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# Characterization and Properties of *Pholas* Luciferase as a Metalloglycoprotein†

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ABSTRACT: The luciferase of the bioluminescent boring mollusc, *Pholas dactylus,* has been purified by a new method which includes centrifugation in cesium chloride gradients. Homogeneous preparations have been obtained and molecular weight determinations and subunit analysis support the idea that this preparation is an oxyluciferin–luciferase complex. The preparation catalyzes oxidation of ascorbic acid in presence of $H_2O_2$, and this peroxidase activity has been used for characterization (thermal and pH stabilities, activity as a function of pH, isoelectric point, turnover number). The existence of two atoms of copper has been established and their involvement in the peroxidase activity indicated. Chemical analyses have shown that *Pholad* luciferase is a glycoprotein and the existence of glucosamine, fucose, mannose, and galactose residues has been demonstrated. The apparent buoyant density (1.340), the sedimentation coefficient (10.7 S), the Stoke's radius (83 Å), the partial specific volume (0.707), and the molecular weight (350,000) have been determined. The frictional ratio ($f/f_0$ = 1.8) derived from the Stoke's radius indicates that the molecule is asymmetric. The quaternary structure has been examined. Subunits of molecular weight 150,000 and 46,000 have been observed. The latter has electrophoretic properties identical with luciferin or oxyluciferin.

In many bioluminescent systems, it has been possible to characterize the enzyme (called luciferase, but different in each case) and the specific substrate (luciferin) which are responsible for the light emission in aerobic conditions. The luciferins are usually small molecules (mol wt <1000) which are oxidized to oxyluciferins which are often (in the excited state) the actual light emitters. In the boring mollusc *Pholas dactylus,* however, it has been shown that luciferin is itself a protein (mol wt ~45,000) with an oxidizable prosthetic group not yet identified (Henry et al., 1970, 1973). The *Pholas* system differs from most other bioluminescent systems in other important ways. Whereas in the absence of the corresponding luciferase, most luciferins can at best be induced to emit light under a very limited set of reaction conditions, *Pholas* luciferin is luminescent when treated with a striking variety of reagents (Henry and Michelson, 1970, 1973). With several of these reagents, it has been verified that the quantum yield and the spectral distribution of the emission thus obtained were not different from those of the enzymatic reaction. Moreover, the integrity of the protein is needed and in no case could light emission be induced in a tryptic digest of luciferin or from thermally denatured protein. We have also shown that the superoxide ion $O_2\cdot^-$ is a reactive intermediate common to the enzymatic as well as the nonenzymatic luminescence of *Pholas* (Henry and Michelson, 1973; Michelson and Isambert, 1973, Michelson, 1973a,b).

We have earlier described the limited activity of luciferase from *Pholas dactylus* as a peroxidase capable of oxidizing ascorbic acid in presence of $H_2O_2$ (Henry et al., 1973). In this respect luciferase shows a behavior very similar to that of horseradish peroxidase, one molecule of $H_2O_2$ oxidizing one molecule of ascorbic acid. Kinetic characteristics of this reaction have been described previously (Henry et al., 1973). The present communication describes further studies of this luciferase as a peroxidase and characterization of the enzyme as a metalloglycoprotein.

## Experimental Section

*Luciferase Assays.* Peroxidase Activity. The absorption at 265 nm of ascorbic acid was used to measure its oxidation by luciferase, in presence of hydrogen peroxide. The standard assay mixture contained 0.15 μmol of $H_2O_2$, 0.1

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Table I: Purification Procedure for *Pholas* Luciferase.

| | Volume (ml) | Protein Concn (mg/ml) | Total Protein (mg) | Activity (U/ml) | Total Activity[a] | Specific Activity (U/mg) |
|---|---|---|---|---|---|---|
| I Crude extract | 207 | 12.30 | 2550 | 32 | 6,620 | 2.6 |
| II Acid extract | 231 | 9.43 | 2180 | 37.6 | 8,690 | 4.0 |
| III DEAE-Sephadex | | | | | | |
| Peak A | 107 | 1.26 | 135 | 48 | 5,136 | 38.1 |
| Peak B | 58 | 1.86 | 108 | 184 | 10,670 | 99.0 |
| IV Ammonium sulfate fractionation (peak B) | | | | | | |
| 0–30% | 9.3 | 3.95 | 36.7 | 18.8 | 175 | 4.8 |
| 30–60% | 32 | 2.32 | 74.2 | 423 | 13,540 | 182.3 |
| Supernatant | 69.8 | 0.11 | 7.7 | 29.6[b] | 2,070 | 269.1 |
| V Cesium chloride gradient and Sephadex G-100 | 50.6 | 0.30 | 15.2 | 203 | 10,270 | 676.7 |

[a] The increase in total activity has been observed in all preparations. [b] This activity is displayed by nonpeptidic material and may be assigned to metallic cations.

μmol of ascorbic acid, 10 μmol of Tris-HCl (pH 8.5), and variable amounts of luciferase in a volume of 1.0 ml. The ascorbic acid and the hydrogen peroxide were mixed just before the assay and were obtained from 0.01 *M* stock solutions, in water for $H_2O_2$ and in 0.01 *M* Tris-HCl (pH 7.5) for ascorbic acid. Under these conditions, nonenzymatic oxidation of ascorbic acid was minimized.

As the linearity with respect to time was poor, due to the use of nonsaturating concentrations of ascorbic acid, the variation of absorption was recorded on a Cary 15 spectrophotometer and the initial velocity used as a measure of activity. Before addition of the enzyme, the nonenzymatic reaction was recorded and when necessary its rate was substracted from that obtained in presence of the enzyme. A unit of activity is defined as the amount of enzyme which induces a decrease of 0.1 OD/min at 25°. The assay is linear between 0.2 and 10 units.

Luciferase Activity. Oxidation of luciferin and measures of light emission were performed by injection of the enzyme in 1.0 ml of 0.1 *M* phosphate buffer (pH 7.0) containing 0.5 *M* NaCl into a cuvet placed in front of the phototube and containing given amounts of luciferin in 2.0 ml of the same buffer.

The active complex was assayed in the same way except that the luciferin in the cuvet was omitted.

*Apparatus for Measurement of Light Emission.* Light emission was characterized by maximal intensity ($I_{max}$) and by total light emitted ($L$), both of which were measured simultaneously with the instrument previously described (Henry et al., 1970; Henry and Michelson, 1973). The apparatus has been calibrated with the standard of Hastings and Weber (Hastings and Weber, 1963). This standard gives total light values that are about three times too high.

*Purification of Pholad Luciferase.* All operations were done at 4°. Acetone powder (10 g), obtained from the luminous organs of dissected animals, was homogenized with 0.01 *M* Tris-HCl buffer (pH 7.5) in a Polytron grinder (Kinematica GMBH, Lucerne) operating at full speed for 60 sec. The first homogenization was carried out with 150 ml of buffer and was followed by a second one with 80 ml of buffer. During the grinding, care was taken to avoid excessive heating. After each homogenization, the extract was centrifuged for 15 min at 28,000*g* in a refrigerated Sorvall centrifuge. The supernatants from the two homogenizations were pooled and assayed (crude extract; step I). The crude extract was adjusted to 0.1 *M* NaCl and to 0.05 *M* acetate with 2 *M* NaCl solution and 1 *M* acetate buffer (pH 4.8). The suspension was then centrifuged (15 min at 28,000*g*) and the white precipitate discarded. The supernatant was adjusted to pH 6.5 with 0.6 *N* $NH_4OH$ and is referred to as the acid extract (step II).

The acid extract was immediately poured onto a column (3 × 24 cm) of DEAE-Sephadex which had been previously equilibrated with 0.05 *M* Tris-HCl buffer (pH 7.5) containing 0.1 *M* NaCl. The column was washed with 200 ml of the same buffer and then a gradient of NaCl (from 0.1 to 0.6 *M*) was applied. Two peaks of activity were eluted, but only materil from the second (peak B), which was the major one, was submitted to further purification (step III).

Solid ammonium sulfate (210 g/l.) was added to the pooled fractions from peak B. The mixture was stirred and then centrifuged at 28,000*g* for 15 min. The pellet was discarded and to the supernatant solid ammonium sulfate (210 g/l.) was again added. After centrifugation, the pellet was resuspended in 0.05 *M* Tris-HCl buffer (pH 7.5) and dialyzed overnight against a large volume of the same buffer (step IV).

To the dialyzed solution, solid cesium chloride (400 g/l.) was added with magnetic stirring. The clear solution was then transferred into polycarbonate tubes and centrifuged for 45 hr at 50,000 rpm in a Type 65 fixed angle rotor in a Spinco L2-65 B ultracentrifuge. The content of the tubes was sucked off using a peristaltic pump and fractionated using a Mini Seive fraction collector. Fractions (0.2 ml) were collected and assayed for protein content and peroxidase activity after dilution (Figure 2). The active fractions were pooled and directly poured onto a column (3 × 50 cm) of Sepharose 6B equilibrated in 0.05 *M* Tris-HCl (pH 7.5) (Figure 3) and the column was eluted with the same buffer. Active fractions were pooled, concentrated with Diaflo PM-30 membranes, and kept frozen in small tubes at −20° (step V). Alternatively Sephadex G-100 was used instead of Sepharose 6B.

The results of a typical preparation are summarized in Table I.

*Protein Determination.* Various techniques were used depending on the purity of the sample. Protein content at steps I and II was determined by the biuret reaction using

Table II: Metal Content of Purified Luciferase Preparations.[a]

| Origin of the Sample | Treatment before Dialysis | Iron Content (g-atom/molecule) | Copper Content (g-atom/molecule) |
|---|---|---|---|
| Prep. $A_6$ (step IV + sucrose gradient) | Chelex column | | 2.39 |
| Prep. $A_8$ (step IV; peak A) | Dialyzed–mineralized | 0.86 | 2.28 |
| Prep. $A_8$ (step IV; peak B) | Dialyzed | 0.87 | 2.41 |
| Prep. $A_8$ (step IV; peak B) | Chelex column | 0.25 | 2.43 |
| Prep. $A_{10}$ (step V) | Chelex column | 0 | 1.98 |
| Prep. $A_{12}$ (step V) | Dialyzed | 0 | 1.99 |
| Prep. $A_{12}$ (step V) fraction from Sepharose column | Dialyzed | | |
| Tube 97 | | | 2.13 |
| 101 | | | 1.81 |
| 105 | | | 2.14 |
| 109 | | | 2.14 |
| 113 | | | 2.14 |
| 117 | | | 2.28 |
| Av | | | 2.18 ± 0.13 |

[a] The metal content was determined by atomic absorption measurements as described in the Experimental Section. The results of this table were computed assuming a molecular weight of 350,000 and $E_{1\%}$ (1 cm) 14.8. If the value of copper is taken precisely as 2 g-atoms/molecule this gives a value of $E_{1\%}$ (1 cm) equal to 13.6. Student's test ($p = 0.05 - n = 12$) was used for calculation of the average.

crystalline bovine serum albumin as standard. Steps III and IV were analyzed by measuring the absorbance at 280 and 260 nm (Layne, 1957). The protein concentration of highly purified preparations (step V) was determined from its absorbance at 280 nm, assuming $E_{1\%}$(1 cm) (280 nm) 13.6 (derived from determination of copper.) A value of 14.8 was derived from sugar and amino acid analyses.

*Determination of the Isoelectric Point of Luciferase by Electrofocalization.* A LKB 8101 electrofocusing column was used with ampholine giving a range of pH 3.5–10. The sucrose density gradient was formed by layering 24 solutions of decreasing sucrose concentrations. The sample (3 mg of protein) which had been previously dialyzed against 1% glycine solution, was introduced in layer 13. The column was cooled to 3° and a voltage of 300 V was applied for 40 hr, the cathode being at the top of the column. Fractions (3 ml) were then collected and assayed for activity.

A peak of activity was observed at pH 3.5, at the limit of the pH range.

*Metal Analyses.* Preparation of the Samples. About 1-ml samples of solutions containing 0.25–0.8 mg/ml of protein were either dialyzed extensively against large volumes of buffer or passed through a small column (0.9 × 17 cm) of Chelex. The resin (30 g) was prepared in the following way: it was first suspended in 500 ml of 0.5 *M* ammonia for 1 hr and then washed ten times with 500 ml of water on a Büchner funnel. It was then treated with 0.1 *N* HCl in the same way and finally equilibrated with 0.01 *M* Tris-HCl (pH 7.5). Spectra of the samples were recorded on a Cary 15 spectrophotometer, prior to the metal analyses.

Copper and Iron Determination. The samples were routinely assayed without mineralization, since mineralization with $H_2O_2$ and $H_2SO_4$ (Van de Bogart and Beinert, 1967) gave identical results. The metal contents were determined by atomic absorption spectrophotometry using an Instrumentation Laboratory Model 253 spectrophotometer. The apparatus was calibrated with copper and iron standard solutions (Titrisol, Merck). Chelex-treated buffer or dialysates were used as blanks.

In the calculation of the metal content of the enzyme, a molecular weight of 350,000 and a $E_{1\%}$(1 cm) (280 nm) of 13.6 (Table II excepted) were assumed.

*Amino Acid Analysis.* Amino acid analyses were performed on protein samples hydrolyzed for 16 hr at 110° with 6 *N* hydrochloric acid in tubes sealed in vacuo. Analyses were conducted either with a Unichrom amino acid analyzer or with a Technicon TSM analyzer modified for accelerated operations. The results were corrected for destruction of threonine by extrapolating to zero time the results obtained after 24, 36, and 48 hr of hydrolysis. Cysteine was determined as cysteic acid after oxidation with performic acid. The tryptophan content was obtained by the spectrophotometric method of Bencze and Schmid (Bencze and Schmid, 1957).

*Sugar Analysis.* Sugars were analyzed by gas–liquid chromatography of the trimethylsilyl derivatives of the *O*-methyl glucosides obtained by methanolysis of the protein (Chambers and Clamp, 1971). They were identified by comparison with known markers and estimated quantitatively using a standard of mannitol.

Sialic acid was also assayed by the colorimetric technique of Warren (Warren, 1959) and glucosamine by that of Elson (Elson and Morgan, 1963) and by the ninhydrin reaction on the Unichrom analyzer after a 4-hr hydrolysis at 100° with 3 *N* HCl.

*Gel Electrophoresis.* For disc gel electrophoresis the technique of Davis (Davis, 1964) was used, but generally with an acrylamide concentration of 5%. The proteins were stained with Coomassie Brilliant Blue.

The subunits were analyzed and their molecular weights determined by dodecyl sulfate polyacrylamide gel electrophoresis, using a 5% concentration of acrylamide (Weber and Osborn, 1969). The same molecular weight was obtained in 7.5% gels. Samples were prepared by boiling solutions of protein containing 1% dodecyl sulfate, 1% $\beta$-mercaptoethanol, and 8 *M* urea for a few minutes. Carboxymethylation of the samples was according to Craine (Craine et al., 1973). The following proteins were used as markers in the molecular weight determination: RNA polymerase, $\beta$ (mol wt 155,000) $\beta'$ (mol wt 160,000) $\sigma$ (mol wt 90,000), $\alpha$ (mol wt 39,000) subunits, bovine serum albumin (mol wt 67,000; 135,000; 202,000), yeast alcohol dehydrogenase (mol wt 37,000).

Glycoprotein staining following electrophoresis was done either on dodecyl sulfate gels or on ordinary gels. In the first case, the technique of Neville (Neville, 1971) was modified by performing the staining with Schiff's reagent at 25°. With ordinary gels the same technique was used but the first step, washing with 40% methanol (to remove dodecyl sulfate), was omitted. Alcohol dehydrogenase (yeast) was used a control and was not stained by these techniques.

*Molecular Weight Determinations.* Ultracentrifuge Studies. Sedimentation equilibrium studies were performed with a Beckman Spinco analytical ultracentrifuge equipped with an electronic speed control and thermistor assemblies for control of temperature. Interference optics were used and the double sector cell was filled to a column height of 3 mm. The experimental conditions maintained protein throughout the solution and gave a small but finite concen-

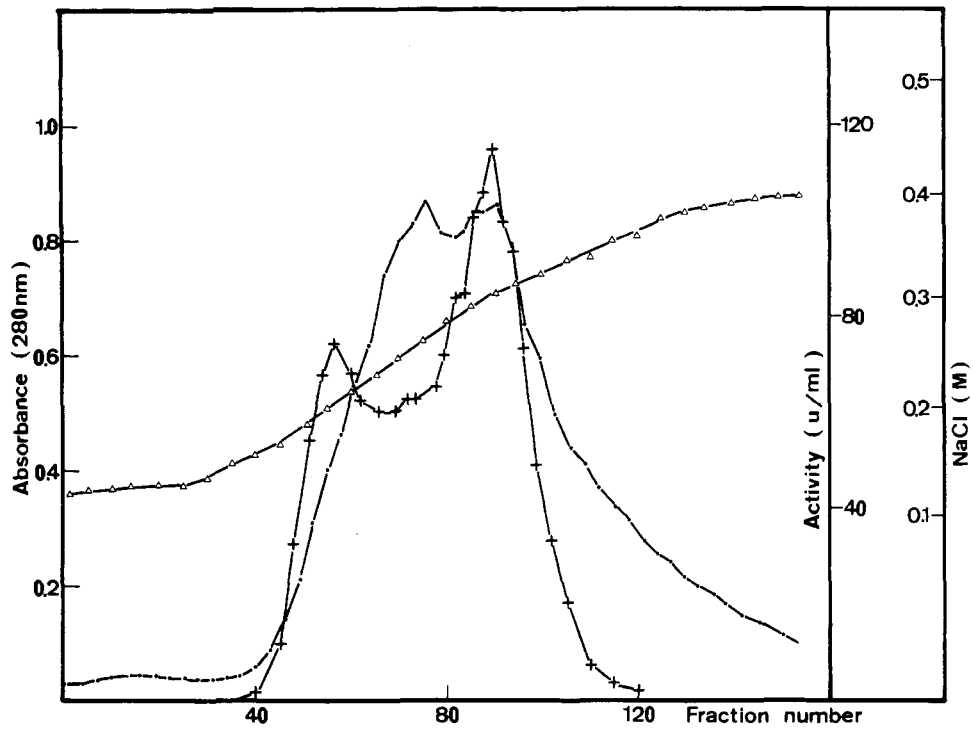


FIGURE 1: Chromatography on DEAE-Sephadex (A-50). The sample (53 mg of protein) in 0.05 *M* potassium phosphate (pH 7.0)–0.1 *M* NaCl was applied to a column (1.4 × 18 cm) of DEAE-Sephadex equlibrated with the same buffer. The column was washed with 50 ml of buffer and the luciferase eluted with a salt gradient formed from 90 ml of 0.1 *M* and 90 ml of 0.5 *M* NaCl in the phosphate buffer. Fractions (1.2 ml) were collected and assayed for peroxidase activity (+); absorbance at 280 nm (●) and NaCl concentration (△) were also measured. Oxyluciferin was not eluted with this salt concentration.

tration of protein at the meniscus at equilibrium. The buffer used was 0.05 *M* Tris-HCl (pH 7.5) with 0.1 *M* NaCl. The results were plotted as log of the protein concentration vs. the square of the distance from the center of rotation. The line obtained was the least-squares fit of experimental points. The protein was studied at several concentrations and at two rotor speeds.

In the calculation of molecular weight, a partial specific volume of 0.707 was used. This value was derived from the chemical composition (Cohn and Edsall, 1943). A $\bar{v}$ of 0.61 was assumed for all sugars.

Determination by Gel Filtration and Density Gradient Centrifugation (Siegel and Monty, 1966). The Stoke's radius was determined by filtration on Bio-Gel A 1.5m (200–400 mesh) in the buffer described for ultracentrifuge studies. Samples containing either one or a mixture of markers and 15% glycerol in a final volume of 0.2 ml were layered on the column (1.5 × 53 cm). Fractions (0.55 ml) were collected and assayed for enzymatic activities. The following markers were used: fibrinogen ($a$ = 107 Å), $\beta$-galactosidase (a = 69 Å), xanthine oxidase ($a$ = 55 Å), catalase ($a$ = 52 Å), yeast alcohol dehydrogenase ($a$ = 46 Å). Dextran Blue and $MnCl_2$ were used for the determination of the characteristics of the column. The Stoke's radius was calculated according to Ackers (Ackers, 1964) but a Porath's plot (Porath, 1963) gave the same result.

The sedimentation coefficient was determined by centrifugation on sucrose gradients (Martin and Ames, 1961). Samples in 0.1 *M* NaCl–0.05 *M* Tris-HCl (pH 7.5) were centrifuged for 10 hr at 40,000 rpm in the SW 65 rotor. Yeast alcohol dehydrogenase and $\beta$-galactosidase were used as markers.

## Results and Discussion

The purification of luciferase described in this article (Table I) is a considerable improvement on the earlier published methods (Henry et al., 1970, 1973; Michelson and Isambert, 1973). Chromatography on DEAE-Sephadex using activity either as luciferase to cause light emission from luciferin, or catalysis of the oxidation of ascorbic acid with $H_2O_2$, to locate the enzyme shows two peaks of activity (Figure 1). The results described are those obtained after complete purification of the second peak which under the conditions used is the major component. It appears that the first peak is free luciferase whereas the second peak as isolated in a final homogeneous state is a complex between luciferase and oxyluciferin. (This will be discussed later in more detail.) Final purification of this complex was achieved by preparative centrifugation in a cesium chloride gradient followed by filtration through Sepharose 6B (Figures 2 and 3).

The purified enzyme shows a single component in ultracentrifugation and a single band of protein in polyacrylamide (5 and 7.5%) gel electrophoresis. In addition specific activity is constant throughout the peak of protein on Sepharose chromatography. Purification was followed quantitatively using ascorbic acid and $H_2O_2$ as substrates since owing to the complex and variable kinetics of light emission specific activity as luciferase was not reliable (see Table I). It may be noted, however, that peroxidase and luciferase activities either on column chromatography or sucrose gradients were always associated. At the earlier stages of purification the ratio of the two activities was not constant and indeed a considerable difference was observed between the first and second peaks separated by chromatography on DEAE-Sephadex, the second peak showing a ratio of activities luciferase/peroxidase of 0.58 (using arbitrary units) compared with 1.21 for the first peak. Although addition of luciferin was strictly necessary for light emission from the first peak, at early stages light could be obtained from the

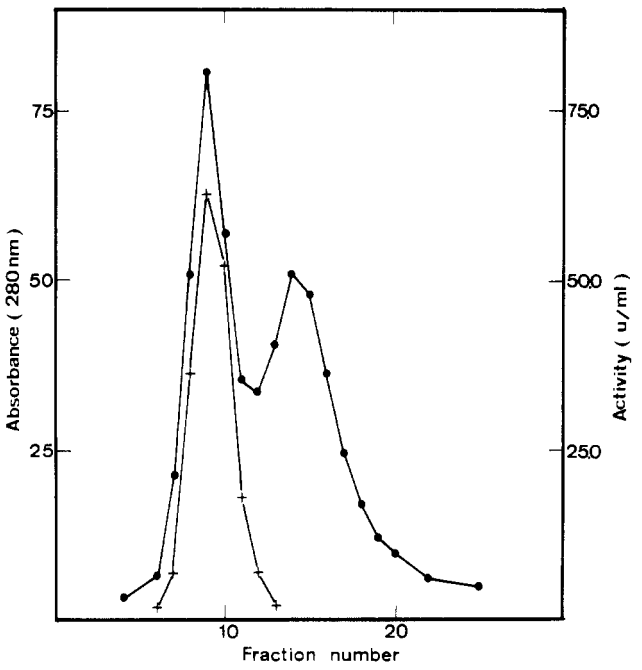


FIGURE 2: Centrifugation on a caesium chloride gradient. The centrifugation was conducted as described in the Experimental Section, under Purification of *Pholad* Luciferase. Each tube (9 ml) contained about 18 mg of proteins. The absorbance and the peroxidase activity were assayed after a 30-fold dilution. The second peak of protein corresponded to several proteins with electrophoretic properties similar to that of luciferase, as judged by polyacrylamide gel electrophoresis. Recentrifugation at 20° of the first peak with yeast alcohol dehydrogenase as a marker gave an apparent buoyant density of 1.340 for luciferase.

second peak not only on addition of luciferin but simply by dilution of the protein into buffer of the same pH and ionic strength. Since we have earlier described the formation of stable complexes between luciferase and luciferin at acid pH or under anaerobic conditions (Henry et al., 1970), this again suggested that the first peak was free luciferase whereas the second was a complex between luciferase and luciferin which during the course of further purification was converted into a luciferase–oxyluciferin complex. In confirmation of this, gel electrophoresis in sodium dodecyl sulfate of protein from the first peak gave a single subunit band of molecular weight 150,000 whereas the second peak gave two bands, one of 150,000 and the other 46,000 corresponding to luciferin or oxyluciferin, both of which possess quite different chromatographic properties from the enzyme and in free form are completely separated from both first and second peaks of activity. The extreme stability of the complex is shown by the fact that rechromatography on DEAE-Sephadex does not give rise to the first peak (which also shows unchanged characteristics on rechromatography).

*Characteristics of Luciferase.* The enzyme is relatively stable up to 45° but at higher temperatures denaturation becomes rapid. Activity was measured both by reaction with luciferin and by oxidation of ascorbic acid in presence of $H_2O_2$. Both techniques gave identical results with a $\Delta H^*$ of activation for denaturation of 55 kcal/mol (Figure 4).

Examination the pH stability (30 min at 0° at different pHs) showed that the enzyme was stable between pH 3 and 9. The activity as a function of pH is shown in Figure 5. Here it can be seen that two distinct pH optima exist, one at pH 5 and a second at pH 8 when activity is measured using $H_2O_2$ mediated oxidation of ascorbic acid. In contrast, as described previously, the luciferase activity measured by light emission due to oxidation of the luciferin shows only the second basic optimum of activity (Henry et al., 1970). This may well be a characteristic of the luciferase, but since the luciferin is itself a protein, it may also contribute to the profile of pH–activity. With respect to activity as an ascor-

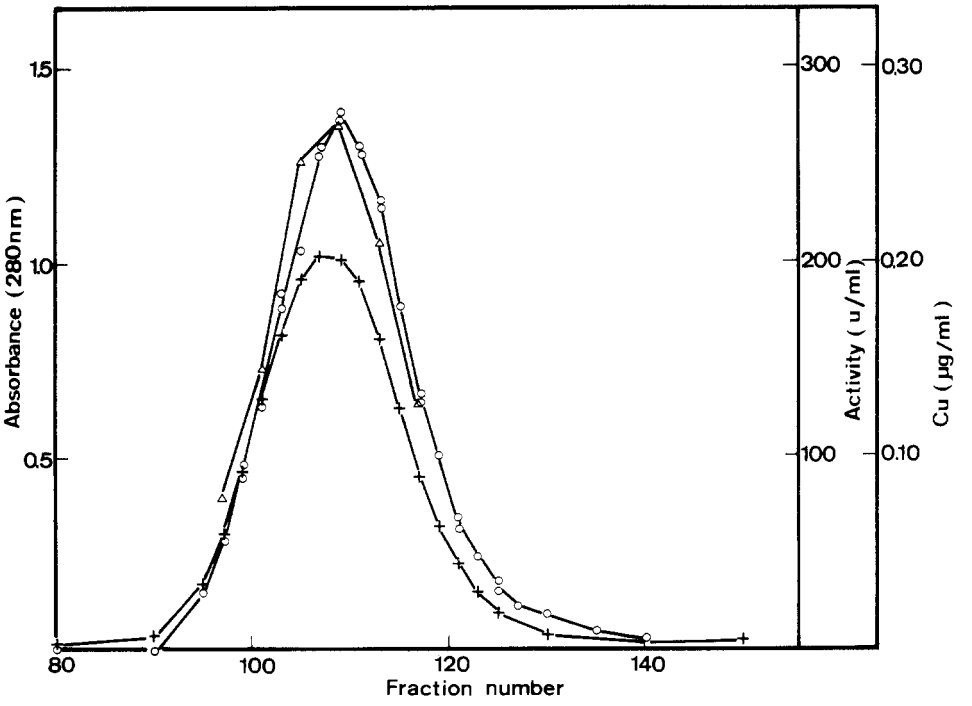


FIGURE 3: Filtration on Sepharose 6B. Luciferase (20 mg of proteins in 4.7 ml) purified by cesium chloride gradient centrifugation was applied to a column of Sepharose 6B (3 × 50 cm) equilibrated with 0.05 *M* Tris-HCl buffer (pH 7.5). The column was eluted with the same buffer and fractions (1.45 ml) were collected. The absorbance at 280 nm (+) was measured and the luciferase was assayed by its peroxidase activity (○). The copper content (△) was analyzed by atomic absorption spectrophotometry as described in the Experimental Section. Fractions from the void volume were used as blanks.

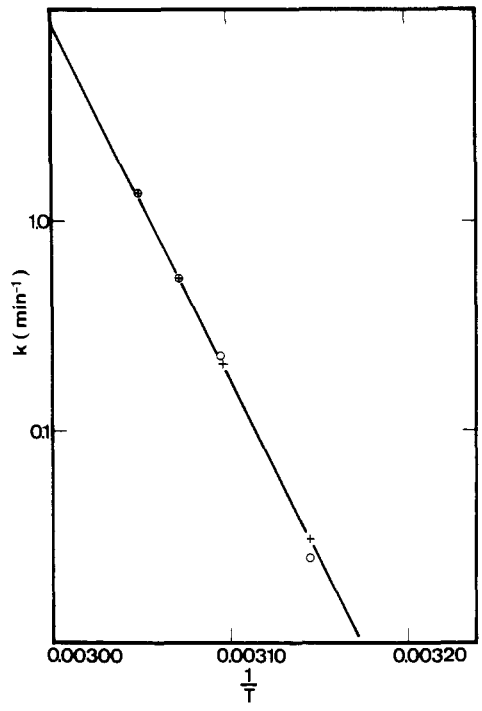


FIGURE 4: Thermal stability. Luciferase (specific activity 132 U/mg, 34 $\mu$g/ml) was incubated at various temperatures in 0.05 *M* potassium phosphate (pH 7.0). Aliquots (10 $\mu$l) were withdrawn and immediately assayed at 25°. Luciferase and peroxidase activities were assayed in two independent experiments. The results were plotted according to Arrhenius and a $\Delta H$ of 55 kcal/mol was derived (luciferase +; peroxidase O).

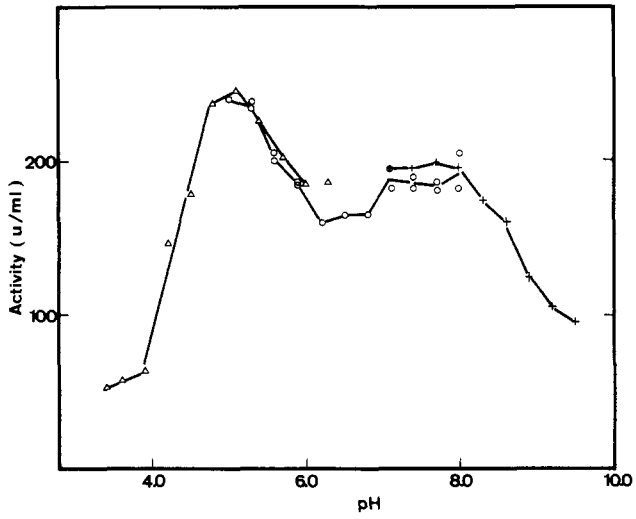


FIGURE 5: pH dependence of the peroxidase activity. Purified luciferase (specific activity 365 U/mg; 22 $\mu$g/ml) was assayed in 0.1 *M* acetate (Δ), potassium phosphate (O), or Tris-HCl (+) buffers. The enzymatic activity was obtained directly in the spectrophotometer using as blank the nonenzymatic same reaction mixture in the absence of enzyme.

bic acid peroxidase, it can be seen that the enzyme is functional even at pH 4.5–4. The isoelectric point is extremely acidic and is difficult to determine since the value is equal to or less than 3.5.

Experimental determination of the turnover number under optimal conditions for oxidation of the substrate gives a figure of 43,400 molecules of ascorbic acid oxidized per min per molecule of enzyme. This may be compared with

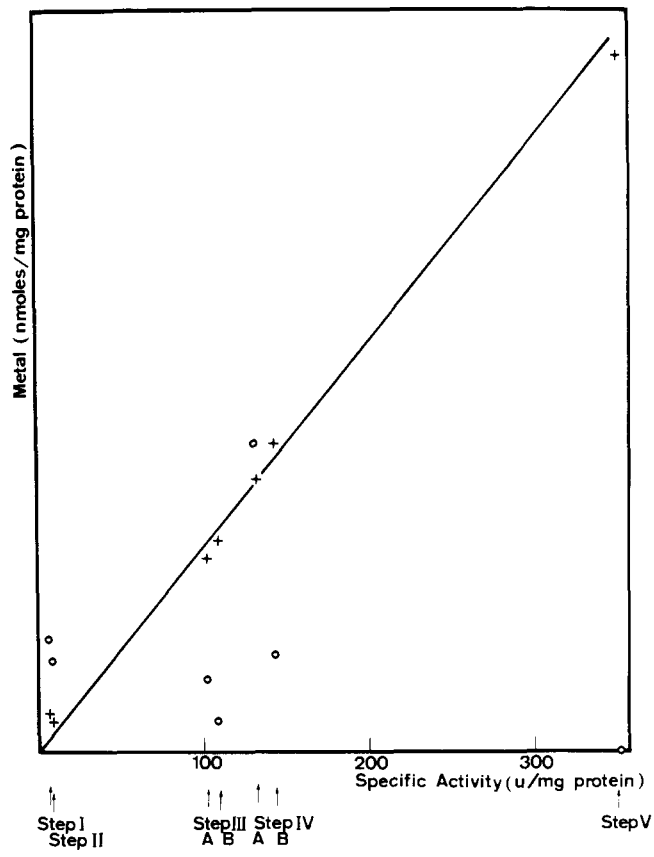


FIGURE 6: Relations between metal (copper and iron) contents and specific activity. The correlation existing between the copper content (+) and the peroxidase activity is shown at the various steps of purification described in the Experimental Section under Purification of *Pholad* Luciferase. Steps $III_A$ and $IV_A$ refer to purification of the A peak of the DEAE-Sephadex chromatography. The specific activities of step I and step II were calculated assuming a total activity constant and equal to sum of $III_A$ and $III_B$ total activites. The lack of correlation existing between iron content (O) and specific activity is also shown. The metals were analyzed by atomic absorption spectrophotometry. The samples were dialyzed against 0.01 *M* Tris-HCl buffer (pH 7.5) prior to the analysis and the dialysates used as blanks.

ascorbic acid oxidase for which the corresponding value (Mapson, 1961) is 246,000.

*Metals.* We have previously mentioned the presence of iron in *Pholad* luciferase (Michelson and Isambert, 1973). However, passage of the enzyme through a column of Chelex considerably diminished the iron content but had no effect on the activity of the enzyme, and indeed led to a slight increase whether tested as luciferase or as ascorbic acid peroxidase. In the case of reaction with luciferin, when all components (enzyme, luciferin, buffers) were pretreated with Chelex an increase in activity of about 25% was observed.

Since preparations of the enzyme with extremely low iron content were fully active we therefore regarded the presence of other metals. In fact all preparations contain copper (approximately 2 atoms of copper in the pure complex) which is not affected on treatment with Chelex. That this copper represents an integral part of the active site is indicated by the fact that correlation between copper content and specific activity at the various steps of purification is linear (Figure 6). Secondly, the amount of copper precisely follows activity when the enzyme is passed through a column of Sepharose (Figure 3). Finally, determination of copper in a number of preparations of pure enzyme invariably gives a value near 2 (see Table II) and zero iron. These are three

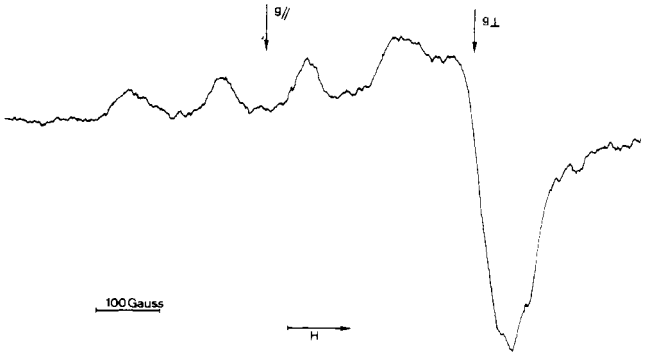


FIGURE 7: ESR spectrum of *Pholad* luciferase. The spectrum of the purified enzyme (17.4 mg/ml, 0.12 m*M* copper as determined from atomic absorption) was recorded at about 120°K on a Varian E9 spectrometer operating under the following conditions: microwave frequency, 9.312 GHz; microwave power 50 mW; modulation amplitude, 10 G; modulation frequency, 100 kHz. The following parameters were derived from the spectrum: $A_{\parallel} = 14.9$ mK, $g_{\parallel} = 2.295$, $g_{\perp} = 2.063$.

criteria cited by Vallée (Vallée, 1955) to define a metalloprotein.

The copper in luciferase is at least partially in cupric form as shown by the electron spin resonance spectra (Figure 7). However, no visible absorption in the region of 600 nm could be observed with the concentrations of enzyme available, indicating an $\epsilon$ value of less than 1000 in the visible.

*Action of Chelating Agents.* Enzymic activity (using oxidation of ascorbic acid as test) is inhibited by high concentrations of cyanide ion ($10^{-2}$ *M*) (Table III). However, this is not due to removal of copper from the protein since the inhibition (in presence or absence of ascorbic acid) is reversible (50%) either by dialysis or by precipitation with ammonium sulfate. Further increase in activity is not obtained by subsequent incubation with $10^{-6}$ *M* $Cu^{2+}$. At the present time we have not succeeded in obtaining an apoprotein by classical techniques.

Typical iron chelators such as 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid $\alpha,\alpha'$-dipyridyl, or 3,5-pyrocatechol disulfonic acid at concentrations up to $10^{-3}$ *M* in presence or absence of ascorbic acid or $H_2O_2$ had no effect. Inhibition was observed in presence of 8-hydroxyquinoline and iodohydroxyquinolinesulfonate both of which chelate ferric ion but also complex with copper. Dialysis did not significantly reverse this inhibition. Bathocupreine sulfonate (specific for cuprous copper) was without effect, even in the presence of ascorbic acid. However, strong (100%) inhibition was observed with sodium diethyldithiocarbamate at $5 \times 10^{-4}$ *M* which is known to be a reagent for cupric copper. Concentrations of $1.1 \times 10^{-5}$ *M* diethyldithiocarbamate caused 50% inhibition of $1.8 \times 10^{-6}$ *M* enzyme (Figure 8). This inhibition cannot be reversed and the diethyldithiocarbamate remains attached to the protein after dialysis, as shown by the absorption spectrum of the dialyzed complex. Inhibition is thus due to chelation rather than to removal of metal ion. Addition of free cupric ion followed by passage of the enzyme through Chelex gave rise only to 20% reactivation. These results suggest a cupric form of the native protein but do not present unambiguous evidence, particularly since specificity of chelating agents can differ between free metal ions and the same ion liganded in a protein.

It may be noted that very similar results (see Table III) have been reported by Cormier et al. for a copper containing luciferase from *Diplocardia* (Bellisario et al., 1972).

Table III: Action of Chelating Agents.[a]

| Chelating Agent | Concn (m*M*) | Activity after Incubation (%) | Activity after Dialysis (%) | *Diplocardia* Luciferase[d] |
|---|---|---|---|---|
| 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid (PDT disulfonate) | 0.4 | 107 (20 hr) | | |
| $\alpha,\alpha'$-Dipyridyl | 0.5 | 88 (1 hr) | 79 | |
| 3,5-Pyrocatechol disulfonic acid (Tiron) | 0.5 | 141 (18 hr) | | |
| Tiron + $H_2O_2$ (0.2 m*M*) | 0.5 | 105 (18 hr) | | |
| $H_2O_2$ | 0.2 | 79 (18 hr) | | |
| Bathocuproine sulfonate | 0.5 | 109 (1 hr) | | |
| Bathocuproine sulfonate + ascorbate (0.2 m*M*) | 0.2 | 110 (1 hr) | | |
| KCN | 10 | 6 (2.5 hr) | 56 (18 hr) | 0 |
| KCN + ascorbate (20 m*M*) | 20 | 5 (2.5 hr) | 63 (18 hr) | |
| 8-Hydroxyquinoline | 0.5 | 1 (3.5 hr) | 19 (21 hr) | 50 |
| 7-Iodo-8-hydroxyquinoline-5-sulfonic acid | 0.5 | 6 (3 hr) | 9[b] | |
| Diethyldithiocarbamate | 0.5 | 0 (1 hr) | 0[c] (15 hr) | 0 |

[a] Samples (0.3 mg of protein) were incubated at 0° with the compound tested in 0.5 ml of 0.05 *M* Tris-HCl (pH 7.5). Aliquots (0.025 ml) were withdrawn at various times and assayed in the normal manner in which the inhibitor is diluted 40-fold. When a variation was observed, the irreversible effect was checked by dialysis against 100 ml of Tris-buffer. In both types of experiments, the results are expressed as percent of a control sample treated in the same way. [b] The reagent was removed by Sephadex G-100 filtration. [c] After addition of 0.01 m*M* $CuSO_4$, 1-hr incubation, and removal of the excess copper on a Chelex column, a 20% recovery was observed. [d] Results from Bellisario et al. (1972). The enzyme was incubated for 30 min with 0.5 m*M* reagent.

*Chemical Studies.* The amino acid composition is presented in Table IV. The relative abundance of aspartic and glutamic acids may explain the acidic isoelectric point.

In addition to amino acids, *Pholas* luciferase contains sugar residues. Their existence was suspected because the luminous products are part of a secretion which is expelled by the animal. The mucous nature of this secretion has been shown by histochemical techniques (Bassot, 1966). Staining of luciferase with Schiff's reagent after periodate oxidation on polyacrylamide gels shows presence of a glucidic moiety. That luciferase is a glycoprotein is also shown by positive staining of the subunit (mol wt 150,000) obtained by dodecyl sulfate polyacrylamide gel electrophoresis using the same techniques. Additonal support is given by the apparent buoyant density of 1.340, determined by centrifugation at 20° in a cesium chloride gradient. Proteins generally give a lower value (e.g., yeast alcohol dehydrogenase, $d = 1.287$) and the observed difference allowed a very efficient purification step.

The glucidic moiety was then analyzed by various techniques and the results are presented in Table IV. Gas-liquid chromatography of the trimethylsilyl derivatives of the *O*-methyl glucosides provided identification and quantitative

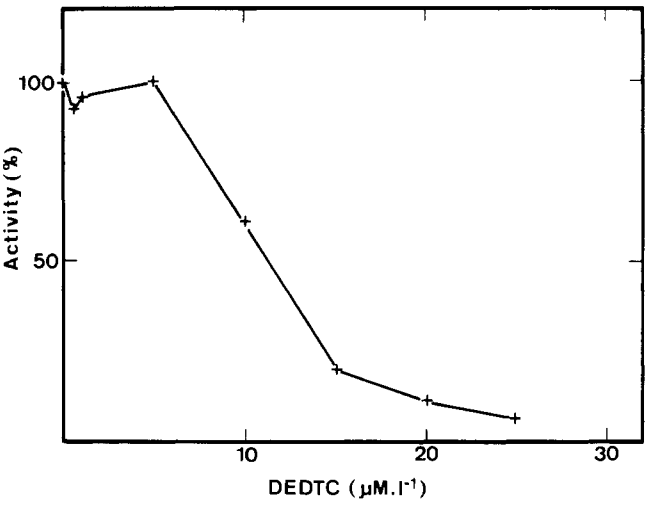


FIGURE 8: Effect of sodium diethyldithiocarbamate on the peroxidase activity. Purified luciferase (365 U/mg; $1.8 \times 10^{-6}$ *M*) was incubated for 15 min with various concentrations of the reagent in 0.05 Tris-HCl (pH 7.5) (final volume 0.075 ml) at 0°. The enzymatic activity was then assayed on 25-μl aliquots.

determination of the sugar components. Various specific colorimetric assays were also used to confirm the results. Glucosamine (and/or its *N*-acetyl derivative) was the only osamine detected whereas fucose, mannose, and galactose were the only neutral hexoses present in significative amounts. Traces of glucose were detected in some analyses but may have been impurities. *N*-Acetylneuraminic acid was not observed, a result which was confirmed by direct colorimetric assay for sialic acid and by the lack of effect of neuraminidase on activity.

*Molecular Weight Determinations.* The molecular weight was estimated from sedimentation equilibrium studies. When the log of the protein concentration was plotted against the square of the distance to the center of rotation, a straight line was obtained indicating a homogeneous preparation. The results obtained at several protein concentrations were plotted and extrapolated to zero concentration. A weight average molecular weight of 352,000 was calculated, assuming a partial specific volume of 0.707, derived from the chemical analysis.

The molecular weight was also derived from independent measurements of the sedimentation coefficient and Stoke's radius, where luciferase was detected by its peroxidasic activity. The sedimentation coefficient (10.7 S) was estimated by centrifugation on sucrose gradients and the Stoke's radius ($a$ = 83 Å) by filtration on a Bio-Gel A 1.5m column. The results when taken together gave a molecular weight of 344,000. The same preparation was used for this study and for the ultracentrifugation studies. The agreement which is observed between the two techniques is again indicative of a homogeneous preparation. The frictional ratio ($f/f_0$) was also determined from the Stoke's radius. A value of 1.8 was obtained, indicating qualitatively high asymmetry of the molecule.

As previous noted, purified preparations of luciferase analyzed by dodecyl sulfate polyacrylamide gel electrophoresis showed two bands unequally stained by Coomassie Blue with molecular weights respectively 150,000 (intense) and 46,000 (weak) which could not be changed by use of various reducing agents or by carboxymethylation. Since the light band, which was absent from preparations of free luciferase, had the electrophoretic properties of free luciferin (or oxyluciferin) these results suggest that the 350,000 molecule is a homogeneous 1:1 complex of luciferase and luciferin (oxyluciferin).

Table IV: Chemical Analyses of *Pholas* Luciferase.[a]

| Component | No. of Residues | Percentage of Total Weight |
|---|---|---|
| Amino acids | | |
| Aspartic acid | 369.5 | 12.2 |
| Threonine | 195.8 | 5.7 |
| Serine | 174.1 | 4.4 |
| Glutamic acid | 282.7 | 10.5 |
| Proline | 114.3 | 3.2 |
| Glycine | 270.3 | 4.4 |
| Alanine | 160.7 | 3.3 |
| Valine | 116.2 | 3.3 |
| Methionine | 85.2 | 3.2 |
| Isoleucine | 139.2 | 4.5 |
| Leucine | 183.0 | 6.0 |
| Tyrosine | 97.6 | 4.6 |
| Phenylalanine | 98.6 | 4.2 |
| Histidine | 53.4 | 2.1 |
| Lysine | 134.8 | 5.0 |
| Arginine | 160.7 | 7.2 |
| Cysteine | 70.1 | 2.1 |
| Tryptophan | 62.7 | 3.4 |
| Sugars | | |
| Glucosamine | 50.5 | 2.9 |
| Fucose | 96.2 | 4.0 |
| Mannose | 64.1 | 3.0 |
| Galactose | 26.1 | 1.4 |

[a] The results were calculated assuming a molecular weight of 350,000. The amino acid and sugar compositions are the average of 11 and 4 analyses, respectively.

## Conclusion

It is of considerable interest to compare the luciferase of *Pholas dactylus* with the only other copper containing luciferase as yet reported. Cormier and coworkers have described purification of luciferase from the earthworm *Diplocardia longa* (Bellisario et al., 1972; Bellisario and Cormier, 1971). This protein has a sedimentation coefficient of 7.3 S (*Pholas* enzyme 10.7 S). Sedimentation equilibrium techniques gave a weight average molecular weight of 320,000 for the *Diplocardia* enzyme (a partial specific volume of 0.73 was assumed) whereas the value for the *Pholas* enzyme was 350,000 using a partial specific volume of 0.707 calculated from the composition (this lower value reflects the presence of sugars in the protein). The Stoke's radius (using the same techniques) was respectively 95 Å (*Diplocardia*) and 83 Å (*Pholas*). That both proteins are highly asymmetric is shown by the calculated frictional ratios of 2.10 (*Diplocardia*) and 1.8, respectively. The two enzymes thus show considerable similarities. However, differences appear in the quaternary structure since *Pholas* luciferase appears to be composed of two subunits of 150,000 (plus 50,000 for the attached oxyluciferin molecule) whereas the *Diplocardia* enzyme is reported to contain three pairs of subunits of molecular weight 71,000, 58,000, and 14,500. A significant difference is also seen in the number of copper atoms per molecule in that *Diplocardia* luciferase contains only one atom of cupric copper. This is perhaps also related to the similarities and differences in the behavior of the two proteins with chelating agents (see Table III). It has also been established that the *Pholad* enzyme is a glycoprotein and perhaps differs in this respect from the *Diplocardia* enzyme.

Whereas the *Diplocardia* enzyme is a luciferin peroxidase with a strict requirement for $H_2O_2$ (indeed $O_2$ inhibits bioluminescence in vitro) the *Pholas* enzyme is an oxidase with respect to oxidation of the corresponding luciferin. However, oxidation of ascorbic acid by $H_2O_2$ is catalyzed by the latter enzyme and in fact compared with ascorbic acid oxidase, a comparable peroxidasic activity is shown. It would be of interest to determine whether the *Diplocardia* enzyme exhibits a similar activity with respect to ascorbic acid, even though it is known that other common substrates for peroxidases in general are not oxidized (which is also the case for the *Pholad* enzyme).

A reasonable mechanism for the oxidation of ascorbic acid by $H_2O_2$ catalyzed by *Pholas* luciferase involves reduction of a $Cu^{2+}$-enzyme by ascorbate to $Cu^{+}$, followed by reoxidation to $Cu^{2+}$ by $H_2O_2$. In contrast, the reaction as luciferase in the oxidation of luciferin with emission of light involves formation of $O_2\cdot^-$ as a critical step in mechanism. As we have earlier suggested (Henry et al., 1973; Henry and Michelson, 1973; Michelson and Isambert, 1973; Michelson and Henry, 1974) this could involve formation of a luciferin radical with reduction of the metal to $Cu^{+}$, followed by reaction of a cuprous enzyme with molecular oxygen to give $Cu^{2+}$ and $O_2\cdot^-$. Interaction of the two radicals then gives rise to a luciferin peroxide intermediate, the decomposition of which results in light emission.

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