Biochemistry of Dinoflagellate Bioluminescence: Purification and Characterization of Dinoflagellate Luciferin from *Pyrocystis lunula*†

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ABSTRACT: Bioluminescence in all dinoflagellate species studied to date is produced by the luciferase-catalyzed oxidation of a newly elucidated type of luciferin, hypothesized to have a substituted polypyrrole-type structure. This paper presents the purification and characterization of the luciferin from *Pyrocystis lunula* along with evidence that it is a polypyrrole-type molecule. Luciferin is extremely labile at low pH, at high salt concentration, and to O_2, so, where possible, the purification steps were carried out in the presence of a buffered reducing agent and under argon. Purified luciferin is soluble in water and polar organic solvents. It is yellow (λ_{max} 245 and 390 nm with a shoulder at 290 nm in neutral or basic aqueous solution) and displays a strong blue fluorescence (λ_{max} for excitation at 390 nm, for emission at 474 nm) that closely matches the bioluminescence emission spectrum [Bode, V. C., & Hastings, J. W. (1963) *Arch. Biochem. Biophys. 103*, 488–499]. Autoxidation leads to concomitant decreases in the 390-nm absorbance, 474-nm fluorescence, and biological activity; similar changes occurred with oxidation by $K_3Fe(CN)_6$, thus allowing a quantitation of luciferin by titration. Luciferin has a molecular weight between 500 and 600, displays positive Ehrlich and Schlesinger reactions, and yields on acid chromate oxidation fragments apparently resembling substituted maleimides; these data support the proposal that dinoflagellate luciferin contains a substituted polypyrrole of the bile pigment type.

Bioluminescent dinoflagellates are ubiquitous in the euphotic zone of the oceans of the world and are responsible for much of the sparkling luminescence, the so-called "phosphorescence" of the sea, elicited upon disturbing surface waters (Harvey, 1952). The light-emitting reaction apparently involves a classical oxidative luciferin–luciferase reaction requiring O_2 but no cofactors (Hastings & Sweeney, 1957; Bode & Hastings, 1963; Fogel & Hastings, 1971). Although many biochemical aspects of the reaction have been elucidated (Hastings, 1978; Dunlap et al., 1981), including its occurrence in both soluble and particulate fractions (De Sa et al., 1963; Fogel & Hastings, 1972; Fogel et al., 1972; Henry & Hastings, 1974), the structure of the substrate, dinoflagellate luciferin, has not yet been determined. Efforts toward this goal have been made difficult by the small quantities of material available and, more importantly, by the extreme instability of this luciferin to autoxidation. In the present work, a species which contains relatively large amounts of luciferin was used and grown in mass cultures; autoxidation during purification was minimized by carrying out the purification under an atmosphere of argon. We report here the purification of dinoflagellate luciferin and present evidence that it contains a polypyrrole, a chemical structure only recently reported to function in bioluminescent systems (Dunlap et al., 1980).

Materials and Methods

Unialgal but not axenic cultures of the dinoflagellate *Pyrocystis lunula* (clone T37) were grown at 19 ± 2 °C in 2.8-L Fernbach flasks with 1500 mL of f/2 medium (Guillard & Ryther, 1962), omitting the silicate and adding 0.5% v/v soil extract (Guillard, 1974). Constant light (100–150 μEinsteins m^{-1} s^{-1}; measured with a LI-192S sensor, LiCor Inc., Lincoln, NE) for growth was provided by cool and warm white fluorescent bulbs. Doubling occurred about every 3.5 days, and cells were harvested during the stationary phase (15 000–20 000 cells mL^{-1}) within 50 days after inoculation.

Luciferin was assayed by one of two methods (Fogel & Hastings, 1971). Determinations by the light intensity method were routinely used to estimate relative amounts and yields of luciferin at the different purification steps. For more rigorous quantitation, the integration assay was employed, using sufficient luciferase to exhaust light emission within less than 3 min. In some cases, such assays were performed by numerical integration, utilizing an 8-bit analgoue to digital converter (Interactive Systems Inc., Philadelphia, PA) sampled and logged approximately 50 000 times/s by an on-line Apple II microcomputer (Apple Computers, Cupertino, CA). In any case, the total photon yield was linear with the amount of luciferin added over at least 2 orders of magnitude (Fogel & Hastings, 1971). The absolute concentration of luciferin was calculated by dividing the photon yield (0.21; Dunlap, 1979) by the quantum yield for luciferin by using *Gonyaulax* luciferase. In both assays, light emission was calibrated with the standard of Hastings & Weber (1963); values reported here were adjusted to the luminol standard (Lee et al., 1966) by applying a factor of 2.8 (Shimomura & Johnson, 1967; Hastings & Reynolds, 1966; Michael & Faulkner, 1976). Data for total quanta per cell were determined by acid stimulation (Schmitter et al., 1976).

Reagent or spectrograde solvents or chemicals were used without further purification except where noted otherwise. Organic solvents came from Fisher and inorganic chemicals from either J. T. Baker or Mallinckrodt. Crystallized and lyophilized bovine serum albumin for assays was obtained from Sigma. Fine grade DEAE–cellulose came from Reeve Angel (Whatman DE52 or DE32) and coarse grade from Brown and Co. (Selectacel); Woelm basic alumina was from Alupharm Chemicals (New Orleans, LA), Florisil was from Fisher, and prepoured TLC plates (silica gel with indicator, cellulose) were from Eastman Kodak. Argon was obtained from MedTech (Medford, MA).

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Table I: Purification Summary for *Pyrocystis* Luciferin[a]

step	solvent	volume	total quanta	% yield
40 flasks of cells	seawater	60 L	1.4×10^{18}	100
boiled cells	2 mM $K_xH_yPO_4$, 5 mM mercaptoethanol, pH 8	500 mL	5.2×10^{17}	37
centrifuged extract	5 mM mercaptoethanol, pH 8	450 mL	5.2×10^{17}	37
DEAE-(coarse) cellulose	50% ethanol, 0.25 M $K_xH_yPO_4$ (approx), 5 mM mercaptoethanol, pH 8	168 mL	3.3×10^{17}	24
alumina column load	70–80% 1-butanol, 20–30% ethanol/H_2O/$K_xH_yPO_4$	100 mL	2.5×10^{17}	18
alumina column eluant-(after concentration)	ethanol, 2.5% NH_4OH	14 mL	2.4×10^{17}	17
DEAE-(fine) cellulose	50% ethanol, 5 mM $K_xH_yPO_4$, 100 mM NaCl, pH 8	90 mL	1.9×10^{17}	14
final sample, concentrated, desalted, extracted into organic solvent for analytical work	(e.g.) ethanol	5 mL	1.6×10^{17}	11

[a] Data reported here are typical for an extraction procedure involving 40 flasks (60 L) of cells at 15 000 cells mL^{-1}. Total quanta were estimated by the light intensity assay.

Ultraviolet absorption spectra were obtained on a Cary 15 spectrometer, fluorescence spectra on a Perkin-Elmer MPF-44 spectrometer, and low-resolution mass spectra at various temperatures on an AEI MS 9 mass spectrometer. Conductivity was measured with a Radiometer Type CDM conductivity meter.

Gel filtration for the determination of the inferred (from Stokes radius) molecular weight of luciferin was performed by employing a 0.4 × 25 cm column of −400 mesh Bio-Gel P2 (Bio-Rad Laboratories, Richmond, CA) equilibrated in 0.2 M sodium phosphate and 5 mM dithiothreitol, pH 8, with a pressure head of 25 cm and a flow rate of about 4 mL h^{-1}. The eluted volume was determined gravimetrically. Partially purified luciferin, as well as void volume and molecular weight markers, was applied in a volume of 250 μL and assayed as already described or as follows: catalase (Sigma) by absorption at 280 nm, vitamin B_{12} (Calbiochem) by absorbance at 550 nm, NAD and NADP (Calbiochem) by absorbance at 280 nm, folate (Calbiochem) by absorbance at 368 nm, and [^{3}H]cAMP (New England Nuclear) by scintillation counting. Similar techniques were employed in experiments utilizing Sephadex G10 (Pharmacia).

Acid chromate cleavage of luciferin for mass spectral analysis was performed by the method of Rudiger (1970). Here, luciferin, in 200 μL or less of methanol (or water plus a small amount of KOH), was mixed with 1–2 mL of an aqueous solution of 1% CrO_3 in either 1% $KHSO_4$ (pH 1.7) or 2 N H_2SO_4. The mixture was allowed to react for 2–3 h at room temperature (or up to 35 °C), and the degradation products were extracted 3 times with 2 mL of diethyl ether (redistilled just before use). The pooled ether fraction was extracted with 0.5 mL of ether-extracted water and concentrated for mass spectral analysis.

The Ehrlich reaction (diagnostic for pyrroles) was performed according to the modification of Mauzerall & Granick (1956), utilizing 2 N perchloric acid in the reaction mixed with *p*-(dimethylamino)benzaldehyde (Fisher). A 200-μL sample of freshly prepared, highly purified luciferin (concentrated from the peak tube of the final DEAE column, 52 μM based on absorbance at 390 nm) in argon-saturated water (plus high NaCl concentration) was placed in a microcuvette; the absorption spectrum was recorded against a distilled water blank to determine that the luciferin was free of degradation products. The blank cuvette was refilled wth 5 N HCl, and at time zero 200 μL of argon-saturated modified Ehrlich's reagent was added to the cuvette with sufficient force to ensure rapid and complete mixing. Then immediately and for the next 22 min, the absorption spectrum was repeatedly scanned from 700 to 400 nm.

The Schlesinger reaction, diagnostic of dipyrrolylmethanes (Gray, 1953), was performed as follows. Several milliliters of concentrated, partially purified luciferin was diluted approximately 1:20 into 100% ethanol to yield a solution of 8 μM luciferin (based on absorbance at 390 nm). To 100 μL of this luciferin were added approximately 10 μL of 1% zinc acetate in 100% ethanol and an equal volume of dilute I_2 also in 100% ethanol. The sample was then checked for fluorescence under a hand-held UV lamp and the absorption spectrum recorded against a blank of 1% I_2 and zinc acetate in ethanol.

Results

Purification. The steps used and results of a typical purification of luciferin are shown in Table I.

The quantity of luciferin in the cells prior to harvesting was estimated by measuring the total light emitted upon acid stimulation. Our measurements, which were taken as the 100% (in vivo) value for later calculation of yields during purification, gave about 1.2×10^9 quanta/cell, a value at the low end of previously reported numbers (Hamman & Seliger, 1972; Swift & Meunier, 1976; Schmitter et al., 1976). This was probably due to the fact that the cell counts included some dead but not yet disintegrated cells.

Cells were harvested by filtration onto Whatman 541 filter paper. Cells were scraped off the filter paper, dispersed into boiling extraction buffer (2 mM potassium phosphate and 5 mM 2-mercaptoethanol, pH 8.5, 5 mL/L of culture), heated for 10 s, and immediately chilled in ice. The solution was saturated with argon while being cooled. All subsequent operations were performed at 0 °C and, whenever possible, under an argon atmosphere. Solutions and reagents were degassed and then saturated with argon, and all chromatography was performed under a positive argon pressure.

The boiled cell suspension was readjusted to pH 8.5 with 0.1 M NaOH, resulting in the formation of a white flocculent precipitate and a slight bathychromic shift from green to reddish yellow. This slurry was centrifuged for 20 min at 27000*g* in a Sorvall RC2B refrigerated centrifuge at 0 °C. The reddish yellow supernatant contained the luciferin.

Following the addition of 1–2 volumes of ice-cold 95% ethanol to reduce the conductivity to less than 5 $m\Omega^{-1}$, the

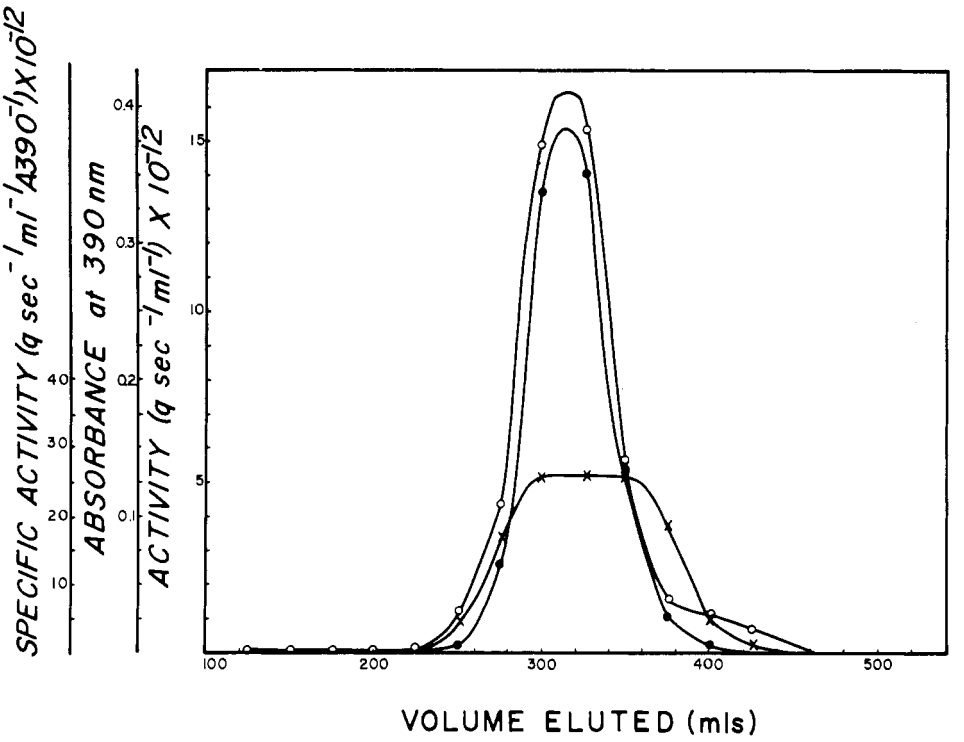

FIGURE 1: Final DEAE-cellulose column, showing elution of purified luciferin. (Closed circles) Absorbance at 390 nm; (open circles) activity, determined by the intensity assay method; (×) specific activity, calculated as activity/absorbance (quanta per second in a light intensity assay/absorbance at 390 nm).

crude luciferin was applied to a 5 × 25 cm DEAE-cellulose column equilibrated in 2 mM potassium phosphate and 5 mM 2-mercaptoethanol in 50% ethanol, washed with 200 mL of equilibration buffer, and eluted with a gradient of 400 mL each of equilibration buffer and 0.2 M potassium phosphate and 5 mM 2-mercaptoethanol, pH 8. A relatively high flow rate of several milliliters per minute was maintained, and the luciferin was eluted at a conductivity of approximately 7 $m\Omega^{-1}$, corresponding to about 70 mM potassium phosphate. Unless experiments were planned immediately for the luciferin preparation, it was adjusted to 50 mM 2-mercaptoethanol and stored at −80 °C under argon with a subsequent activity loss of less than 5% per month.

Additional column chromatography steps used to prepare luciferin for analytical chemical studies were modified from those used by Shimomura & Johnson (1967) for the euphausid (*Meganyctiphanes*) fluorescent substance. Columns were loaded and eluted with a positive argon pressure of 5–10 psi.

The DEAE eluate was concentrated by rotary evaporation at 30–40 °C to approximately 10–20 mL and then diluted with 4 volumes of cold 1-butanol.[1] The powdery precipitate (containing salt from the elution buffer) was removed by centrifugation (5000*g*, 10 min), and the greenish yellow highly blue fluorescent supernatant was loaded onto a 2.5 × 15 cm column of basic alumina in ice-cold 1-butanol, washed with 100 mL of cold 1-butanol, and eluted with approximately 200 mL of 50% ethanol containing 2.5% ammonium hydroxide. The luciferin appeared on the column as a highly fluorescent band, and its elution in this and subsequent chromatographic steps was thereby monitored; activity and blue fluorescence were always correlated.

The activity peak from the alumina column was concentrated as before to 5–10 mL and diluted with 1 volume of DEAE equilibration buffer. The material (greenish or yellowish, depending on the extent of prior oxidation) was loaded onto a 2.5 × 8 cm column of DEAE-cellulose (Whatman DE32 or equivalent grade, chosen for high binding capacity) equilibrated in 10 mM sodium arsenate, pH 8, in 50% ethanol, washed with 100 mL of equilibration buffer, and eluted with 300 mL of the same buffer containing 0.1 M NaCl added (Figure 1). If the luciferin prior to this step was noticeably green, the material was resolved into a blue compound (probably oxidized luciferin, as judged by its absorption spectrum) and the yellow highly fluorescent luciferin which eluted later. Care was taken to exclude the blue compound from the peak pool.

Characterization. Luciferin in the absence of a reducing agent (such as in the final two column chromatography steps) is highly susceptible to oxidation, and this instability is further potentiated by extremes of pH and ionic strength, as shown in Figure 2. This stability condition was particularly significant in steps where concentration was achieved either by in vacuo evaporation of an aqueous solution containing salt or by lowering the pH and extracting into an organic solvent.

During the course of this work, a number of TLC systems were tried in an effort to find one that would repeatedly move the active component as a single spot. None was found,[2] and

[1] Alternatively, concentration was achieved by lowering the pH to 3.8 with 1 M citrate and extracting the luciferin into an organic solvent and subsequently back into about 0.1 volume of water at pH 9 (0.1 M Tris). Luciferin from an aqueous solution will partition greater than 90% into small volumes of ethyl acetate or methylene chloride, about 40% into equal volumes of benzene or 3:1 chloroform/methanol, but not at all into anisole or carbon tetrachloride. Some salt (>50 mM) in the aqueous phase is necessary to achieve this separation, and the pH dependence displays a p*K* of around 4.3 (Njus, 1975). Luciferin was more stable in ethyl acetate than in methylene chloride, so its use was preferred.

[2] The following TLC materials were examined, the numbers in parentheses denoting the number of conditions tried with that medium: silica gel (10), aged (deactivated) silica gel plates (4), cellulose (3), Florisil (5), basic alumina (2), sucrose (3). In all these cases, R_f values were less than 0.15 or greater than 0.9, or resolution was poor due to trailing or smearing of the spot.

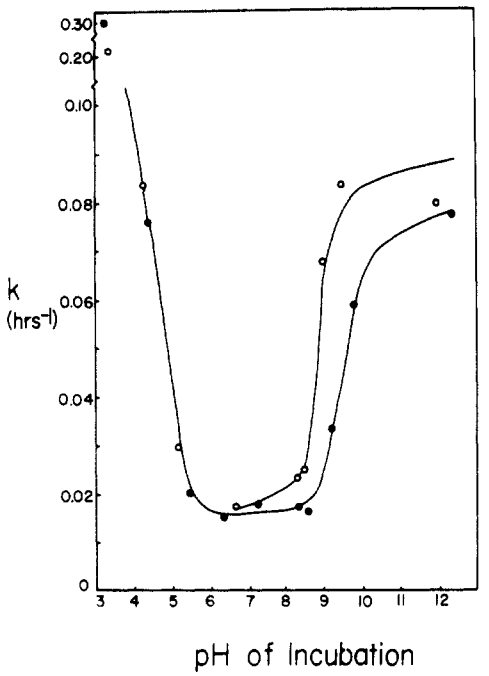

FIGURE 2: Stability of luciferin as a function of pH with (closed circles) or without (open circles) 0.67 M NaCl. Luciferin from the first DEAE column was concentrated by extraction into ethyl acetate and back into degassed, argon-saturated 10 mM Tris and 5 mM dithiothreitol, pH 9. Equal volumes of the luciferin solution and various degassed, argon-saturated buffers (listed below), at a final concentration of 0.1 M, were mixed and the resulting pHs determined. The luciferin solutions were kept under argon on ice and assayed at 0, 0.5, 2, 3.5, 13.75, 24.75, and 43.25 h after the initial pH change (using the intensity method by utilizing approximately 1 μg of purified *Gonyaulax* luciferase). The exponential decay curves [of the form activity = (initial activity)e^{-kt}] were fitted by least-squares regression to straight lines of the form $\ln y = a - kx$, and the exponential decay constants (k) were plotted vs. the pH of incubation. The average regression coefficient was >0.97. The buffers used were as follows: pH 3–8, 0.05 M citrate plus 0.05 M phosphate; pH 8–11, 0.05 M carbonate plus 0.05 M veronal; pH 12, 0.1 M NaOH. Decay rates about 10-fold higher than those reported here were reported by Njus (1975). This is attributed to the higher O_2 concentration, as the buffers and samples were not stored under argon in those experiments.

even the best (aged silica gel plates) apparently catalyzed the decomposition of luciferin, since a single spot which after elution was shown to have activity did not run as a single spot when eluted and immediately run again. Even though this standard criterion for purity was not available to us, it is inferred for several reasons that the compound is luciferin and was essentially pure. The near constancy of the specific activity (ratio of activity to A_{390}) across the peak in Figure 1 indicates that little increase in specific activity is to be expected with further treatments. Fluorescence data also speak to this question. The corrected fluorescence emission spectrum for the partially purified compound (Bode & Hastings, 1963; Njus, 1975; Hastings, 1978) peaked at 474 nm and appeared within the precision of the measurement to be coincident with the in vivo bioluminescence emission noted for this and related species of dinoflagellates. No other fluorescent compounds were noted after the final stage of purification. The excitation peak for this compound corresponds to the maximum in the UV absorption spectrum (Figure 3A). The pH dependence of fluorescence shows a pK of around 4.7 for the fluorescer (Njus, 1975). This is near the pK already noted for extraction of the active compound into organic solvents, and is also near the drop-off point in the stability vs. pH plot (Figure 2).

The absorption spectrum of highly purified luciferin in aqueous solution [Figure 3A and Dunlap et al. (1980)] exhibits

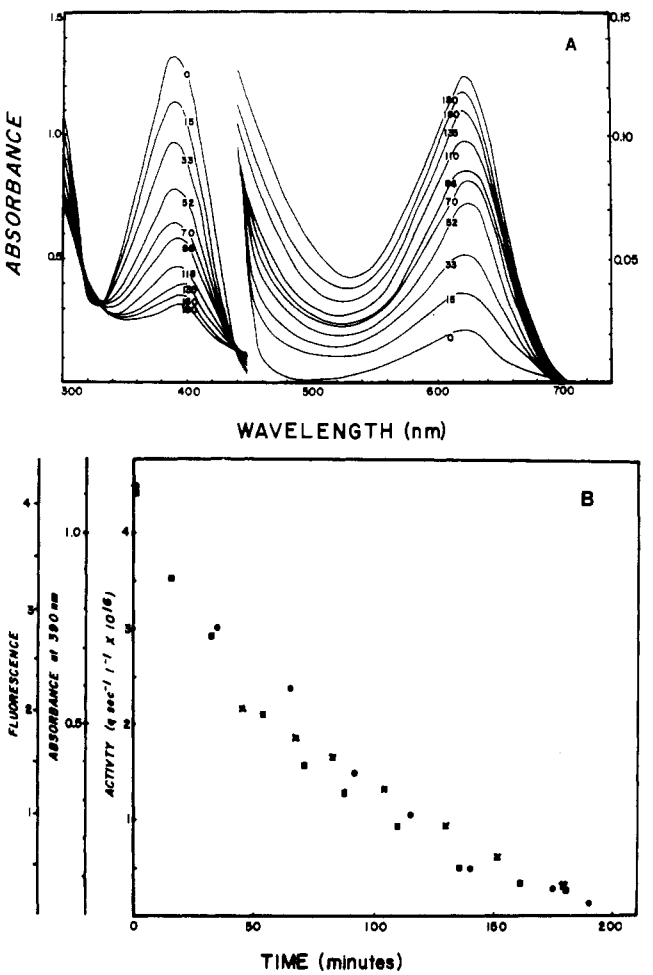

FIGURE 3: (A) Changes in the absorption spectrum of highly purified luciferin upon air oxidation. The eluate from the final DEAE column was concentrated about 13-fold in vacuo; a 2-mL sample containing about 0.1 μmol of luciferin (determined by A_{390}) was placed in a fluorescence cuvette, and the absorption spectrum was scanned repeatedly as it gradually equilibrated with air at room temperature. At various times, the fluorescence of the same sample was determined, and aliquots were withdrawn for the bioluminescence assay (B). The numbers superimposed on the lines indicate the time elapsed in minutes after the first spectrum. Note that all the spectra on the right-hand side are plotted on a 10× scale and that a small amount of oxidation was already evident at the beginning of the experiment. (B) Fluorescence emission (×, 390-nm excitation; emission measured at 475 nm) and activity (circles, measured by intensity assay with *Gonyaulax* luciferase) changes coincident with the absorption changes (squares) taken from (A). Both these and the fluorescence data were corrected before plotting by subtracting the background (determined at 230 min), and the fluorescence data were corrected for self-absorption of the emitted light by using the data from (A).

characteristic peaks at 245 and 390 nm with a shoulder at 290 nm. In the presence of ethanol and ethyl acetate, the 390-nm absorption maximum was shifted to shorter wavelengths. Acidity (below about pH 3.8) virtually eliminated the 390-nm peak although it was recoverable if the pH was promptly returned to greater than 5. Above 400 nm, the absorbance trails off and under optimal conditions was undetectable above 550 nm. But despite all precautions, a long-wavelength absorption (λ_{max} about 620 nm with a shoulder at 585 nm), apparently resulting from spontaneous oxidation, began to appear soon after the last purification step. Such material was already present at zero time in Figure 3. Because of its instability, the luciferin solutions used in the following experiments (except where specifically noted) were purified through the alumina and final DEAE steps immediately before use and were used within the next few hours, before the long-wave-

length absorbance equalled 3% of the 390-nm peak.

When luciferin was allowed to oxidize in air, there occurred simultaneous changes in absorbance (Figure 3A), fluorescence, and activity (Figure 3B). The isosbestic points at 315, 332, and 442 nm suggest (but do not prove) that there were no long-lived intermediates and apparently that the spectral changes were due to the oxidation of a single compound. The parallel decrease in absorption and activity implies that the compound was the active one, and the concomitant loss of fluorescence indicates that this same compound was the one responsible for the fluorescence that matches the in vivo and in vitro light emission.[3]

Luciferin is stoichiometrically oxidized by $K_3Fe(CN)_6$, resulting in spectral changes very similar to those produced by air oxidation. While the stoichiometries of these two oxidative processes have not been rigorously compared, the oxidation by $K_3Fe(CN)_6$ does allow a quantitation by titration of the amount of luciferin contained in a sample. The results of two such determinations are presented in Figure 4A and allow the assignments of 2.8×10^4 and 8.4×10^4 for the molar extinction coefficients at 390 and 245 nm, respectively (the latter calculated from the peak height at 245 nm relative to that at 390 nm). These values assume a two-electron-transfer oxidation as is typical for the potassium ferricyanide oxidation of organic compounds [see, however, Johnson et al. (1962)]. Figure 4B illustrates the spectral changes that occurred upon $K_3Fe(CN)_6$ oxidation.

The Stokes radius and apparent molecular weight of luciferin were determined by gel filtration by employing Bio-Gel P2 (exclusion limit 1800 daltons). An inferred molecular weight of 535 was determined by interpolation on the regression-fitted line from three separate runs. No activity was eluted in several attempts with a Sephadex G10 column, probably because the luciferin was nonspecifically adsorbed by the resin as a result of its hydrophobic nature, resulting in the oxidation of luciferin on the column prior to elution. Although acrylamide resins such as P2 are generally less troublesome in this regard, particularly under the buffer conditions employed here, the value is considered accurate to within only 15%. The data thus indicate an upper limit for the molecular weight of luciferin of about 600.

The absorption spectrum, extinction coefficient, and molecular weight of luciferin are similar to many reported for polypyrrole-type structures (Gray, 1953; Fuhrop & Smith, 1975); several chemical tests exist that allow further evaluation of this hypothesis. A common one employs the Ehrlich aldehyde reagent [*p*-(dimethylamino)benzaldehyde: Mauzerall & Granick (1956); Feigl (1966)], which will react with several types of pyrroles and polypyrroles to give a reddish violet color. The spectral changes with luciferin in this test are presented in Figure 5. The color (λ_{max} 557 nm with no shoulder on the low-wavelength side) develops fully within 10 min. The extinction coefficient of the diaminobenzyl adduct was calculated

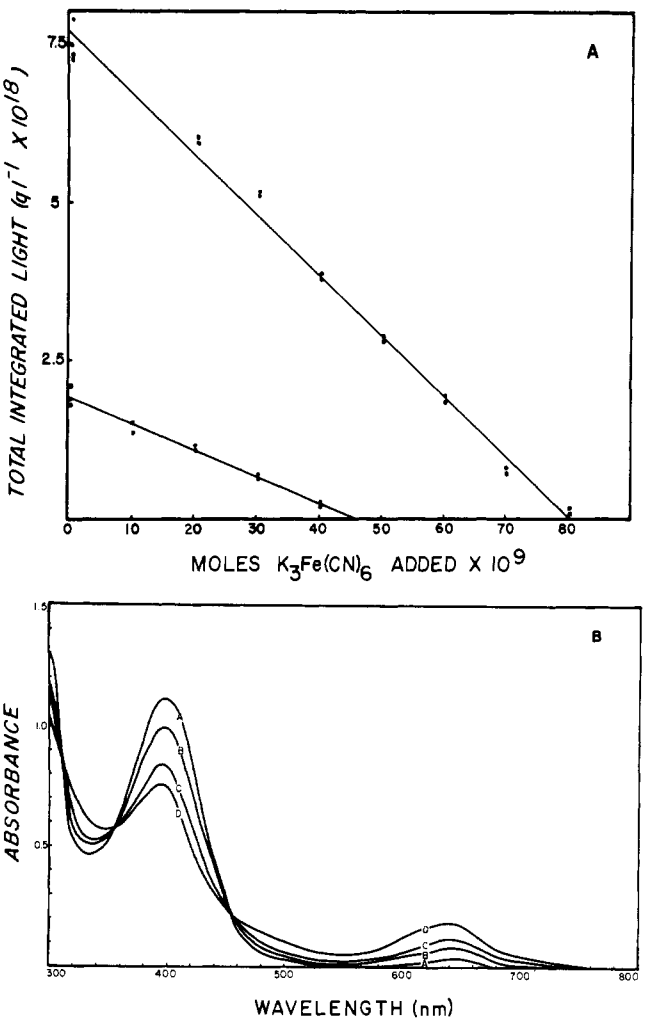

FIGURE 4: (A) Titration of *Pyrocystis* luciferin with $K_3Fe(CN)_6$. The amount of biologically active luciferin in a sample was determined by titration of its ability to make light with luciferase. Total light output was measured by the computer-monitored numerical integration assay; individual assays required 2–3 min and used about 10 μg of purified luciferase each. Two different solutions of freshly purified luciferin were used: one (lower curve) in 2 mL of 50% ethanol, 10 mM potassium phosphate, and 100 mM NaCl, pH 8 (A_{390} = 0.54), and a second (upper curve) concentrated about 10-fold from the final DEAE column to remove the ethanol and resuspended to yield a final volume of 400 μL (A_{390} = 1.65). Both the luciferin solutions and the oxidant were saturated with argon and held at 0 °C under argon. In each case, the concentration of potassium ferricyanide was adjusted so that the addition of about 10% of the starting volume of luciferin would result in complete loss of luciferin activity. A correction was then applied to account for volume changes due to $K_3Fe(CN)_6$ additions and luciferin removals (for assay). After the data were plotted, a least-squares linear regression line was fitted through all the points, and the abscissal intercept was assumed to equal the number of moles of luciferin initially present. The extinction coefficients were calculated to be 2.72×10^4 and 2.86×10^4 for the upper and lower curves, respectively. In both cases, the titration was complete in less than 90 min so that, with the precautions employed, oxidation by sources other than potassium ferricyanide should have been less than 10%. (B) Spectral changes upon titration of luciferin with $K_3Fe(CN)_6$. To 200 μL of 0.16 M luciferin (50% ethanol, 10 mM potassium phosphate, and 100 mM NaCl, pH 8) in a microcuvette (A) were made sequential additions of 1.5 (B), 2 (C), and 2 μL (D) of 10 mM $K_3Fe(CN)_6$. The spectra were recorded immediately after the additions. Other precautions were the same as those stated above.

[3] The decrease in fluorescence did not exactly parallel the changes in activity and absorption. This may have been due to an error arising from the subtraction of the background fluorescence; this value changed with time and could be estimated with only considerable uncertainty due to the fact that the products of luciferin air oxidation may be complex and unstable, and also are fluorescent themselves. In Figure 3B, the background for both absorbance and fluorescence was determined at 230 min, when the absorption at 390 nm was lowest, and before the maximum shifted to other wavelengths. By selection of a fluorescence background level taken at a different time, a better fit could be obtained. In addition, another experiment (not shown) was performed in which 800 nM luciferin in a 2-mm path-length fluorescence cuvette was titrated with 100 μM $K_3Fe(CN)_6$ under conditions similar to those given in Figure 4. Self-absorption was negligible, and activity and fluorescence could be monitored on the same sample. Since the titration required only a few minutes, the luciferin concentration was decreased quickly in a controlled fashion, presumably limiting the number of reaction products. Thus, the end point and the fluorescence background could be determined very accurately. Under these conditions, the decrease in activity and fluorescence tracked within the precision of the measurements (to within less than 5%).

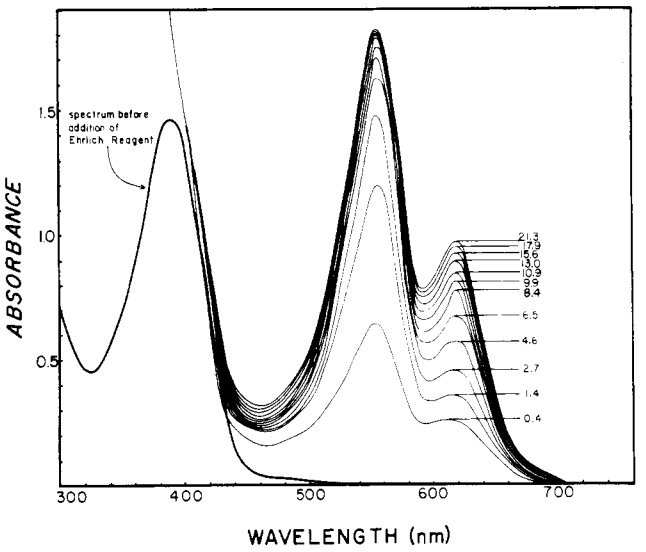

FIGURE 5: Spectra taken during the course of the reaction of highly purified luciferin (52 μM; see Materials and Methods) with Ehrlich's reagent. The thick line (λ_{max} 390 nm) shows the absorption spectrum of luciferin prior to the addition of the aldehyde reagent. The thinner lines at the right show the changes in the spectrum over time, the numbers denoting the number of minutes subsequent to the Ehrlich reagent addition that the monochromator passed that wavelength. The development of the diagnostic absorption at 557 nm was 90% complete within 5 min, and the color remained stable for 15 min thereafter.

to be 3.5×10^4 on the assumption that all of the luciferin contributing to the initial absorbance at 390 nm is available for reaction. This is somewhat lower than literature values for model compounds [about 6×10^4: Mauzerall & Granick (1956); Fuhrop & Smith (1975)] and suggests that the luciferin, which should be very unstable under the 2 N perchloric and 17 N acetic acid conditions of the reaction, may in fact have been partially destroyed prior to reaction. While the Ehrlich reagent is capable of reacting with certain other chemicals (Lightner, 1979), the kinetics of these reactions and the final colors they produce are much different than those seen in the present case. Thus, the reaction with luciferin described here suggests that this molecule contains a pyrrole or a polypyrrole.

Another more specific test is the Schlesinger reaction (Schlesinger, 1903), which is specific for dipyrrolylmethenes, dipyrrolylmethanes capable of being dehydrogenated by I_2 (Gray, 1953), or any other structure containing these moieties and therefore capable of complexing with zinc. Thus, the reaction of luciferin with alcoholic zinc acetate and iodine to form a yellow-green fluorescence and a greenish color (λ_{max} 480 nm) is diagnostic of one of these types of structures. A yellow-green rather than a red fluorescence further indicates that luciferin does not contain several pyrrolyl groups in conjugation.

If luciferin contains a polypyrrole-type structure, then oxidative cleavage of the separate pyrroles from the ring or chain should result in the formation of substituted maleimides and aldehydes, the stoichiometry and type depending on the oxidation conditions and the linear sequence of the pyrroles (Rudiger, 1970). While an analysis of this type has not yet been completed, preliminary mass spectral data from samples of luciferin subjected to acid chromate oxidation showed fragments whose sizes (m/e 137, 139) were consistent with the presence of methylvinylmaleimide and methylethylmaleimide. Futhermore, recent work (Shimomura, 1980) has shown that a related and partially cross-reacting molecule (*Meganyctiphanes* fluorescent substance, see Discussion) is an open-chain tetrapyrrole containing a methylethylmaleimide, a methylvinylmaleimide, a modified methyl propionate maleimide, and a complex fused ring fourth pyrrole.

Discussion

At least nine chemically distinct luciferins have been purified; the dinoflagellate molecule is evidently one of the most unstable luciferins yet to be encountered. Its nonenzymatic reaction with oxygen is very rapid, involving a concomitant chemiluminescence (unpublished observations) and the formation of the bluish compound (λ_{max} about 620 nm, Figure 3A). A similar instability was also noted during the course of purification of the fluorescent substance involved in light emission in the euphausid *Meganyctiphanes norvegica* (Shimomura & Johnson, 1967). Further similarities in behavior during purification, bioluminescence emission spectra, and chemical characteristics led us to examine the systems for cross-reactivity, the positive results of which are reported elsewhere (Dunlap et al., 1980).

Pyrocystis lunula was chosen for these studies from among the several bioluminscent dinoflagellates available to us because of its high bioluminescence yield per cell (Seliger et al., 1969; Swift & Meunier, 1976; Schmitter et al., 1976). Also, previous studies have indicated that dinoflagellate luciferin is the same or a very similar molecule in different species (Hastings & Bode, 1961; Hamman & Seliger, 1972). While interspecies differences in the absolute levels of various biochemical components have been noted (Seliger et al., 1969; Swift et al., 1973; Schmitter et al., 1976), the luciferins and luciferases from all species thus far examined have been found to cross-react in all combinations (Schmitter et al., 1976). Furthermore, the in vivo bioluminescence emission spectra all peak near 474 nm (Bode & Hastings, 1963; Seliger et al., 1969; Swift et al., 1973), indicating apparent uniformity in both the protein environment and the actual chemistry of the emitter.

Knowledge of the structure of this luciferin is important in establishing the identity of the emitter in the dinoflagellate bioluminescent reaction. The apparent identity of the bioluminescence emission spectrum with the fluorescence emission spectrum of the purified but unreacted luciferin suggests that the emitter must have a structure very similar to if not identical with the structure of the unreacted luciferin. However, the oxidation of luciferin must precede the formation of the excited state, and at the same time must alter the chemical structure of the luciferin. This poses a dilemma, then, since the oxidized luciferin itself (Figure 3A) probably cannot be the emitter. One way to explain this is to propose that the energy of the primary excited state is transferred to an unoxidized luciferin molecule which then actually emits the light.

A more likely explanation, however, is that the emitter occurs only as a transient intermediate and, though oxidized, retains structural components that make its emission properties very similar to those of the unreacted luciferin: the differences might be so small as to have not been detected in previous measurements. For example, the first oxidative steps releasing the energy for the population of the excited state could involve a part of the luciferin molecule not in conjugation with the chromophore (vide infra), and the energy could be transferred to the chromophore portion and emitted as light. The presence or absence of such a small unconjugated oxidizable moiety could be expected to make only a minor contribution to the shape of the fluorescence emission spectrum, so that the chromophore would appear largely unchanged when chemically excited in the bioluminescent reaction. After emission, the emitter would then rapidly decompose to oxidized luciferin following the pattern noted in Figure 3A. More experiments

are required to test these hypotheses.

Spectral, physical, and chemical data have been presented consistent with the hypothesis that *Pyrocystis* luciferin contains a substituted bile pigment type structure. The de novo production rather than the mere enhancement of a long-wavelength (λ_{max} 620 nm) absorption band suggests that oxidation, either by molecular oxygen or by $K_3Fe(CN)_6$ (vide supra), is bringing into conjugation previously unconjugated electronic systems. Whether this is by removal of hydrogen to create methene bridges (by analogy to the air oxidation of the linear tetrapyrrole bilirubin to biliverdin) or by some other means is yet to be elucidated. In this context, it is interesting to note that the molecular weights and spectral data for several linear tetrapyrroles are similar to those presented here for luciferin.[4] Furthermore, in light of the marked instability of luciferin in air, it is interesting that Matheson et al. (1974) found bilirubin to be oxidized by singlet oxygen at a rate 30 times faster than that of any previously studied compound. However, luciferase was not at all retarded by affinity columns bearing either bilirubin or biliverdin (J. C. Dunlap, unpublished experiments), suggesting that the putative basic pyrrole structure of luciferin differs significantly from these molecules.

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[4] Although spectral comparisons are useful, they are made difficult in this instance by the lack of data for all compounds in any one solvent. The absorption maximum of bilirubin in chloroform (λ_{max} 450 nm, extinction coefficient 5×10^4) is lowered in both wavelength and intensity upon transfer to aqueous alkali (λ_{max} 420 nm, extinction coefficient "weaker"). Oxidation of bilirubin to biliverdin yields a compound (λ_{max} 392 and 640 nm in methanol; molar extinction coefficients 2.5×10^4 and 1×10^4, respectively) spectrally similar to oxidized luciferin (Figure 3A; λ_{max} about 395 and 620 nm; extinction coefficients 2.8×10^4 and not determined). Of less significance are the similarities in molecular weights (585 for bilirubin, 583 for biliverdin, and 500–600 for luciferin). Tetrapyrrole data cited here were taken from Lemberg & Legge (1949).

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