Purification and Properties of the Luciferase and of a Protein Cofactor in the Bioluminescence System of *Latia neritoides**

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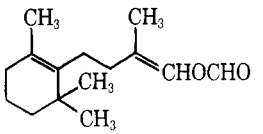
ABSTRACT: By procedures described, luciferase and a "purple protein" cofactor were isolated, their properties were studied, and kinetics of the light-emitting reaction with the purified, highly hydrophobic, *Latia* luciferin were investigated. The luciferase has a molecular weight of 173,000, and its catalytic activity is quickly destroyed at 95° or in 90% alcohol. The purple protein has a molecular weight of 39,000 and it is stable for some minutes at 100° or in 90% alcohol. Quantum yields, *i.e.* (photons emitted)/(molecules reacted), were computed as 0.63 for luciferase (probably with product inhibition), as 0.0030 and 0.0068 for luciferin at 25 and 8°, respectively, and as 8 or more for the recycling purple protein. Among inhibitors and activators, 10^{-4} M Fe^{2+} facilitated the increase in luminescence by the purple protein but at the same time catalyzed a nonluminescent decomposition of the luciferin; 10^{-4} M ascorbate, and to a lesser extent 2×10^{-4} M reduced diphosphopyridine nucleotide, activated luciferase when incubated with it for periods up to 5 min before the luminescence reaction; 10^{-4} M KCN and 10^{-4} M *o*-phenanthroline each inactivated luciferase; and 10^{-3} M cysteine caused a 50% inhibition of luminescence.

On the basis of mass spectra, the structure of the oxidation product of luciferin is proposed, as well as an over-all reaction scheme wherein this product and formic acid are produced in an oxygenase type of luminescence reaction.

In the bioluminescence system of the freshwater limpet, *Latia neritoides*, oxygen plus at least two organic components have been shown to be required for a light-emitting reaction in aqueous solution (Shimomura *et al.*, 1966): (1) an enzyme, "luciferase," which acts on (2) a specific substrate, "luciferin," of the proposed structure I (Shimomura and Johnson, 1968a). The present investigation has resulted in purification of the luciferase and has revealed that a third organic component, a "purple protein," is also essential for light emission. Such small amounts of the latter are required to saturate the system that it was not detected in the partially purified luciferase preparations in previous studies. After purification, precipitates of this protein in saturated ammonium sulfate are deep purple. Pure luciferase, on the other hand, is colorless. Kinetic data in this study indicate that luciferase acts in the manner of an enzyme in the bioluminescence reaction, whereas the purple protein acts more in the manner of a cofactor.



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Materials and Methods

Purified luciferin and crude luciferase were obtained from specimens frozen in Dry Ice as described previously (Shimomura *et al.*, 1966; Shimomura and Johnson, 1968a). Except when stated otherwise, two types of buffer solutions were used throughout: (1) 0.005 M sodium phosphate, hereafter referred to as "buffer," and (2) the same, but with the addition of 0.0002 M EDTA, hereinafter referred to as "EDTA buffer." The final pH of all buffered solutions was 6.8.

The following assay procedures were used with all solutions (except the luciferin assay; *vide infra*, no. 4) in buffer. (1) For crude luciferase, 2.5 ml of the luciferase solution was added to 2.5 ml of a standard amount of luciferin in buffer, freshly diluted from a stock ethanolic luciferin solution each time; (2) for purified luciferase, 2.5 ml of the luciferase solution plus a standard amount of purple protein were added to 2.5 ml of standard luciferin solution; (3) for the purple protein, 2.5 ml of a mixture of this protein with a standard amount of luciferase was added to 2.5 ml of standard luciferin solution (in this assay the intensity of emitted light is not proportional to the amount of purple protein); and (4) for luciferin, a small volume of an ethanolic solution was placed in a cuvet and 5 ml of standardized crude luciferase was added. All assays were made at 25°, in terms of "light units" (LU) with a photomultiplier light-integrating apparatus on which 1 LU represented 10^{10} photons. All purification procedures were carried out at, or very slightly above, 0°.

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Extraction of Luciferase and Purple Protein. A 10-g batch of frozen specimens was added to a pint mason jar containing a small amount of 5-mm glass beads and 200 ml of cold buffer and stirred for 15 min with a Teflon-bladed Servall Omnimixer at 30 V at room temperature. Turbidity of the solution was eliminated by centrifugation. The supernatants from four such batches were combined and poured onto a 3.5 × 15 cm column of DEAE-cellulose prepared with buffer. The adsorbed active material was washed with buffer containing 0.12 M NaCl and was then eluted with buffer containing 0.3 M NaCl. The active fractions, containing both luciferase and purple protein, were saturated with ammonium sulfate, the precipitate was centrifuged, and the supernatant was discarded.

Separation of Luciferase and Purple Protein. The ammonium sulfate precipitate from a total of 120–160 g of specimens was dissolved in a small amount of EDTA buffer containing 0.2 M NaCl and was chromatographed on a 2.5 × 70 cm column of Sephadex G-200, prepared with the same buffer mixture. The effluent fractions were checked for optical density at 280 mμ, for luciferase activity, and for purple protein activity. Luciferase activity was found in the early part of the effluent, immediately after a strong peak of protein impurity. The purple protein, distinguishable by red fluorescence under ultraviolet light, followed the luciferase.

The combined fractions having luciferase activity, but still containing appreciable amounts of purple protein, were diluted with an equal volume of cold water and chromatographed on a 1.8 × 11 cm column of DEAE-cellulose prepared with EDTA buffer containing 0.1 M NaCl. A gradient concentration elution was carried out with 300 ml of buffer in a constant-volume mixing chamber and with EDTA buffer containing 0.55 M NaCl as the high concentration solution. The purple protein was eluted first and the active luciferase considerably later, showing complete separation of the two bands.

Purification of Luciferase. From a total of 400 g of specimens, luciferase fractions separated from the purple protein were combined, saturated with ammonium sulfate, and the precipitate was centrifuged. The precipitate was dissolved in 5 ml of 0.015 M buffer containing 0.00025 M EDTA and 0.05 M NaCl. The solution was desalted by passing through a 1.8 × 15 cm column of Sephadex G-25 prepared with EDTA buffer containing 0.1 M NaCl. The active effluent was chromatographed on a 1 × 10 cm column of DEAE-cellulose, similarly prepared. Gradient concentration elution was carried out with 170 ml of the same buffer mixture in the constant-volume mixing chamber, and with EDTA buffer containing 0.5 M NaCl as the high concentration solution.

The elution curve (Figure 1) shows the presence of an inactive protein (denatured luciferase?) just before the activity peak. The purest part of the eluate (between the vertical broken lines in the figure), which was colorless and had a nearly constant specific activity (activity/optical density) was estimated to contain approximately 10 mg of protein and was used for all quantitative experiments reported here, including ultracentrifugal analyses. Luciferase activity could be preserved without loss at −25° as the ammonium sulfate precipitate.

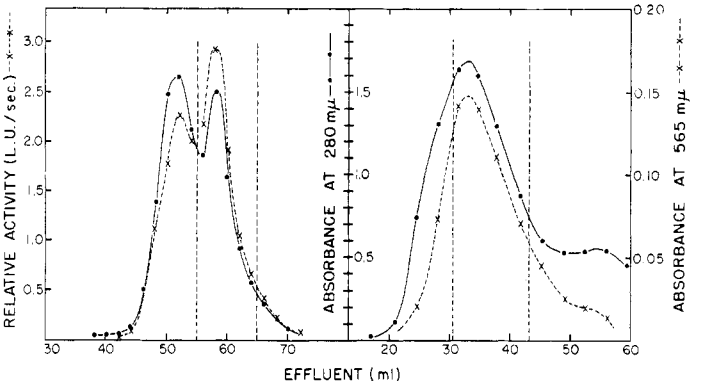


FIGURE 1: Elution curves. Left: of luciferase from the final DEAE-cellulose column. Right: of purple protein from the final Bio-Gel P-100 column. Since activity of the purple protein is not proportional to concentration, absorbance at 565 mμ is plotted instead of activity.

Purification of the Purple Protein. The fractions separated from luciferase by chromatography first on Sephadex G-200 and then on DEAE-cellulose were combined, saturated with $(NH_4)_2SO_4$, and centrifuged. The precipitate was dissolved in a small amount of buffer, then purified by two successive gel filtrations, first on a 2 × 55 cm column of Bio-Gel P-100 (50–100 mesh) and second on a 1.8 × 40 cm column of Bio-Gel P-100 (100–200 mesh). A buffer solution of EDTA-phosphate containing 0.1 M NaCl was used throughout.

The elution curve of the second column is shown in Figure 1. The purest part of the eluate (between the two vertical broken lines) contained approximately 18 mg of protein and was used for ultracentrifugal analyses and quantitative experiments.

Properties of Luciferase. Concentrated solutions of luciferase are colorless (Figure 2). The activity is satisfactorily stable for a few days at about 0° in buffer containing 10^{-3}–10^{-4} M EDTA, but with 10^{-3} M cysteine or 10^{-3} M ascorbic acid instead of EDTA complete loss of activity occurred in 2 days. The addition of ten volumes of ethanol to a luciferase solution caused 85% loss of

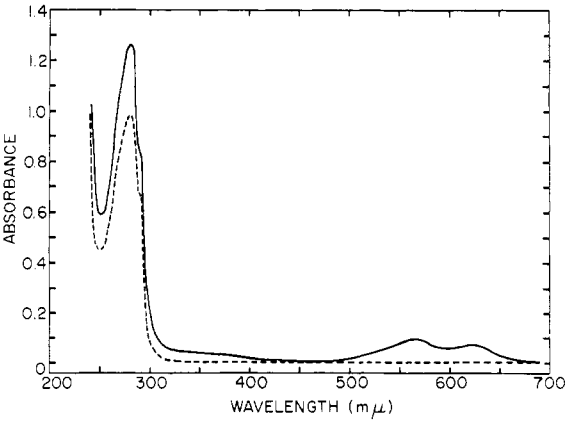


FIGURE 2: Absorption spectra of luciferase (broken line) and purple protein (solid line), measured on a Perkin-Elmer 202 spectrophotometer.

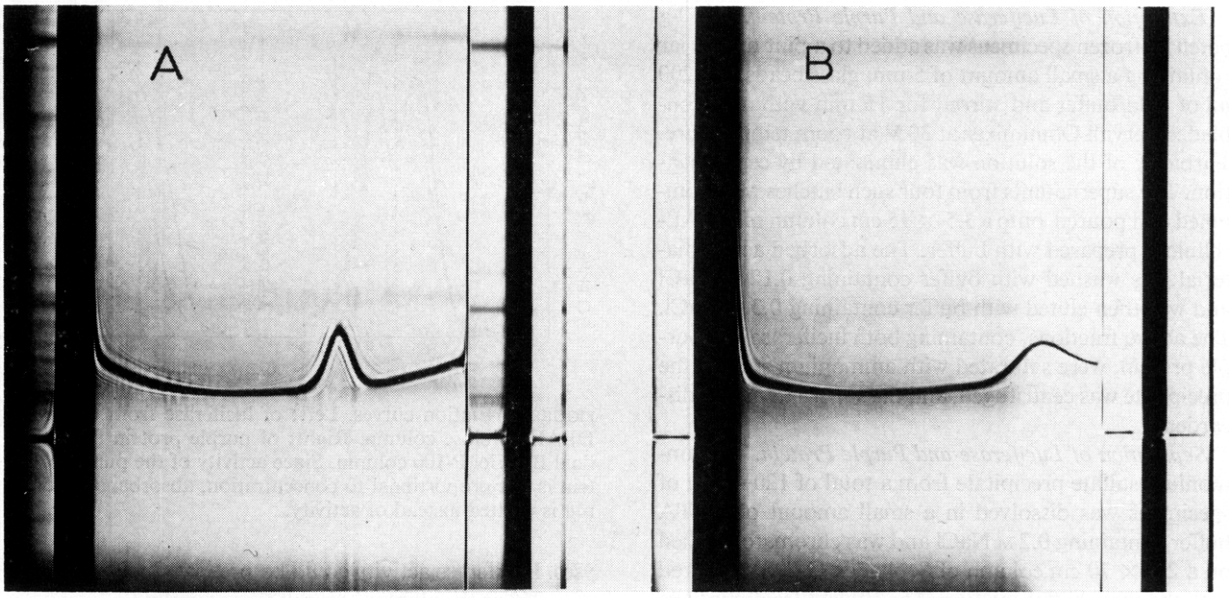


FIGURE 3: Ultracentrifugal pattern of luciferase at 2.8° (left) and of purple protein at 3.4° (right), both at 59,780 rpm, in 0.015 M sodium phosphate–0.00025 M EDTA– 0.1 M NaCl (pH 6.8) (after 56 min).

activity at once. Heating at 95° for 1 min caused complete loss of activity.

An ultracentrifugal run at 2.8° showed a single peak (Figure 3A). The sedimentation constant for 20° was computed as 6.9×10^{-13}. By the sedimentation equilibrium method, with the luciferase in EDTA buffer containing 0.1 M NaCl at 3° and 6166 rpm, the molecular weight was computed as 173,000. In both calculations, the partial specific volume was assumed to be 0.73.

Properties of the Purple Protein. The purple protein is reddish in buffer and purple in the $(NH_4)_2SO_4$ precipitate. The absorption spectrum (Figure 2) has two peaks in the visible region at 568 and 620 mμ. The chromophore could be a bile pigment but it evidently is not a porphyrin compound since the Soret band is lacking. Under ultraviolet light, the solution is strongly red fluorescent with two emission peaks at 570 and 640 mμ (Figure 4).[1] The color and the fluorescence irreversibly disappear on addition of $Na_2S_2O_4$. The activity of the purple protein in solution was not affected by treatment with ten volumes of ethanol or by heating at 100° for a few minutes.

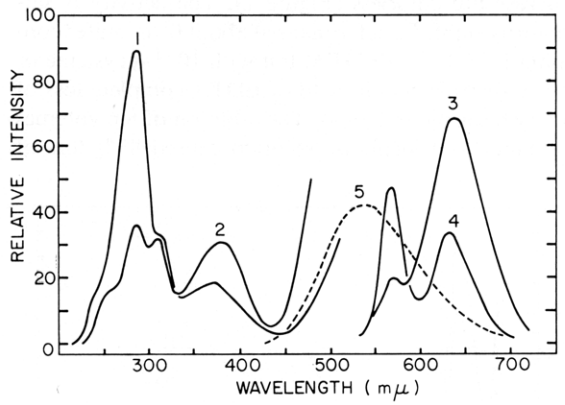


FIGURE 4: Fluorescence spectra of purple protein solution, optical density 0.45/cm at 280 mμ (solid line), and bioluminescence spectrum of the purified system (broken line), measured on an Aminco-Bowman spectrophotofluorometer with the corrected spectra attachment. (1) Excitation spectrum recorded at 630 mμ; (2) excitation spectrum recorded at 565 mμ; (3) emission spectrum excited at 285 mμ; (4) emission spectrum excited at 380 mμ; and (5) bioluminescence spectrum of a reaction mixture of 10,000 LU of luciferin, 1 μl of purple protein (optical density 0.45/cm at 280 mμ) and 10 μl of luciferase solution (optical density 2.7/cm at 280 mμ) in 5 ml of buffer.

An ultracentrifugal run at 3.4° showed a single peak (Figure 3B) and s_{20} was computed as 2.7×10^{-13}. The sedimentation equilibrium method, with the protein in EDTA buffer containing 0.1 M NaCl, at 3° and 8225 rpm, gave a molecular weight of 39,000, again assuming the partial specific volume as 0.73.

Kinetics of Bioluminescence Reaction. The change in total light and intensity against time is shown in Figure 5 for various amounts of luciferase added to a mixture of purple protein and luciferin, the latter two in fixed amounts.

The relationship between log intensity and time (broken lines) indicates that light emission is essentially a first-order reaction, and that the rate constants are practically the same for various concentrations of luciferase, except for curve 1 pertaining to the highest concentration. Since a reaction rate constant should, in general, be proportional to the concentration of an enzyme, this property of luciferase is rather unusual, as if it func-

[1] In principle, the peak at 640 mμ could be a sensitized fluorescence, since proteins containing tryptophan generally exhibit fluorescence at 340 mμ when excited at 280 mμ, and the 380-mμ excitation peak in Figure 4 could represent a transfer of energy giving rise to the 640-mμ fluorescence. It will be noted in Figure 4 that the 570- and 640-mμ fluorescence peaks do not retain their relative intensity when excited at the two different excitation peaks, suggesting the presence of two fluorescence emission groups of the purple protein.

TABLE I

Incubation Mixture	Mixture Added to, after *ca.* 2 min	Result
A. Luciferin + purple protein + O_2	Luciferase + O_2	No lag
B. Luciferin + luciferase + O_2	Purple protein + O_2	Lag
C. Luciferin + luciferase + purple protein	O_2	Lag
D. Purple protein + luciferase + O_2	Luciferin + O_2	Lag

tioned as a substrate rather than an enzyme during the initial few minutes included in Figure 5. At 60 min, however, the total light of curves 1, 2, 3, and 4 reached 120, 119, 102, and 78 LU, respectively, showing a gradual approach to a certain maximum, suggesting that the final total light is independent of the luciferase concentration and that two different rate constants are involved, one for the initial faster reaction and another for a slower reaction thereafter.

Various amounts of purple protein and fixed amounts of luciferase and luciferin were used in the experiment of Figure 6. The relationship between total light and time (solid lines) shows an initial lag which increases with decreasing concentrations of purple protein. The relationship between log intensity and time shows that, except for the lag period, the reaction is nearly first order. Moreover, at low concentrations of purple protein, the first-order rate constant decreases with decrease in concentration of this protein (curves 3–6) as if it acted in the manner of an enzyme. For the high concentrations (curves 1–3), the rate constant is nearly the same, probably indicating saturation of the system. It will be noted also that the total light decreases. While the decrease is only about 10% in the experiment illustrated in Figure 6, at still higher concentrations of purple protein relative to luciferase the reduction amounted to as much as 35%. In these respects the purple protein appears not to act in the manner of an enzyme.

With regard to the low concentrations, it is evident from Figure 6 (curve 6) that considerable light emission occurred when no purple protein was added. This fact can be interpreted in two ways: (1) purple protein is a nonessential, activating factor, or (2) the luciferase contained small amounts of it, not removable by the method employed; the actual amount present can be estimated as approximately 1% or less by weight of the proteins. The second interpretation appears more likely, because light intensity increased more than in proportion to increases in concentration of luciferase when no purple protein was added.

Some evidence regarding the sequence of reactions and the cause of the observed lag (Figure 6) was derived from the effects of preincubation of reaction mixtures, summarized in a qualitative way in Table I. The maximum effect was attained by preincubation for 1–2 min. In Table I O_2 refers to oxygen in solution equilibrated with air, and the length of the lag was approximately the same in (B–D). Solutions of the three components in mixture C were evacuated prior to mixing under vacuum.

It thus appears that (1) the slow reaction is a combination of the purple protein with luciferin, since preincubation of these two components plus O_2 abolishes the lag; (2) this combination is probably an equilibrium reaction, since the maximum effect occurs in about 2 min; and (3) the combination either requires the presence of oxygen or else oxygen combines somewhat slowly with a luciferin–purple protein or luciferin–purple protein–luciferase complex as the case may be (mixture C). Unfortunately, insufficient material was available for a more detailed study of the reaction mechanism, *e.g.*, by a rapid-flow method.

Because of the hydrophobic nature of luciferin, the method of adding it to the buffer influenced the time course as well as efficiency of light emission. Usually, 10 μl of an ethanolic solution was added to 2.5 ml of buffer by a micropipet. When the same amount of luciferin in a much smaller volume of ethanol was added,

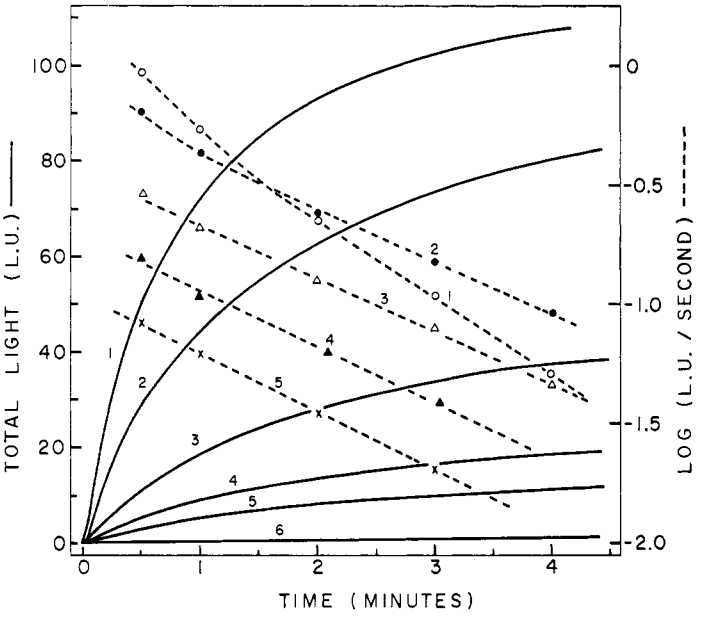


FIGURE 5: Influence of luciferase concentration on total light produced and intensity of luminescence as a function of time, with constant initial concentrations of luciferin and purple protein. Reaction mixtures in 5 ml of buffer contained 10 μl of luciferin solution, 10 μl of purple protein solution of optical density = 0.6/cm at 280 mμ, and luciferase solution of optical density = 1.2/cm at 280 mμ in the following amounts: curve 1, 10 μl; curve 2, 5 μl; curve 3, 2 μl; curve 4, 1 μl; curve 5, 0.5 μl; and curve 6, none added. The just detectable light produced in the absence of added luciferase is most probably attributable to a trace of luciferase contaminating the purple protein solution.

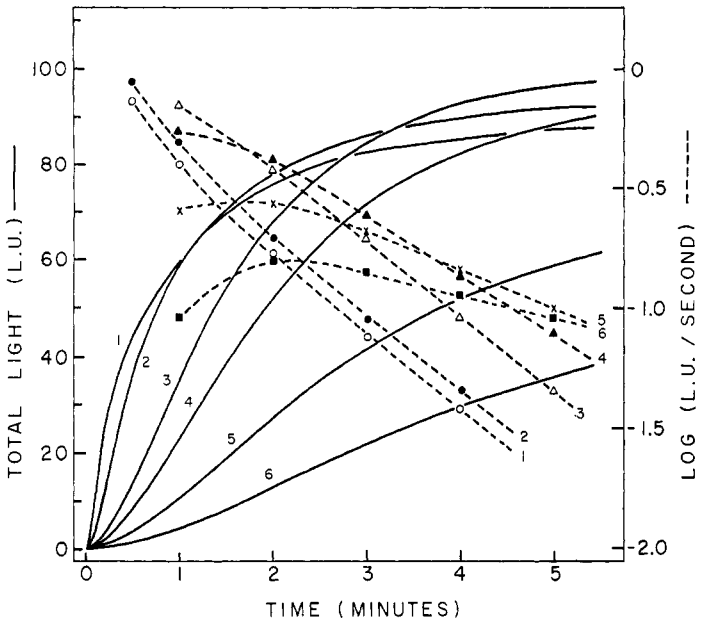


FIGURE 6: Influence of purple protein concentration on total light produced and intensity of luminescence as a function of time, with constant initial concentrations of luciferin and luciferase. Reaction mixtures in 5 ml of buffer contained 10 μl of luciferase of optical density = 1.2/cm at 280 mμ, 10 μl of luciferin solution, and the following amounts of purple protein of optical density = 0.6/cm at 280 mμ: curve 1, 20 μl; curve 2, 5 μl; curve 3, 1 μl; curve 4, 0.5 μl; curve 5, 0.2 μl; and curve 6, none added.

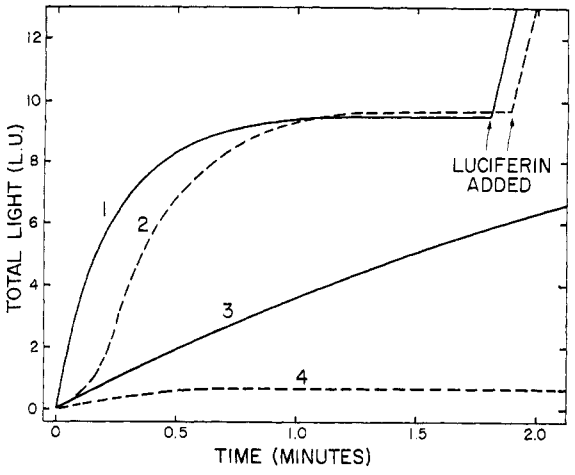


FIGURE 7: Influence of 2×10^{-4} M Fe^{2+} as $Fe(NH_4)_2(SO_4)_2$ on the time course and total light produced after incubating for 2 min with different combinations of reactants as follows: curve 1, (purple protein + luciferase + Fe^{2+}) + (luciferin), or (purple protein + Fe^{2+}) + (luciferase + luciferin); curve 2, (luciferase + Fe^{2+}) + (purple protein + luciferin); curve 3, (luciferase) + (purple protein + luciferin) and curve 4, (purple protein + luciferin + Fe^{2+}) + (luciferase). In each mixture, the amount of luciferase was sufficiently large for a fast reaction, and the amount of purple protein was small compared with that of luciferin.

or if the luciferin solution was not added rapidly and immediately dispersed by shaking, a dimmer, longer lasting luminescence resulted, probably because of uneven dispersal, in the form of an emulsion. On the other hand, if the same amount of luciferin in a considerably larger volume of ethanol was added, inhibition due to ethanol resulted. Addition of luciferin in *n*-hexane gave rise to virtually no light emission.

Inhibitors and Activators. Further study of the previously reported (Shimomura *et al.*, 1966) inhibition by Fe^{2+} indicates that low concentrations of this ion have the dual action of (1) augmenting the effect of the purple protein, and (2) causing a destruction of luciferin with little or no light emission. Referring to Figure 7, incubation of purple protein with Fe^{2+} and luciferase before mixing with luciferin, or incubation of purple protein with Fe^{2+} before mixing with luciferase plus luciferin, leads to an immediate, rapidly rising but short-lived luminescence (curve 1). Incubation of Fe^{2+} with luciferase before mixing with luciferin plus purple protein (curve 2) results in the same initial rate of light production as in a control with no added Fe^{2+} (curve 3), but the short initial period is followed by a rapid rise to the same total as that of curve 1. The total light of curve 3, not shown in the figure, was several times greater than that of curves 1 and 2. Abortive light emission, however, could be repeatedly elicited from the reaction mixtures of curves 1 and 2 by repeated additions of luciferin. Finally, addition of Fe^{2+} always reduced the total light produced, and prior incubation of luciferin and purple protein with Fe^{2+} resulted in practically no light production when the solution was mixed with luciferase (curve 4).

Cyanide, as previously reported (Shimomura *et al.*, 1966), is a potent inhibitor of this system. In the present study it was found that the inhibition by 10^{-4}–10^{-3} M KCN could not be reversed by dilution. Addition of luciferase to the inhibited system momentarily restored light emission, whereas the addition of luciferin or purple protein had practically no effect. Thus CN^- evidently inactivates luciferase.

o-Phenanthroline, which is strongly inhibitory at 10^{-4} M (approximately 80% inhibition), was found to inactivate luciferase in the same manner as CN^-. The presence of Fe^{2+} in luciferase is suspected from these results, although no further evidence for this has been obtained thus far.

Ascorbic acid showed a strong activation, with a maximum effect when luciferase was incubated with 10^{-3}–10^{-4} M ascorbate in buffer for 5 min; further incubation up to 1 hr showed no difference. The degree of activation, which varied with the relative amounts of each component, ranged from 5- to 50-fold, on the basis of the maximum intensity of luminescence which resulted.

Reduced diphosphopyridine nucleotide at 2×10^{-4} M, similarly incubated, showed some activation (approximately one-fourth of ascorbate), but cysteine at 10^{-3} M caused an approximately 50% inhibition.

Quantum Yields of Luciferase, Purple Protein, and Luciferin. The quantum yield, *i.e.*, (number of photons emitted)/(number of molecules reacted), with respect to luciferase can be expected to vary with the reaction time involved. For the faster phase of the reaction (see Figure 5) and with a large excess of luciferin, the quantum yield for luciferase was calculated to be 0.63; it could be

much higher for the slower phase (*vide infra*). The experimental details were as follows.

The luciferase solution containing 10^{-3} M ascorbate in 2.5 ml of buffer was cooled to 12° and incubated 5 min before mixing with 2.5 ml of buffer containing luciferin equivalent to 11,000 LU, plus 0.8 μl of purple protein solution having an optical density of 0.48/cm at 280 mμ, which had been similarly cooled and incubated. The amount of protein in the luciferase solution, according to the method of Warburg and Christian (1941), was 2.97×10^{-4} mg, by dilution of a stock solution of optical density = 2.7/cm at 280 mμ, and the molecular weight was taken as 173,000 from the present study. The averaged total light emitted in four experiments amounted to 5 LU in 1 min, 21 LU in 3 min, 40 LU in 5 min, 59 LU in 10 min, and 65 LU in 15 min. The quantum yield of 0.63 is based on the 15-min total of 65×10^{10} photons. With sufficient time for completion of the slower phase of the reaction, the maximum total quantum yield could be several times greater.

The turnover number of luciferase, defined as the number of molecules of luciferin which give a light-emitting reaction with one molecule of luciferase per second, averages 0.14 for the first 15 min, or a total of 126 luciferin to 1 luciferase molecule during the 15-min period. These figures are based on a value of 0.005 for the quantum yield of luciferin at 12°, obtained by interpolation of the data for 8 and 25° (*vide infra*).

The quantum yield of the purple protein was computed on the basis of the increased amount of light over and above the amount emitted by a control to which none of this protein was added. The quantum yield was 3.2, 7.0, and 8.1 after 5, 10, and 15 min, respectively, from the start of the reaction. The experimental details were as follows.

The first mixture consisted of 2.5 ml of buffer containing 2 μl of luciferase of optical density = 2.7/cm at 280 mμ, and 10^{-3} M ascorbate. The second mixture consisted of 2.5 ml of buffer containing luciferin equivalent to 11,000 LU, with or without purple protein added. The amount of purple protein used was 5.28×10^{-5} mg, according to the same method as with luciferase, and the molecular weight was taken as 39,000 from the data of this study. The first and second mixtures were cooled, held at 12° for 5 min, and then mixed. The amount of light emitted without purple protein added was 62, 152, and 280 LU in 5, 10, and 15 min, respectively; with purple protein added, it was 320, 720, and 940 LU for the same respective periods.

The quantum yield of luciferin turned out to be surprisingly low, *viz.*, 0.0030 at 25° and 0.0068 at 8°, based on total light of 110 and 250×10^{10} photons produced by 0.145 μg of luciferin at the respective temperatures. The weight of the luciferin used was adjusted by dilution of a stock solution calibrated in terms of light units with the previously reported value that 3.8×10^{5} LU = 0.50 mg of luciferin (Shimomura and Johnson, 1968a). Crude luciferase which was used for luciferin assays gave the same quantum yields for luciferin as sufficient concentrations of purified luciferase solutions, containing optimum concentrations of purple protein, to give a fast reaction.

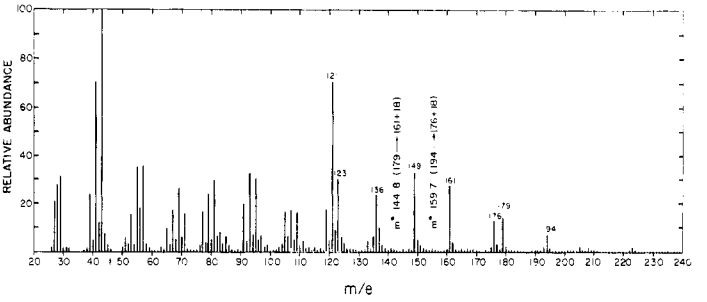
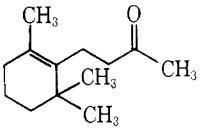


FIGURE 8: Mass spectrum of the product of luciferin in the bioluminescence reaction, 70 eV. The water used in the reaction was found to contain traces of impurities which give an intense peak at *m/e* 149 and also several peaks in the low *m/e* region with the highest at *m/e* 57.

Luminescence Product of Luciferin. Evidence concerning the structure of the product of luciferin in the luminescence reaction was sought by mass spectrometry. The procedure for obtaining the product was as follows. At 10°, 0.2 ml of 0.1 M sodium ascorbate and 1.5 ml of luciferase solution (optical density 2.7/cm at 280 mμ) were added to 100 ml of buffer, allowed to stand for 10 min, then 50 μl of purple protein solution (optical density 0.45/cm at 280 mμ) and 0.2 ml of luciferin solution (2.2×10^{6} LU) were added to the mixture to start the luminescence reaction. After 10 min, 0.5 ml of luciferase solution (the same as before) was added, and allowed to stand for 40 min at room temperature to complete the reaction. This solution was added to 20 ml of ethanol and extracted with *n*-hexane. The water layer was acidified to pH 2.5 with H_2SO_4, and reextracted with *n*-hexane. The *n*-hexane extracts were combined, evaporated, and subjected to mass spectrometry (Figure 8).

The main component with a molecular weight of 194 is most probably the product of the luminescence reaction of luciferin. This compound evidently has at least one oxygen atom, as judged by the metastable peaks. The structure of luciferin and the fragmentation pattern suggest dihydro-β-ionone (II) for this compound. For



II

further evidence, Figure 8 was compared with the mass spectrum of the synthetic compound of II (Shimomura and Johnson, 1968a). Considering the possiblity of contamination in treating very small amounts of luciferin with relatively large amounts of proteins, chemicals, and solvents, in addition to the fact that the water was actually contaminated, the spectrum of the luminescence product is judged to be virtually the same as that of the synthetic compound, strongly supporting the conclusion that the two compounds are indeed the same.

Discussion

Apart from oxygen, which is required in the great

majority of bioluminescence reactions, the reactants essential for light-emission in the *Latia* system are notably different from those which have been isolated thus far from any other type of luminescent organism (for recent synopsis, see Johnson, 1967). The roles of the components, the applicability of the terminology used, and the chemistry of the over-all reaction call for brief comment, as follows.

Role of Luciferin, Luciferase, and Purple Protein. First, in regard to luciferin, the evidence indicates that it functions as a specific substrate in an enzyme-catalyzed oxidation which results in light emission. Numerous structurally related compounds which were tested in this study for possible activity gave negative results. Under appropriate conditions, the total light produced is proportional to the amount of luciferin, over a wide range of concentrations. Moreover, analysis of the product indicates that this substrate is quantitatively oxidized in the reaction. Thus the term "luciferin" appears to be clearly applicable, despite the fact that under certain conditions, *e.g.*, low enzyme concentration or the presence of small concentrations of Fe^{2+}, the total light is not proportional to the initial concentration of luciferin. The low quantum yield is very likely attributable to the highly hydrophobic nature of this substrate, a property which is not shared by any other known type of luciferin.

Second, in regard to luciferase, the data on reaction kinetics and quantum yield in this and a previous study (Shimomura *et al.*, 1966) show that it catalyzes the luminescence reaction, though the activity decreases after an initial period of rapid reaction, possibly because of product inhibition. Moreover, it has the properties of specificity of action, a fairly high molecular weight protein, instability to heat and alcohol, etc., largely common to enzymes. Thus the term "luciferase" appears applicable.

Third, in regard to the purple protein, the quantum yield shows that it recycles, as does an enzyme and as does the nonprotein fluorescent substance in the photoprotein type of bioluminescence system of the shrimp *Meganyctiphanes* (Shimomura and Johnson, 1967, 1968b). However, the purple protein evidently combines with luciferin in the presence of oxygen, in a reaction which comes to equilibrium within 2 min at room temperature. In this respect it does not act in the manner of an enzyme which would be expected to result in a continuing accumulation of the reactive intermediate with continued incubation times. Moreover, the first-order kinetics of the luminescence reaction would not normally be expected with two sequential enzyme reactions limiting the production of light. Finally, the purple protein differs from most enzymes in its stability to heat and to such denaturants as alcohol. Thus, from the evidence at hand, it seems appropriate to consider this component as a specific cofactor.

The Light-Emitting Molecular Species. Of the three organic components, only the purple protein shows any significant fluorescence under ultraviolet light, and its fluorescence maxima at 570 and 640 mμ are quite different from the bioluminescence emission maximum at 535 mμ. The structure of the luciferin component makes the possibility that luciferin is converted into a fluorescent compound highly unlikely, and the evidence is against such a possibility. Thus it seems that the light-emitting species or complex must be formed after the reaction is initiated, from the luciferase or purple protein or a complex of the two.

Oxidation Scheme of Luciferin. The luminescence reaction requires oxygen, as previously reported for the incompletely purified system and confirmed in the present study with the purified components. From the structure of luciferin and of its product in the luminescence reaction, eq 1 is proposed. Because of the hydrophobic

$$\text{luciferin (I)} + O_2 \xrightarrow[\text{purple protein}]{\text{luciferase, } H_2O} h\nu + \text{II} + 2H\cdot COOH \quad (1)$$

nature of luciferin, concentrations sufficiently high to measure oxygen consumption by manometric or oxygen electrode methods can scarcely be attained in aqueous solutions of luciferase. Sensitive tests for the presence of formaldehyde as a product of the reaction with a maximum concentration of luciferin, however, were negative, thus indirectly supporting the above scheme.

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