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Extraction, Purification, and Properties of the Bioluminescence System of the Euphausid Shrimp *Meganyctiphanes norvegica**

Osamu Shimomura and Frank H. Johnson

ABSTRACT: By the procedures described, two components, a protein (P) and a fluorescent substance (F), have been extracted and highly purified from homogenized specimens of the euphausid shrimp *Meganyctiphanes norvegica*. P and F react in aqueous solution containing molecular oxygen, producing blue light which is qualitatively indistinguishable from the fluorescence emission of F under ultraviolet light. The reaction is extremely sensitive to pH near neutrality, *e.g.*, the light intensity rising from 4% of the maximum to 95% of the maximum in going from pH 7.4 to 7.6 in phosphate buffer. Substance F is unstable at acid pH and is oxidized by atmospheric oxygen or stoichiometrically by $K_3Fe(CN)_6$ at alkaline pH, the product(s) being reducible, in part, by $Na_2S_2O_4$. In neutral and alkaline aqueous solution the absorption spectrum has a peak at 393 mμ, the peak decreasing gradually during autoxidation, with a proportional decrease in luminescence activity. Substance P is a protein of relatively high molecular weight, *ca.* 200,000 or more, and extremely sensitive to heat, dilute solutions having a half-life of 10–25 min at 0° in Tris at pH 7.5, the optimum pH for the light-emitting reaction in the presence of F. In this reaction, oxidative decomposition of P is evidently the major source of energy for luminescence. The quantum yield with respect to P varies from 0 in the absence of F to 0.1 or more (depending on a definitive value for molecular weight of P) in the presence of adequate amounts of F. Under favorable conditions the quantum yield of F can be 10 or more, showing that F recycles in the light-emitting reaction. Since the protein component does not act as an enzyme and F does not act as a substrate, therefore this system is regarded as a new type of photoprotein system, rather than as a new type of luciferin–luciferase system analogous to those which have been previously demonstrated in extracts of various other types of luminescent organisms.

Euphausid shrimps, including species of all genera except *Bentheuphausia* which is nonluminescent, possess intricate, precisely organized, eye-like photophores, the structure of which has been extensively studied (Vallentin and Cunningham, 1888; Chun, 1896; Trojan, 1907; Dahlgren, 1916; Bassot, 1960a,b; 1966), while the biochemistry of the light-emitting process has remained unknown. Unsuccessful efforts have been made (Harvey, 1931) to extract a specific, heat-labile enzyme and heat-stable substrate analogous to those which by now have been obtained from various other types of luminescent organisms and referred to since the early work of Dubois (1887) by the general terms luciferase and luciferin, respectively (briefly summarized and discussed by Johnson (1966, 1967) and Hastings (1966)).

Recently, evidence has been found (J. D. Doyle, personal communication) that extracts of the photophores of two euphausids, *Meganyctiphanes norvegica* and *Thysanoessa raschii*, contain heat-labile and heat-stable components which when mixed in cool aqueous solution result in light emission. The present investigation, begun independently of Doyle's, has resulted in the isolation, from homogenized specimens of *M. norvegica*, of a heat-labile and a heat-stable component which, though of uncertain identity with those in Doyle's experiments, also give a luminescence reaction on mixing. Our heat-labile substance is a protein with molecular weight of 200,000 or more; the heat-stable substance is diffusible and under ultraviolet light emits a blue fluorescence which is qualitatively indistinguish-

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able from the bioluminescence emission in the reaction with the protein.

The evidence in this paper indicates that oxidative decomposition of the protein is the chief source of energy for the visible radiation of the luminescence reaction, whereas the fluorescent substance recycles. The system, therefore, is not analogous to luciferase–luciferin systems, but represents a new example of a type for which the term "photoprotein" has been suggested on the basis that no enzyme activity is directly involved in the luminescence reaction. Thus the *Meganyctiphanes* system is analogous to two previously known photoprotein systems, *viz.*, those of the jellyfish *Aequorea* and *Halistaura* (Shimomura *et al.*, 1962, 1963a,b) and the polychaete annelid worm *Chaetopterus* (Shimomura and Johnson, 1966), and the term photoprotein is used in this paper for the heat-labile component.

Materials and Methods

Live specimens were initially studied at the Kristinebergs Zoologiska Station in Sweden. Subsequently, specimens preserved with Dry Ice were obtained from Bergen, Norway, and at Millport, Scotland.

Because of the extreme sensitivity of this luminescence system to heat, a temperature as close to 0° as feasible was maintained in all experiments except those dealing directly with the quantitative effects of higher temperatures. A refrigerated Sorvall RC-2B with SS-34 head was used for centrifugation. A refrigerated Buchler fraction collector was used for chromatography, and in practically every instance a moderate pressure of argon was used to accelerate the rate of flow because of instability of the active material. For study of kinetics, the solutions were kept in an ice bath and removed for a few seconds at a time to measure light intensity during the course of the reaction.

Light intensity was measured in terms of arbitrarily defined light units by means of a photomultiplier–amplifier apparatus with automatic recorder for integrated light. Calibration of this apparatus in absolute units by the chemiluminescence of luminol according to the method of Lee *et al.* (1966) showed that 1 light unit was equivalent to 0.99×10^{10} photons. In close agreement, a value of 1 light unit = 1.05×10^{10} photons was found with the luminescence reaction of purified *Cypridina* luciferin and luciferase, in accordance with the quantum efficiency determinations of Johnson *et al.* (1962). Calibration by the secondary liquid scintillation method of Hastings and Reynolds (1966) indicated 2.8×10^{10} photons/light unit. In the present paper, 1 light unit is taken equal to 1×10^{10} photons.

Deionized distilled water was used throughout, and organic solvents, principally ethanol, were redistilled before using. Assays for luminescence activity were usually made by adding a small volume, 5–100 μl of the test materials, as specified, to 5 ml of 0.02 M Tris buffer containing 0.2 M NaCl, with the pH adjusted to 7.5 by means of HCl. With the arsenate buffer used for other purposes, the pH was also adjusted by HCl. In experiments involving precipitation by ammonium sulfate, the stated extent saturation is the value from a table of solubility in water at 25°, although the actual value was somewhat different, chiefly due to the fact that the solution was maintained close to 0°. Further details pertaining to methods are given with reference to specific experiments and procedures in the text.

Experiments and Results

Purification of the Photoprotein (P)[1] *and Fluorescent Substance (F).* The best procedure which was finally devised for extraction and purification of the active components is diagrammed in Figure 1 and described in the following paragraphs in accordance with the numbered steps.

(1) The frozen shrimps (68 g) were removed from Dry Ice and divided into four 17-g portions, and each portion was stirred with 150 ml of water at room temperature for a few seconds to separate and partially thaw the individual specimens. The turbid supernatant was drained, and the shrimps were stirred again with 150 ml of cold water for a few seconds to remove more of the salts. The water was again drained and the specimens were transferred to a cold mortar and ground with some sand for a few minutes. Cold water (60 ml) was added to the paste and thoroughly mixed resulting in a brei which was not luminous because of slight acidity due to residual carbon dioxide from storage with Dry Ice. Four batches simultaneously prepared by the above method were combined and centrifuged at 15,000 rpm for 10 min. The precipitate was discarded.

(2) The pH of the supernatant was brought down to 5.6–5.7 by addition of 19.5 ml of 0.2 M acetic acid and the cloudiness that formed was removed by centrifugation at 15,000 rpm for 10 min.

(3) To the clear supernatant, 25 g of DEAE-cellulose, by wet weight of filter cake, with pH adjusted to 5.75 before filtration, was added and stirred for 1 hr at 0°. The slush was then filtered on a cold Büchner funnel. The filter cake was temporarily stored in Dry Ice until the next step. Most of the active components were adsorbed on DEAE-cellulose, and luminescence could be elicited simply by mixing the cellulose with Tris buffer (pH 7.5) with some NaCl added.

(4) Two frozen filter cakes were broken into small pieces and mixed with 10 g of plain cellulose powder and 150 ml of 0.01 M sodium arsenate–0.01 M sodium azide buffer (pH 6.5). When completely thawed, the mixture was poured and packed into a chromatographic column, 3-cm i.d., which had been packed with a 1-cm layer of DEAE-cellulose in advance. Argon pressure was used to increase the flow rate. After the column was tightly packed, the active components were eluted

[1] Abbreviations used: P, photoprotein; F, fluorescent substance; DPN^+ and DPNH, oxidized and reduced diphosphopyridine nucleotides; FAD, flavin–adenine dinucleotide; BSA, bovine serum albumin; BAL (British Anti-Lewisite), 2,3-dimercapto-1-propanol.

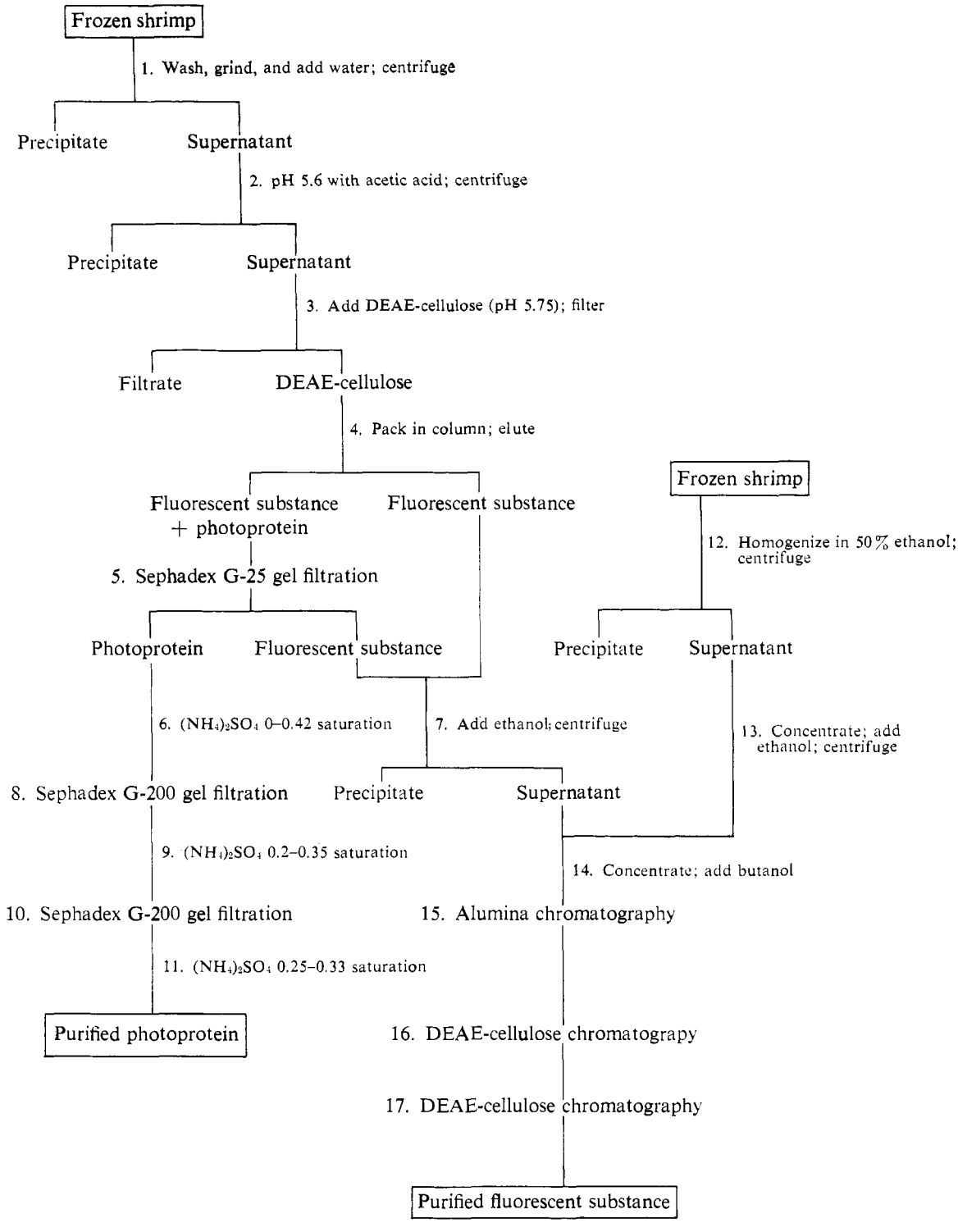

FIGURE 1: Diagram of the purification procedure for the photoprotein and the fluorescent substance. Details are given in the text.

with 0.5 M KCl–0.01 M sodium arsenate–0.01 M sodium azide (pH 6.5) and the eluate was collected in approximately 10-ml fractions. The luminescence activity of each fraction was checked by adding 0.05 ml of eluate to 5 ml of 0.02 M Tris–0.2 M NaCl (pH 7.5). The strongest activity was usually found in the second fraction counted from the front end of the eluting solvent. The solution was cloudy, light yellowish brown in

color, and blue fluorescent under ultraviolet light. Nearly 90% of the activity was found in the first four or five fractions. The next four to five fractions, which showed fairly strong fluorescence but had very little activity, were mixed with an equal volume of ethanol and stored in a deep freezer; these fractions were later mixed with the fluorescent material separated in step 5 from the Sephadex G-25 column.

(5) The first four to five fractions, containing nearly 90% of the luminescence activity, were combined and poured into a column of Sephadex G-25 (fine) (2.5 × 22 cm) packed with 0.5 M KCl–0.01 M sodium arsenate–0.01 M sodium azide (pH 6.5). Elution was done with the same buffer, and the eluate was collected in fractions. The active protein component passed through the column with no detectable retention, whereas the fluorescent component was considerably retained, resulting in an effective separation of the two, although they remained slightly contaminated with each other. The activity of the protein fractions was checked by the usual procedure, *i.e.*, by adding to 5 ml of the Tris buffer a small amount (0.05 ml) of the fluorescent solution and a small amount (0.05-0.1 ml) of the solution to be tested. The activity of the fluorescent components was visually judged by the intensity of fluorescence under a Mineralight, without testing luminescence activity.

(6) The protein fractions, which contained approximately 90% of the total activity, were combined and the active protein was precipitated at 0° with ammonium sulfate in an amount to give 0.42 saturation at 25°. The mixture was centrifuged at 12,000 rpm for 10 min, and the precipitate was stored in Dry Ice in an evacuated flask before further purification.

(7) The fluorescent fractions were combined and an equal volume of cold ethanol was added. This solution was combined with the fluorescent solution from the previous DEAE-cellulose column (from step 4) kept at −25° for a period of from 2 hr to 1 night. The precipitate that formed was removed by centrifugation.

The supernatant was vacuum evaporated to a volume of 10 ml, with the aid of a rotating flask and Dry Ice–acetone trap. To this concentrate, two volumes of cold ethanol was added, and the resulting precipitate was removed by centrifugation. The supernatant was temporarily stored in an evacuated flask in Dry Ice before further purification.

(8) Five to seven batches of frozen precipitate of protein were first partially thawed, then dissolved in 20 ml of 0.02 M sodium arsenate–0.5 M KCl buffer (pH 6.6), the insoluble matter was centrifuged at 18,000 rpm for 10 min, and the supernatant was filtered through a Sephadex G-200 column (2.5 × 20 cm) previously equilibrated with the same buffer which was used to dissolve the protein. The eluate was collected in 5-ml fractions. Each fraction was immediately checked for specific activity after suitable dilution of those fractions having an optical density greater than 2 OD/cm, in order to avoid errors due to a poor linearity between the measured luminescence activity and various concentrations of the photoprotein in the undiluted solutions.

(9) The fractions which had the highest specific activity, adding up to 75–80% of the total eluted, were combined and fractionated by ammonium sulfate precipitation.

(10) The protein which precipitated at between 0.2 and 0.35 saturation was further purified by repeating the gel filtration through the Sephadex G-200 column; the results are summarized in Table I.

TABLE I: Final Purification of Photoprotein with Sephadex G-200.

Fraction No. (4 ml each)	OD/cm (280 mμ)	OD after Dilution (*A*)	Initial Intensity (*B*) (light units/min)	Sp Act. (*B*/*A*)
1	0.03	0.03	0.5	16.6
2	0.43	0.43	8.5	19.8
3	2.42	2.42	47	19.4
4	6.60	2.20	40	18.2
5	8.40	2.24	37	16.5
6	7.50	2.40	34	14.2
7	3.85	2.30	16	7.0
8	2.7	2.20	7	3.2

(11) Finally, only fractions 3–6 of Table I were further fractionated with ammonium sulfate and only the material that came down at 0.25–0.33 saturation was saved.

(14) Five batches of the fluorescent substance prepared in step 7 (or by the alternative method of 12 and 13, described below) were combined and evaporated *in vacuo* to 25–30 ml, followed by addition of 40 ml of cold ethanol, and the resulting precipitate, mostly salts, was removed by centrifugation. To this solution, 200 ml of cold 1-butanol was added, and the cloudiness that formed was removed by centrifugation.

(15) The clear butanol solution was now poured into a chromatographic column, 3.5-cm i.d., packed to a height of 10 cm with alumina (basic) using 1-butanol. The fluorescent compound adsorbed on the upper half of the alumina. It was eluted with 0.35 N ammonium hydroxide in 50% ethanol, and the eluate was collected in fractions. The activity of each fraction was checked by adding 5 ml of the usual Tris buffer and 0.05 ml of active protein solution to 0.05 ml of eluate fraction. The luminescence activity of F usually corresponded to the intensity of fluorescence visually observed. The active fractions were combined, a few milligrams of Tris crystals were added, and the solution was vacuum evaporated to 5–6 ml, followed by the addition of 10 ml of ethanol, and keeping at −25° for approximately 20 min. The precipitate that formed was removed by

centrifugation, and the greenish supernatant was diluted with ten volumes of 50% cold ethanol which had been deaerated with argon in advance to remove oxygen.

TABLE II: Final DEAE-Cellulose Chromatography of Fluorescent Substance.

Fraction No. (5 ml each)	OD/cm (390 mμ)	OD after Dilution (*A*)	Initial Intensity (*B*) (light units/min)	Sp Act. (*B*/*A*)
1	0.04	0.04	0	0
2	2.60	2.0	9.0	4.5
3	10.2	2.14	14.0	6.55
4	6.40	2.30	15.2	6.61
5	1.70	1.70	11.3	6.65
6	0.40	0.40	2.6	6.5

(16) The above solution was poured into a column (3.5 × 15 cm) packed with DEAE-cellulose in 50% ethanol. The fluorescent compound was adsorbed on the upper half of this column and was eluted by 20 ml of 0.2 M NaCl–0.01 M sodium arsenate in 50% ethanol (pH 7.3) (the pH was checked before addition of ethanol) followed by 0.3 M NaCl–0.01 M sodium arsenate in 50% ethanol (pH 7.3). The eluate was collected in fractions, each of which was checked for luminescence activity, and the active fractions were combined by the same method as the one for the eluate of the previous chromatography. In the process, a bluish pigment, which along with a yellowish one (F) gave a greenish supernatant referred to above, separated from the mixture and passed through the column.

The combined active, fluorescent solutions were again evaporated to give 5–6 ml of yellow solution, and a major part of the salt was removed by centrifugation after addition of 10 ml of cold ethanol.

(17) The solution was purified again by DEAE-cellulose chromatography. In this chromatography, a 2 × 8 cm DEAE-cellulose column was used, and the

TABLE III: Stability of the Fluorescent Substance and Photoprotein during Storage under Various Conditions.

Substance (in moderate concn)	Solution (M)	Storage Conditions (°C)	Act. Lost (%) (approx)
Fluorescent substance	Tris (0.02)–NaCl (0.2), pH 7.5	In air (25)	In 1.5 hr (50)
	Tris (0.02)–NaCl (0.2), pH 7.5	In air (0)	In 1.5 hr (15)
	50% ethanol	In air (0)	In 18 hr (50–60)
	50% ethanol	Air evacuated (0)	In 18 hr (10)
	BAL (0.01) or cysteine in 50% ethanol	In air (0)	In 18 hr (10–20)
	HCl (0.01) in 30% ethanol	In air (0)	In 2 min (95)
	75% ethanol	Air evacuated, in Dry Ice	Not detectable after 1 week
Photoprotein	Tris (0.02)–NaCl (0.2), pH 7.5	In air (0)	In 10–25 min (50)
	Sodium phosphate (0.05), pH 6.3	In air (0)	In 18 hr (90)
	Sodium arsenate (0.05), pH 6.3	In air (0)	In 18 hr (86)
	Sodium arsenate (0.02)–KCl (0.5), pH 6.0	In air (0)	In 18 hr (80)
	Sodium arsenate (0.02)–KCl (0.5), pH 6.3	In air (0)	In 18 hr (70)
	Sodium arsenate (0.02)–KCl (0.5), pH 6.6	Air evacuated (0)	In 18 hr (60)
		In air (0)	In 18 hr (60)
		In air (15)	In 10 min (50)
	Sodium arsenate (0.02)–KCl (0.5), pH 6.6, plus $(NH_4)_2SO_4$ (0.4 saturation)	In air (0)	In 18 hr (45)
		In vacuo, in Dry Ice	After 3 weeks (50)
	Sodium arsenate (0.02)–KCl (0.5), pH 6.6, plus 1.5% BSA	In air (0)	In 18 hr (25)

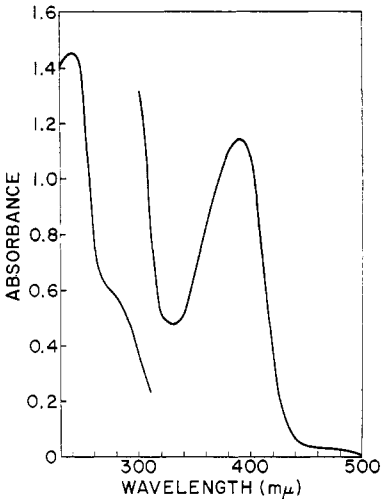

FIGURE 2: Absorption spectrum of the fluorescent substance in 50% ethanol.

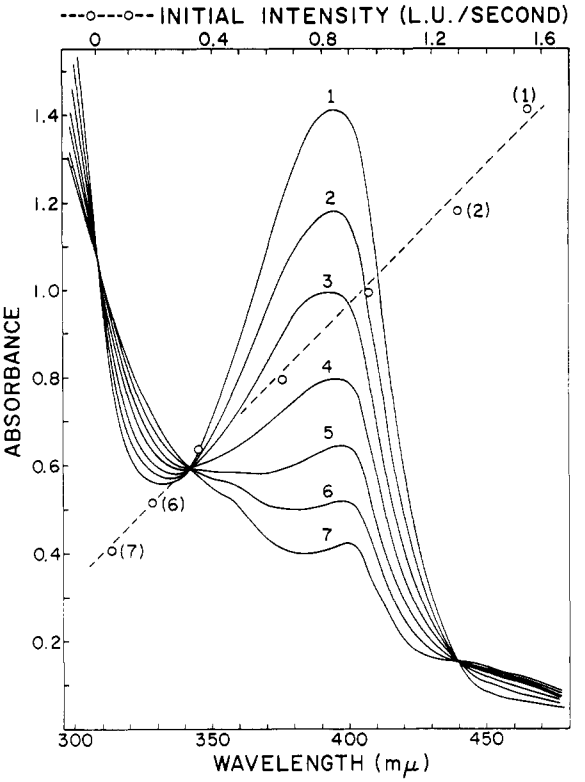

FIGURE 3: Changes in the absorption spectrum of the fluorescent substance in 0.02 M Tris–0.2 M NaCl (pH 7.45) while standing exposed to air in a 1-cm light-path cell. Curve 1: 3 min; 2, 45 min; 3, 1 hr and 35 min; 4, 3 hr and 15 min; 5, 5 hr and 10 min; 6, 7 hr and 50 min; and 7, 17 hr. The relation between activity and optical density at 390 mμ at various times is indicated by open circles and a broken line. Activity was measured using 0.05 ml of test solution and 0.05 ml of photoprotein solution. An approximate correction for activity was made in accordance with the rate of spontaneous inactivation of photoprotein while standing.

sample was added to the column after dilution with ten volumes of 50% ethanol, the same as in the previous run. Elution, however, was carried out using only 0.1 M NaCl–0.01 M sodium arsenate in 50% ethanol (pH 7.3). For each fraction collected, absorbance at 390 mμ was measured as well as luminescence activity (Table II). Solutions having an optical density greater than 2 OD/cm were suitably diluted before measuring their luminescence activity and the specific activity was computed from solutions having an absorbance close to 2, as with the photoprotein solutions referred to above. The fractions having the highest and almost constant specific activities were combined, yielding about 75% of the activity of the total eluted. The yellowish solution was concentrated *in vacuo*, two volumes of cold ethanol was added, and precipitated salt was removed by centrifugation and then stored in an evacuated flask in Dry Ice until used.

Alternative Purification Method for F. (12) The fluorescent substance could be conveniently extracted and purified by the following alternative method which, though simpler and in some ways better, involved sacrificing the accompanying protein. Frozen shrimps (85 g) were homogenized with 50 ml of ethanol and 200 ml of 50% ethanol using a Servall Omni-Mixer at 70 v for 3 min. The homogenate was centrifuged at 15,000 rpm for 15 min, and the precipitate was discarded.

(13) The supernatant was evaporated to 15–20 ml *in vacuo*, two volumes of cold ethanol was added, and the resulting precipitate was removed by centrifugation. Six to seven batches of solution prepared by this method were purified by the same method as described above, using alumina and DEAE-cellulose (steps 14–17).

Properties of the Photoprotein. The ultraviolet spectrum of photoprotein purified by the method described is that of a colorless simple protein, with a value of 1.4 for the ratio of optical density at 280 mμ/optical density at 260 mμ, and with a slight bulge at 290 mμ. Photoprotein solution (1 ml) with optical density of 1.0/cm at 280 mμ, was found to contain approximately 1.0 mg of protein after removal of salts and drying. The photoprotein was precipitated by ammonium sulfate at 0–4° in a relatively narrow range of concentrations, *i.e.*, between 0.25 and 0.33 saturation. Judging from the results of gel filtration through Sephadex G-200 during purification, the molecular weight is expected to be relatively large, perhaps over 200,000.

The stability of the photoprotein under several conditions is summarized in Table III. In general, it is most stable at pH 6.6 with some salts added, the stability decreasing at higher or lower pH, and in higher or lower salt concentrations. NaCl and KCl, both in 0.5 M concentrations, had stabilizing effects to about equal extents. KCl was employed in most cases, however, because it was more favorable than was NaCl for gel

TABLE IV: Effects of Various Substances on Initial Light Intensity.

Reagent	Concn (M)[a]	Reduction of Intensity (%)
$HgCl_2$ (NaCl added)	10^{-6}	96[b]
p-Mercuribenzoic acid	10^{-6}	55[b]
$CuSO_4$	2×10^{-5}	30
	10^{-4}	80
$Zn(CH_3COO)_2$	2×10^{-5}	60
	10^{-4}	94
$CdCl_2$	10^{-4}	80
$NiCl_2$	10^{-4}	70
$Co(CH_3COO)_2$	10^{-3}	65
$FeCl_3$	2×10^{-4}	15
$K_3Fe(CN)_6$	3×10^{-7}	50[c]
$K_4Fe(CN)_6$	10^{-4}	10
KCN	10^{-3}	0
EDTA	10^{-3}	0
$NaHSO_3$	10^{-3}	0
Menadione	10^{-4}	5
Hydroquinone	10^{-3}	35
H_2O_2	10^{-2}	15
Cysteine	10^{-3}	0
Glutathione (reduced)	10^{-3}	0
ATP	5×10^{-4}	15
DPN^+	3×10^{-4}	0
DPNH	3×10^{-4}	12
FAD	10^{-6}	6
Coenzyme A	5×10^{-4}	20
Pyridoxal phosphate	10^{-4}	20
Folic acid	4×10^{-4}	35
Cytochrome *c*	1 mg/5 ml	45
BSA	0.5%	50

[a] Final concentration in reaction mixture. [b] Partially reversed by BAL. [c] Per cent of reduction varies with the amount of fluorescent substance used.

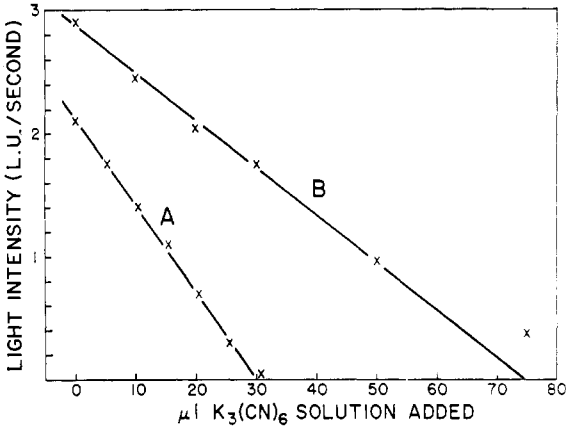

FIGURE 4: Titration of the fluorescent substance with $K_3Fe(CN)_6$ solution. (A) A 50% ethanol solution (1 ml) of the fluorescent substance, OD 4.48/cm at 390 mμ, plus 1 ml of 0.02 M Tris–0.2 M NaCl (pH 7.5) was titrated with 1.05×10^{-2} M $K_3Fe(CN)_6$. At each addition of 5 μl of $K_3Fe(CN)_6$, a 50-μl portion was taken out and used for checking luminescence activity, using 0.1 mg of photoprotein which had lost about 30% of its initial activity on standing. A correction for the amount of the solution which was taken out was made before plotting. The straight line falls to zero intensity at 30 μl. (B) To seven portions of 50 μl each of the solution of fluorescent substance in 50% ethanol, OD 2.24/cm at 390 mμ, 5 ml each of the usual Tris–NaCl buffer (pH 7.5) and various amounts of 1.05×10^{-4} M $K_3Fe(CN)_6$ were added, then luminescent activities were checked by adding 0.1 mg of photoprotein to each portion. The straight line falls to zero intensity at 75 μl.

filtration. Attempts to stabilize the protein at pH 7.5 (favorable for the luminescence reaction), by addition of various agents such as EDTA, cysteine, 2-mercaptoethanol, etc, were unsuccessful. BSA was found to be an effective stabilizer at pH 6.6, but a strong inhibitor at the more alkaline pH required for luminescence. Ammonium sulfate precipitation had not much effect in increasing stability when the precipitate was kept at 0°. When the precipitate was kept under vacuum in Dry Ice, not much inactivation was observed in 2–3 days, although a large amount of insoluble, inactive protein was formed after 2–3 weeks. Unlike various other systems, stability of the protein decreased after purification, perhaps due to the removal of stabilizing components. For least loss of activity, the purified ammonium sulfate precipitate was stored under vacuum in Dry Ice. For use, a portion of the precipitate was dissolved in 0.02 M sodium arsenate–0.5 M KCl (pH 6.6).

Properties of the Fluorescent Substance. The highly purified fluorescent substance in solution is yellow, with a tinge of green, and has a strong, sky blue fluorescence under a Mineralite. The molecular weight, judged by gel filtration, is probably less than 1000. The substance is soluble in water, methanol, and ethanol, slightly soluble in butanol, insoluble in ethyl acetate, ether, or chloroform, and quickly decomposes at acid pH. When a neutral aqueous solution of the substance is shaken with ethyl acetate, the substance stays in the water layer. After acidification, however, it decomposes to a product which has a somewhat different blue fluorescence and no luminescence activity. This product goes into the ethyl acetate layer and is recovered in the water layer after alkalization. These facts suggest that the decomposition product, and perhaps the original fluorescent substance as well, has an acidic nature. The active fluorescent substance is unstable in air, presumably because of oxidation. The stabilities under several principal conditions are summarized in Table III.

The absorption spectrum in 50% ethanol, recorded on a Perkin-Elmer 202 spectrophotometer and illustrated

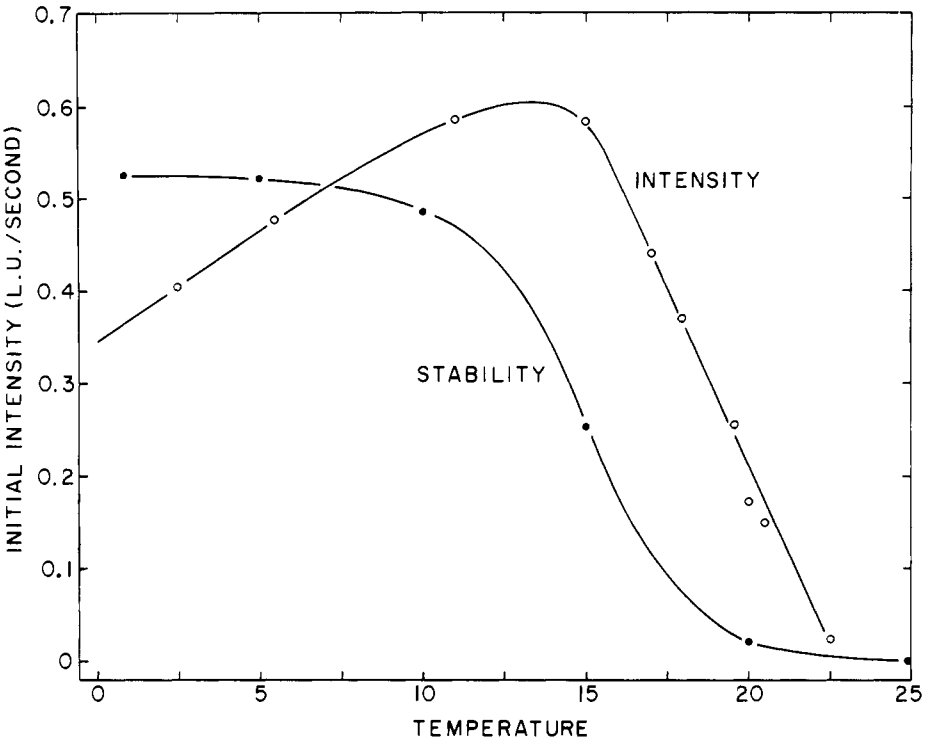

FIGURE 5: Effect of temperature on stability and luminescence intensity. Stability: 0.1 ml of photoprotein in 0.02 M sodium arsenate–0.5 M KCl (pH 6.6) was incubated in a water bath for 10 min, then cooled in ice, and activity was checked by mixing 5 ml of 0.025 M Tris–1 M NaCl (pH 7.59) with 0.05 ml of fluorescent substance solution. Intensity: 0.05 ml of fluorescent substance solution was added to 5 ml of 0.02 M Tris–0.15 M NaCl (pH 7.5) at various temperatures, followed by addition of 0.1 ml of photoprotein solution. Temperature was measured with a thermister.

in Figure 2, has a characteristic peak at 390 mμ. Although the absorption spectrum in 0.05 N NaOH in 50% ethanol is practically identical with the one at neutrality, acidification with 0.05 N HCl in 50% ethanol results in the disappearance of the peak at 390 mμ along with disappearance of luminescence activity.

Figure 3 shows the changes in absorbance of a solution of the fluorescent substance left in air at 25°, as well as the relation between optical density and luminescence activity on standing. Approximately 90% of the activity was destroyed in 8 hr. From Figure 3, a proportionality is evident between the decrease of activity and the decrease of optical density at 390 mμ, despite certain difficulties in the corrections made for spontaneous inactivation of the photoprotein used in the assays; although kept under the most stable conditions, this loss amounted to about 60% in 18 hr (Table III). In this experiment, relatively large amounts of photoprotein were used in the assays, in order to minimize errors due to the nonlinear relation between concentration of reactants and luminescence intensity. An unknown, but evidently not large, error could arise from possible further decomposition of the primary oxidation product of the fluorescent substance. Although the change of fluorescence was not measured quantitatively, visual observations indicated that the decrease of the characteristic blue fluorescence is accompanied by decrease of luminescence activity and decrease of optical density at 390 mμ. In the experiment of Figure 3, after 17 hr the solution showed only a weak bluish purple fluorescence.

The fluorescent substance is also oxidized by ferricyanide. The inhibition of the luminescence reaction by $K_3Fe(CN)_6$ (Table IV) is extremely strong, and it was found that whereas this reagent quickly inactivates the fluorescence substance, it has very little effect on the photoprotein. The results of titration of the fluorescent substance with $K_3Fe(CN)_6$ are shown in Figure 4. The titrations were carried out at two concentrations of the substance, differing by a factor of 100, and the amount of the substance remaining was determined by luminescence activity. At both concentrations the ratio between the amount of the fluorescent substance used and the amount of ferricyanide needed to inactivate it shows remarkable agreement, *i.e.*, in Figure 4, the ratio of the two total amounts of F ($B:A$, 2.5×10^{-2}) is equivalent to the extrapolated total $K_3Fe(CN)_6$ requirements ($B:A$, 2.5×10^{-2}), proving that the oxidation by ferricyanide is stoichiometric. Assuming that the reaction is an oxidation of 2 equiv/mole, which is usual for ordinary organic compounds, the molecular extinction coefficient of the fluorescent substance was calculated as 2.84×10^4. There remains the possibility, however, of a one-electron transfer which

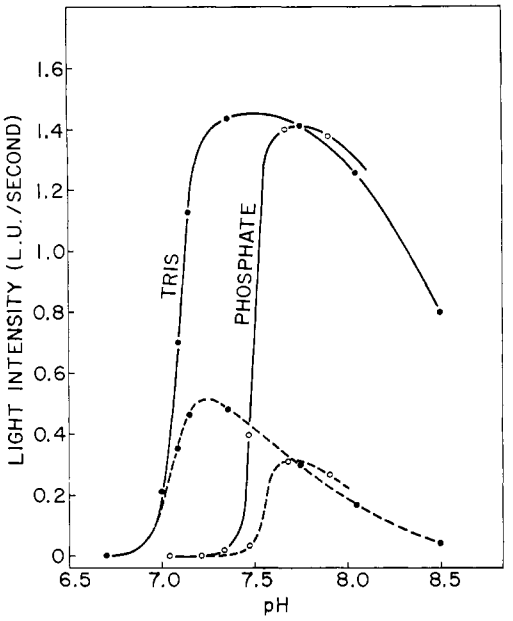

FIGURE 6: Effect of pH on the luminescence reaction in 0.025 M Tris–1 M NaCl (solid circles) and in 0.025 M sodium phosphate–1 M NaCl (open circles). The pH of the former was adjusted by the concentration of HCl, and that of the latter by the ratio of monobasic and dibasic salts. Photoprotein and fluorescent substance solutions (0.05 ml each) were added for the luminescence reaction near 0° and the pH was measured at 25° after finishing measurement of luminescence. Solid lines: effect on initial light; broken lines: after 10 min.

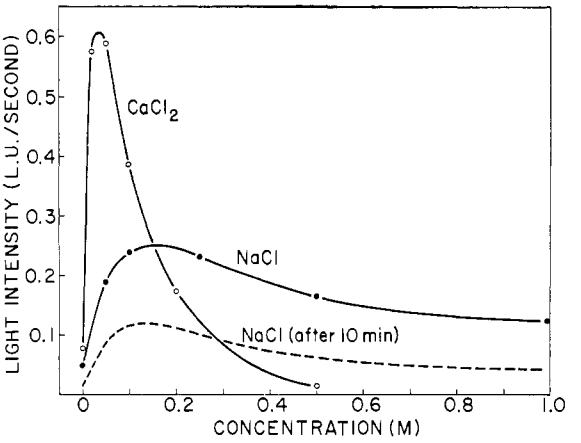

FIGURE 7: Influence of concentration of salts. The various concentrations of salts were dissolved in 0.02 M Tris, followed by adjusting pH to 7.6 with HCl, and the initial light intensities were measured after adding 0.05 ml each of photoprotein and fluorescent substance solution. Intensity after 10 min in NaCl buffer is shown by the broken line.

occurs under certain conditions with *Cypridina* luciferin. If such were the case, the coefficient would be 1.42×10^4.

After oxidation by atmospheric oxygen or by ferricyanide, the fluorescent substance could be partially reduced to the active form by $Na_2S_2O_4$. The active form could not be similarly recovered, however, after inactivation by acid.

Influence of Temperature, pH, Concentration of Salts, and Inhibitors on the Luminescence System. The photoprotein is extremely sensitive to heat, and a momentary exposure to room temperature usually results in nearly total loss of luminescence activity. The fluorescent substance, on the other hand, is stable at the same temperature, in the absence of oxygen. The extent of inactivation of the photoprotein on exposing to various temperatures for 10 min, as well as the initial light intensity at various temperatures, are shown in Figure 5. The optimum temperature for initial intensity was found to be 13°.

One of the most characteristic features of this luminescence system is a strong dependence on pH. Four kinds of buffer systems were tested, namely, Tris-HCl, triethanolamine-HCl, Veronal-HCl, and sodium phosphate, at a concentration of 0.02 M with 1 M NaCl added. In regard to initial light intensity, the first three buffers gave no light at pH 6.8. With rise in pH, the intensity increased sharply, reaching a maximum at around 7.5, then decreasing at more alkaline pH. The rise of intensity on the acidic side of the maximum was very steep, requiring only about 0.2 pH to go from 10% of the maximum to 90% of the maximum intensity.

With Veronal buffer some inhibition was observed, but the same inhibition covered the entire pH region tested, thus leading to almost no change in relative shape of the curve. With phosphate buffer, the intensity

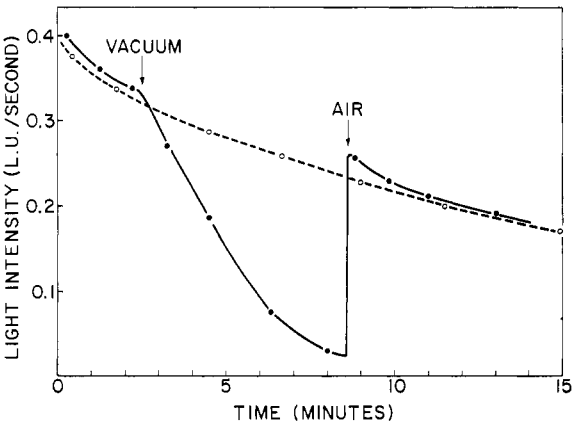

FIGURE 8: Necessity of oxygen for the luminescence reaction. Fluorescent substance solution (0.06 ml) and photoprotein solution (0.1 ml) were added to 5 ml of buffer (pH 7.45) in a tube specially designed for the purpose of evacuation while in place opposite the window of the phototube.

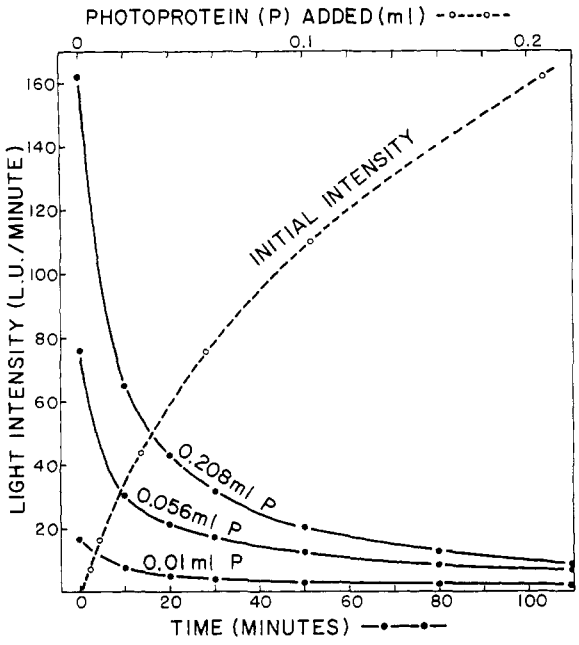

FIGURE 9: Influence of photoprotein concentration on initial light intensity (broken line) and on the course of the luminescence reaction (three solid lines). To start the luminescence reaction, 0.052 ml of fluorescent substance solution, OD 1.2/cm at 390 mμ, and various amounts of 35%-inactivated photoprotein solution (2.5 mg/ml) were added to 5 ml of 0.02 M Tris–0.15 M NaCl (pH 7.6).

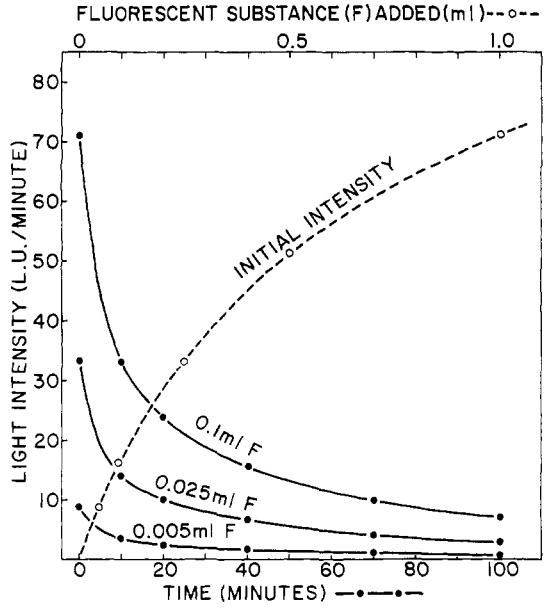

FIGURE 10: Influence of concentration of the fluorescent substance on initial light intensity (broken line) and on the course of the luminescence reaction (three solid lines). To start the luminescence reaction, various amounts of fluorescent substance solution, OD 1.2/cm at 390 mμ, and 0.14 mg of 60%-inactivated photoprotein were added to 5 ml of 0.02 M Tris–0.15 M NaCl (pH 7.6).

was almost zero at pH 7.2 and reached a maximum at pH 7.75, possibly indicating an inhibition by $H_2PO_4^-$; in fact, in concentrations of more than 0.04 M, phosphate buffer at any pH begins to be strongly inhibitory. The results for Tris-HCl buffer and sodium phosphate buffer are shown in Figure 6. In each of the four buffers, luminescence intensity showed a considerable drop after 10 min, accompanied by a slight shift of the maximum toward the acid side, indicating, as expected, more inactivation on the alkaline than on the acid side.

The extraordinary sensitivity of the light-emitting reaction to pH in the region of neutrality suggests the possibility that small changes in pH constitute part of the controlling mechanism of the flash *in vivo*.

Concentration of salts also was found to have strong influence on this luminescence system. Although extremely low concentration of salts could not be tested because of instability of the photoprotein, very weak light was produced in solutions of fairly low salt concentration. Relations between initial light intensity and different concentrations of NaCl and $CaCl_2$, each with 0.02 M Tris at pH 7.6, are shown in Figure 7. The optimum concentration of NaCl was found at 0.15 M and of $CaCl_2$ at around 0.04 M. At the start, the maximum intensity in the buffered $CaCl_2$ is much higher than that in the buffered NaCl, but after the luminescence reaction had proceeded for 10 min, the intensity in the two salts had dropped to the same level. Initial intensities in 0.1 M solutions of KCl, LiCl, and $MgCl_2$, in the same buffer as used in the experiment of Figure 7, were found to be 0.24, 0.29, and 0.20, respectively, whereas under the same conditions, in NaCl and $CaCl_2$ the initial intensities were 0.24 and 0.39, respectively. Bromine salts gave less activation than chlorine salts.

Table IV summarizes the results obtained in tests for inhibitory effects by a variety of substances. Relatively few substances caused strong inhibitions, the most outstanding being heavy metal ions, *p*-mercuribenzoic acid, and ferricyanide.

Oxygen Requirement. The data of Figure 8 illustrate the necessity of oxygen for the light-emitting reaction.

Influence of Concentration of P and F on Light Emission. The time course of the luminescence reaction and the influence of initial concentrations on light intensity, when a fixed amount of fluorescence substance and various amounts of photoprotein are used, and *vice versa*, are illustrated in Figures 9 and 10. The broken lines of Figures 9 and 10 show the lack of linearity between initial intensity and amount of P and between initial intensity and amount of F, respectively. In these experiments a 50-fold range in concentration was included, and in each instance the light intensity dropped to half after approximately 8 min. (Curves for all but three concentrations of P and F are omitted from these figures to avoid crowding.) If very high concen-

trations of P, *e.g.*, 2 mg/ml, are used together with an adequate amount of F in the reaction mixture, the time for decrease to half the initial intensity may be greatly prolonged, to as much as 45 min. Conversely, with minimal amounts of P and a large excess of F, the time for decrease to half the initial intensity may be considerably shortened, to as little as 3.5 min. Such results are opposite to what would be expected if the photoprotein acted as an enzyme. Curves for intensity–time relations, plotted from Figures 9 and 10 using a logarithmic scale on the ordinate, are practically parallel (Figure 11), indicating that the apparent specific rate is essentially the same in each case. A likely interpretation is that the rate of inactivation of the photoprotein is great enough to overshadow all other factors involved. Figure 11 shows also that the luminescence reaction involves at least two specific rates, indicted by the change in slope for the initial and later periods.

Quantum Yield of the Fluorescent Substance. Figure 12 gives the results of an experiment with two solutions, each with a fixed, minimum amount of fluorescent substance to which repetitive additions of a large amount of photoprotein were made. The solid line refers to solution A, wherein the reaction was initiated at time zero, and the broken line to solution B, wherein the reaction was initiated 60 min later by mixing aliquots of the same solutions used at first for solution A. The intention of this experiment was to measure all the light which could be elicited from the fluorescent substance, and the conditions were such that the intensity of the light should be practically proportional to the amount of this substance (*cf.* Figure 10), whereas the effect of beginning with a different amount of photoprotein should be small (*cf.* Figure 9). As shown in Figure 12, the luminescence response was strong even at the fourth addition of photoprotein to solution A at 90 min. Further repetitive additions were abandoned as unnecessary and perhaps unfeasible, especially in view of the difficulty in maintaining a constant pH, as well as the continual spontaneous loss in activity of both components as discussed below.

In Figure 12, the progressively decreasing maximum intensity on successive additions of the photoprotein to solution A (solid line) could reasonably be due chiefly to inhibitory products formed either as the result of the luminescence reaction or spontaneous decomposition of the reactants, or both. Inhibitory products are evidently formed in the luminescence reaction mixture, as shown by the fact that the maximum intensity attained in solution B (broken line) is practically the same when, at 60 min, photoprotein is added to both solutions, even though solution A then contains a greater amount of active photoprotein (the amount responsible for residual luminescence plus the amount in the new addition). At this time, the only inhibitory products initially present in B consisted of small amounts formed by spontaneous decomposition in the stock solution.

At 90 min, photoprotein was added to both solutions, both of which contained inhibitory products, and the maximum intensity attained was very nearly the same. The total amount of fluorescent substance that disappeared in solution A during the first 60 min can be estimated as the difference between the two maxima at 90 min, and this amounts to not more than 2% of the amount present at zero time. A minimum quantum yield can therefore be computed as the ratio between the photons produced and the number of molecules of fluorescent substance disappearing in this period. On the basis of the molecular extinction coefficient and a two-electron transfer in titration with $K_3Fe(CN)_6$, the number of fluorescent substance molecules at the start was 4.2×10^{13}, of which 2%, or 0.084×10^{13}, disappeared during the first 60 min of the reaction. In the same interval, a total of 0.78×10^{13} photons was emitted. The minimum quantum yield according to these figures thus averages 9.3 photons/fluorescent molecule. In repeated experiments, no detectable difference was sometimes found for the amount of fluorescent substance disappearing in the luminescence reaction and in spontaneous decomposition. It follows that the quantum yield is potentially unlimited.

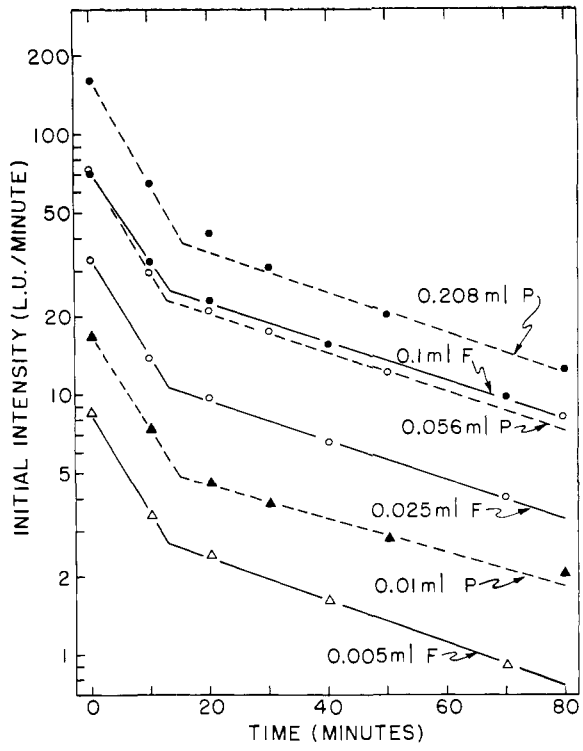

FIGURE 11: Change of intensity with time. Replotted from Figures 9 (broken lines) and 10 (solid lines) using a log scale for light intensity.

Quantum Yield of the Photoprotein. The converse of the preceding experiment (Figure 12) was carried out with a minimum amount of photoprotein and a large excess of fluorescent substance (Figure 13), under conditions such that the luminescence intensity should be nearly proportional to the amount of active photo-

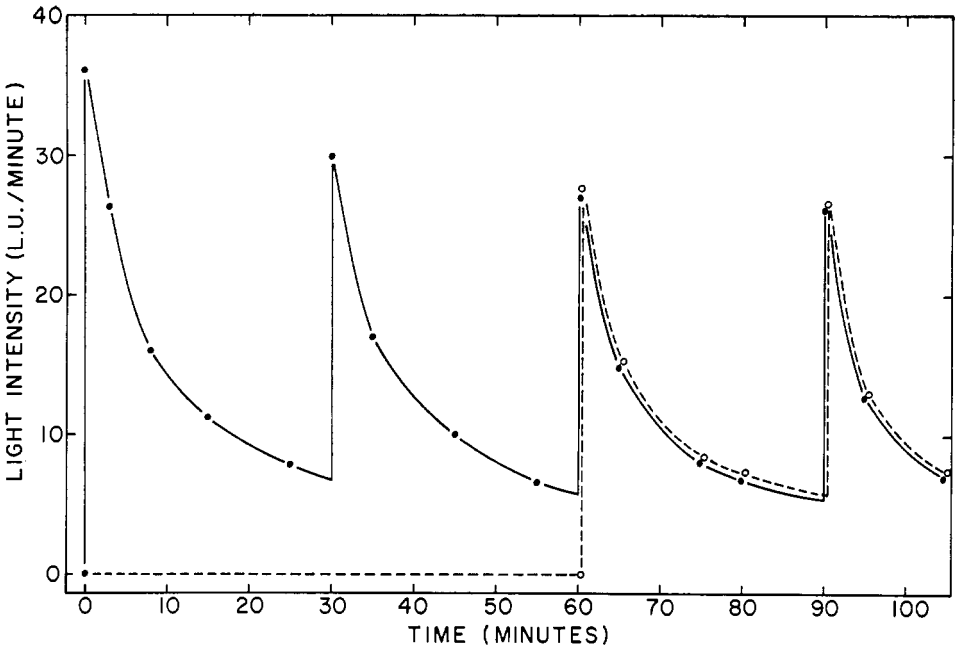

FIGURE 12: Course of the luminescence reaction using a small amount of fluorescent substance and successive additions of a large amount of photoprotein. Solid line (solution A): 10 μl of fluorescent substance solution, OD 0.2/cm at 390 mμ, and 1.1 mg of photoprotein were added to 5 ml of buffer (pH 7.5) at zero time, and 1.1 mg each of photoprotein (partially inactivated) was added at 30, 60, and 90 min. Broken line (solution B): same conditions as with the solid line, except additions of photoprotein at 0 and 30 min were omitted. At 30 and 90 min for the solid line, and at 90 min for the broken line, 10 μl of 0.1 M Tris was added to adjust the pH of the test solutions.

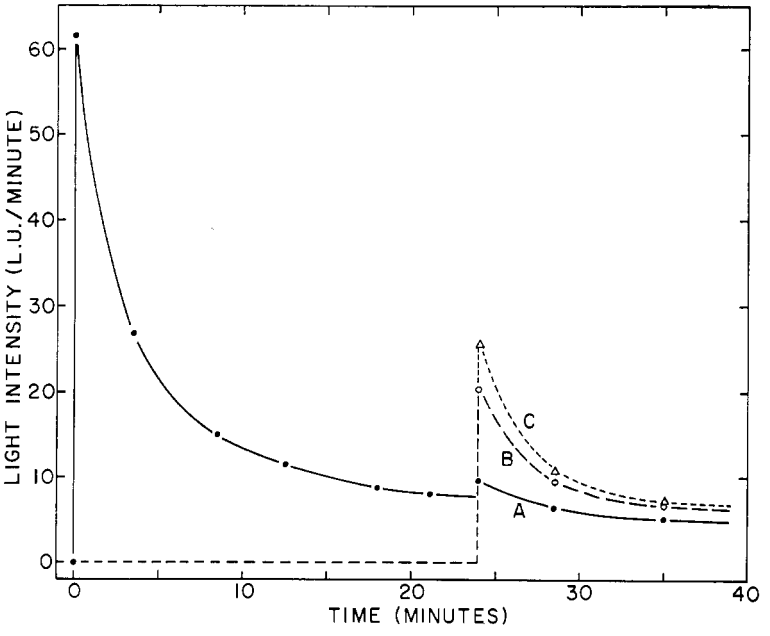

FIGURE 13: Time course of luminescence intensity using a small amount of photoprotein and a large amount of fluorescent substance. Curve A: 25 μl of fluorescent substance solution, OD 6.0/cm at 390 mμ, and 17 μg of photoprotein were added to 5 ml of buffer (pH 7.5) at zero time, and additional 25 μl of fluorescent substance solution was added at 24 min. Curves B and C: same as A, except addition of fluorescent substance solution at zero time was omitted for both curves, and 50 μl of fluorescent substance solution instead of 25 μl was added in curve C at 24 min.

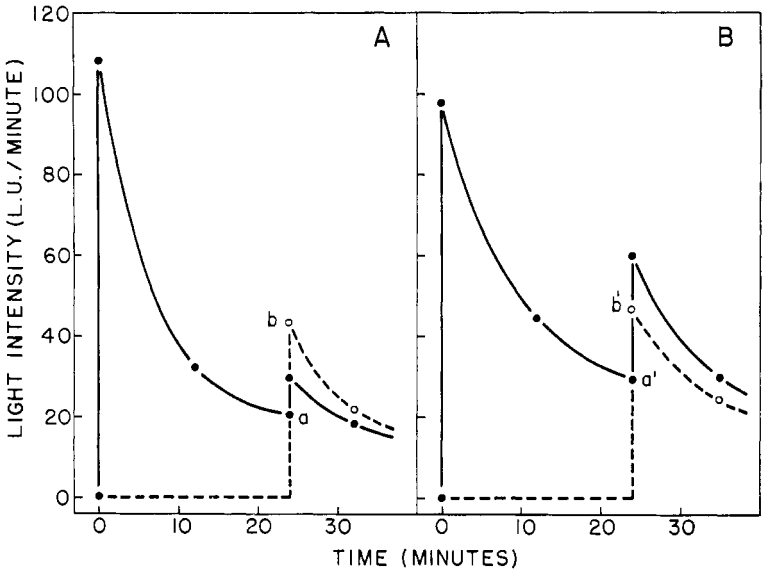

FIGURE 14: Difference in destruction of photoprotein during the luminescence reaction when different ratios of photoprotein and fluorescent substance are used. (A) (solid line) Fluorescent substance solution (25 μl), OD 4.48 at 390 mμ, and 75%-inactivated photoprotein (0.25 mg) were added to 5 ml of buffer (pH 7.35) at zero time, and 25 μl of fluorescent substance solution was added at 24 min. (A) (broken line): same, but first addition of fluorescent substance at zero time was omitted. (B) Fluorescent substance (2.5 μl) and 75%-inactivated photoprotein (2.5 mg) were used, respectively, in the same procedure as in A.

protein present, while changes in the amount of fluorescent substance should have a relatively small effect. In solution A (solid line), light intensity dropped to 8 light units/min in 24 min. At this time a second addition of fluorescent substance was made to solution A and the light rose to 9.5 light units/min, indicating that a large excess of fluorescent substance remained. Also at this time, the luminescence reaction was initiated in control solution B by mixing the same amount of F with the same amount of P used initially for solution A. Control solution C corresponded to B except that twice the amount of F was added in C. In B the light rose to 20.5 light units/min, and in C to 25.5 light units/min. The relatively small difference between the maxima in B and C is again indicative of a large excess of fluorescent substance compared to photoprotein. The difference between the maximum intensity of B at 24 min (uncorrected for the difference in rate of spontaneous inactivation of fluorescent substance in buffer and in 50% ethanol, which, as shown by Table III, would amount to only a few per cent) and the intensity of A just prior to the second addition of fluorescent substance represents the loss of photoprotein during the 24-min interval, this loss being $((20.5 - 8)/20.5) \times 100$, or 61%. The total number of photons emitted during this interval amounted to 0.38×10^{13}. Assuming the molecular weight of the photoprotein to be 200,000, the total initial number of molecules of photoprotein would be 5×10^{13}. The quantum yield would then be $(0.38 \times 10^{13})/(0.61 \times 5 \times 10^{13}) = 0.12$. It is interesting to note that this value, even though only a first approximation, is very close to that which was estimated for the luminescence reaction of the photoprotein aequorin from the jellyfish *Aequorea* (Johnson *et al.*, 1962). In the present instance a higher quantum yield would result, of course, if the molecular weight of the photoprotein is actually higher; in the event that it is as much as one million, the yield would be increased to 0.62. Data from the analytical centrifuge, though complicated by instability of the photoprotein, indicated a probably maximum value of one million as the molecular weight.

Further experiments carried out in the manner just discussed indicated that the quantum yield of the photoprotein varies widely with the amount of fluorescent substance added. Representative data are illustrated in Figure 14A,B, involving (Figure 14A) small amounts of photoprotein compared to fluorescent substance. For Figure 14A the amount of photoprotein was one-tenth, and the amount of fluorescent substance ten times, the amounts of these respective components used for Figure 14B. Control runs (broken lines) show the results of mixing the components at 24 min and of adding at this time a second aliquot of fluorescent substance to the first solution (solid line). During the 24-min interval, the spontaneous inactivation of the fluorescent substance should be about 4% (*cf.* Table III) so this source of error was neglected in the calculations which follow.

In Figure 14A the light intensity in 24 min dropped to *a*, whereas the control gave intensity *b*. The fraction of photoprotein spent in light emission $= (b - a)/b = 0.52$, and the photons emitted was 1×10^{13}. Again assuming a molecular weight of 200,000, the quantum

yield of the photoprotein would be 0.107. In Figure 14B it would be expected that the data for the corresponding fraction $(b' - a')/b'$ would give a value somewhat too small because of the nonlinearity between light intensity and concentration of reactants (*cf.* Figures 9 and 10). Instead of the value of 0.37 indicated by the data, a roughly estimated value of 0.45 was taken as being more nearly correct. The number of photons produced in the 24-min interval was 1.2×10^{13}, and the quantum yield, computed as before, amounted to 0.015. Thus, with ten times the fluorescent substance used in the experiment of Figure 14A as in that of Figure 14B, the quantum yield of the photoprotein was some seven times greater, regardless of the value used for the molecular weight of the protein.

Because of the nature of this system, such differences in quantum yields are rather to be expected. The chief reason is that although the fluorescent substance, F, is essential for the luminescence reaction with the photoprotein, P, F recycles rather than being consumed in this reaction. Consequently, at concentrations of F which are low relative to the amount of P, the quantum yield of P within a given interval of time is limited by the number of molecules of F. Thus, the quantum yield of the photoprotein can be negligible at extremely low concentrations of F, and can rise, with increasing concentrations of F, to some value which, in principle, could be unity.

Acknowledgments

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