

Description of the system in vitro

Apart from the two major constituents described above (luciferin and luciferase) both of which are proteins, O_2 is also necessary. The spectrum of emission (noncorrected) of the system (using a continuous interference filter) is shown in Fig. 5. λ_{max} is at 505 nm with a half width of the band of 95 nm.

In general, two measurements were made, peak intensity (I_{max}) and total light emitted (L). The ionic concentration has an effect on I_{max} with an optimum of 0.5 M NaCl, but L is relatively insensitive. The variation of I_{max} and L as functions of pH is shown in Fig. 6. A marked pH optimum for I_{max} can be noted at pH 8.8, whereas for L the effect is much smaller though again diminution at pH 9 or higher is important.

Kinetics

We have previously mentioned that the relationship between L and luciferin concentration (with constant luciferase) is linear. The kinetics with variation of luciferase and constant luciferin are non-Michaelian, and L and I_{max} are not proportional to the concentration of luciferase (for this reason it was not possible to describe the purification of luciferase in terms of specific activity). The variations of I_{max} with enzyme concentration (constant luciferin) and with luciferin concentration (constant luciferase) are shown in Figs. 7a and 7b.

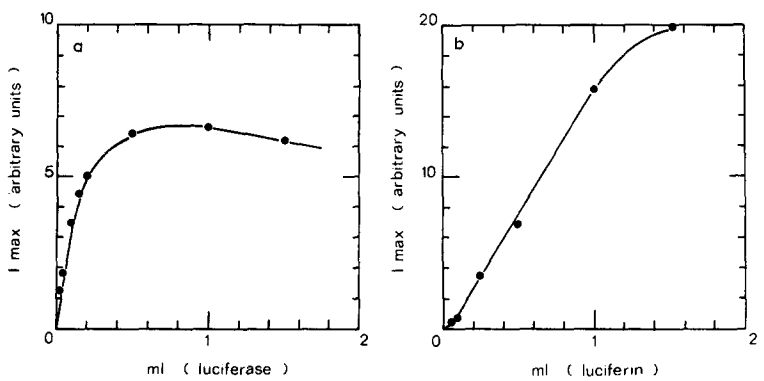

Fig. 7. Variation of I_{max} with luciferase concentration (a) and luciferin concentration (b). Same conditions as in Fig. 1.

Effect of O_2

When luciferin and luciferase are mixed in the presence of nonlimiting quantities of O_2, the rate of emission of light indicates a reaction which is approximately first order. This reaction, in contrast with many other bioluminescent systems, continues for several hours. However, if the luciferin and luciferase are mixed under anaerobic conditions followed by admission of O_2 after a few minutes incubation, the emission becomes markedly biphasic with an initial high intensity flash in the 1st min followed by a slow reaction corresponding to that described above. These results suggested that an accumulation of an intermediate occurs in the absence of O_2. This intermediate could also be demonstrated by incubation of the luciferin–luciferase mixture in acetate buffer (pH 4.8) at 0°, followed by injection into phosphate

buffer (pH 7.0) at 20°. Again a biphasic emission was observed (Fig. 8). The kinetics of the slow reaction remained unchanged (first order with a small rate constant), but both the intensity of the flash and the total light emitted in the fast reaction increased with the time of the preincubation at acid pH. As a function of the time of preincubation, I_{max} of the fast reaction increases up to a limiting value obtained after about 30 min of preincubation.

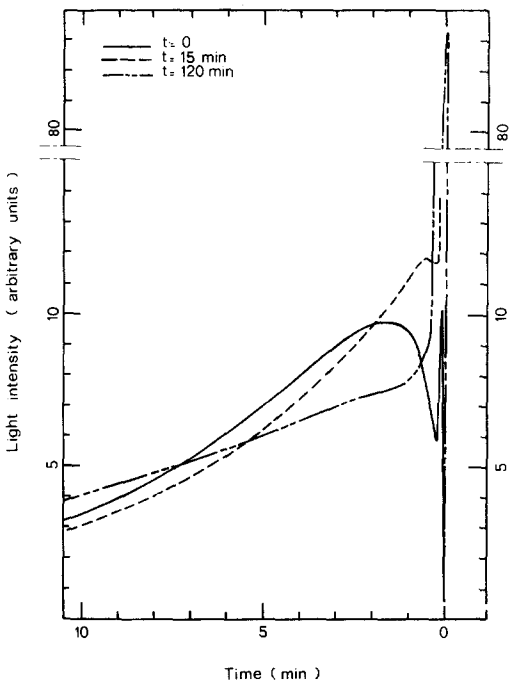

Fig. 8. Incubation of luciferin and luciferase at pH 4.8 (acetate buffer). Luciferin and luciferase were mixed at 0° in a 0.05 M acetate buffer with 0.5 M NaCl. Aliquots (1 ml) were injected into a cuvette containing 2 ml of 0.1 M phosphate buffer (pH 7.0) and 0.5 M NaCl thermostated at 25°.

This value depends on the concentration of luciferase, both I_{max} and L increasing with increases in luciferase up to a final value of L_{fast}/L_{total} equal to 0.12. Under optimal conditions, the intensity of the fast reaction is some 10 times the value of I_{max} of the slow reaction. The rate of decay of the fast reaction is exponential and the time constant (rate constant) is independent of the concentration of luciferase.

An indication of the formation of a luciferin–luciferase complex was obtained by filtration of the mixture on Sephadex G-100 at pH 4.8. Under these conditions, the fraction giving rise to a rapid emission of light on addition of phosphate buffer (pH 7.0) was excluded, whereas the residual luciferin (tested by addition of luciferase to the fractions after neutralisation) was eluted in the same volume as control luciferin. One can thus conclude that the fast reaction is due essentially to preformation and accumulation of the luciferin–luciferase complex. Electrophoresis on acrylamide gel showed that luciferin was indeed present in the excluded fraction. Simple 'carry over' would not, of course, result in changed kinetics of light emission.

Action of reducing agents

The effect of reducing agents on a crude system has previously been discussed by McELROY AND SELIGER[3] and by PLESNER[6]. NADH had no effect on the emission of the system *in vitro*, whereas injection of $FMNH_2$ during the slow reaction occurring with luciferin–luciferase in phosphate buffer resulted in a flash. The I_{max} of this flash

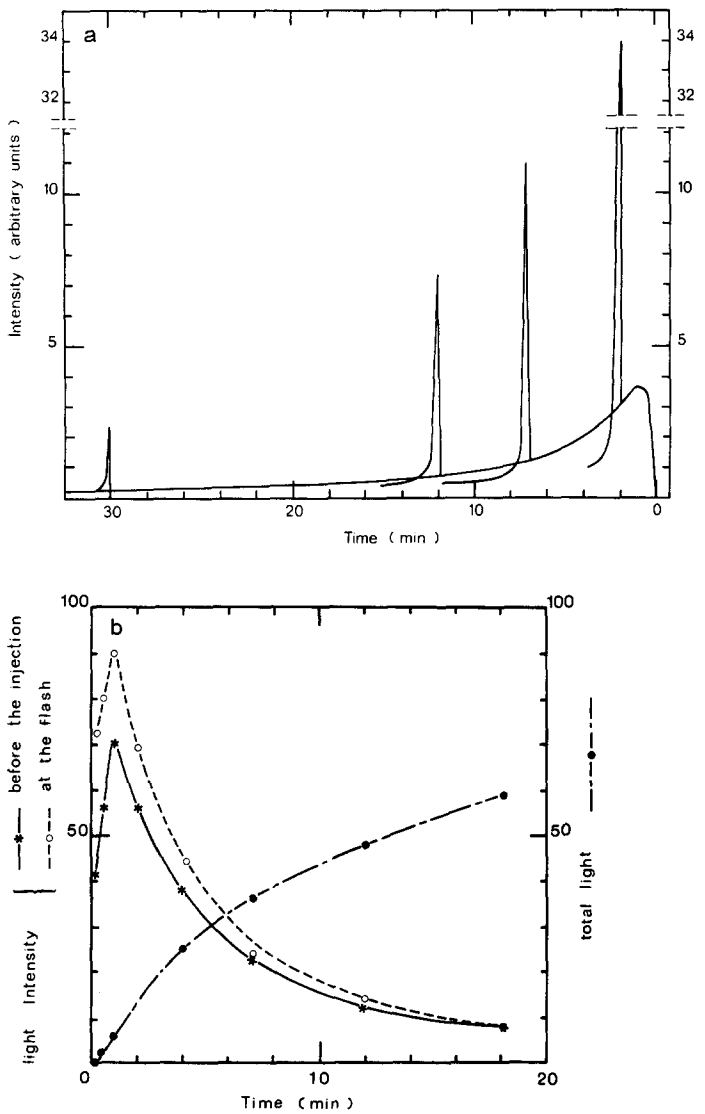

Fig. 9. a. Injection of 0.5 ml of $FMNH_2$ (1 mM) into a cuvette containing luciferin (0.5 ml) and luciferase (0.5 ml) mixed at zero time in 0.1 M phosphate buffer (pH 7.0) and NaCl (0.5 M), final volume 3 ml. The injections were 1, 7, 12 and 30 min after the beginning of the reaction. The flavin had been reduced by H_2 in the presence of platinized asbestos in a phosphate buffer (0.01 M) containing 1 mM EDTA. b. Conditions as in a. Total light as a function of time (——) and comparison of light intensity before and after injection of $FMNH_2$, showing the absence of a correlation between the quantity of oxidised luciferin and the effect of the reducing agent.

reached a limiting value with increases in the quantity of $FMNH_2$ but was not correlated with the amount of luciferin consumed during the incubation before injection. Thus there is a diminution rather than an increase in the flash emitted with an increase in the time of preincubation. This is also shown clearly in Figs. 9a and 9b where it can be seen that the trace of I_{max} of the flash induced by injection of $FMNH_2$ at different times follows closely the normal curve of diminution of light intensity of the system. This clearly eliminates the possibility of reversal by reduction of a possible 'oxyluciferin'. It may be noted that $FMNH_2$ produced only a very feeble emission of light when added to solutions of luciferin or to solutions of a mixture of luciferase and luciferin which had previously been incubated to completion of light emission.

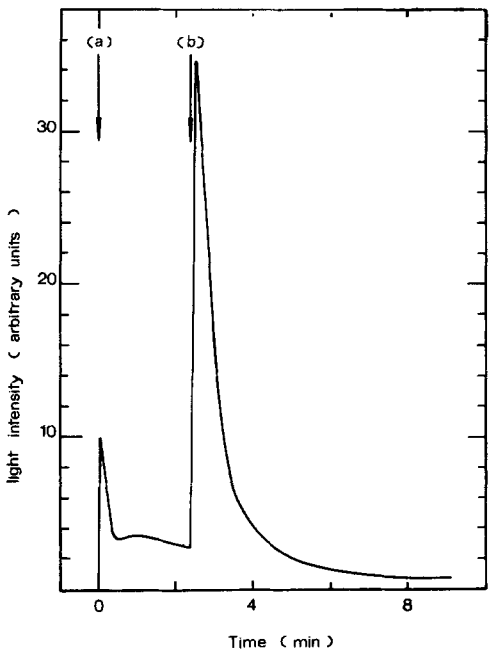

Fig. 10. Effect of injection of Fe^{2+} during the reaction. The reaction was initiated by mixing purified luciferin (7 μg/ml) with luciferase, in a phosphate buffer (pH 7.0) (0.05 M) containing 0.5 M NaCl. In b, Fe^{2+} are injected at a final concentration of 0.1 mM.

Similar results were obtained with dithionite at low concentration (40 μg/ml), though at high concentration (2 mg/ml) an inhibition was observed.

Effect of metal ions

No effect was observed on addition of Ca^{2+}, Cu^{2+}, Cu^{3+}, Mg^{2+}, Mn^{2+} or Zn^{2+} to the system. A strong inhibition occurred with Hg^{2+}, while a remarkable stimulation was obtained on the addition of Fe^{2+} as shown in Fig. 10.

CONCLUSION

From ancient times up to the beginning of the present century, *P. dactylus* has aroused the curiosity and interest of a number of workers. However, since the studies

by DUBOIS[2] no detailed investigations have been described. One possible explanation lies in the gastronomic attractions of this mollusc as a result of which the ecology has been profoundly modified. Thus, once prevalent in the entire Mediterranean Basin as well as on the Atlantic coast of Europe, the species can now only be found in two small areas (to the knowledge of the present investigators). Whereas the voluminous and often contradictory publications of DUBOIS provide a number of explanations for the luminescence of *P. dactylus* (indeed, the general terms luciferin and luciferase were introduced by DUBOIS largely as a result of his work with this organism), certain difficulties are immediately evident. Thus, although the results of DUBOIS accord well with the scheme proposed by SELIGER AND MCELROY[7],

$$\text{Luciferin} + O_2 \xrightarrow[\text{luciferase}]{} P^*$$
$$P^* \rightarrow P + h\nu$$

unlike the Cypridina system, purification of Pholade luciferin yields a macromolecule from which no small molecule with activity can be isolated. While the possibility exists that a true luciferin function is associated with a prosthetic group of the protein called luciferin in this study, it is remarkable that no characteristic absorption or fluorescence is discernable, other than those of a typical protein.

Studies of the anaerobic incubation of luciferin and luciferase followed by admission of O_2 suggest the formation of an enzyme–substrate complex, the formation of which is a rate-limiting step in the emission of light under normal conditions. While a similar supposition may be made to explain the effects of preincubation of luciferin–luciferase at acid pH (in the presence of O_2), the problem is somewhat more complicated. Thus the rate constant for the decrease in intensity of the flash obtained on neutralisation is first order and independent of the concentration of luciferase. One must therefore postulate not a Michaelian complex but rather the accumulation of an activated intermediate as in the case of bacterial systems[9]. Nevertheless, an increase in the time of preincubation or in the concentration of luciferase does not lead to complete transformation of luciferin to an active intermediate, indicating that for the preformation of this intermediate some kind of equilibrium is involved.

Finally, it may be noted that the action of reduced flavin nucleotide in the system does not appear to be due simply to the reconversion of an oxidised product by a reductive process. Further studies on this point and on the role of luciferase are in progress. A number of resemblances between the Pholade system and that of Latia[10] may be noted.

ACKNOWLEDGEMENTS

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