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Isolation and Properties of Luciferase, a Non-Heme Peroxidase, from the Bioluminescent Earthworm, *Diplocardia longa*†

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ABSTRACT: It has been demonstrated that *in vivo* luminescence from the bioluminescent earthworm, *Diplocardia longa*, arises from the coelomic fluid exuded by the worm following mechanical or electrical stimulation. The blue-green luminescence has an unstructured peak emission at 19,700 cm^{-1} (507 nm) with a half-width of 3070 cm^{-1} (78 nm). The source of the luminescence has been shown to be cells present in the coelomic fluid of the worms; these coelomic cells, about 40 μ in diameter, have been isolated by sucrose gradient centrifugation. Luciferase has been isolated in highly purified form from the coelomic cells of *D. longa* and has been shown to have an approximate molecular weight of 300,000 and a frictional ratio (f/f_0) of 2.10 indicating that it is highly asymmetric. Data from sodium dodecyl sulfate gel electrophoresis indicates that luciferase is composed of three pairs of nonidentical subunits of molecular weight 71,000, 58,000, and 14,500. The absorption and fluorescence spectra of luciferase are those of a typical protein with no evidence of any ultraviolet or visible absorbing prosthetic groups. Luciferase is inhibited by a variety of metal-binding agents such as potassium cyanide, *o*-phenanthroline, and sodium diethyldithiocarbamate, but the activity of luciferase could not be correlated with the metal content of the protein. The enzyme is also irreversably inactivated by H_2O_2 and kinetic data suggest that one molecule of H_2O_2 per luciferase site is required for this inactivation. During the light reaction, therefore, luciferase does not exhibit normal enzymatic catalysis and "turnover."

It has been previously demonstrated that luciferin, luciferase, and H_2O_2 are required for *in vitro* light emission from the earthworm, *Diplocardia longa* (Cormier *et al.*, 1966; Bellisario and Cormier, 1971). These requirements for luminescence are expressed in the following reaction scheme

$$\text{luciferin} + H_2O_2 \xrightarrow{\text{luciferase}} \text{light (max} = 507 \text{ nm)} + \text{products}$$

Thus, the *Diplocardia* luminescent system belongs to a class of peroxidative bioluminescent reactions in which H_2O_2 is utilized for light emission instead of molecular oxygen (Cormier and Totter, 1968; Hastings, 1968).

We present evidence that luciferase and luciferin are located within the coelomic cells of this bioluminescent worm. *Diplocardia* luciferase has been isolated in the present study in order to determine the physical properties of a luciferase of the peroxidative type. Furthermore, we show that *Diplocardia* luciferase is a non-heme protein and exhibits no activity toward a number of typical peroxidase substrates such as guaiacol, benzidine, or pyrogallol. In addition, luciferase is irreversibly inactivated by H_2O_2 and kinetic data indicates that luciferase does not exhibit normal enzymatic catalysis and "turnover."

Materials and Methods

The following materials were purchased from the suppliers indicated: crystalline bovine serum albumin and crystalline egg albumin (Pentex); twice crystallized catalase (Worthington); fumarase (Nutritional Biochemicals); glutamic dehydrogenase (Boeringer); fibrinogen (Mann); lysozyme and twice crystallized catalase (Sigma); Sephadex gels and Blue Dextran 2000 (Pharmacia); acrylamide, Agarose 1.5 m gel, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylenediamine (California Biochemicals); sodium lauryl sulfate (Mann); Sepraphore III cellulose acetate electrophoresis strips and Tris-barbital (pH 8.8) buffer (Gelman); riboflavin and Bromophenol Blue (Eastman); Coomassie Blue (Colab Laboratories); DEAE-cellulose (Whatman); hydroxylapatite (Serva); hydrogen peroxide (Baker); succinyl peroxide and ethyl hydroperoxide (K & K Labs); dimethylbenzyl hydroperoxide (Matheson, Coleman & Bell); *o*-phenanthroline (G. F. Smith Co.); sodium diethyldithiocarbamate (Eastman); 8-hydroxyquinoline, benzidine, *o*-dianisidine, guaiacol (Sigma). All other chemicals used were from commercial sources and are the highest quality available.

Isolation of Coelomic Cells. Coelomic fluid from *D. longa* was collected by electrically stimulating two worms with a manually operated magneto generator in 30 ml of an earthworm Ringer solution (Block *et al.*, 1964) which was made hypertonic with 0.1 M KCl and 0.1 M NaCl. Following this stimulus, the coelomic fluid was exuded from the mouth and dorsal pores of the worms and had a milky appearance due to the presence of numerous cells suspended in the fluid. All following operations were carried out at 4°.

When the worms ceased exuding coelomic fluid, they were washed and homogenized in 60 ml of 0.1 M sodium borate

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buffer (pH 7.6) in a tissue grinder with a motor-driven Teflon pestle. The whole worm homogenate was centrifuged at 3000*g* for 5 min and the supernatant assayed (fraction I). The suspension of coelomic cells in the saline solution was also assayed (fraction II) and then allowed to sit for 15 min. During this time the coelomic cells settled to the bottom of the container. The supernatant (fraction IIIA), which contained few coelomic cells, was carefully decanted from the sedimented layer of coelomic cells (fraction IIIB). Both fractions were assayed. Two milliliters of the settled coelomic cells (fraction IIIB) was placed on a 30% (w/v) to 70% (w/v) linear sucrose gradient. The volume of the gradient was 40 ml and the sucrose solutions were prepared in the modified Ringer solution. Following centrifugation at 1800*g* for 10 min, the coelomic cells migrated as a single broad band approximately two-thirds into the gradient. The bottom of the centrifuge tube was punctured and 5-ml fractions were collected. The band of coelomic cells was collected as nearly as possible in a single fraction.

Preparation of Acetone Powder. Normally about 400 worms were collected and separated into eight groups of equal weight for processing. Each group of about 50 worms was electrically stimulated in 250 ml of 0.1 M EDTA at 4° with the magneto generator until the worms ceased to exude coelomic fluid. The EDTA suspension of coelomic cells was centrifuged at 480*g* for 5 min. The coelomic pellets from 200 worms were combined and an acetone powder prepared from them. The procedure yielded about 1 g of acetone powder from 20 worms. The acetone powder was stored at −80°. Portions of a single batch of the acetone powder stored in this manner were extracted for luciferin and luciferase at various times during the period of 1 year. The luciferin and luciferase activity did not decrease over this period of time.

Luciferase Assay. The standard assay mixture contained luciferin, luciferase (usually 5–25 μg of luciferase was employed), 4.4 μmoles of H_2O_2, and 60 μmoles of potassium phosphate (pH 7.5) in a volume of 1.0 ml. The reaction was initiated by injecting H_2O_2 into the buffered mixture of luciferin and luciferase. Luciferin eluted from DEAE-cellulose column chromatography (step 2 in luciferase purification) was used for routine luciferase assays. The luciferin used in some assays was further purified by alumina column chromatography as previously described (Bellisario and Cormier, 1971). When used, the more purified preparation is referred to as alumina luciferin.

The flash peak of luminescence was used to measure luciferase activity. A unit of luciferase activity is defined as that amount of luciferase that produces a flash peak of 10^{11} quanta sec^{-1}. The light intensity was calibrated in quanta sec^{-1} by use of the luminol secondary light standard described by Lee *et al.* (1966). Light emission was measured with 1P21 photomultiplier tube operated at 1000 V and the output amplified with a MacNichol photometer (Chase, 1963). The signal was recorded on an Esterline Angus recorder. Total light was measured with a separate integration circuit with a digital readout.

Purification of Luciferase. Acetone powder (1.5 g) was homogenized four times with 0.1 M sodium borate buffer (pH 7.6) with a glass tissue grinder equipped with a motor-driven Teflon pestle. All operations were done at 4°. The first homogenization was carried out with 80 ml of the borate buffer followed by three homogenizations each utilizing 70 ml of borate buffer. After each homogenization, the crude extract was centrifuged at 16,000*g* for 5 min, the supernatant saved, and the pellet reextracted. Three separate 1.5-g portions of acetone powder were carried through the above procedure and the supernatants pooled (step 1).

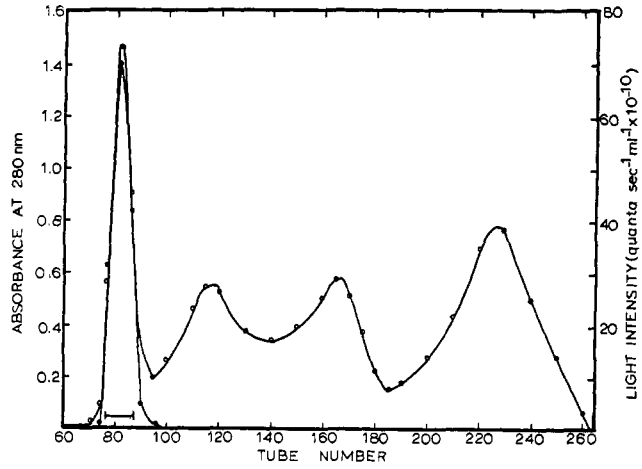

FIGURE 1: Sephadex G-150 column chromatography of partially purified luciferase. Luciferase (28 ml) from step 3 of the purification procedure was applied to a 4.9 × 82.5 cm Sephadex G-150 column equilibrated with 0.1 M potassium phosphate (pH 7.5). The column was eluted with the same buffer at 4°; fractions (5.4 ml) were collected at a flow rate of 27 ml/hr. Luciferase was assayed as in Methods. (○) Absorbance at 280 nm; (●) luciferase activity; (|—|) fractions pooled.

The viscous pooled supernatants from 4.5 g of acetone powder were immediately poured onto a DEAE-cellulose column (9 × 14 cm) which had been previously equilibrated with the extraction buffer. The luciferin did not adsorb and passed through the column free of luciferase activity. The luciferin from the column was stable for 1 year at −80° and was used for routine luciferase assays. The bound luciferase was eluted from the column with 0.1 M Na_2HPO_4. Fractions of 200 ml were collected and the fractions with an absorbance at 280 nm greater than 0.5 were pooled (step 2).

Solid ammonium sulfate (600 g/l.) was added to the pooled fractions from step 2 and the mixture was stirred for 1 hr before being centrifuged at 18,000*g* for 15 min. The pellet was stirred for 10 min with 40 ml of 40% saturated ammonium sulfate which had been previously brought to pH 7.5 with ammonium hydroxide. The mixture was centrifuged at 18,000*g* for 15 min and the supernatant which contained inactive protein was discarded. The pellet was extracted with 25 ml of 0.1 M potassium phosphate buffer (pH 7.5) for 2 hr; not all of the protein was solubilized during this period. The suspension was centrifuged at 27,000*g* for 20 min and the pellet which contained less than 3% of the luciferase activity was discarded (step 3).

The yellow-brown supernatant from step 3 was applied to a G-150 column (Figure 1). The luciferase was excluded and eluted with the void volume of the column. The most active fractions were pooled (step 4).

The pooled fractions from step 4 were concentrated to a volume of about 10 ml with an ultrafiltration cell (Amicon Corp.) and dialyzed against 1 l. of 0.05 M potassium phosphate (pH 7.0) for 48 hr with three changes of buffer. The dialyzed luciferase was applied to a 2.1 × 10 cm hydroxylapatite column equilibrated with the dialysis buffer. The bound luciferase was eluted with a linear gradient formed from 150 ml of 0.05 M potassium phosphate (pH 7.0) and 150 ml of 0.4 M potassium phosphate (pH 7.0). A single peak of protein coinciding with the luciferase activity was eluted

at the beginning of the gradient (step 5). The same results were obtained by eliminating the gradient and stepwise eluting luciferase with 0.2 M potassium phosphate (pH 7.5).

Ultracentrifuge Studies. Sedimentation velocity and equilibrium studies were performed with a Spinco Model E centrifuge equipped with schlieren and absorption optics. The sedimentation coefficients were determined by the method of Schachman (1957). Sedimentation coefficients at low protein concentrations were determined with absorption optics and photoelectric scanner. All sedimentation coefficients were corrected to water and 20°.

High-speed sedimentation equilibrium experiments were done by the short-column method of Yphantis (1964) using the absorption optics and a photoelectric scanning system.

Electrophoretic Methods. Disc gel electrophoresis followed the procedure of Davis (1964). In addition to the standard pH 9.5 system, disc electrophoresis at a running pH of 8.5 was obtained using the system of Hedrick and Smith (1968). The gel electrophoresis system of Epstein *et al* (1968) utilizing a polyacrylamide pore size gradient was also employed. All of the polyacrylamide gels were polymerized with riboflavin (Brewer, 1967). The electrophoresis was run at room temperature with a current of 2 mA/tube and the proteins were stained with Amido Black.

Electrophoresis on cellulose acetate membranes (Sephraphore III) was done using the method of Greenbaum *et al.* (1963). Electrophoresis was carried out for 1 hr with a current of 5 mA/strip using 0.1 M potassium phosphate buffer (pH 7.5) or 0.05 M Tris-barbital buffer (pH 8.8). The proteins were stained with Ponceau S.

Molecular weight determinations in polyacrylamide gels containing sodium dodecyl sulfate were performed according to the method of Weber and Osborn (1969) except that the gels were polymerized with one-fifth of the concentration of ammonium persulfate. The samples were incubated in 1% sodium dodecyl sulfate and 1% HOC_2H_4SH in 0.01 M sodium phosphate (pH 7.0) for 4 hr and then dialyzed overnight against the same buffer containing 0.1% sodium dodecyl sulfate and 1% HOC_2H_4SH. In some cases 8 M urea was included in the dialysis buffer. Electrophoresis in the 10% acrylamide gels was carried out at 8 mA/tube for 5 hr at room temperature. The mobility of the proteins was relative to that of the tracking dye; the mobility of the tracking dye was fixed by slicing the gel through the center of the tracking dye prior to staining the gels with Coomassie Blue.

Protein Determination. Protein concentrations were determined by the biuret reaction (Gornall *et al.*, 1949) using crystalline bovine serum albumin as the standard or by the absorbance at 280 nm. With purified luciferase preparations, the absorbance at 278 nm was found to be 1.8 at a protein concentration of 1 mg/ml based on biuret. Molar concentrations of luciferase were based on a molecular weight of 300,000.

Metal Analyses. Analyses for metals were done by atomic absorption spectroscopy using a Perkin-Elmer Model 303 or 403 atomic absorption spectrometer equipped with a deuterium arc assembly. The standard metal solutions were obtained from Fisher Scientific Co.

Electron paramagnetic measurements of luciferase were made at −180° with a Varian Model 4052-10a spectrometer equipped with 100-kcycles modulation and an automatic low-temperature accessory.

Microscopic Studies. Photomicrographs of the coelomic cells were taken with the Nomarski differential interference microscope (Carl Zeiss).

Spectral Analyses. The *in vivo* bioluminescence spectra were taken from the luminous slime exuded by the worm following electrical stimulation. The slime was scraped onto a piece of filter paper which was placed in a cuvet and the spectra immediately taken. The light emission lasted for several minutes.

The *in vitro* bioluminescence spectra were obtained from the following reaction mixture contained in a cuvet: 1.1 mg of luciferase, 0.1 ml of alumina luciferin, 0.04 μmole of potassium phosphate (pH 7.5), and 0.15 μmole of H_2O_2 in a total volume of 1.0 ml. Again the light emission lasted for several minutes.

The luminescence measurements were made at 25° with a component spectrofluorimeter (Cormier and Prichard, 1968), and the data recorded on line with a Nova minicomputer (Data General). The slit width was 3 mm. Each spectrum is an accumulated average of eight or more scans. The spectra were corrected for photomultiplier sensitivity, monochrometer efficiency, and decay of the light emission during the scan (Wampler and DeSa, 1972).

Absorption spectra were recorded with a Cary Model 14 recording spectrophotometer. Fluorescence measurements of luciferin and luciferase were made with an Amino-Keirs spectrophosphorimeter (American Instrument Co.).

Results

Isolation of Coelomic Cells. Table I shows the luminescence activity of the various fractions obtained during the isolation of the coelomic cells (as described in Methods). It is evident from Table I that only about 1% of the luciferin or luciferase activity remained in worms which had been depleted of their coelomic fluid (fraction I). The luminescent activity is seen to occur in the fraction of coelomic cells (fraction II). When the settled suspension of coelomic cells was decanted, nearly all of the luminescence activity was found in the sediment of coelomic cells (fraction IIIB); less than 2% of luciferin or luciferase activity remained in the supernatant (fraction IIIA). The data in Table I show that a negligible amount of light emission occurred before the cells were osmotically lysed with distilled water. Mechanical disintegration of the coelomic cells also resulted in a brilliant burst of light. Therefore the luminescence activity is associated with the coelomic cell fraction and negligible light emission occurs until the cells are lysed.

A portion (2 ml) of the coelomic cells from faction IIIB (Table I) was further purified by sucrose gradient centrifugation. Two bands of cells were observed in the sucrose gradient. A thin band of small cells was present near the top of the gradient. These cells differed in appearance from the main band of coelomic cells and were devoid of luminescence activity. The majority of the coelomic cells migrated as a single broad band about two-thirds of the distance into the gradient and were collected in a single tube. When osmotically lysed with distilled water these cells produced a burst of light. Both the luciferin and the luciferase activity were contained in this fraction of coelomic cells indicating that neither component had been separated by sucrose gradient centrifugation.

Only 10% of the total luminescence activity applied to the sucrose gradient was recovered. This low recovery of activity was probably due to the difficulty encountered in lysing the coelomic cells in the presence of sucrose. When the cells in the sucrose were diluted fivefold further with distilled water and assayed in the presence of luciferase, the total lumines-

TABLE I: Isolation of Coelomic Cells.

Fraction	Total Vol (ml)	Total Luminescence Units	
I. Whole worm homogenate	60	+Water[a]	0.2
		+Luciferin[b]	4.4
		+Luciferase[b]	1.6
		Endogenous activity[c]	ND
II. Suspension of coelomic cells	30	+Water	260
		+Luciferin	420
		+Luciferase	380
		Endogenous activity	0.7
III. A. Supernatant from step II	23	+Water	0.4
		+Luciferin	5.6
		+Luciferase	5.5
		Endogenous activity	0.01
III. B. Sediment of coelomic cells from step II	7	+Water	390
		Endogenous activity	0.2

[a] Fractions from each of the isolation steps prepared in Methods were assayed for luminescence by mixing 0.2 ml of the fraction with 0.6 ml of glass distilled H_2O and shaking vigorously to lyse as many cells as possible. To this solution was immediately injected 0.2 ml of 0.022 M H_2O_2 and the flash peak of the light emission recorded. One luminescence unit is taken as a flash peak of 10^{11} quanta sec^{-1}. The total volume was 1.0 ml. [b] These assays were as described in footnote *a*, except that 0.1 ml of luciferin or luciferase was added to the assay to determine if either component had been selectively lost during the isolation procedure. [c] Endogenous activity refers to light emitted by 0.2 ml of the fraction without lysis with distilled water. ND, not detected.

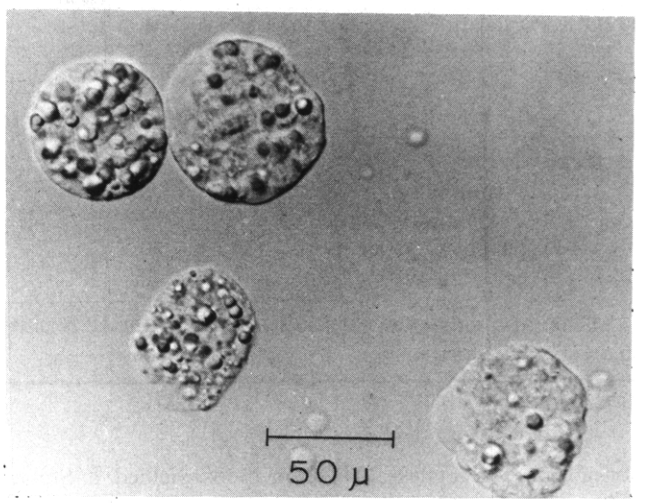

FIGURE 2: Photomicrograph of coelomic cells. The coelomic cells isolated from sucrose gradient centrifugation were diluted with modified Ringer solution, centrifuged, and then resuspended in the modified Ringer solution. The photomicrograph was taken with the Nomarski differential interference microscope (Smith, 1971). The scale line is 50 μ; magnification 160×.

cence activity increased 2,4-fold, probably due to the increased cell lysis.

A photomicrograph of the suspension of coelomic cells obtained from sucrose gradient centrifugation is shown in Figure 2. The cells shown in Figure 2 contain numerous granules and are about 45–50 μ in diameter.

Purification of Luciferase. Table II summarizes the steps used during the purification of *Diplocardia* luciferase. Luciferase was purified approximately 20-fold with about 12% yield. It is estimated that luciferase makes up about 5% of the total extractable protein from coelomic cells. Other purification steps such as fractional ammonium sulfate precipitation or ethanol precipitation resulted in additional losses of luciferase activity with no further purification as judged by dodecyl sulfate gel electrophoresis.

Luciferase is relatively unstable and lost 20–60% of the original activity when stored at 4° in 0.1 M potassium phosphate (pH 7.5) for 2 weeks. The loss of activity was not prevented by dithiothreitol or 2-mercaptoethanol which inhibit luciferase. However, luciferase was somewhat stabilized when stored frozen at −80° and routinely remained 90% active for 1 month under these conditions.

Ultracentrifuge Studies. Figure 3A shows the sedimentation velocity behavior of luciferase at a concentration of 5 mg/ml. Luciferase appeared as a single schlieren peak contaminated with a small amount of a faster moving component. The major peak had a sedimentation coefficient ($s_{20,w}$) of 6.9 S, and corresponded to the luciferase activity as judged by sucrose gradient centrifugation. The sedimentation coefficient is dependent on protein concentration; under the same conditions as Figure 3A but at a concentration of 0.07 mg/ml, luciferase exhibited a sedimentation coefficient of 7.3 S as determined with absorption optics. The latter figure of 7.3 S is used for all calculations involving the sedimentation coefficient since it approximates the sedimentation coefficient at infinite dilution.

Dialysis of luciferase at a concentration of 2.5 mg/ml *vs.* 0.001 M potassium phosphate (pH 7.0) for 4 days resulted in a 50% loss of activity. As shown in Figure 3B in the lower ionic strength buffer luciferase no longer appeared as one major schlieren peak but was composed of at least three components of 5.9, 7.35, and 8.7 S. The aggregation phenomenon was shown to be reversible. When the luciferase in the 0.001 M phosphate buffer was reincubated in 0.1 M potassium phosphate for 5 hr and recentrifuged, only a single 7.2S peak appeared (Figure 3C). However, the activity was not restored.

These results showed that the activity and the state of aggregation of luciferase are influenced by differences in ionic strength and buffer composition. For these reasons, most of the experiments presented here were carried out in 0.1 M potassium phosphate buffer (pH 7.5) utilizing freshly prepared luciferase preparations.

Sedimentation equilibrium data on luciferase by the short-column Yphantis method produced a linear relationship when the log of the protein concentration was plotted against the square of the distance from the center of rotation. A weight-average molecular weight of 320,000 was calculated from the slope assuming a partial specific volume of 0.73. The linearity of the slope indicated the homogeneity of the luciferase preparation.

A Stokes radius of 100 Å was calculated for luciferase from the above values of the molecular weight (320,000), sedimentation coefficient (7.3 S), and assumed partial specific volume (0.73). The Stokes radius of luciferase was also estimated from Sephadex G-200 gel chromatography by the method of Ackers (1964) and from Agarose 1.5 m gel chromatography by comparing the elution of luciferase with

TABLE II: Purification Procedure for *Diplocardia* Luciferase.[a]

	Total Vol (ml)	Total Protein (mg)	Total Units	Sp Act. (Units/mg)	Recov (%)	Purificn
I. Crude extract	800	2500	900	0.36	100	1
II. DEAE-cellulose	1600	455	280	0.62	31	1.7
III. $(NH_4)_2SO_4$ precipitate	28	260	270	1.04	30	2.9
IV. Sephadex G-150	70	23	190	5.8	21	16
V. Hydroxylapatite	40	15	110	7.3	12	20

[a] Details on individual steps are given in Methods. A unit of luciferase activity produced a flash peak of 10^{11} quantum sec^{-1} in the standard assay.

fibrinogen and catalase. These methods yielded a Stokes radius of about 95 Å for luciferase which is in good agreement with the centrifugation data. A diffusion constant ($D_{20,w}$) of 2.25×10^{-7} cm^2 sec^{-1} was calculated using 95 Å as the Stokes radius. Using this diffusion constant and the sedimentation coefficient (7.3 S) a molecular weight of 291,000 was calculated, in good agreement with the sedimentation equilibrium data.

The frictional ratio (f/f_0) of luciferase was determined by comparing the Stokes radius of luciferase (95 Å) with the radius of an unhydrated spherical molecule of the same molecular weight and partial specific volume. The calculated frictional ratio is 2.10; deviation of the frictional ratio from unity arises from the combined effects of asymmetry and degree of hydration of the molecule (Scheraga and Mandelkern, 1953), and a frictional ratio of 2.10 qualitatively indicates that luciferase is highly asymmetric.

Electrophoresis and Ion-Exchange Chromatography of Luciferase. Electrophoresis of luciferase on cellulose acetate membranes with 0.1 M potassium phosphate at pH 7.5 resulted in a single band which corresponded to the activity (Figure 4). In contrast, partially purified luciferase from step 3 of the purification procedure, yielded five protein bands in the pH 7.5 buffer system.

Electrophoresis of luciferase at pH 9.0 by the method of Epstein *et al.* (1968) resulted in the pattern shown in Figure 5C. A broad band of protein entered slightly into the porous upper region of the gel.

As shown in Figure 5A, disc gel electrophoresis of luciferase on 5.5% polyacrylamide gels with a running pH of 8.5 resulted in two bands near the top of the lower gel. The same results were obtained with 3.75% polyacrylamide gels. The standard pH 9.5 system of Davis (1964) produced a single broad smeared band of protein in the same region (Figure 5B). In all cases, the luciferase activity was associated with the stained areas.

A possible explanation for the gel electrophoretic heterogeneity exhibited by luciferase is due to the fact that luciferase occurs in at least two ionic species. As shown in Figure 6, DEAE-cellulose chromatography of luciferase yielded two chromatographically separable active forms of luciferase.

Subunit Structure of Luciferase. Electrophoresis of luciferase in the presence of dodecyl sulfate and 2-mercaptoethanol by the procedure of Weber and Osborn (1969) revealed the presence of three components staining with approximately equal intensity (Figure 7). The electrophoretic pattern was not altered by alkylation of luciferase with ICH_2CO_2H, by the addition of 8 M urea to the usual denaturing conditions of 0.1% dodecyl sulfate and 1% HOC_2H_4SH, or by heating the samples to 95° for 2 min prior to electrophoresis. The molecular weights of the subunits were determined by comparison of their mobilities with the migration of standard proteins such as ovalbumin dimer, bovine serum albumin, catalase, fumarase, ovalbumin, and lysozyme. The molecular weights of two large subunits were determined from gels prepared with two-thirds of the normal amount of cross-linker; under these conditions the subunits fell within the linear range of the standard curve and were found to have molecular weights of 71,000 and 58,000. The molecular weight of the third subunit was determined to be 14,500 with gels containing the normal amount of cross-linker. The minimum molecular weight of luciferase calculated from the sum of the subunit molecular weight is 143,500. This is approximately half of the molecular weight (320,000) determined by ultracentrifugation. Thus, the native protein most likely consists of three pairs of nonidentical subunits.

Spectral Properties of Luciferase. As shown in the inset to Figure 8, luciferase has an absorption maximum at 278 nm with a 278:260 nm ratio of 1.8. The absorption spectrum of a colorless solution of luciferase (5 mg/ml) is shown in Figure 8. Much of the absorption extending into the visible is due to light scattering and was eliminated by blanking luciferase against fibrinogen. When blanked against an equal concentration of fibrinogen, luciferase showed no absorbancy beyond 450 nm. The lack of any characteristic heme Soret absorption bands or flavin absorbance in the visible region of the luciferase spectrum indicates that luciferase is not a heme peroxidase (Saunders *et al.*, 1964) or a flavoprotein peroxidase such a DPNH peroxidase (Dolin, 1960). A Soret peak of about 1.5 absorbancy units would be expected at this luciferase concentration if luciferase contained an equimolar ratio of heme based on a molecular weight of 300,000 for luciferase and a Soret extinction coefficient of 90 mM^{-1} cm^{-1} of horseradish peroxidase (Keilin and Hartree, 1951).

The fluorescence spectrum of luciferase is that of a typical protein with an emission peak at 345 nm when excited at 290 nm (uncorrected). There is no evidence of any other fluorescence emission peaks.

Metal Requirements for Luciferase. As shown in Table III, luciferase is inhibited by a variety of metal-binding agents with the exception of EDTA. As shown with *o*-phenanthroline and 8-hydroxyquinoline, the extent of inhibition depended on the time of incubation of luciferase with the inhibitor. In a separate experiment, it was further shown that the decrease in light intensity by KCN was due to a time-dependent inhibition of luciferase and not luciferin. Incubation of luciferase with 0.02 mM KCN for 10 min resulted in 84% decrease in light intensity while incubation of the

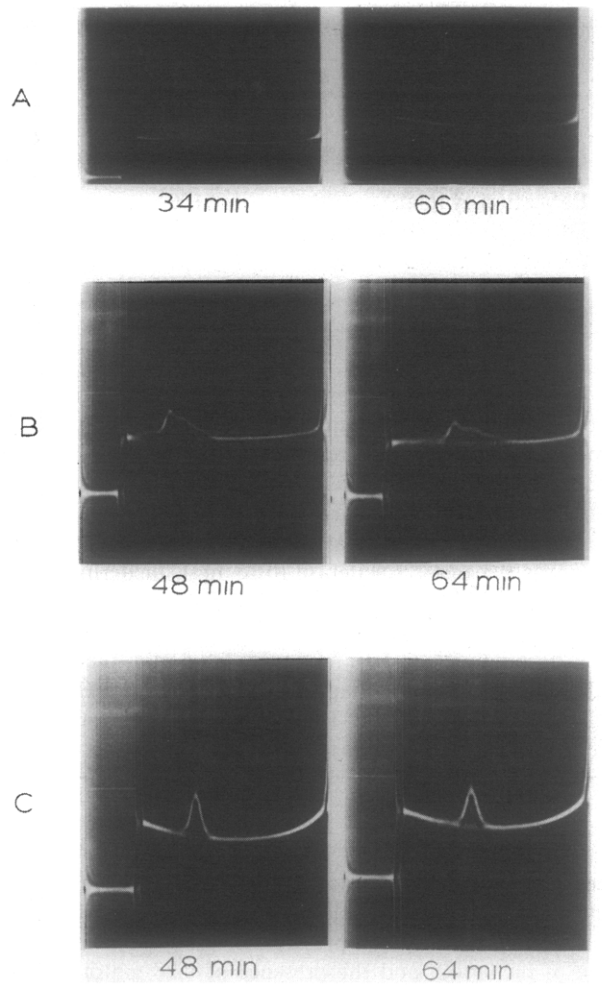

FIGURE 3: Sedimentation velocity of luciferase. (A) Schlieren pattern of luciferase (5 mg/ml) in 0.05 M potassium phosphate (pH 7.0). The photographs were taken 34 and 66 min at a bar angle of 70 and 65°, respectively, after reaching a rotor speed of 60,000 rpm. The temperature was 6°. (B) Luciferase (2.5 mg/ml) was dialyzed at 4° for 48 hr *vs.* 0.001 M potassium phosphate (pH 7.0). Sedimentation was then carried out in the 0.001 M potassium phosphate buffer (pH 7.0) at 6°. (C) Sufficient 1 M potassium phosphate (pH 7.5) was added to the luciferase preparation in 0.001 M potassium phosphate (pH 7.0) to bring the final buffer concentration to 0.1 M and the preparation was incubated at 25° for 8 hr prior to centrifugation at 21°. The photographs were taken 48 and 64 min after reaching speed (60,000 rpm). The bar angle was 60° and the direction of sedimentation from left to right.

same concentration of KCN with luciferin for 10 min prior to assay only resulted in 20% loss of activity. These assays were carried out with a rapid-mixing device (Prichard, 1968) and the final concentration of KCN in both cases was 0.01 mM.

The inhibition of luciferase by 0.01 mM *o*-phenanthroline was completely reversed by the presence of 0.05 mM $CuSO_4$ or 0.05 mM $Fe(NH_4SO_4)_2$ in the assay mixture. The inhibition by 0.01 mM KCN could only be partly reversed, 10% of the original activity being restored with 0.5 mM $CuSO_4$. The addition of several concentrations of $CuSO_4$, CuCl, $Fe(NH_4SO_4)_2$, $FeCl_3$, or a variety of other metals to untreated luciferase did not increase luciferase activity.

The inhibition of luciferase by the metal binding agents could also be reversed by removal of the inhibitor from the luciferase preparation by dialysis. When luciferase was dialyzed against 2 mM KCN or 2 mM *o*-phenanthroline for 36

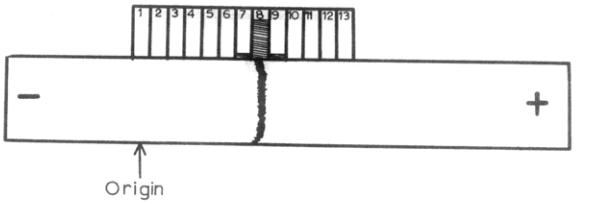

FIGURE 4: Electrophoretic pattern of luciferase on cellulose acetate membranes. Luciferase (60 μg) was applied in 0.1 M potassium phosphate buffer (pH 7.5) and electrophoresed in the same buffer. The figure represents a tracing of one of the stained strips; the relative luciferase activity eluted from sections of the strip is illustrated diagrammatically on top of the tracing.

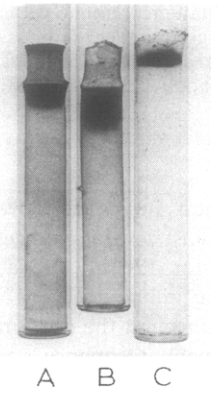

FIGURE 5: Polyacrylamide gel electrophoresis of luciferase. (A) Disc gel electrophoresis of luciferase (39 μg) with a running pH of 8.5 on 5.5% polyacrylamide gel according to Hedrick and Smith (1968). (B) Disc gel electrophoresis of luciferase (31 μg) on 5.5% polyacrylamide gels with the standard pH 9.5 system of Davis (1964). (C) Electrophoresis of luciferase with a running pH of 9.0 by the acrylamide pore size gradient method of Epstein *et al.* (1968). The direction of migration was from the cathode (top) to the anode (bottom). The gels were destained with a charcoal diffusion destaining apparatus (Hoeffer Scientific Instruments). In all cases, the protein completely entered into the gel; the material on top of some of the upper gels is charcoal dust from the destainer.

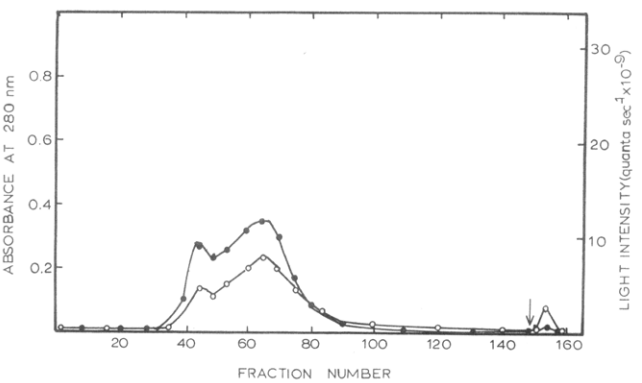

FIGURE 6: Elution profile of luciferase from DEAE-cellulose column chromatography. Luciferase (22 mg) in a volume of 22 ml was dialyzed *vs.* 2000 ml of 0.02 M sodium borate (pH 8.1) for 48 hr with two changes of buffer. A 1.9 × 5.3 cm Whatman DE-52 column was equilibrated with the borate buffer until the pH and conductivity of the effluent exactly matched the dialysis buffer. The luciferase was eluted with a linear salt gradient formed from 300 ml of 0.02 M sodium borate (pH 8.1) buffer and 300 ml of 0.15 M NaCl in the same buffer. The flow rate was 20 ml/hr and 4-ml fractions were collected. When the gradient was finished, 1 M NaCl in the borate buffer was added at the point indicated with the *arrow*. Luciferase was assayed as in Methods. (○) Luciferase activity; (●) absorbance at 280 nm.

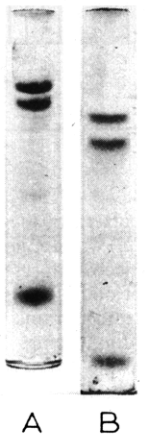

FIGURE 7: Dodecyl sulfate acrylamide gel electrophoresis of luciferase. The procedure is given under Methods except in part B where two-thirds of the normal amount of cross-linker was used. In part A, 25 μg of luciferase was applied and in part B, 10 μg. The anode is at the bottom of the gel.

hr all activity was lost as indicated above. When this was followed by dialysis against several changes of 0.1 M potassium phosphate (pH 7.5) for 120 hr, full activity was restored. Inhibition by carbon monoxide was completely reversed by flushing nitrogen through the assay mixture for 2 min.

Electron paramagnetic resonance spectroscopy of active luciferase showed the presence of a large cupric signal with a g value of 2.045. Double integration of the cupric signal, when compared to a cupric EDTA standard, gave a cupric content of 0.88 g-atom/mole of enzyme. Atomic absorption spectroscopy revealed the presence of 0.8 g-atom of copper/mole of luciferase, and the presence of 0.28 g-atom of iron and 0.21 g-atom of zinc. When luciferase was dialyzed against 1 mM *o*-phenanthroline and then against metal free 0.1 M potassium phosphate (pH 7.5), all of the original activity was restored. However, 90% of the copper was removed by the *o*-phenanthroline treatment.

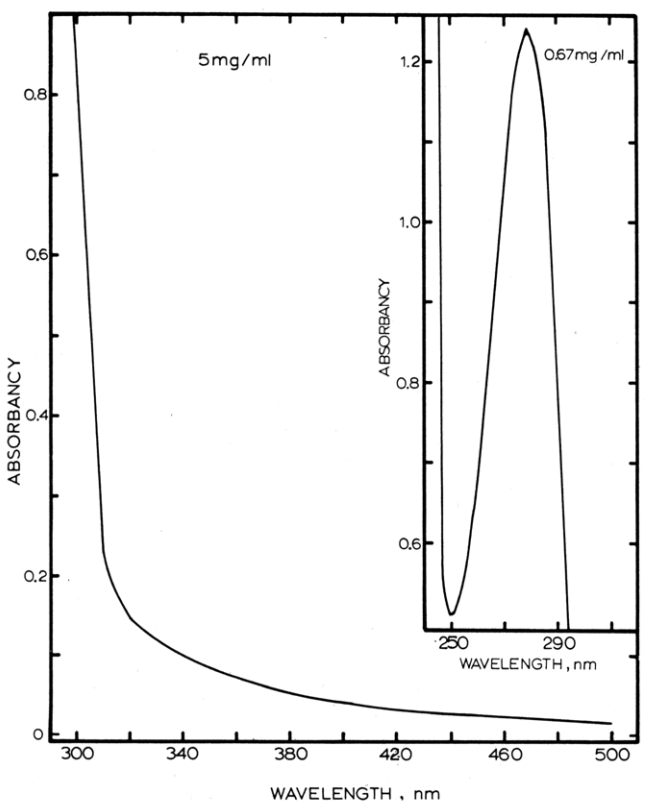

FIGURE 8: Absorption spectrum of luciferase. Luciferase (5 mg/ml) was dissolved in 0.05 M potassium phosphate (pH 7.5). The inset shows the absorption maximum of luciferase at a protein concentration of 0.67 mg/ml. The spectra were taken with a Cary 14 spectrophotometer at 25°.

TABLE III: Inhibition of Luciferase by Metal-Binding Agents.

Inhibitor (5×10^{-4} M)[a]	% Inhibn: 0 Time	% Inhibn: 30-Min Incubn
Potassium cyanide	100	100
Diethyldithiocarbamate	100	100
o-Phenanthroline	75	100
8-Hydroxyquinoline	20	50
EDTA	0	0
Carbon monoxide[b]	88	

[a] Luciferase (0.22 mg) was incubated at room temperature with 0.04 mmole of potassium phosphate (pH 7.5) and 0.4 mmole of the compounds tested in a volume of 0.4 ml. The incubation mixture of luciferase and inhibitor were immediately assayed in the standard assay (0 time) or after 30-min incubation at 4°. The final inhibitor concentration in the assay was 0.5 mM. [b] Luciferase (0.16 mg), luciferin, and 60 μmoles of potassium phosphate (pH 7.5) were incubated in a Thunberg tube with 0.2 ml of 0.37 mM H_2O_2 in the sidearm. After flushing with carbon monoxide for 5 min, the tube was sealed and the reaction started by tipping in the H_2O_2. The final volume was 0.8 ml. The same procedure was carried out in the absence of carbon monoxide as a control.

Properties of the in Vitro Light Reaction

Bioluminescent Spectra. As shown in Figure 9, the *in vitro* light emission from purified luciferin and luciferase is very similar to the *in vivo* luminescence from freshly exuded slime. The *in vivo* wavenumber spectrum has an unstructured peak emission at 19,700 cm^{-1} (507 nm) with a half-bandwidth of 3070 cm^{-1} (78 nm). The slight differences between the *in vitro* and the *in vivo* spectra are not considered to be significant.

Dependence of Light Emission on Luciferase Concentration. There is a linear relationship between the initial flash peak and the luciferase concentration. The pH dependence of light emission exhibited a broad optimum from pH 7.0 to 8.5. The addition of albumin at a final concentration of 0.2 mg/ml resulted in a 10% increase in light emission; higher concentrations of albumin yielded no further increases. The decay of the light intensity was first order with the half-time for decay about 12 sec. The half-time for decay was independent of luciferase concentration indicating luciferase was probably not turning over (Chase, 1963). The total light emitted was also proportional to luciferase concentration, further indicating that luciferase did not behave as a normal enzyme. The quantum yield for luciferase was fairly constant over the range of luciferase concentrations assayed and was extremely low (0.2%) under these conditions. (A

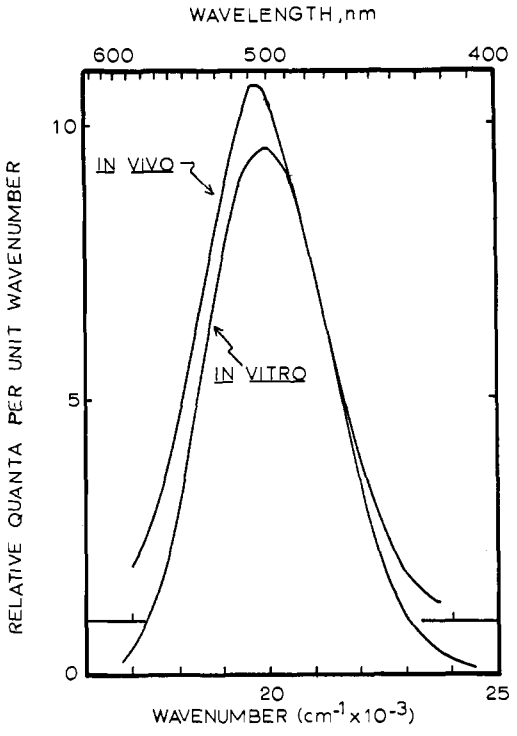

FIGURE 9: Wavenumber plot of *Diplocardia in vivo* and *in vitro* luminescence. Details are given in Methods.

100% quantum yield indicates one photon is emitted per luciferase molecule reacted.)

When the reaction was over, the injection of fresh luciferin or H_2O_2 into the spent reaction mixture resulted in no further light emission. However, the injection of an equal amount of luciferase into the spent reaction mixture resulted in a flash of light of approximately equal intensity and total light as the original flash. Incubation of luciferase with luciferin for 1 min prior to assay had no effect on luciferase activity. However, the incubation of luciferase with the assay concentration of H_2O_2 for 1 min, resulted in a loss of 90% of the luciferase activity. It therefore appeared that although necessary for light emission, H_2O_2 also inactivated luciferase. For this reason H_2O_2 was always added last in the standard assay.

Dependence of Light Emission on H_2O_2 Concentration. As shown in Figure 10, H_2O_2 exhibited an apparent saturation phenomenon. The data yielded an apparent K_m for H_2O_2 of 2 mM. Although the value obtained for the K_m is probably not valid in terms of normal enzyme kinetics, it indicates the relatively high concentration of H_2O_2 necessary for maximum activity. As shown in Figure 11, semilogarithmic plots of light intensity against time at varying H_2O_2 concentrations were linear for about 90% of the reaction; thereafter the decay deviated from linearity (the rate of decay decreased) and no longer followed pseudo-first-order kinetics. The pseudo-first-order rate constants calculated from the slopes in Figure 11 were proportional to the H_2O_2 concentration. Except for the two highest concentrations of H_2O_2, the quantum yield for luciferase was fairly constant around, 0.38%, over the range of H_2O_2 concentrations studied.

Inactivation of Luciferase by H_2O_2. Previous results indicated that luciferase is inactivated by H_2O_2. To test this hypothesis, luciferase was incubated with three different concentrations of H_2O_2 and the kinetics of inactivation of luciferase were followed by withdrawing aliquots at various time intervals and assaying the remaining luciferase activity in the standard assay. These results are shown by the open circles in Figure 11B. Following a rapid initial 10–20% drop of luciferase activity (not shown in the figure), luciferase incubated with H_2O_2 was inactivated at the same pseudo-first-order rate as the decay of the light emission. The rate of inactivation of luciferase at higher H_2O_2 concentrations than those shown in Figure 11B was not determined because

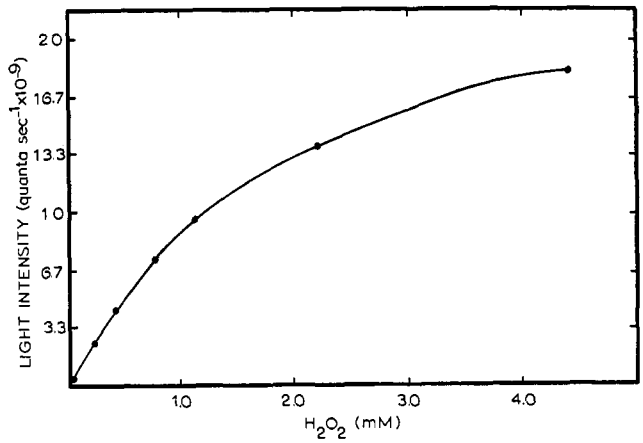

FIGURE 10: H_2O_2 concentration curve. The reaction mixture contained 0.575 ml of 0.1 M potassium phosphate (pH 7.5), 0.025 ml of alumina luciferin, and 0.2 ml of luciferase (0.12 mg). The reaction was initiated by injecting 0.2 ml of H_2O_2 (0.044–4.4 mmoles) into the assay mixture. The final volume was 1.0 ml. (O) Intensity of flash peak.

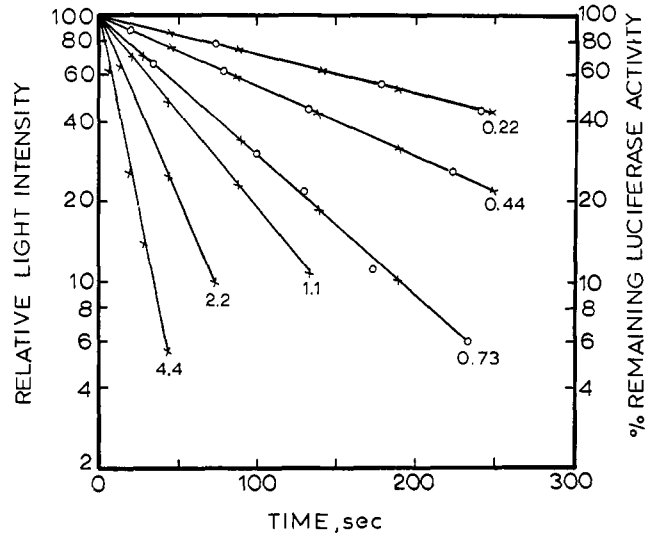

FIGURE 11: (A) Dependence of the first-order decay of light intensity on H_2O_2 concentration. The conditions for light emission are exactly as given in Figure 10. The H_2O_2 concentrations (mM) in the assay are given alongside the respective lines. Zero time represents the flash peak of light intensity. (×) Light intensity at various time intervals following the initial flask peak. (B) Pseudo-first-order inactivation of luciferase with different concentrations of H_2O_2. Luciferase was incubated in 0.1 M potassium phosphate (pH 7.5) at 25° with the mM concentrations of H_2O_2 shown alongside the *upper* three curves (*i.e.*, 0.22, 0.44, and 0.73 mM H_2O_2). The incubation mixture contained 75 μg of luciferase in a volume of 1.0 ml. Aliquots were withdrawn at the times shown and assayed for remaining luciferase activity with the standard luciferase assay given in Methods. The curves have been adjusted to 100% activity.

the rapid inactivation of luciferase prevented making accurate measurements of remaining luciferase activity. From these results it is concluded that luciferase is inactivated by H_2O_2 and the inactivation of luciferase is the determining factor in the light reaction such that the decay of light intensity is not due to depletion of luciferin but rather results from the destruction of luciferase by H_2O_2.

An attempt was made to determine the rate constant for the inactivation of luciferase by H_2O_2 based on the assumption that the rate of inactivation of luciferase is the same as the rate of decay of light intensity as shown in the upper three curves of Figure 11. Following the treatment of Burton and Josse (1970) based on the earlier work of Levy *et al.* (1963), the inactivation of luciferase by H_2O_2 can be expressed as

$$\text{luciferase} + n\text{H}_2\text{O}_2 \xrightarrow{k} \text{inactive luciferase}$$

where n is the number of H_2O_2 molecules necessary to react with each inactivation site on luciferase, and k is the reaction rate constant. Following the treatment of Levy *et al.* (1963), the rate of irreversible inactivation is given by

$$-\text{d(E)}/\text{d}t = k(\text{H}_2\text{O}_2)^n(\text{E}) \quad (1)$$

where E is the active luciferase remaining at time t. Since the H_2O_2 concentration is in great excess over the luciferase concentration and therefore does not change appreciably during the reaction, the inactivation of luciferase follows pseudo-first-order kinetics at a given H_2O_2 concentration.

The relationship of the pseudo-first-order reaction rate constant (k') to the H_2O_2 concentration is given by

$$k' = k(\text{H}_2\text{O}_2)^n \quad (2)$$

and a value of n can be therefore obtained experimentally from a logarithmic plot of eq 2.

$$\log k' = \log k + n \log \text{H}_2\text{O}_2 \quad (3)$$

where n is the slope when the log of k' is plotted against the log of the H_2O_2 concentration. The values for the pseudo-first-order rate constants, k', at a given H_2O_2 concentration were obtained from the slopes of the lines in Figure 11. The logarithmic data were then plotted according to eq 3. A value for n of 0.99 was obtained from the slope of this plot indicating one molecule of H_2O_2 inactivates each luciferase site. Since n is equal to unity, the inactivation of the luciferase is second order overall as expressed in eq 1. As predicted from eq 2 a plot of k' *vs.* H_2O_2 yielded a straight line whose slope is equal to k, the second-order rate constant for the luciferase inactivation reaction. A value of $k = 13.8\ \text{M}^{-1}\ \text{sec}^{-1}$ was obtained.

Luciferase (0.5 mg/ml) was only 1% active after being incubated with 1 mM H_2O_2 for 10 min at 4°. The H_2O_2-inactivated luciferase exhibited no spectral or fluorescence changes. The loss of activity was not reversed by dialysis or by the addition of ascorbate, dithionite, ferrocyanide, ferricyanide, CuCl, $CuSO_4$, or $Fe(NH_4SO_4)_2$ to the dialyzed luciferase. Dithionite or ascorbate added in the presence of $CuSO_4$ was further inhibitory.

The addition of 0.2 mM KCN or 0.5 mM *o*-phenanthroline to luciferase resulted in complete but reversable inhibition as indicated earlier in the text. If H_2O_2 were added secondarily, at a concentration required for complete inhibition, it was found that luciferase was protected from inactivation by H_2O_2. This was shown by dialysis of the above mixture against 0.1 M potassium phosphate buffer (pH 7.5) and assaying for luciferase. After such dialysis complete recovery of luciferase was obtained when pretreated with KCN and 50% recovery was obtained when pretreated with *o*-phenanthroline.

Effects of Luciferin Concentration. Both the initial flash peak and the total light is proportional to the luciferin concentration. The first-order decay constant is independent of the luciferin concentration. As previously indicated, the total light emitted from a given amount of luciferin is limited by the inactivation of luciferase by H_2O_2 and is an artifact of the assay; that is, all of the luciferin is not utilized during the reaction. Furthermore, the quantum yield with respect to luciferase is proportional to the luciferin concentration. Therefore, attempts to estimate total light emission from luciferin using low concentrations of luciferin were unsuccessful due to the low quantum yield of luciferase under these conditions.

Specificity of Luciferin and Luciferase. Diplocardia luciferase shows no activity toward a variety of typical peroxidase substrates such as guaiacol, *o*-dianisidine, pyrogallol, uric acid, or 3,4-dihydroxphenylanine. Luciferase also exhibits no demonstrable DPNH peroxidase activity (Dolin, 1960) or catalase activity. Furthermore, horseradish peroxidase does react with *Diplocardia* luciferin to produce light.

Organic peroxides do not replace H_2O_2 in the light reaction. Cumene hydroperoxide and succinyl peroxide were completely inactive in the standard luciferase assay. Ethyl hydroperoxide had slight activity and was 0.2% as effective as H_2O_2. This trace of activity is probably due to a small amount of H_2O_2 contamination in the ethyl hydroperoxide preparation since the addition of catalase to the assay immediately abolished the light emission. The negligible rate of decomposition of ethyl hydroperoxide by catalase (Chance, 1949) is not sufficient to account for the immediate inhibition. All three organic peroxides inactivated luciferase; as with H_2O_2 inactivation, the luciferase activity was not restored following dialysis.

As previously reported (Bellisario and Cormier, 1971), there is no evidence for an oxygen requirement for *in vitro* light emission. As shown in Figure 12, the presence of oxygen in the reaction mixture resulted in decreased light emission when compared with the anaerobic system. The oxygen inhibition could be reversed by flushing nitrogen through the system indicating the decreased light emission was not due to inactivation of luciferase or to destruction of the luciferin.

Discussion

It has been well established that light emission from bioluminescent earthworms arises from the slime exuded when the worms are sufficiently disturbed (Harvey, 1952). Skowron (1928) studied this process in the luminescent earthworm, *Microscolix phosphoreus*, and postulated that the luminous slime was derived from the lysis of granule containing cells present in the worms' coelomic fluid. Gilchrist (1919) made similar observations on a South African earthworm which contains coelomic cells of about 20 μ in diameter. In the present investigation, the coelomic cells from *D. longa* have been isolated by sucrose gradient centrifugation and it has been confirmed that the components of the light-emitting reaction are contained in the coelomic cells of *D. longa* and that lysis of the cells is necessary for light emission. The physiological significance of earthworm luminescence re-

mains unknown although Gilchrist (1919) has proposed the sudden ejection of luminous slime serves as a defensive mechanism, and Friend (1919) has suggested that luminescence may serve to attract mates.

Diplocardia luciferase has been purified to apparent homogeneity as judged by sedimentation equilibrium analysis. However, luciferase exhibits heterogeneity on disc gel electrophoresis, appearing as two bands (Figure 5). DEAE-cellulose chromatography partially separated two active species of luciferase indicating luciferase exists in at least two different ionic forms. The reasons for the charge differences of luciferase are unknown, but the DEAE-cellulose data provide an explanation for the two bands evident on disc gels and support the proposition that luciferase has been highly purified.

Sedimentation velocity and equilibrium centrifugation data yield a molecular weight between 291,000 and 320,000 for luciferase. Dodecyl sulfate gel electrophoresis data indicate that luciferase is composed of three pairs of nonidentical subunits of molecular weight 71,000, 58,000, and 14,500. Furthermore, the frictional ratio of luciferase was estimated to be 2.10 indicating the molecule is highly asymmetric. The absorption and fluorescence spectra of luciferase are those of a typical protein and indicate that luciferase is not a flavoprotein peroxidase, as DPNH peroxidase (Dolin, 1960) or a typical heme peroxidase (Saunders *et al.*, 1964). Luciferase does not exhibit activity toward a number of typical peroxidase substrates such as pyrogallol, luminol, benzidine, or *o*-dianisidine. All of the above data indicate the *Diplocardia* luciferase does not structurally or functionally resemble the widespread typical heme peroxidases (Saunders *et al.*, 1964).

The discovery that luciferase is a non-heme protein was striking in view of the early observations by Cormier *et al.* (1966) that luciferase is strongly inhibited by KCN. It was therefore felt that the KCN inhibition might be indicative of a metal requirement for luciferase and the effect of a variety of metal-binding agents on luciferase activity was then determined. As shown in Table III, luciferase is inhibited by several metal-binding agents such as KCN, *o*-phenanthroline, sodium diethyldithiocarbamate, and 8-hydroxyquinoline. Atomic absorption spectroscopy results yielded a value of 0.8 atom of copper/luciferase molecule and lesser amounts (0.21 and 0.28 atom per luciferase molecule) of zinc and iron, respectively. According to the results obtained with electron paramagnetic resonance spectroscopy all of the copper in luciferase exist in the cupric state.

From these results, it was suggested (Bellisario and Cormier, 1971) that luciferase might be a metalloprotein which contained copper. However, it is now reported that the removal of 90% of the copper from luciferase with *o*-phenanthroline did not result in a corresponding loss of luciferase activity. A metal requirement for luciferase has therefore not been established even though it is reversibly inhibited by a variety of metal-binding agents. Metal-binding agents could inhibit luciferase by means other than by forming metal complexes. For example, *o*-phenanthroline has been postulated to inhibit flavoprotein monooxygenases by hydrophobic interactions rather than metal chelation (Yamamoto *et al.*, 1969) and KCN is known to bind to carbonyl groups (Dixon and Webb, 1964) and to inhibit enzymes which form Schiff base intermediates (Wishnick and Lane, 1969).

The requirements of the *Diplocardia* bioluminescent reaction fit the classical definition by Dubois (1887) of a "luciferin-luciferase" reaction utilizing H_2O_2 instead of molecular oxygen. However, kinetic studies of the *Diplocardia* system

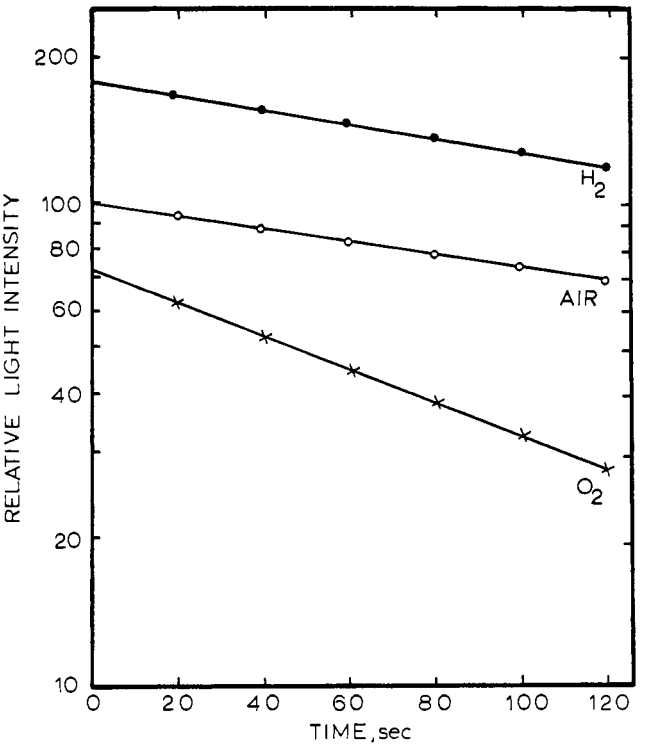

FIGURE 12: Effects of oxygen on *in vitro* light emission. These experiments were carried out in a modified Thunberg tube which could be alternately evacuated and flushed with various gases, and then sealed. The center well contained 0.45 ml of 0.1 M potassium phosphate (pH 7.5), 0.16 mg of luciferase, and 50 μl of alumina luciferin. The side arm contained 0.2 ml of 0.37 mM H_2O_2 and the reaction was initiated by tipping the H_2O_2 into the center well. In the control (air) there were no additions. For the oxygen (O_2) and hydrogen (H_2) experiments, the vessel was cycled six to eight times with alternate vacuum evacuation and flushing with the proper gas before the light reaction was initiated.

do not fit the classical luminescent reaction. In the classical system the oxidation of the substrate, luciferin, is catalyzed by the enzyme, luciferase, and the luciferin is limiting when the reaction has gone to completion. The total light is proportional to the luciferin concentration and is independent of luciferase concentration. Anomalous results were obtained with the *Diplocardia* system; the total light emitted was proportional to the luciferase concentration and luciferase, not luciferin, was limiting when the reaction had gone to completion. The highest quantum yield obtained for luciferase was 3%; *i.e.*, only 3 photons were emitted per 100 luciferase molecules. Assuming the luciferin quantum yield to be greater than 3% luciferase does not exhibit normal enzymatic catalysis and turnover. Because of the high light intensities produced in the reaction we can estimate that the luciferin quantum yield must be significantly greater than 3%. Because we do not know its purity or molecular weight an absolute value is impossible to obtain at this time.

It is assumed that the low efficiency of the luciferase in the light reaction has been an artifact due to the irreversible destruction of luciferase by H_2O_2 and in fact the rate of decay of the light emission has been shown to correspond to the rate of inactivation of luciferase by H_2O_2 (Figure 11). It is therefore felt that the destruction of luciferase by H_2O_2 is the overriding factor in the light reaction which resulted in the anomalous kinetic results and which prevented the demonstration that the *Diplocardia* protein exhibited normal catalysis. Because of the inactivation and concomitant low

quantum efficiency of the luciferase, it could not be demonstrated that luciferin is consumed in the reaction as predicted by the classical luciferin–luciferase luminescent reaction.

Although kinetic evidence indicates that one molecule of H_2O_2 inactivates each site on luciferase, the identity of the luciferase inactivation site has not been elucidated. Since KCN and *o*-phenanthroline protect luciferase from inactivation by H_2O_2 the same binding site may be involved for both H_2O_2 and metal chelator.

Because of the destructive effect of H_2O_2 on luciferase the requirements for O_2 in the crude coelomic fluid must play an important role in the bioluminescence of earthworms. We believe that the crude coelomic fluid contains an oxidase which acts as an H_2O_2 generating system. That is H_2O_2 must not be present in the coelomic cells until they lyse in the presence of O_2. Thus luciferase would be protected from inactivation from H_2O_2 until there were a need for the worm to bioluminesce. It seems possible that the observations on oxygen requirements for bioluminescence in other earthworms (Harvey, 1952; Johnson *et al.*, 1966) may involve the same kind of phenomena.

Acknowledgments

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