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STUDIES ON THE BIOLUMINESCENCE OF *RENILLA RENIFORMIS*

XI. LOCATION OF THE SULFATE GROUP IN LUCIFERYL SULFATE*

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SUMMARY

1. Luciferin, in the coelenterate *Renilla reniformis*, is stored as a sulfate derivative which we have termed luciferyl sulfate. The chemical behavior of luciferyl sulfate, and its absorption characteristics in the infrared, suggest that the sulfate moiety is attached to luciferin *via* an acid sulfate linkage equivalent to that of ascorbic acid sulfate. This has been confirmed by the chemical synthesis of luciferyl sulfate from luciferin and sulfamic acid. The properties of the synthetic compound are identical to those of the natural one. In both cases luciferin sulfokinase and 3′,5′-diphosphoadenosine are required for light production, in addition to luciferase and O_2, and the kinetics of light emission are identical.

2. A simplified procedure for the isolation of luciferyl sulfate is also given which results in the extraction of essentially all of this compound from Renilla. This procedure has been used to isolate Renilla-like luciferyl sulfates from other coelenterates such as Cavernularia, suggesting that the method has general application.

INTRODUCTION

There are two enzymes involved in bioluminescence of the sea pansy, Renilla[1,2]. The reactions catalyzed by these enzymes are given in the following equations:

$$\text{Luciferyl sulfate} + \text{3′,5′-diphosphoadenosine} \underset{\longleftarrow}{\xrightarrow{\text{Luciferin sulfokinase}}} \text{luciferin} + \text{3′-phosphoadenosine-5′-phosphosulfate (PAPS)} \quad (1)$$

$$\text{Luciferin} + O_2 \xrightarrow{\text{Luciferase}} \text{light} + CO_2 + \text{product} \quad (2)$$

Eqn. 1 represents the 3′,5′-diphosphoadenosine-linked conversion of luciferyl sulfate to luciferin catalyzed by the enzyme luciferin sulfokinase (3′-phosphoadenylyl sulfate:luciferin sulfotransferase)[1]. In Eqn. 2 luciferase, at low concentrations, produces a blue structureless emission with a maximum at 20500 cm^{-1} (488 nm)[3].

Abbreviation: PAPS, 3′-phosphoadenosine-5′-phosphosulfate.

* Contribution from the University of Georgia Marine Institute, Sapelo Island, Ga., U.S.A.

In addition to light only two other products are produced, CO_2 and a blue fluorescent compound which represents the product excited state in the reaction[3,4].

Since luciferyl sulfate represents the storage form of luciferin in Renilla, its isolation and structural determination is of importance. We have previously reported on the structure of Renilla luciferin[4]. Here we present data which shows that the sulfate group in luciferyl sulfate is attached via an acid sulfate type linkage. This is confirmed by the chemical synthesis of luciferyl sulfate from luciferin. The isolation of luciferyl sulfate has been previously reported[5]. This procedure is rather involved, however, and the yields are lower than can be obtained by present methods. We, therefore, present a simplified procedure for the isolation of luciferyl sulfate resulting in the extraction of essentially all of this compound from Renilla. This procedure has been used to isolate Renilla-like luciferyl sulfates from other coelenterates such as Cavernularia, suggesting that the method has general application.

METHODS

Isolation of luciferyl sulfate

Methanol was made 0.01 mM in Tris–HCl buffer, pH 7.5 (Methanol–Tris). Healthy animals were added to containers two thirds filled with methanol–Tris until such containers were filled. After standing 5 min the mixture was processed through a meat grinder followed by homogenization at 3° in a Servall Omni-mix for 3 min at top speed. The homogenate was kept at —20° until further processing. The supernatant was used as the crude starting material for luciferyl sulfate isolation and contained about 50–75 % of the active material in the residue. The residue can be again extracted with methanol–Tris to recover the bulk of the remaining activity. In order to extract all of the luciferyl sulfate from the residue we found it convenient to pour the crude homogenate into a 10 cm × 90 cm glass column. After settling, the Renilla residue itself becomes the column bed and the methanol–Tris the eluant. After the first portion of eluant passed through the column an equivalent amount of fresh methanol–Tris was added and the residue eluted a second time. Essentially all of the luciferyl sulfate was found in the combined eluates.

Approximately 15 l of supernatant (representing about 4000 animals) were concentrated by flash evaporation at 38° until the methanol was removed leaving a cloudy aqueous solution representing about 1/5 the initial supernatant volume. This solution was extracted 4 times with equal volumes of ethyl acetate which was saturated with 1 % Na_2CO_3. The pooled ethyl acetate layers were flash evaporated at 38° resulting in a yellow oily residue. Ethanol (95 %) was made 1 mM with Tris–HCl buffer, pH 7.5 (ethanol–Tris) and the yellow oily residue was dissolved in 60 ml of this solution (Fraction I).

Water was added to Fraction I to make a 30 % solution (v/v). This solution was extracted two times with 1/3 of a volume of benzene. The ethanol–water layer was flash evaporated at 38° to dryness and the pale yellow residue was dissolved in 18 ml of ethanol–Tris (Fraction II). This material was stored at —20° and is stable indefinitely.

Fraction II was concentrated to dryness by flash evaporation at 38° and the residue dissolved in 5 ml of methanol–phosphate (80 % spectrograde methanol made 0.01 M with 1 M potassium phosphate buffer, pH 7.5). This solution was chroma-

tographed on a 4 cm × 20 cm LH-20 column that had been equilibrated with methanol–phosphate. The LH-20 was obtained from Sigma Chemical Company. Using methanol–phosphate as the solvent 65 ml fractions were collected at the rate of 20 ml/h. Essentially all of the contaminents preceed the elution of luciferyl sulfate on LH-20. The active material appears in Fractions 80–90 ± 5. These active fractions were combined, evaporated to dryness by flash evaporation at 38° and the residue dissolved in 2 ml of methanol-phosphate. This material was rechromatographed on a 2 cm × 18 cm LH-20 column as outlined above except that 2-ml fractions were collected at the rate of 8 ml/h. No loss of activity occurred during chromatography on LH-20.

At pH 7.5 luciferyl sulfate absorbs at 272 nm. The above column fractions were thus monitored by following absorbance at 272 nm.

Absorbance measurements

Ultraviolet and visible absorption measurements were made using a Cary model 14 recording spectrophotometer. Infrared measurements were made using a Beckman IR-10. KBr pellets were utilized and they were made from a micro pellet die.

Synthesis of luciferyl sulfate

The procedure used for the synthesis of luciferyl sulfate was a modification of one reported for the synthesis of sulfate esters of alcohols and phenols[10,11]. All operations were carried out under a He atmosphere. To 50 mg of sulfamic acid in a test tube 1 ml of anhydrous pyridine was added. The tube was placed in a dry ice–acetone bath and sealed. The sealed tube was heated at 110° for 1 h and allowed to cool. 0.5 ml of the supernatant from this tube was added to 50 μg of luciferin in a test tube which was placed in a dry ice–acetone bath and sealed. The sealed tube was heated at 110° for 30 min. The tube was cooled, opened and the pyridine removed under a stream of He. To the residue, 0.1 ml of a solvent was added which consisted of 80 % methanol containing 10 mM potassium phosphate, pH 7.5. To this solution 0.1 ml of a 1 % solution of ammonium acetate was added. The mixture was heated at 80° for 5 min. The solution was cooled and a second addition of 0.1 ml 80 % methanol–10 mM potassium phosphate was made. At this point the solution was checked for luciferyl sulfate activity. When such activity was confirmed the product was purified by column chromatography on LH-20 as described above.

RESULTS AND DISCUSSION

Isolation of luciferyl sulfate

Fig. 1 shows the results of the second chromatography of luciferyl sulfate on LH-20. Prior to fraction 44 the fractions showed no absorption in the ultraviolet or visible and no fluorescence. The biological activity of fractions 44–58 was also determined by measuring the initial rate of light production under assay conditions previously described[7]. In all such cases the concentration of luciferyl sulfate was adjusted such that it was on the linear portion of the saturation curve. Note that the biological activity is proportional to the absorbance at 272 nm. Thus the specific activity (luciferin activity per $A_{272\,nm}$) of these luciferyl sulfate fractions is constant across the peak and is taken as one index of purity. In addition the absorption

spectrum of each active fraction has the same shape and all show a peak at 272 nm. Furthermore the active material can be converted by mild acid treatment to luciferin as previously described[8] and the resulting luciferin appears to be very pure by high resolution mass analysis (K. Hori, M. J. Cormier and R. Lovins, unpublished data).

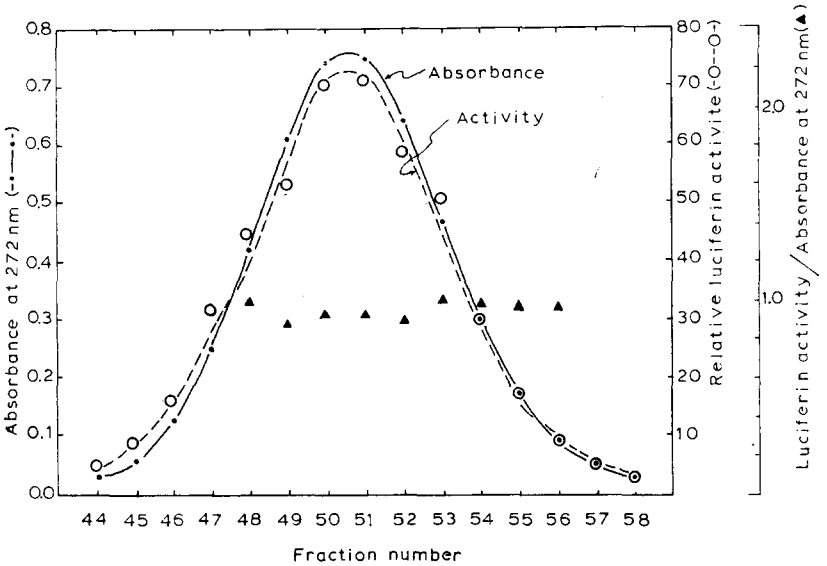

Fig. 1. An elution profile of luciferyl sulfate from LH-20. Luciferyl sulfate fractions were monitored by absorbancy at 272 nm and by light production in the presence of luciferase. See text for details.

Approximately 5 mg of luciferyl sulfate can be obtained from 40000 animals. The method given here is relatively simple and results in the extraction of essentially all of the detectable luciferyl sulfate from Renilla.

The method described here should be considered a general one for the isolation of luciferyl sulfate from those bioluminescent forms which contain structurally related luciferyl sulfates. For example luciferyl sulfate has been isolated by the above procedure from another coelenterate, *Cavernularia obesa*, which appears to be identical to the luciferyl sulfate isolated from the coelenterate Renilla. That is their chromatographic behavior on LH-20 (see METHODS) are identical as well as their absorption characteristics. Furthermore luciferyl sulfate isolated from Cavernularia will replace Renilla luciferyl sulfate as the substrate for light production in the presence of Renilla luciferyl sulfate, Renilla luciferase, 3′,5′-diphosphoadenosine, Ca^{2+}, and O_2 (ref. 13). Luciferase and luciferin sulfokinase obtained from Cavernularia will also produce light in the presence of Renilla luciferyl sulfate, 3′,5′-diphosphoadenosine, Ca^{2+}, and O_2. Thus the bioluminescent systems of these two coelenterates appear to be very similar in their biochemical requirements. Cavernularia has also been found to contain the characteristic "green *in vivo* emitter" recently described as being responsible for the *in vivo* light emission of *Renilla reniformis*[3]. It is of interest that the *in vivo* emission of other coelenterates have this characteristic green emission[15] suggesting possible similarities in the biochemical requirements for bioluminescence among the coelenterates.

Spectral characteristics of luciferyl sulfate

As shown in Fig. 2 the absorption spectrum of luciferyl sulfate at pH 7.5 shows a peak at 272 nm which shifts to 295 nm when the pH is changed to 12. These shifts are reversible. At pH 10 the compound shows a peak at 280 nm corresponding to that reported earlier[5].

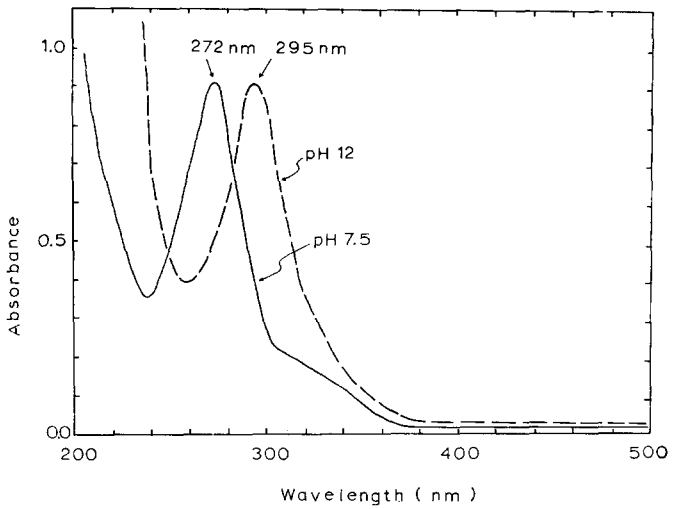

Fig. 2. Absorbance of luciferyl sulfate at pH values of 7.5 and 12.

The fluorescence of luciferyl sulfate in spectrograde methanol is very weak as reported previously[5]. Luciferin, however, exhibits a relatively intense fluorescence at 520 nm when excited at 410 nm.

The luciferyl sulfate isolated by the method described here is colorless at 1 mg/ml concentrations but becomes straw yellow at concentrations of 20 mg/ml. In confirmation of our earlier report[6], no amino acids are liberated by acid or alkaline hydrolysis.

Location of sulfate group in luciferyl sulfate

The structure of the major portion of Renilla luciferin has recently been reported[4] and this structure is shown as part of Fig. 3. R_1 is a 3-substituted indole whereas some uncertainties still remain regarding groups R_2 and R_3.

Fig. 3 gives the infrared spectra of luciferyl sulfate and luciferin. In the case of luciferin the absorption at 1630 cm^{-1} is assigned to an amide linkage present in the fused pyrrole ring. As expected this absorption is absent in luciferyl sulfate but new absorption bands appear at 1215 cm^{-1} and 1265 cm^{-1} which are assigned to an acid sulfate type linkage equivalent to such linkages found in indolyl-3-sulfate and ascorbic acid sulfate. By examining the infrared spectrum of indoxyl sulfate we find that it also exhibits absorption in the 1215 cm^{-1} and 1265 cm^{-1} regions of the spectrum. It has been reported that ascorbic acid sulfate has an absorption at 1260 cm^{-1} which has been assigned to the S=O vibration[12,14].

We have synthesized luciferyl sulfate from luciferin and sulfamic acid as given in METHODS. This method results in the synthesis of sulfate esters of alcoholic and

phenolic hydroxyls. The synthesized product is identical to the natural compound. For example their chromatographic behavior on LH-20 columns (see METHODS) are identical as is their absorption characteristics. In addition, requirements for light production with synthetic luciferyl sulfate are identical to those reported for the natural compound and the kinetics are the same[7]. In both cases luciferin sulfokinase

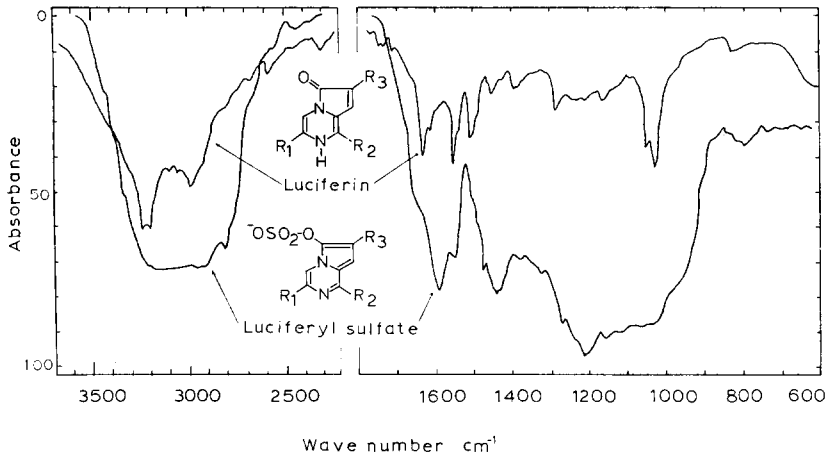

Fig. 3. Comparison of the infrared spectra of luciferin and luciferyl sulfate.

and 3′,5′-diphosphoadenosine are required for light production, in addition to luciferase and oxygen. It was previously shown that sulfate could be eliminated from luciferyl sulfate by mild acid treatment[9]. This is also the case for the synthetic compound. Treatment with mild acid converts both compounds to luciferin plus inorganic sulfate whereby light production occurs upon the addition of luciferase and O_2.

Thus the evidence presented above provides strong support for the existence of an acid sulfate type linkage in luciferyl sulfate as shown in Fig. 3. Such a linkage is also consistent with our earlier finding that luciferin sulfokinase catalyzes the conversion of luciferin to luciferyl sulfate in the presence of PAPS[1].

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REFERENCES

1 M. J. CORMIER, K. HORI AND Y. D. KARKHANIS, *Biochemistry*, 9 (1970) 1184.
2 Y. D. KARKHANIS AND M. J. CORMIER, *Biochemistry*, 10 (1971) 317.
3 J. E. WAMPLER, K. HORI, J. W. LEE AND M. J. CORMIER, *Biochemistry*, 10 (1971) 2903.

4 M. DeLuca, M. Dempsey, K. Hori, J. E. Wampler and M. J. Cormier, *Proc. Natl. Acad. Sci.*, U.S., 68 (1971) 1658.
5 K. Hori and M. J. Cormier, *Biochim. Biophys. Acta*, 102 (1965) 386.
6 K. Hori and M. J. Cormier, *Biochim. Biophys. Acta*, 130 (1966) 420.
7 M. J. Cormier, *J. Biol. Chem.*, 237 (1962) 2032.
8 M. J. Cormier and K. Hori, *Biochim. Biophys. Acta*, 88 (1964) 99.
9 M. J. Cormier, K. Hori and P. Kreiss, in F. H. Johnson and Y. Haneda, *Bioluminescence In Progress*, Princeton Univ. Press, Princeton, N.J., 1966 ,p. 360.
10 S. Yamaguchi, *Nippon Kagaku Zasshi*, 80 (1959) 171.
11 S. Yamaguchi, *Nippon Kagaku Zasshi*, 80 (1959) 204.
12 C. G. Mead and F. J. Finamore, *Biochemistry*, 8 (1969) 2652.
13 M. J. Cormier, J. M. Crane, and Yo Nakano, *Biochem. Biophys. Res. Commun.*, 29 (1967) 747.
14 S. F. D. Orr, *Biochim. Biophys. Acta*, 14 (1954) 173.
15 J. G. Morin and J. W. Hastings, *J. Cell. Physiol.*, 77 (1971) 313.