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# Earthworm Bioluminescence: Characterization of High Specific Activity *Diplocardia longa* Luciferase and the Reaction It Catalyzes†

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ABSTRACT: *Diplocardia longa* luciferase purified by an improved procedure differs from that first described by Bellisario et al. [Bellisario, R., Spencer, T. E., & Cormier, M. J. (1972) *Biochemistry 11*, 2256-2266] in having much higher specific activity (40×) and firmly bound, EPR-silent copper. Improved assay conditions suggest that this protein acts as a catalyst in a bioluminescent reaction involving the degradation of 3-(isovalerylamino)-1-hydroxypropane hydroperoxide. This substrate is formed spontaneously on the addition of hydrogen peroxide to *D. longa* luciferin (3-(isovalerylamino)propanal). The quantum yield of the bioluminescence for this substrate is 3%. Detailed physical and chemical analyses of high specific activity *D. longa* luciferase indicate that it is a large (300 000 daltons), asymmetric ($f/f_0$ = 1.63, with 0.4 g/g hydration), multisubunit enzyme. It contains carbohydrate (6%), lipid (2%), and copper (up to 4 mol/300 000 daltons). The amino acid composition is unusual with 11% by weight of the residues being either proline or hydroxyproline.

In the bioluminescence of the earthworm *Diplocardia longa*, hydrogen peroxide and 3-(isovalerylamino)propanal (earthworm luciferin) react in the presence of the protein *D. longa* luciferase with emission of blue-green light (Bellisario & Cormier, 1971; Bellisario et al., 1972; Ohtsuka et al., 1976; Mulkerrin & Wampler, 1978). Previous work on this protein (Bellisario & Cormier, 1971; Bellisario et al., 1972) has shown that it is large (300 000 daltons), is asymmetric ($f/f_0$ = 2.1; with no hydration and $\bar{v}$ = 0.73) and is composed of multiple subunits. Analysis of the purified protein also revealed a reproducible titer of bound, EPR-detectable copper (Bellisario & Cormier, 1971), but further study (Bellisario et al., 1972) showed that 90% of this copper could be removed with no loss of activity. A second important observation, reported by Bellisario et al. (1972), was the lack of a catalytic role for the protein under their assay conditions. In contrast, the data reported here show that *D. longa* luciferase can be prepared with much higher specific activity and that this protein does indeed contain firmly bound, but EPR-silent, copper. In addition, by alteration of the assay conditions to protect the protein from the denaturing action of hydrogen peroxide, turnover of the enzyme can be demonstrated. However, since the physical data for protein prepared according to this new procedure (Rudie, 1977; Mulkerrin & Wampler, 1978) are nearly identical with those reported for the previous, low specific activity protein (Bellisario et al., 1972), we conclude that the differences between the two preparations are explained by a difference in the fraction of active enzyme.

The physical data reported here extend the physical description of the protein to include the carbohydrate, lipid, and amino acid compositions, and a more accurate evaluation of the physical parameters. Interestingly, amino acid analysis reveals that luciferase contains 5.8% by weight of hydroxyproline.

A reinvestigation of the in vitro reaction shows that the true substrate of the bioluminescence is a luciferin peroxide adduct. The stoichiometry and quantum yield data suggest that luciferase catalyzes the luminescent degradation of 3-(isovalerylamino)-1-hydroxypropane hydroperoxide. These data also indicate that the earthworm bioluminescence reaction is quite similar to the copper-catalyzed chemiluminescence which

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we previously reported (Rudie & Wampler, 1979).

## Materials and Methods

*Diplocardia longa* are collected from southern Georgia as previously described (Mulkerrin & Wampler, 1978), where the coelomic cells from the worms are obtained and frozen. Luciferase is prepared from these cells by the improved procedure of Rudie (1977) [see also Mulkerrin & Wampler (1978)]. Luciferin is synthesized by using the revised procedure of Mulkerrin & Wampler (1978). All salts, buffers, and chromatographic media are of the best grade available, and solvents are spectrograde.

The assay for *D. longa* luciferase activity has been standardized in response to the data reported in this work with a different order of addition than previously used (Bellisario & Cormier, 1971; Bellisario et al., 1972; Ohtsuka et al., 1976; Mulkerrin & Wampler, 1978; Rudie, 1977), and concentrations were optimized for luciferase determination rather than peroxide determination (Mulkerrin & Wampler, 1978). The standard assay involves preincubation of luciferin (3.2 $\mu$mol = 0.02 mL of 25 mg/mL luciferin in methanol) and hydrogen peroxide (3.2 $\mu$mol = 0.2 mL of 0.16 M hydrogen peroxide in pH 7.5 phosphate buffer) in a total volume of 0.9 mL for at least 30 s followed by injection of 0.1 mL of luciferase-containing solution by using a Hamilton automatic syringe (model CR-700-200). The assay buffer is 0.1 M potassium phosphate at pH 7.5. Peak light intensity is used as the assay parameter and is obtained with a calibrated photometer of our own design (Anderson et al., 1978). The photometer is calibrated by using the luminol chemiluminescence reactions according to Lee et al. (1966), with spectral-response corrections based on the manufacture's sensitivity curve for our photomultiplier. Assays are performed by using 1 × 7.5 cm test tubes, with the instrument viewing the bottom of the tube. The quantum yields reported here are based on the total calibrated photon yield and are subject to the uncertainties recently discussed by Wampler (1978).

A unit of luciferase activity is defined in the same way as reported previously (the amount of luciferase which gives $10^{11}$ photons/s of peak light intensity) but with the new assay procedure. In our previous papers (Ohtsuka et al., 1976; Mulkerrin & Wampler, 1978; Rudie, 1977; Rudie et al., 1976; Wampler et al., 1979), luciferase units are based on assays involving a lower concentration of peroxide with the peroxide added last. Therefore, these new units of activity are larger by a factor of 5.2. Comparing our units of activity with those of Bellisario et al. (1972) is difficult, however, since the luciferin concentration used in their work is unknown. On the basis of data obtained with fresh coelomic fluid by both groups and assays of acetone powders prepared by Bellisario, we estimate that their units are smaller than ours by a factor of 0.13.

The low amounts of luciferase isolated in a given preparation, its large molecular weight, and the low solubility of the enzyme make determination of copper content difficult. Analysis of the copper in luciferase by using the procedure of Van de Bogart & Beinert (1967) was carried out with copper detected at the limit of sensitivity of the assay and poor reproducibility. The following procedure was adopted to allow estimation of the copper content on very small (0.1–0.3 mL) volumes of luciferase solution. For this procedure, the water used in the buffers and the washing procedures was doubly distilled, deionized water. All glassware was rigorously cleaned with final washes with 0.001 M EDTA solutions. Copper standards were prepared by dissolving copper metal in acid and volumetric dilution. Protein samples were prepared by dialysis vs. the standard buffer in the presence of various metal chelators (EDTA, *o*-phenanthroline, diethyl dithiocarbamate). Under these conditions, Bellisario et al. (1972) reported 90% loss of their detectable copper. Protein samples were placed in EPR tubes (approximately 4.0-mm diameter, containing 0.3 mL of solution), and the tubes were scribed to indicate the position of the solution meniscus. EPR spectra were obtained, and then the tubes were slowly dried. The residue was dissolved in 12 N HCl and incubated for 48 h at 50 °C. Following this treatment, HCl was added to bring the contents back to the original volume. A second EPR spectrum was then obtained. Controls included portions of the buffer used for dialysis of the luciferase samples prior to this procedure, 12 N HCl with and without added copper, and luciferase samples with copper added. Following this procedure, each tube was cleaned and filled to the scribed level with standard 10 $\mu$M CuEDTA solution, and the spectra were taken a third time. The relative values of the double integrals of these latter spectra were used to adjust for variations between tubes. Difference spectra generated numerically from the luciferase samples and the controls were used for copper determination by using double integration following base line correction and, if necessary, recorrection of the base line of the intergrated spectrum by using a second-order correction.

The EPR spectra and the spectral manipulations described above were obtained by using a Varian Model 4502 spectrometer (Palo Alto, CA) and an EPR data system designed in this laboratory (J. E. Wampler, unpublished design). In this instrument system, the *x*- and *y*-axis scan signals from the 4502 are monitored by the computer (Nova 820, Data General Corp., Southboro, MA) by using an interface designed by On-line Instrument Systems (Jefferson, GA). Since the *x*-axis scan signal is a voltage ramp, the computer collects data corresponding to specific *x*- voltage levels. The program for control and data acquisition allows continuous repetitive scanning with scan averaging and an arbitrary number of data points in a given spectrum. The data system included a complete set of peripheral equipment (Ball Computer Products dual-drive floppy diskette system, Sunnydale, CA; Tektronics Model 603 display oscilloscope, Beaverton, OR; Digital Equipment Corp., Model VT-55 video terminal, Maynard, MA) mounted in a portable cabinet rack. The software which controls this system was written by utilizing the assembly language packaged routines of *SPECOS* (Spectroscopy Operating System; J. E. Wampler, unpublished experiments). Features of this software have been previously described (Wampler, 1976; Wampler et al., 1979) and include a variety of numerical procedures, interactive display support, and *x*,-*y*-plotting routines.

Protein determinations were performed by several methods: dye binding following the procedure of Bradford (1976), the microbiuret method of Goa (1953), fringe displacement in the ultracentrifuge (Babul & Stellwagon, 1969), and dry weight. Dry weight determinations were done by using platinum boats cleaned by boiling in detergent solution for 2 h, soaking in 1.0 N HCl for 2 h, rinsing in distilled water, soaking for 2 h in chloroform, and drying at 120 °C to constant weight (2 days). Samples (30 $\mu$L) of luciferase solution (in pH 7.5 phosphate buffer, 0.001 M) were dried in boats for 24 h at 60 °C, weighed, and then dried again for 24 h at 110 °C. From the dry weight measurements, the extinction coefficient of luciferase was evaluated as $\epsilon_{280}^{0.1\%} = 1.25$. For most work, the absorbance at 280 nm was, therefore, used to determine protein concentration.

Amino acid analyses were performed on acid hydrolysates (Moore & Stein, 1963). Tryptophan was protected in a 24-h hydrolysis by thioglycolic acid, and α-amino-β-guanidinopropionic acid was used as an internal standard. Serine and threonine were determined by extrapolation to zero time of hydrolysis while valine and isoleucine were recorded as the values after 48 h of hydrolysis. Due to possible interferences by luciferin and the corresponding alcohol analogue of luciferin, these compounds were hydrolyzed under the same conditions as well as a mixture of luciferin and ribonuclease A (Sigma). No interferences due to luciferin or its hydrolysis products under these conditions were found.

Carbohydrate analysis of luciferase was performed esssentially by the method of Dubois et al. (1956). Samples of luciferase (0.09–0.15 mg) were lyophilized and resolubilized in 40 mL of distilled water. Phenol (40 μL of 4% phenol in water) was added to each sample and to standards of galactose (0–12 μg in 4 μL). Samples were warmed in an 80 °C constant-temperature block, and 200 μL of concentrated sulfuric acid was added to each. The samples were mixed and heated for 20 min at 80 °C and then cooled. Absorbance at 490 nm was recorded. Amino sugars were determined and detected in the amino acid analyzer.

Lipids were extracted with chloroform–methanol–water (95:35:1 v/v), and phosphate was quantitated by the procedure of Bartlet (1959). Neutral lipids were resolved on silica gel H thin-layer plates with petroleum either–diethyl ether–glacial acetic acid (85:15:1 v/v) and quantitated by the procedure of Scott et al. (1976).

The physical parameters describing *D. longa* luciferase were measured by standard techniques. The sedimentation coefficient was measured by a sedimentation velocity with absorption optics in a Beckman Model E analytical ultracentrifuge (Fullerton, CA) (Schachman, 1957). Measurements were made at 5 °C in 0.1 M phosphate buffer, pH 7.5, and corrected to 20 °C and water. The molecular weight was measured by sedimentation equilibrium in a model E analytical ultracentrifuge with absorption optics (Yphantis, 1964). Partial specific volume was measured by the difference in sedimentation equilibrium of luciferase in $H_2O$ and in 95% $D_2O$ (Edelstein & Schachman, 1967). The diffusion constant was determined by using a synthetic boundary cell and interference optics in the model E analytical untracentrifuge (Schachman, 1957; Schumaker & Schachman, 1957). Measurements were made at 5 °C in 0.1 M phosphate buffer, pH 7.5, and corrected to 20 °C and water.

Polyacrylamide disc gel electrophoresis was run as described by Gabriel (1971) by using 3.75% gels at pH 4.3 and 8.9 in 5 × 100 mm tubes. Sodium dodecyl sulfate–polyacrylamide gels were run according to the method outlined by Neville (1971). Sodium dodecyl sulfate ($NaDodSO_4$) gels were fixed and washed in 20% (w/v) sulfosalicylic acid prior to staining. All gels were stained for protein with Coomassie brilliant blue G-250 in 10% (w/v) perchloric acid (Reisner et al., 1975). Luciferase activity was localized on alkaline and acid disc gels by slicing the gel immediately after electrophoresis and washing the gel slices in assay tubes containing 0.7 mL of 0.1 M phosphate buffer, pH 7.5, for 24 h at 5 °C. The gel slices were then assayed by the addition of 0.02 mL of 2.5 mg/mL luciferin in methanol and the injection of 0.2 mL of 0.22 M $H_2O_2$.

## Results

The modified purification procedure of Rudie (1977) yields much higher specific activity than that of Bellisario et al. (1972). With the adjustment in units of activity mentioned above, this purification routinely yields luciferase with specific activity greater than 500 units/mg, and under optimum conditions with freshly frozen coelomic fluid, average specific activity approaches 1500 units/mg. This can be compared with the specific activity of the protein purified by Bellisario et al. (1972), which was 56 units/mg in terms of the new units of activity. Representative purification data covering a 4-month period (nine preparations) are summarized as follows, with the average value given and the range of values indicated in parentheses: total units of activity in crude extracts per 100 worms = 78 000 (6800–208 000); purification = 129-fold (7–531-fold); recovery of activity = 16% (5–41%); and final specific activity = 1498 units/mg (832–2184 units/mg).

Table I: Purification Data for *D. longa* Luciferase

| step[a] | total protein (mg) | sp act. (units/mg) | % recovery | purification |
|---|---|---|---|---|
| extract | 6304 | 34 | 100 | 1 |
| DEAE-cellulose | 575 | 120 | 30 | 3.5 |
| CM-cellulose | 28 | 1350 | 18 | 40 |
| HAP | 7.5 | 1920 | 7 | 56 |

[a] Purification by chromatography from an extract of frozen coelomic cells from 100 worms. Extraction buffer was 0.1 M sodium borate, pH 7.5, containing 0.125 g/L sodium azide, 33 mg/L catalase, and 75 mg/L dithiothreitol. Chromatography on diethylaminoethyl- (DEAE-) cellulose, carboxymethyl- (CM-) cellulose, and hydroxylapatite (HAP) was carried out by the procedures of Rudie (1977) and Mulkerrin & Wampler (1978).

The results of one such purification are shown in Table I. In this procedure, we begin with coelomic fluid as opposed to whole earthworms, thereby eliminating a great deal of material and requiring only a 56-fold purification of luciferase. The percent recovery in each step is somewhat low due to highly selective cuts, where only very few of the fractions are retained. Side fractions are recombined and rechromatographed. Luciferase strongly adsorbs to *N*-isovaleryldiaminohexane-Sepharose in 0.01 M phosphate (pH 7.5). Elution with 2.5 mg/mL 3-(isovalerylamino)propanol, the alcohol analogue of luciferin, results in the elution of a peak of activity. Luciferase can also be eluted from this gel with 0.2 M NaCl. This affinity chromatography of luciferase following CM-cellulose (carboxymethylcellulose) chromatography by using either salt or luciferol elution gives a preparation of luciferase with a specific activity of about 1500 units/mg of protein, which is constant across the peak. However, recovery from this step was too low and variable to allow its routine use in the purification scheme. Luciferase from the hydroxylapatite step (the final step) of the purification scheme could also be processed by the affinity step, but no significant improvement in specific activity was obtained.

Reevaluation of the Stokes radius was carried out by using a calibrated Bio-gel A5 column and gave a calculated Stokes radius (Akers, 1967) of 85.5 ± 0.9 Å with a frictional coefficient of $16.7 \times 10^{-8}$ and $f/f_0$ of 1.63 (using 0.4 g/g hydration).

The physical data obtained for *D. longa* luciferase by the new purification procedure and the data from Bellisario et al. (1972) are compared in Table II, The diffusion coefficient, $D_w^{20}$, for luciferase is $2.3 \times 10^{-7}$ cm$^2$/s, with a frictional coefficient of $1.7 \times 10^{-8}$, and is in excellent agreement with that measured by gel filtration. The sedimentation coefficient of luciferase is 7.6 ± 0.6 S and displays no concentration dependence between 0.3 and 0.8 mg/mL. Luciferase concentration of 1.0 mg/mL is not attainable due to solubility. The partial specific volume from sedimentation in 95% $D_2O$ and 100% $H_2O$ (0.78 ± 0.06 cm$^3$/g) agrees with the value of

Table II: Physical Characteristics of *D. longa* Luciferase

| | this work | from Bellisario[a] |
|---|---|---|
| composition | | |
| amino acids | 92% (w/w) | |
| carbohydrate | 6 ± 0.6% (w/w) | 7% (w/w) |
| neutral lipid | 0.5% (w/w) | |
| phospholipid | 1.4% (w/w) | |
| hydration | 0.29–0.48 g/g | |
| copper | 4 g-atom/mol | |
| extinction coeff ($\epsilon_{280}^{0.1\%}$) | | |
| by dry wt | 1.25 | |
| by composition | 1.2 | |
| by biuret | 1.78 | 1.8 |
| mol wt | | |
| by sed equil | $(2.6 \pm 0.22) \times 10^5$ | $3.2 \times 10^5$ |
| by calculation | $(2.9 \pm 0.24) \times 10^5$ | |
| hydrodynamic parameters | | |
| Stokes radius (A) | | |
| by molecular sieve | 85.5 ± 0.9 | 100 ± 5 |
| by diffusion | 89 ± 5 | |
| diffusion const ($cm^2/s$) at 20 °C in water | $(2.3 \pm 0.15) \times 10^{-7}$ | |
| sedimentation const (s) at 20 °C in water | $(7.6 \pm 0.6) \times 10^{-13}$ | $7.3 \times 10^{-13}$ |
| frictional coeff | | |
| by molecular sieve | $(16.7 \pm 0.2) \times 10^{-8}$ | |
| by diffusion | $(17 \pm 1) \times 10^{-8}$ | |
| partial specific volume | | |
| by composition | 0.73 ± 0.01 | |
| by $D_2O/H_2O$ sed equil | 0.78 ± 0.06 | |

[a] Data from Bellisario et al. (1972).

Table III: Amino Acid Composition of *D. longa* Luciferase

| amino acid | wt % | residues/ 300 000 daltons |
|---|---|---|
| lysine | 8.86 | 207 |
| histidine | 2.63 | 58 |
| arginine | 4.86 | 94 |
| aspartic acid | 13.25 | 345 |
| threonine | 7.43 | 220 |
| serine | 2.99 | 103 |
| glutamic acid | 10.93 | 254 |
| proline | 5.27 | 163 |
| hydroxyproline | 5.81 | 134 |
| glycine | 3.61 | 190 |
| alanine | 5.09 | 215 |
| cysteine | 0.44 | 12 |
| valine | 3.91 | 118 |
| methionine | 0.12 | 3 |
| isoleucine | 5.53 | 147 |
| leucine | 6.59 | 175 |
| tyrosine | 6.42 | 118 |
| phenylalanine | 3.98 | 81 |
| tryptophan | 2.2 | 36 |

0.73 $cm^3/g$ calculated from composition. Similarly, acrylamide gel electrophoresis under denaturing conditions shows the same subunit structure for luciferase as that reported previously (Bellisario et al., 1972).

The amino acid analysis of luciferase (Table III) is unusual in the high proline content and hydroxyproline content (11% of the total residues and total mass). A relatively high hydrophobicity [1.157 kcal/residue by the method of Bigelow (1967)] is calculated by using this amino acid composition, but the calculated values for hydration and partial specific volume are in the normal range (Table II).

Native, active luciferase prepared via this new procedure has no EPR-detectable copper (Figure 1). When the protein is incubated at room temperature with 0.5% hydrogen peroxide, however, an EPR copper signal does appear (Figure 1). While this effect of peroxide is consistent and may, in part, explain the denaturing effects of peroxide noted by Bellisario et al. (1972), quantitation of this copper signal by double integration of the signal showed that the amount of copper(II) produced was not reproducible.

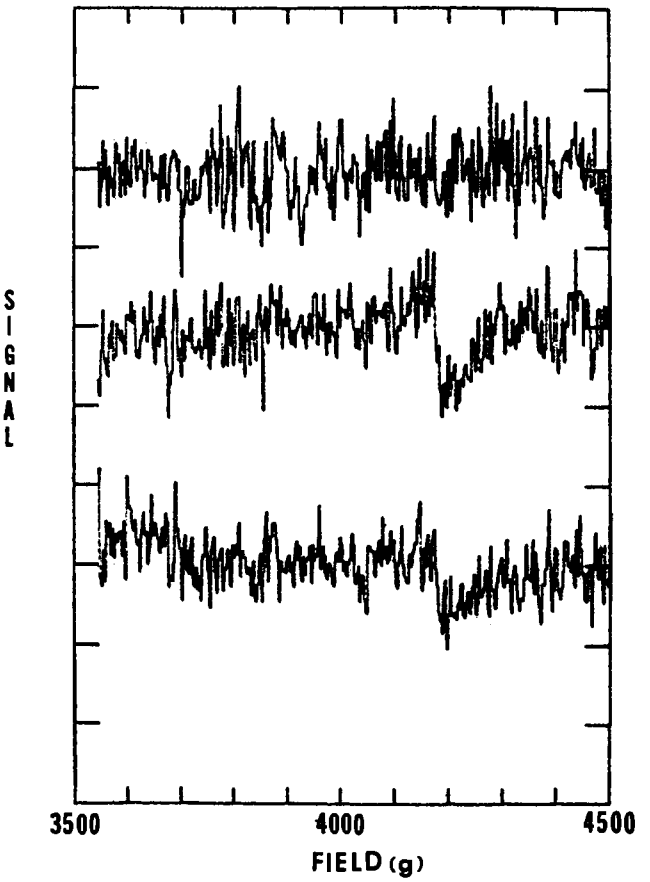



FIGURE 1: Difference EPR spectra of *D. longa* luciferase vs. the dialysis buffer (0.1 M potassium phosphate, pH 7.5, containing 0.001 M *o*-phenanthroline). (Top) Sample (0.3 mL) of luciferase, 1.0 mg/mL, specific activity of 1920 units/mg. (Middle) Same sample with 0.005 mL of 30% hydrogen peroxide added to the thawed sample with 10 min of incubation. (Bottom) standard CuEDTA. Experimental conditions: difference spectra generated numerically from 8 separate scan-averaged spectra, microwave frequency = 9.28 GHz, microwave power = 3 mW, cavity temperature = 103 K.

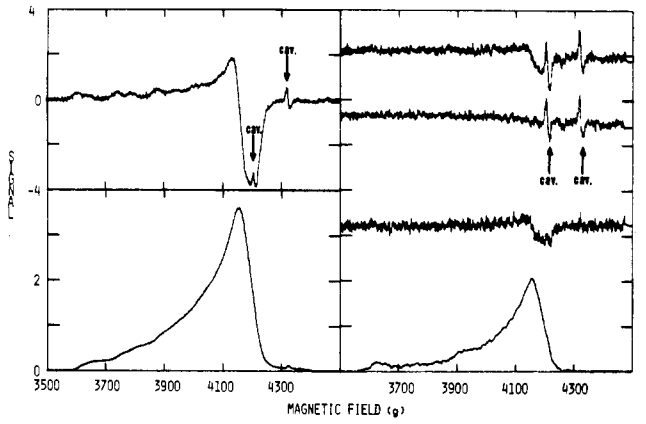



FIGURE 2: EPR spectra representative of the determination of copper(II) in *D. longa* luciferase. (Left panel, top) Spectrum of copper standard (10 μM CuEDTA) (note the cavity signal, cav.); (left panel, bottom) integrated spectrum. (Right panel, top to bottom) Spectrum of hydrolyzed luciferase (0.9 mg/mL), specific activity = 1200 units/mg) following the treatment described in the text; the spectrum of the dialysis buffer; the difference spectrum generated from these two; and the integrated difference spectrum. Experimental conditions are described in the legend of Figure 1.

Table IV: Variation of Copper Content of *D. longa* Luciferase

| prepn | sp act.[a] | Cu per mol |
|---|---|---|
| A | 416 | 0.33 |
| B | 468 | 0.7 |
| C | 1196 | 1.2 |
| D | 1508 | 1.5 |
| E | 1924 | 4.3 |

[a] Units/mg of luciferase, where a unit of luciferase activity is the amount of protein which gives $10^{11}$ photons/s of peak light intensity in the standard assay.

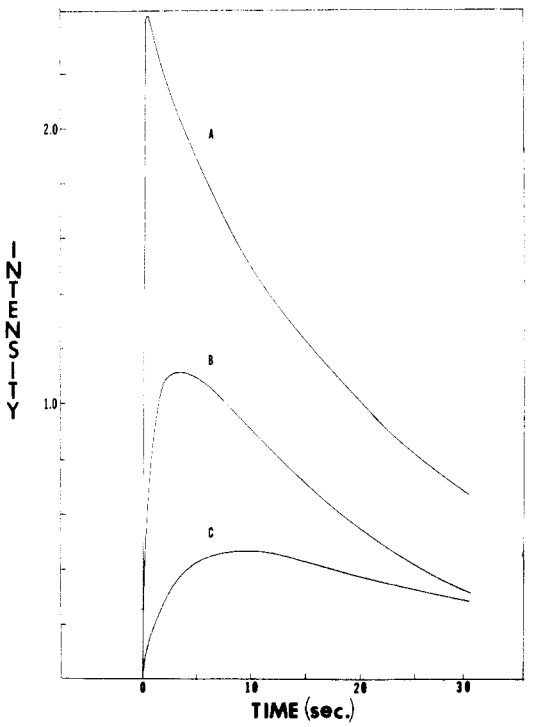



FIGURE 3: Kinetic profiles for in vitro *D. longa* bioluminescence reactions. (A) Luciferase injected last; (B) hydrogen peroxide injected last; (C) luciferin injected last. In each case, 0.1 mL of the appropriate concentration was injected into 0.9 mL with final concentrations as follows: luciferase (0.1 unit/mL); luciferin (0.032 M), hydrogen peroxide (0.032 M), pH 7.5 potassium phosphate buffer (0.1 M).

When the protein is hydrolyzed with strong hydrochloric acid (12 N HCl at 51 °C for 41 h), copper(II) EPR appears. The procedure outlined by Figure 2 was used to quantitate the copper content of luciferase. At these low signal levels, the error due to the base line correction and double integration is estimated to be 30%. However, the reproducibility of the determination between different samples of the protein was not good. A possible explanation for this lack of reproducibility is indicated by the data of Table IV, where the copper content and specific activity for various enzyme preparations are listed.

The light yield and kinetics of in vitro reactions using the components of the *D. longa* bioluminescence system vary with the order of addition as shown in Figure 3. The highest light yield and initial rate are obtained when luciferin and peroxide are preincubated and the reaction is initiated by injection of luciferase (curve A, Figure 3). Since initial reaction rate is monitored by the peak light intensity [for a discussion of the assumptions underlying this use of peak light, see Wampler (1978)], a continuous variation experiment should reveal the stoichiometry of the reaction. Figure 4 shows the data for variation of luciferin and peroxide and indicates a 1:1 stoichiometry. The combined data of Figures 3 and 4 suggest that a luciferin peroxide adduct is the true substrate of the bioluminescence reaction. The data of Figure 5 show

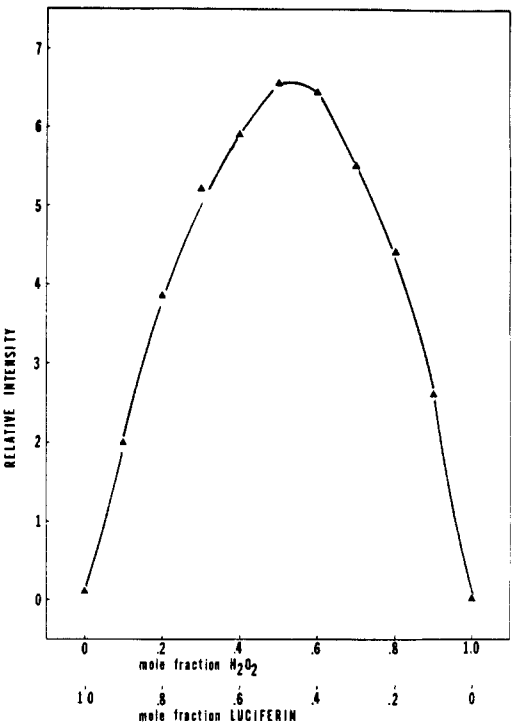



FIGURE 4: Continuous variation study of the *D. longa* in vitro bioluminescence reaction. The concentrations of luciferin and hydrogen peroxide were varied as indicated so that the sum of their concentrations in each assay was 0.0064 M. Luciferin and hydrogen peroxide were mixed prior to assay into a final volume of 0.9 mL and incubated for 1 min. Each sample was then analyzed by injection of 0.1 mL of luciferase solution (0.1 unit).

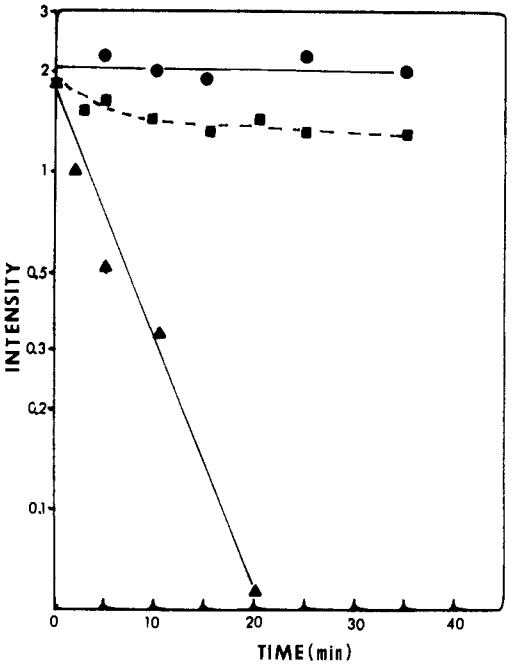



FIGURE 5: Competition between catalase and *D. longa* luciferin for hydrogen peroxide. (●) Luciferin and peroxide were mixed at time zero and assayed by injection of luciferase into an aliquot removed at the indicated times. (—) Luciferin and hydrogen peroxide were preincubated for 2 min, followed by addition of catalase at time zero and analyzed by injection of luciferase in an aliquot removed at the indicated times. (▲) Peroxide and catalase were mixed at time zero, and samples were removed at 30-s intervals, incubated with luciferin for 30 s, and then assayed by injection of luciferase. Concentrations in all assays were the following: luciferin and hydrogen peroxide (0.01 M), luciferase (0.1 unit/mL), and catalase (34 units/mL).

that this is indeed true since catalase is a very competitive inhibitor of the assay performed by injection of luciferase plus

luciferin into catalase plus hydrogen peroxide but is a very poor inhibitor when catalase is added to a preincubated mixture of luciferin and peroxide. Similarly, while catalase can compete very effectively with luciferase for peroxide introduced by injection, it cannot compete effectively for the intermediate formed by preincubation. For example, if peroxide and the adduct ($\lambda_{max}$ = 235 nm) are both monitored by absorbance at 240 nm, the rate of catalase action is highly dependent on the presence of luciferin in the assay mixture. As the reaction proceeds, the spectrum shifts to that of the adduct, and further changes occur at a much slower rate.

By protection of luciferase from reaction and hydrogen peroxide by using excess luciferin preincubated with peroxide, the quantum yields for the peroxide adduct and for luciferase can be evaluated. The values obtained are 3% for the quantum yield for the peroxide adduct and 63% for luciferase. This is the first evidence that *D. longa* luciferase acts in a catalytic role in this bioluminescence reaction.

## Discussion

*D. longa* is a very large earthworm (some specimens over 24 in. long), but the size varies considerably both within a given collection and during different parts of the collection season. In addition, little is known of the biology of bioluminescence in earthworms or whether the bioluminescence activity exhibits seasonal variation or is perturbed by other factors. These considerations and the possible roles of bioluminescence in earthworms have been discussed by Jamieson & Wampler (1979). Regardless of the cause, of the nine enzyme preparations discussed above, there was a wide variation in initial total activity per 100 worms and the degree of purification required to isolate the pure luciferase. There was, however, no correlation between degree of purification, percent recovery, or initial activity and the final specific activity of the enzyme. The preparation summarized in Table I is therefore considered to be typical. All physical studies and composition analyses were performed by using enzyme with specific activity greater than 1500 units/mg (five preparations).

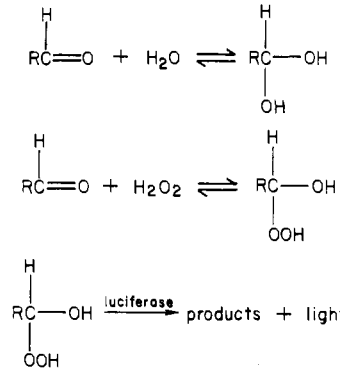
High specific activity *D. longa* luciferase is very similar to the protein of Bellisario et al. (1972) (Table II). By consideration of the size and asymmetry of the enzyme ($f/f_0$ = 1.63, for $\bar{v}$ = 0.73 and hydration of 0.48 g/g), and the fraction of the total protein which it represents (in the best cases, 5–10% of the protein in the crude extract), it seems unlikely that the two purification schemes would isolate any major contaminating protein fraction. Gel electrophoresis bears this out. Therefore, Bellisario's preparation probably contained a high percentage of inactive protein.

Comparison of copper content and copper removal data indicates a possible explanation for the observed differences. Bellisario et al. (1972) reported that approximately 1 g-atom of EPR-detectable copper was found per 300 000 daltons but that this copper was removable with no loss of activity. Since peroxide oxidizes the copper in our preparations under some conditions and also inactivates the protein, it is likely that EPR-silent copper, which is not removable by dialysis vs. chelators, is characteristic of the active enzyme and that removable copper(II) is associated with inactive, oxidized luciferase. This conclusion is supported by the data of Table IV where specific activity correlates with copper content (unweighted linear correlation coefficient = 0.87). These data predict 3.7 g-atom of copper/mol for the highest specific activity (2100 units/mg) luciferase. These results must be interpreted with caution, however, since there is considerable uncertainty in the copper determinations and since other factors may influence specific activity. For example, while peroxide totally and irreversibly inhibits luciferase after prolonged exposure, in no case was the full copper EPR signal generated by peroxide treatment. Our purification procedures (Mulkerrin & Wampler, 1978) and storage procedures (Wampler et al., 1979) are designed specifically to protect luciferase from hydrogen peroxide by introducing catalase into the initial extraction buffer, carrying out dialysis procedures with a separate dialysis tube containing catalase suspended in the dialysis buffer, and storing luciferase in the dark in catalase-protected dialysis. In addition, Bellisario et al. (1972) purified luciferase from acetone powders of the coelomic fluid rather than frozen, separated coelomic cells. Since they did not use dithiothreitol to solubilize the slime formed during the initial extract, the first column step took considerably longer and therefore exposed luciferase to peroxide for a considerable length of time. We have verified that peroxide was indeed present under these conditions, since extracts of the acetone powders luminescence. It is therefore likely that the preparation of Bellisario et al. (1972) consisted mainly of inactive luciferase. This conclusion is supported by the physical data and the final corrected specific activities of the two preparations.

A second important difference between our data and that reported previously (Bellisario et al., 1972) is the demonstration of catalysis by *D. longa* luciferase with approximately 21 turnovers demonstrated, and a turnover number of 39 $\mu$mol of peroxide adduct $s^{-1}$ (mg of luciferase).$^{-1}$

The true substrate of the *D. longa* bioluminescence reaction appears to be 3-(isovalerylamino)-1-hydroxypropane hydroperoxide, the 1:1 adduct formed by addition of peroxide to the aldehyde group of luciferin in a reaction analogous to aldehyde hydration. Support for this proposal lies in the order of addition kinetics (Figure 3), the continuous variation experiment (Figure 4), and the experiments with catalase inhibition (Figure 5 and the results described above).

Together, these data lead us to postulate a reaction sequence which includes uncatalyzed reactions of luciferin with water and peroxide and catalysis by *D. longa* luciferase of the breakdown of the hydroperoxide compound. These reactions are listed as follows:




In addition, because of the stronge inhibition by metal binding agents, particularly copper chelators (KCN, *o*-phenanthroline, diethyl dithiocarbamate, 8-hydroxyquinoline, carbon monoxide, and oxygen) (Bellisario et al., 1972; Rudie, 1977), we postulate that the copper in luciferase has an active role in the catalysis. The reaction scheme above must be considered when the complex kinetic behavior of the in vitro bioluminescence reaction is analyzed (Wampler et al., 1979). Effects of salts and other solute species (Mulkerrin & Wampler, 1978; Wampler et al., 1979) must be interpreted both in terms of effects on luciferase and in terms of effects on the hydration and peroxide addition reactions.

Earthworm bioluminescence is more common than it was previously thought to be. Recent comparative studies (Jamieson & Wampler, 1979; Wampler & Jamieson, 1980) indicate that the mechanism of bioluminescence for over 13 species is very similar to that of *D. longa*. These comparisons include the only other species which have been studied biochemically, *Octochaetus multiporus* (Johnson et al., 1966; Wampler & Jamieson, 1980) and *Microscolex phosphoreus* (Skowron, 1926; J. E. Wampler, unpublished experiments). In all cases, either *D. longa* luciferase or luciferin or both act to stimulate the in vitro bioluminescence of extracts of the luminescent fluid of the various worms. In all cases, hydrogen peroxide also stimulates the bioluminescence of such extracts. It seems likely, therefore, that earthworm bioluminescence, in general, involves a similar reaction scheme. Further studies of the comparative biochemistry of earthworm luciferases and the products of the *D. longa* reaction are in progress in this laboratory.

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