Renilla Luciferin as the Substrate for Calcium Induced Photoprotein Bioluminescence. Assignment of Luciferin Tautomers in Aequorin and Mnemiopsin†

Kazuo Hori, James Michael Anderson, William W. Ward, and Milton J. Cormier*

ABSTRACT: A study was made of the effects of pH and protic and aprotic solvents on the spectral properties of *Renilla* (sea pansy) luciferin and a number of its analogs. The results have made possible the assignment of two tautomeric forms of *Renilla* luciferin, one which absorbs maximally at 435 nm and another which exhibits an absorption maximum at 454 nm. Furthermore the results provide an explanation for the visible absorption characteristics of the photoproteins aequorin (λ_{max} 454 nm) and mnemiopsin (λ_{max} 435 nm). In addition a *Renilla*-like luciferin can be extracted from both of these photoproteins. This luciferin produces light with *Renilla* luciferase, at a rate dependent upon the concentration of dissolved oxygen, and in other respects is indistinguishable from *Renilla* luciferin in this bioluminescent reaction. The results suggest that the native chromophore in both photoproteins is *Renilla* luciferin (or a nearly identical derivative). The results also suggest that a hydroperoxide intermediate probably exists in photoproteins, on energetic grounds, and to account for the oxygen concentration independency of the rate of photoprotein reactions. This hydroperoxide may be attached initially to an amino acid side chain (possibly indolyl–OOH, imidazoyl–OOH, or –SOOH) rather than to the luciferin chromophore.

There is considerable evidence now which suggests that the chemical path to light emission is analogous, or perhaps identical, among two of the major classes of bioluminescent coelenterates, the Anthozoans and the Hydrozoans (Cormier et al., 1974). Light emission in the Anthozoans, including the sea pansy *Renilla reniformis*, occurs by an oxygen-dependent, luciferase-catalyzed oxidation of luciferin (Hori et al., 1973):

$$\text{luciferin} + O_2 + \text{luciferase(E)} \rightarrow \text{E-oxyluciferin}^* + CO_2 \quad (1)$$

$$\text{E-oxyluciferin}^* \rightarrow \text{E-oxyluciferin} + h\nu\ (490\ \text{nm}) \quad (2)$$

In *Renilla*, the elucidation of the structure and the chemical synthesis of a fully active analog of luciferin (I, Figure 1) has been achieved (Hori et al., 1973). This analog is identical with native *Renilla* luciferin except that the benzyl side chain at position 2 (see I, Figure 1) is replaced with a substituted phenolic side chain (K. Hori et al., unpublished results). For convenience of discussion this analog will be referred to as *Renilla* luciferin. The products of the light reaction, CO_2 and oxyluciferin (II, Figure 1), have also been determined (Hori et al., 1973; DeLuca et al., 1971).

In contrast to these oxygen-dependent luminescent reactions, the rate of light emission among the Hydrozoans is independent of the concentration of dissolved oxygen. This was first demonstrated in vitro by Shimomura et al. (1962, 1963) for the photoprotein aequorin and the reaction, based on recent data, can be illustrated (Shimomura et al., 1974):

$$\text{protein–chromophore} + Ca^{2+} \rightarrow \text{Ca–protein–oxyluciferin}^* + CO_2 \quad (3)$$

$$\text{Ca–protein–oxyluciferin}^* \rightarrow \text{Ca–protein–oxyluciferin} + h\nu\ (469\ \text{nm}) \quad (4)$$

The product, *Aequorea* oxyluciferin (III, Figure 1), is nearly identical in structure with the oxyluciferin product (II, Figure 1) of *Renilla* bioluminescence. Because of this and other similarities between the *Renilla* and *Aequorea* bioluminescence systems (Hori et al., 1973; Cormier et al., 1974) it was suggested that the structure of the protein-bound chromophore in aequorin (eq 3) was analogous to the structure of *Renilla* luciferin (I, Figure 1). Recent chemical evidence (Shimomura et al., 1974) supports the presence of such a chromophore in aequorin.

The properties of yet another photoprotein have recently been described by Ward and Seliger (1974a,b). This one, isolated from the ctenophore *Mnemiopsis*, and referred to as mnemiopsin (Girsch and Hastings, 1973), is also induced to bioluminescence in the presence of calcium ions and it too does not require the presence of dissolved oxygen. The spectral properties of mnemiopsin, before and after the calcium-triggered luminescent reaction, also suggest the presence of a *Renilla*-like oxyluciferin (II, Figure 1) as a bioluminescent product (Ward and Seliger, 1974b). In addition these bioluminescent ctenophores, as with *Aequorea*, have been found to contain the storage form of *Renilla* luciferin, i.e., *Renilla* luciferyl sulfate (Cormier et al., 1973).

In contrast to aequorin, which exhibits a visible absorbance near 460 nm (Shimomura et al., 1974), mnemiopsin has a visible absorbance near 435 nm (Ward and Seliger, 1974b). In both photoproteins, however, the calcium induced bioluminescence results in the disappearance of these visible absorption bands followed by the appearance of oxyluciferin products with an absorbance (λ_{max} 335 nm) similar to that observed for *Renilla* oxyluciferin (Hori et al.,

† From the Bioluminescence Laboratory, Department of Biochemistry, University of Georgia, Athens, Georgia 30602. *Received December 9, 1974.* This work was supported in part by the National Science Foundation and the U.S. Atomic Energy Commission. M.J.C. is a Career Development Awardee, No. 1-K3-6M-3331-10, of the U.S. Public Health Service. Contribution No. 000 from the University of Georgia Marine Institute, Sapelo Island, Georgia. This is No. XV of a series entitled "Studies on the Bioluminescence of *Renilla reniformis*."

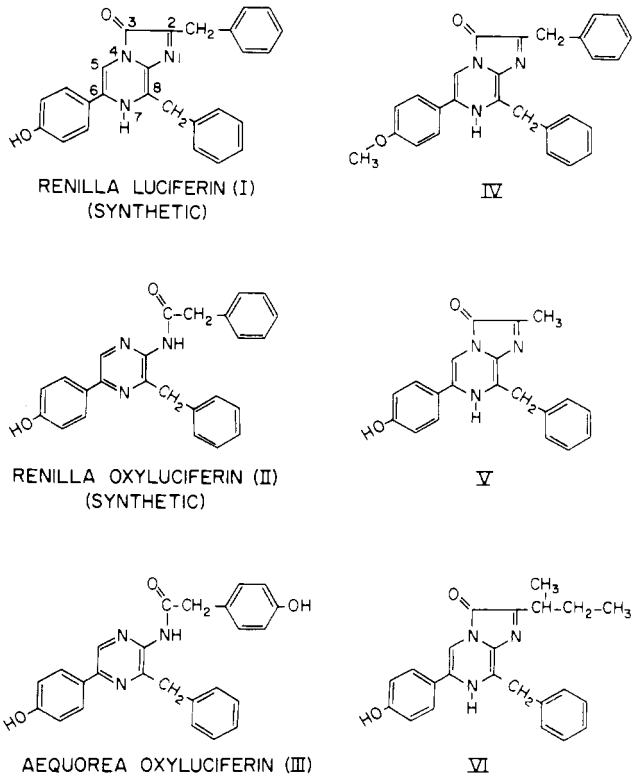


FIGURE 1: Structure of synthetic *Renilla* luciferin, *Renilla* and *Aequorea* oxyluciferins, and *Renilla* luciferin analogs.

1973; Shimomura and Johnson, 1969; Ward and Seliger, 1974b).

We have made a study of the spectral properties of *Renilla* luciferin (I, Figure 1) and certain model compounds the results of which provide an explanation for the visible absorption characteristics of both aequorin and mnemiopsin. We provide evidence that a *Renilla*-like luciferin is the chromophore responsible for these absorption properties. In addition, these studies provide some insight into the nature of the stabilized oxygenated intermediate which exist in these photoproteins.

Experimental Procedure

Materials. All solvents used for spectroscopy were of Spectrograde quality.

Methods. Renilla luciferin (I), luciferin analogs IV and V, and *Renilla* oxyluciferin (II) were synthesized as previously described (Hori et al., 1973). Model compounds VII and VIII were synthesized as described by Sugiura et al. (1970). Physical data (ultraviolet, infrared, nuclear magnetic resonance, and mass spectrum) on these model compounds were consistent with the structures shown.

All absorption measurements were performed under anaerobic conditions in order to prevent oxidation of luciferin and its analogs. These were done in an anaerobic cuvet, using solvents equilibrated with purified argon. Measurements were made with an on-line computer spectrophotometer system described by DeSa and Wampler (1973).

Luminescence measurements and quantum yields were determined as previously described (Hori et al., 1973).

Results and Discussion

Effects of pH on Renilla Luciferin Absorption. Varying the pH has pronounced effects on the absorption spectrum

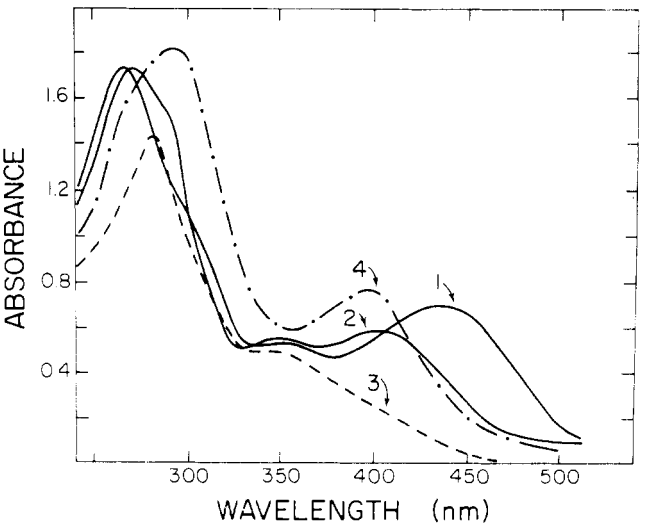
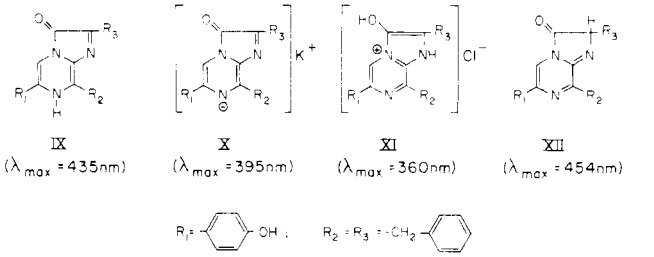


FIGURE 2: Absorption spectra of *Renilla* luciferin as a function of pH. Solutions were kept anaerobic with purified argon. Luciferin was dissolved in the following solvents: curve 1, 80% methanol–20% 5 m*M* citrate buffer (pH 5.0); curve 2, 80% methanol–20% 5 m*M* Tris buffer (pH 7.0); curve 3, methanol made 0.1 *N* with concentrated HCl; curve 4, KOH at pH 11. The pH given for curves 1 and 2 is assumed to be that of the buffers used.

of *Renilla* luciferin as illustrated in Figure 2. The 435-nm absorption band normally observed in methanolic solutions occurs near pH 5 (curve 1, Figure 2) rather than at pH 7 (curve 2, Figure 2) which had been the assumed pH of methanol (Hori and Cormier, 1973). A reversible blue shift, observed at high pH (curve 4, Figure 2), can be assigned to a deprotonation at position 7 (see I, Figure 1) as deduced from a study of model compounds first reported by Sugiura et al. (1970). We synthesized such model compounds (VII and VIII, Figure 3) and examined their absorption properties at neutral and alkaline pH as shown in Figure 3. Note that when position 7 contains methyl rather than hydrogen no absorption shift is observed at alkaline pH. Under strong acidic conditions, however, a blue shift (data not shown) occurs to about 390 nm for both model compounds (VII and VIII) as previously reported (Sugiura et al., 1970). An acid-induced blue shift is also seen in *Renilla* luciferin (curve 3, Figure 2) and this spectrum is similar to that observed for luciferyl sulfate (Hori et al., 1972). Studies with model compounds (Sugiura et al., 1970), with a structurally related luciferin from the marine crustacean *Cypridina* (Kishi et al., 1966a,b), and with *Renilla* luciferin and luciferyl sulfate (Hori et al., 1972), have made it possible to correlate the observed pH induced spectral shifts with certain tautomeric and ionic forms of luciferin. The predominant species near pH 5 can be assigned to IX while that near pH 11 and 1 can be assigned to X and XI, respectively. Based on data presented below structure XII does not make a significant contribution to the spectra shown in Figure 2 although it is an important and predominant species in



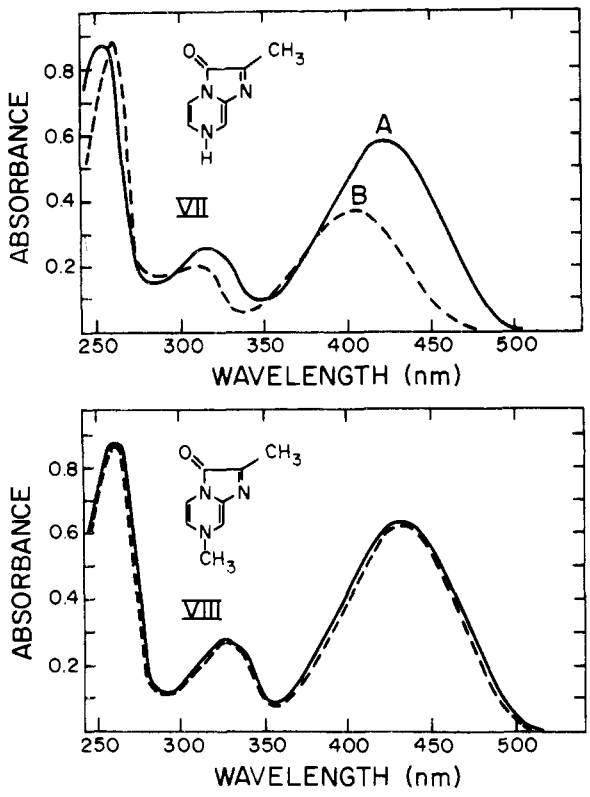


FIGURE 3: Effect of base on the absorption spectra of model luciferin compounds. Both compounds shown were dissolved in 3 ml of anaerobic methanol. Compound VII in methanol (curve A); 50 μl of 2 *N* KOH added (curve B). Compound VIII in methanol (—); 50 μl of 2 *N* KOH added (- - -).

aprotic solvent solutions. Whereas we recognize that the tautomeric forms listed above may be in equilibrium with other ionic forms of a given tautomer it is simpler for discussion purposes to represent them as shown.

Effect of Aprotic Solvents on the Absorption of Renilla Luciferin and Its Analogs. Dramatic changes in the visible absorption of *Renilla* luciferin are observed when one compares methanolic and aprotic solvent solutions of this compound as illustrated in Figure 4. In methanol (curve A) an absorption maximum is seen at 435 nm with a $\epsilon_{mM}(435)$ of 9.7 (Hori et al., 1973). In dimethylformamide, or Me_2SO[1] (data not shown), the visible absorption shifts to 454 nm with a $\epsilon_{mM}(454)$ of 3.7 (curve C). In diglyme (curve B) the visible absorption maximum also occurs at 454 nm but with a lowering of the $\epsilon_{mM}(454)$ to 2.2. These spectra were taken under anaerobic conditions and during the handling of the sample some autoxidation occurred resulting in the formation of *Renilla* etioluciferin (Hori et al., 1973). Thus we believe that this accounts for much of the absorption seen near 350 nm since *Renilla* etioluciferin exhibits an absorption maximum at 354 nm in aprotic solvents.

The effects seen in Figure 4 are not due to dielectric changes in the environment since the dielectric constants for methanol and dimethylforamide are not markedly different. Furthermore a fourfold dilution of a methanolic solution of luciferin with distilled water does not alter the shape of curve A (Figure 4). We suggest that these effects are due to interactions of luciferin with the solvents and additional data provided below can be interpreted in this way.

[1] Abbreviations used are: Me_2SO, dimethyl sulfoxide; HSEtOH, β-mercaptoethanol.

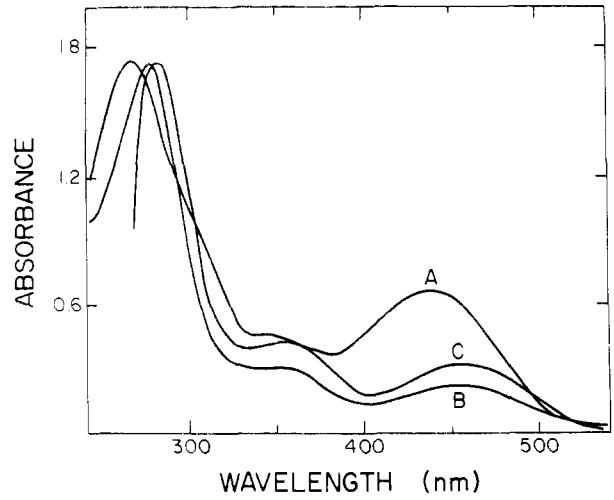


FIGURE 4: Effect of aprotic solvents on *Renilla* luciferin absorption. Anaerobic solutions of luciferin in methanol (curve A); in diglyme (curve B); in dimethylformamide (curve C).

Red shifts in aprotic solvents similar to those observed with *Renilla* luciferin also occur with luciferin analogs IV, V, and VI (see Figure 1). The luciferin analog VI does exhibit an interesting difference in the visible region of the spectrum. In methanol it exhibits a visible absorption maximum at 428 nm (data not shown) which shifts to 444 nm in diglyme (curve 1, Figure 5) and in each case the ϵ_{mM} is relatively high, i.e., near 8. As shown in Figure 5 (curves 2 and 3) a similar effect is observed for *Cypridina* luciferin whose structure is analogous to I (Figure 1). In the case of *Cypridina* luciferin substituents attached to the heterocyclic ring at positions 2, 6, and 8 are replaced with α-methylpropyl, β-indolyl, and γ-guanidinopropyl, respectively (Kishi et al., 1966a,c). The results in methanol (curve 3, Figure 5) are in agreement with Kishi et al., 1966a. In each solvent the ϵ_{mM} remains relatively high, being near 9 at 434 nm in methanol and near 7 in dimethylformamide or diglyme. This is in contrast to *Renilla* luciferin, as discussed above, which shows a $\epsilon_{mM}(435)$ in methanol of 9.7 but a $\epsilon_{mM}(454)$ in diglyme of only 2.2. When position 2 of *Renilla* luciferin is substituted with methyl (as in V) or by benzyl (as in I) the $\epsilon_{mM}(454)$ is near 2.2 in diglyme, but when position 2 is substituted by α-methylpropyl (as in VI) the $\epsilon_{mM}(444)$ is increased about three times. The reason for this is unknown.

As illustrated in Figure 6 the model compounds VII and VIII (Figure 3) also exhibit substantial red shifts when dissolved in aprotic solvents as opposed to methanol (see Figure 3). Studies on the spectroscopy of *Renilla* and *Cypridina* luciferins and model compounds, as outlined above, have allowed the assignment of IX, X, and XI as the major ionic and tautomeric forms of *Renilla* luciferin as a function of pH. The exception is XII which we suggest is the predominant tautomer occurring when luciferin is dissolved in aprotic solvents. We believe that such solvents stabilize XII via solvent interaction (hydrogen bonding, etc.) with luciferin. This change in tautomeric forms, which occurs in going from a methanolic to an aprotic environment, can be illustrated with the model compounds VII and VIII as shown in Figure 7. This suggestion is consistent with the observations that *Renilla* luciferin, *Cypridina* luciferin, or various model compounds such as VII are relatively nonchemiluminescent when dissolved in methanol but become chemiluminescent when base catalyzed in aprotic solvents. This is presumably due to the reactivity of the carbanion of tautomer XII, but

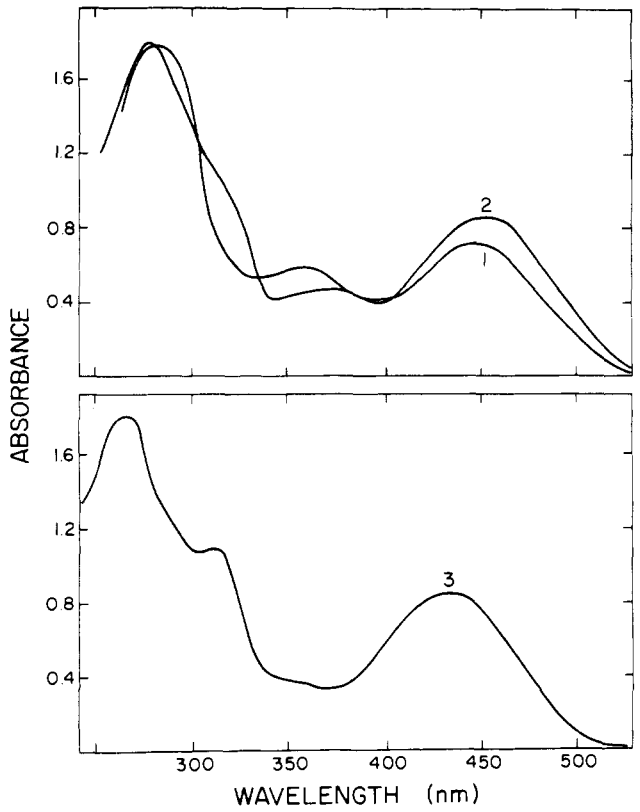


FIGURE 5: Effect of aprotic solvents on *Cypridina* luciferin absorption and an analogous *Renilla* luciferin analog. Anaerobic solutions of VI (see Figure 1) in diglyme (curve 1), of *Cypridina* luciferin in diglyme (curve 2), and of *Cypridina* luciferin in methanol (curve 3).

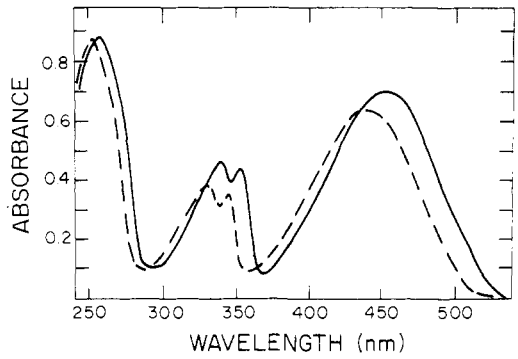
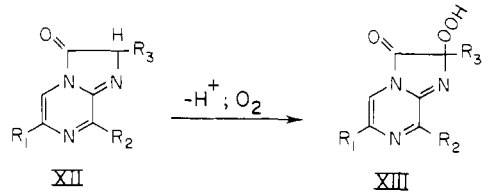


FIGURE 6: Effect of aprotic solvents on luciferin model compounds. Anaerobic solutions of VII (- - -) and VIII (—) in diglyme.

not the anion of IX, with dissolved oxygen to yield the hydroperoxide intermediate (XIII). The formation of XIII as



XII XIII

an intermediate prior to chemi- or bioluminescence is consistent with modern theories and has been discussed in recent reviews (McCapra, 1973; Cormier et al., 1973, 1975).

Observations and Comments on the Nature of Photoproteins. As illustrated in Figure 8, aequorin[2] exhibits an absorption in the visible near 454 nm (curve 1 and inset curve 1) which is similar to that observed for *Renilla* luciferin in diglyme (curve 2 and inset curve 2). Furthermore the observed $\epsilon_{mM}(454)$ of 2.2 for *Renilla* luciferin in diglyme is a close match for the $\epsilon_{mM}(460)$ of 2.4 for aequorin reported by Shimomura et al. (1974). These observations overcome the objection by Shimomura et al. (1974) that a *Renilla*-like luciferin could not account for the visible absorption of aequorin. Their conclusions have been based primarily on the effects of pH on the spectroscopy of model compounds and on *Cypridina* luciferin which we have shown to behave atypically. Their observations have further led them to suggest the presence of a second chromophore in aequorin, termed "yellow compound", to account for the visible absorption observed near 454 nm. They extracted a yellowish compound from aequorin with diethyl ether after pretreatment with $NaHSO_3$. We noted that many of the physical and chemical properties which Shimomura et al. (1974)

[2] The spectrum in Figure 8 (curve 1) agrees in general with the one published by Shimomura and Johnson (1969).

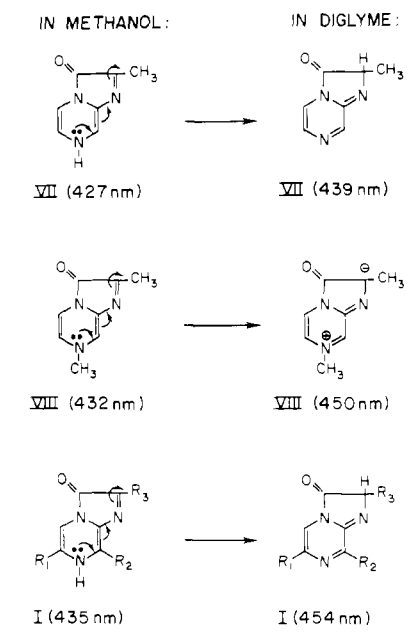


FIGURE 7: Proposed tautomeric or ionic changes in *Renilla* luciferin and model compounds induced by aprotic solvents.

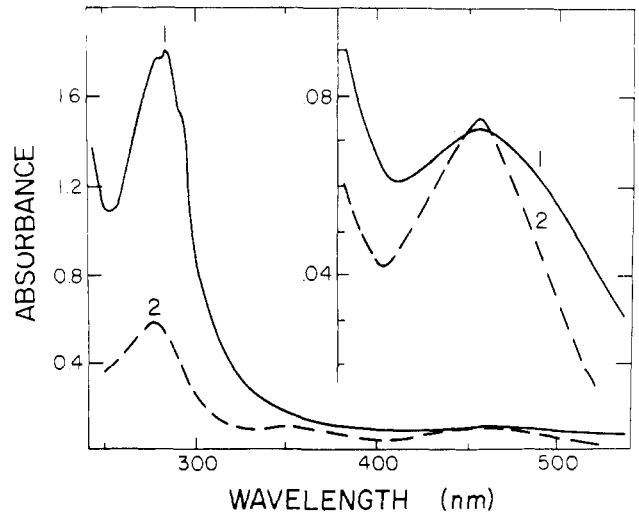


FIGURE 8: Absorption spectra of the photoprotein aequorin (curve 1 and inset curve 1) compared to that of *Renilla* luciferin in diglyme (curve 2 and inset curve 2).

Table I: Extraction of *Renilla*-Like Luciferin from Aequorin and Mnemiopsin.

Experiment	Calcium Dependent Reaction		*Renilla* Luciferase Dependent Reaction		Percent of Photoprotein Chromophore Released as Free Luciferin[d]
	Total Photon Yield[a] (einsteins)	Moles of Chromophore Reacted[b]	Total Photon Yield (einsteins)	Moles of Chromophore Reacted[c]	
A. Native aequorin	5.0×10^{-12}	1.7×10^{-11}	$<2 \times 10^{-18}$	$<4 \times 10^{-17}$	0
B. Aequorin + $NaHSO_3$ + methanol[e]	$<2 \times 10^{-18}$	$<6 \times 10^{-18}$	2.2×10^{-14}	6.0×10^{-13}	3.5
C. B + urea + HSEtOH[f]	$<2 \times 10^{-18}$	$<6 \times 10^{-18}$	1.1×10^{-13}	3.7×10^{-12}	22
D. Native mnemiopsin	4.2×10^{-13}	2.1×10^{-12}	1.5×10^{-16}	3.7×10^{-15}	0.18
E. Mnemiopsin + $NaHSO_3$ + methanol	$<2 \times 10^{-18}$	$<8 \times 10^{-18}$	2.8×10^{-16}	7.6×10^{-15}	0.36
F. E + urea + HSEtOH	$<2 \times 10^{-18}$	$<8 \times 10^{-18}$	7.3×10^{-16}	2.5×10^{-14}	1.2

[a] Aequorin assays were performed with 0.05 *M* calcium acetate (pH 6.0) at 4°. Mnemiopsin assays were performed using 0.10 *M* $CaCl_2$–0.20 *M* Tris (pH 8.5) at 4°. [b] Corrections were made for the 0.29 quantum yield of aequorin (Shimomura and Johnson, 1970) and the quantum yield of mnemiopsin (Ward and Seliger, in preparation). [c] Corrections were made for the 0.04 quantum yield of *Renilla* luciferin (Hori et al., 1973) and for small losses due to autoxidation of luciferin as observed in control experiments. [d] Tabulated percentages were calculated as (moles of chromophore reacted)/(1.7×10^{-11}) for aequorin and (moles of chromophore reacted)/(2.1×10^{-12}) for mnemiopsin. [e] Sodium bisulfite incubations were performed anaerobically as described by Shimomura et al. (1974). After 30-min bisulfite treatment, three volumes of anaerobic methanol were added. [f] Nine volumes of anaerobic 8 *M* urea containing 10^{-2} *M* HSEtOH were added to the methanolic solution and the mixture was incubated anaerobically at room temperature for 3 hr.

listed for "yellow compound" are consistent with those of *Renilla* luciferin. Furthermore, by pretreating aequorin, under conditions used for extracting the "yellow compound" (Shimomura et al., 1974), we are able to obtain a *Renilla*-like luciferin. Similar treatment of the photoprotein mnemiopsin also yielded a *Renilla*-like luciferin. These results are summarized in Table I. Under conditions listed in Table I both aequorin and mnemopsin yield a compound which reacts with *Renilla* luciferase to produce light. This luciferase-catalyzed light emission is dependent on dissolved oxygen and independent of calcium ion. The kinetics of light emission (data not shown) are similar to that observed when the treated photoprotein extract is replaced with *Renilla* luciferin. In addition, the color of the light produced (λ_{max} 490 nm) is the same when either *Renilla* luciferin or the treated photoprotein extract is used as a substrate for *Renilla* luciferase. Since both aequorin and mnemiopsin have been purified to homogeneity (Shimomura and Johnson, 1969; Ward and Seliger, 1974a) and since no luciferin-like activity appears prior to the treatment of aequorin with $NaHSO_3$ it seems that this treatment makes it possible to extract native luciferin from this photoprotein. As shown in Table I the recovery of luciferin from these photoproteins depends on the method of treatment. Treatment of aequorin with $NaHSO_3$ followed by methanol releases 3.6% based on total light measurements. Further treatment of this mixture with 8 *M* urea, made 10 m*M* in β-mercaptoethanol, resulted in the recovery of 22% of the luciferin chromophore. Recovery of luciferin from mnemiopsin was on the order of 1%. In the case of aequorin the luciferin recovery values are sufficiently high so as to leave little doubt that the native chromophore in photoproteins can be extracted and equated with *Renilla* luciferin.[3]

On the basis of the above results, we suggest that the "yellow compound" extracted from aequorin by Shimomura et al. (1974) is not a "new" chromophore but is in fact the same chromophore responsible for the 454-nm absorption band in native aequorin and that it is closely related in structure to native *Renilla* luciferin.

Furthermore, proposing a second chromophore in aequorin to account for its 454-nm absorption does not explain the 435-nm absorption of mnemiopsin (Ward and Seliger, 1974b). Because of the spectral observations on *Renilla* luciferin reported here, coupled with its extraction from both aequorin and mnemiopsin, we suggest a simpler explanation, than the one reported by Shimomura et al. (1974), to account for the visible absorption characteristics of both photoproteins. We propose that the native chromophore in both mnemiopsin and aequorin is native *Renilla* luciferin (or a nearly identical derivative). We further suggest that mnemiopsin provides a methanol-like environment which favors luciferin tautomer IX while aequorin provides a diglyme-like environment which favors tautomer XII.

An interesting prediction emerges from the above suggestion. That is, the oxygen independency of photoprotein luminescent reactions cannot be accounted for in *both* mnemiopsin and aequorin, by assuming the existence of the hydroperoxide intermediate XIII. This is due to the fact that such a hydroperoxide cannot be accommodated by luciferin tautomer IX which apparently exist in mnemiopsin. The extraction from aequorin of a *Renilla*-like luciferin, the oxygen dependency of its luminescent oxidation by *Renilla* luciferase, and the fact that this extracted luciferin is stable for at least 3 days when dissolved in anaerobic methanol rules out the possibility that the extracted chromophore could be equivalent to intermediate XIII. We suggest that a hydroperoxide intermediate must exist in photoproteins, on energetic grounds, and to account for the oxygen independency of these reactions, but that it be attached to a site other than the luciferin chromophore. It would be interesting if this hydroperoxide were found to be attached initially to an amino acid side chain (possibly indolyl-OOH, imidazoyl-OOH, or -SOOH). A similar suggestion of a luciferase-hydroperoxide intermediate has been made by Lee and Murphy (1973) and Murphy et al. (1974) to account for the lack of a flavine hydroperoxide intermediate in bacterial bioluminescence.

[3] Since the completion of this manuscript, extraction procedures have been improved such that luciferin yields of 45, 98, and 85% have been obtained from the photoproteins aequorin, mnemiopsin, and berovin, respectively.

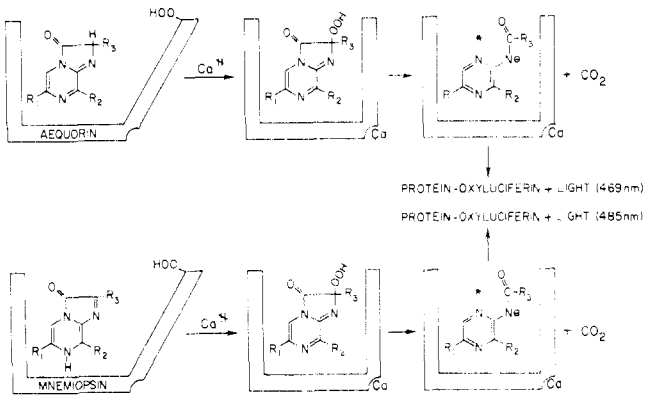


FIGURE 9: Hypothetical scheme for the calcium induced bioluminescent reactions of the photoproteins aequorin and mnemiopsin.

On the basis of the above observations and considerations we suggest a simplified scheme for the calcium-induced photoprotein bioluminescent reactions as illustrated in Figure 9. In this scheme the binding of calcium ion to the photoproteins brings about a conformational change in the protein which results in the *Renilla*-like luciferin chromophores of mnemiopsin and aequorin, respectively, reacting with a protein side chain hydroperoxide to yield a protein bound luciferin hydroperoxide intermediate. This intermediate then decomposes to CO_2 and an electronic excited state of the corresponding protein-oxyluciferin monoanion complex which leads to light emission. The formation of the oxyluciferin monoanion excited state would be analogous to the well-described luminescent reaction in *Renilla* (Hori et al., 1973). In addition, the fluorescence emission of this monoanion in dimethylformamide or diglyme (λ_{max} 470 nm) is similar to the observed bioluminescence emission of aequorin (Morise et al., 1974).

Acknowledgment

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