Blumberg, S., Schechter, I., and Berger, A. (1970), *Eur. J. Biochem. 15*, 97.
Brocklehurst, K., and Kierstan, M. P. J. (1973), *Nature (London), New Biol. 242*, 167.
Brubacher, L. J., and Bender, M. L. (1966), *J. Amer. Chem. Soc. 88*, 5871.
Citri, N. (1973), *Advan. Enzymol. Relat. Areas Mol. Biol. 37*, 397.
Drenth, J., Jansonius, J. N., Koekoek, R., Swen, H. M., and Wolthers, B. G. (1968), *Nature (London) 218*, 929.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.
Faller, L., and Strutevant, J. M. (1966), *J. Biol. Chem. 241*, 4825.
Fersht, A. R. (1972), *J. Mol. Biol. 64*, 497.
Fink, A. L., and Bender, M. L. (1969), *Biochemistry 8*, 5109.
Glazer, A. N. (1966), *J. Biol. Chem. 241*, 3811.
Glazer, A. N., and Smith, E. L. (1971), *Enzymes, 3rd Ed., 3*, 501.
Greenstein, J. P., and Winitz, M. (1961), Chemistry of the Amino Acids, Vol. 2, New York, N. Y., Wiley, p 926.
Hammes, G. G. (1968), *Advan. Protein Chem. 23*, 1.
Herskovits, T. T. (1967), *Methods Enzymol. 11*, 748.
Hinkle, P. M., and Kirsch, J. F. (1970), *Biochemistry 9*, 4633.
Hinkle, P. M., and Kirsch, J. F. (1971a), *Biochemistry 10*, 3700.
Hinkle, P. M., and Kirsch, J. F. (1971b), *Biochemistry 10*, 2717.
Jolley, C. J., and Yankeelov, J. A. (1972), *Biochemistry 11*, 164.
Lake, A. W., and Lowe, G. (1966), *Biochem. J. 101*, 402.
Lowe, G. (1970), *Phil. Trans. Roy. Soc. London, Ser. B 257*, 237.
Mole, J. E., and Horton, R. (1973), *Biochemistry 12*, 818.
Mycek, M. J., and Fruton, J. S. (1957), *J. Biol. Chem. 226*, 165.
Neumann, H., and Shinitzky, M. (1971), *Biochemistry 10*, 4335.
Sluyterman, L. A. AE. (1967), *Biochim. Biophys. Acta 139*, 418.
Steiner, R. (1971), *Biochemistry 10*, 771.
Yang, P. C., and Schwert, G. (1972), *Biochemistry 11*, 2218.

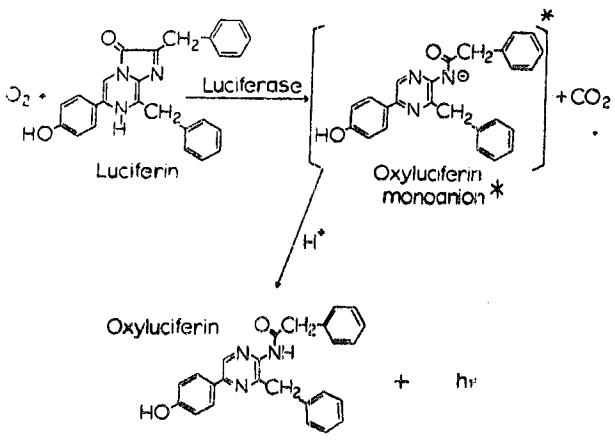
Mechanism of Calcium Induction of *Renilla* Bioluminescence. Involvement of a Calcium-Triggered Luciferin Binding Protein†

James Michael Anderson, Harry Charbonneau, and Milton J. Cormier*

ABSTRACT: When extracts of bioluminescent Anthozoans, such as *Renilla*, are prepared in the presence of metal chelators they can be induced to bioluminesce upon the addition of calcium ions. In this sense such bioluminescence emissions resemble those of the calcium-dependent photoprotein isolated from *Aequorea*. However, in the case of *Renilla* such calcium-induced bioluminescence involves a calcium-triggered release of luciferin from a binding protein prior to catalysis by luciferase. The luciferin binding protein can be completely separated from luciferase and the presence of luciferin bound to the binding protein has been demonstrated by spectroscopy. Light production requires the presence of the charged luciferin binding protein, luciferase, calcium, and oxygen. Luciferin can be removed from the binding protein and the resulting discharged binding protein can be recharged in the presence of excess luciferin. The properties of the recharged binding protein are the same as those of the native one. The calcium triggering of the luciferin binding protein of *Renilla* is the logical choice of mechanism for linking the nerve impulse to the bioluminescent system in this animal. The evidence suggests that a luciferin binding protein exists among the bioluminescent Anthozoans generally but not among the bioluminescent Hydrozoans. Thus, in the Anthozoans the calcium-induced *in vitro* bioluminescence is due to two proteins. One is a calcium-triggered luciferin binding protein and the other is luciferase. In the Hydrozoan, *Aequorea*, the photoprotein is viewed as playing a dual role. That is, it acts as a luciferin binding protein on the one hand while in the presence of calcium it acts like a luciferase.

A brilliant green luminescence is produced by *Renilla reniformis*, an Anthozoan coelenterate, upon appropriate stimulation. In recent years there has been considerable progress in elucidating the biochemical components involved in this bioluminescence. The identity of several of the proteins involved (Cormier and Totter, 1968; Cormier *et al.*, 1970a,b; Karkhanis and Cormier, 1971; Wampler *et al.*, 1971; DeLuca *et al.*, 1971; Hori *et al.*, 1972) as well as the structure of a fully active analog of the light producing substrate, or luciferin (Hori *et al.*, 1973; Hori and Cormier, 1973), have been determined. The light-producing reaction involves the oxidation of the fully active luciferin analog as shown in Scheme I. The above reactions produce a blue emission (λ_{max}

SCHEME I



† From the Department of Biochemistry, University of Georgia, Athens, Georgia 30602. *Received October 9, 1973.* 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-3331-09, of the U. S. Public Health Service. Contribution No. 278 from the University of Georgia Marine Institute, Sapelo Island, Georgia. This is No. XIV of a series entitled Studies on the Bioluminescence of *Renilla reniformis*.

490 nm) which is in contrast to the *in vivo* bioluminescence which is green (λ_{max} 509 nm) (Wampler *et al.*, 1971; Morin and Hastings, 1971b). Recent evidence shows that the green emission is due to the fluorescence of a protein-bound chromophore which acts as an acceptor of energy transferred from the electronic excited state of an oxyluciferin monoanion (Wampler *et al.*, 1971; Hori *et al.*, 1973). The energy transfer is nonradiative making the interaction between luciferase and the green fluorescent protein a logical requirement (Wampler *et al.*, 1972).

The bioluminescent system of *Renilla* has been found to be packaged in discrete subcellular particles termed lumisomes (Anderson and Cormier, 1973a,b). Purified lumisomes appear, when viewed by electron microscopy, as membrane-bound vesicles about 0.2 μm in diameter. They contain luciferase, green fluorescent protein, and a calcium-induced bioluminescent activity. If lumisomes are lysed in the presence of calcium ions they produce a green emission typical of the *Renilla in vivo* bioluminescence. Kinetics of the calcium-induced light emission from lumisomes are independent of lumisome concentration. This is in contrast to the soluble system where the rate is dependent on protein concentration (Anderson and Cormier, 1973a,b).

In the Hydrozoan *Aequorea* bioluminescence arises from the interaction of calcium ions and a photoprotein (Johnson *et al.*, 1962; Shimomura *et al.*, 1962, 1963a,b; Shimomura and Johnson, 1969, 1972). No evidence for a *Renilla* type luciferase has been found although a stabilized form of *Renilla* luciferin, luciferyl sulfate, is found in *Aequorea* as well as in *Renilla* (Hori *et al.*, 1972; Cormier *et al.*, 1973). Light can also be produced by the addition of calcium ions to crude extracts of *Renilla*, and other Anthozoans (Hastings and Morin, 1969; Cormier *et al.*, 1970a,b). This phenomenon has lead to the assumption that photoproteins, similar to those found in *Aequorea*, occur in the Anthozoans (Morin and Hastings, 1971a; Cormier *et al.*, 1973). However, in contrast to *Aequorea* photoprotein (Johnson *et al.*, 1962) and the results of Hastings and Morin (Morin and Hastings, 1971a; Hastings and Morin, 1969) the calcium-activated bioluminescence of *Renilla* has been found to be oxygen dependent (Anderson and Cormier, 1973a,b). In testing the kinetics of *Renilla* calcium-induced luminescence *in vitro* we find that the emission decay rate is dependent on protein concentration. Furthermore, a careful examination of the luminescence decay of this calcium-induced bioluminescence showed that such decays are not first order and that the decay rate depends on oxygen concentration as well as on protein concentration. These results indicated that a multiprotein system is involved in *Renilla* bioluminescence rather than a single photoprotein.

The discrepancies between the *Aequorea* photoprotein system and the soluble system extracted from *Renilla* demonstrated the need for an elucidation of the nature of *Renilla* calcium-induced bioluminescence. This report shows that the calcium induction of bioluminescence in *Renilla* is due to the calcium-induced release of luciferin from a luciferin binding protein. Unless specifically stated otherwise discussions using the term "binding protein" refer to a luciferin-protein complex.

Experimental Methods

Renilla mülleri were obtained from Gulf Specimen Co., Inc., Panacea, Fla. *Renilla reniformis* were obtained by collection at Sapelo Island, Ga.

Preparation of Binding Protein. Animals were soaked for 30 min in the homogenizing medium ($Mg(OAc)_2$ (300 mM), disodium ethylene glycol bis(aminoethyl ether)-*N*,*N*'-tetraacetic acid (EGTA) (20 mM), and Tris-HCl (pH 7.2) at 25° (50 mM)) which prevented the animals from bioluminescing during homogenization. The animals were then placed in fresh, cold, homogenizing medium using about one animal to 10 ml of medium. The tissue was then homogenized using a Willems Polytron PT 35 st, set at full speed, for 30 sec. The resulting brei was centrifuged at 20,000*g* for 20 min to remove lumisomes (Anderson and Cormier, 1973b). The supernatant was then dialyzed extensively against the standard buffer (0.5 mM Na_2-EDTA–5 mM Tris-HCl (pH 7.2) at 25°) followed by centrifugation and removal of the resultant precipitate. The binding protein and some of the luciferase were adsorbed to a DEAE-cellulose column (Whatman DE 22) equilibrated with the standard buffer. The protein was eluted from the column with 1.0 M NaCl, in the standard buffer, followed immediately by G-75 Sephadex chromatography in the standard buffer. The binding protein and luciferase activity eluted in a common peak from the G-75 Sephadex. The protein preparation was then brought to 80 mM with respect to NaCl and absorbed to A-50 DEAE-Sephadex. Binding protein was eluted, separate from luciferase, by a shallow 80–300 mM NaCl gradient. Binding protein from *Renilla mülleri* was over 500-fold purified at this stage. Any remaining luciferase could be removed by a second DEAE-Sephadex step.

Protein Assays. Binding protein was assayed by injecting 0.2 ml of $CaCl_2$ in 0.55 ml of a luciferase-binding protein mixture. The final concentrations of ingredients were as follows: NaCl, 575 mM; $CaCl_2$, 2.67 mM; Na_2-EDTA, 0.43 mM; Tris-HCl (pH 7.5) at 25°, 56.3 mM; potassium phosphate, 1.3 mM (from luciferase); luciferase, 7.5 μg/ml. Initiation of the reaction required the availability of free calcium which was accomplished by using a sixfold excess of calcium over EGTA. The assay was found to be linear over a tenfold concentration of binding protein with constant luciferase. Assays were confined to this concentration range. Highly purified luciferase was prepared from *Renilla reniformis* by an improved method (Hori *et al.*, 1973). Luciferase was assayed by injecting a mixture of 0.1 ml of the enzyme and 0.9 ml of standard buffer, made 1.0 M in NaCl, into a vial containing synthetic luciferin dissolved in 10 μl of methanol (Hori *et al.*, 1973). All references in this paper to synthetic luciferin refer to the fully active benzyl analog. Luminescence measurements were performed as previously described (Hori *et al.*, 1973).

Removing Luciferin from Binding Protein. A concentrated preparation of binding protein was chromatographed on G-25 Sephadex equilibrated with a mixture of $CaCl_2$ (2 mM) and Tris-HCl (pH 7.2, 25°, 5 mM). The void volume effluent containing absorbancy at 280 nm was saved and found to be free of binding protein activity. This material was used as an inhibitor of luciferase and as a source of discharged binding protein.

Recharging Binding Protein. To an anaerobic preparation of the binding protein, which had been discharged of luciferin as described above, was added 50 μl of a methanolic solution of synthetic luciferin. The mixture was immediately chromatographed on G-25 Sephadex, equilibrated with the standard buffer. The effluent from the column which showed binding protein activity was saved.

Absorption and Fluorescence Spectroscopy. Absorption spectra were obtained by using a Spectronic 505 spectrophotometer connected on-line to a Nova computer (DeSa and Wampler, 1973). Fluorescence spectra were obtained

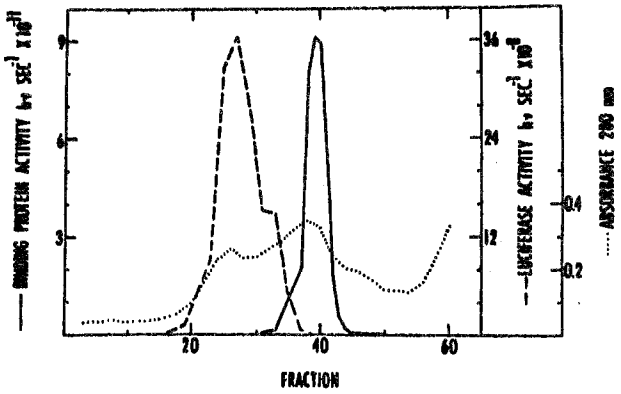


FIGURE 1: Separation of *Renilla mülleri* luciferase and binding protein activities on DEAE-Sephadex. Elution was with an 80–300 mM NaCl gradient at pH 7.2 at 25°.

using a component spectrofluorimeter linked on-line to the same computer (Wampler and DeSa, 1971; DeSa, 1972).

Polyacrylamide Gel Electrophoresis. Ten per cent polyacrylamide gels were formed by the method of Gabriel (1971). The gels were loaded with 0.360 absorbance unit (280 nm) of protein and electrophoresed at 2 mA/gel. The buffer system was Tris (25 mM) and glycine (192 mM) at pH 8.3. One gel was stained with Coomassie Blue following electrophoresis. The other gels were serially sliced into 2-mm sections. The sections were separately soaked in 0.5 ml each of the assay medium for 12 hr. Luciferase and calcium ions were then added and the light emission measured.

Results

Crude extracts of *Renilla*, which are made in the presence of a chelator, produce light simply upon the addition of calcium ions. The specificity for calcium is very high and is similar to that observed for lumisomes (Anderson and Cormier, 1973b). This calcium-induced activity can be partially purified by chromatography on DEAE-cellulose followed by chromatography on G-75 Sephadex and the activity eluted from both columns in the same volume as luciferase. The calcium-induced activity can be inactivated by treatments which destroy luciferase activity, *i.e.*, either by photooxidation in the presence of 0.10% Methylene Blue or incubation for several days in 2 M NaCl. The calcium-induced activity can be completely regenerated by re-addition of purified luciferase to the inactivated preparations. Preparations of the calcium-induced activity from *Renilla mülleri*, which have been partially purified as described above, can be separated into two distinct protein fractions by chromatography on A-50 DEAE-Sephadex (Figure 1). One fraction contains luciferase activity and the other a calcium-induced luciferin activity. Bioluminescence emission with this latter fraction requires added luciferase in addition to calcium ions. Therefore, the calcium-induced activity of *Renilla* was presumed to be a mixture of luciferase and a calcium-triggered luciferin binding protein. The experiments that follow show that this is the case.

Although luciferase and binding protein, extracted from *Renilla reniformis*, did not separate cleanly on DEAE-Sephadex they could be completely separated from each other by a hydrophobic chromatography step prior to DEAE-Sephadex. The hydrophobic chromatography was performed using methods similar to those described by Hofstee (1973). The protein preparation was bound to agarose and substituted with aromatic groups, followed by elution of the

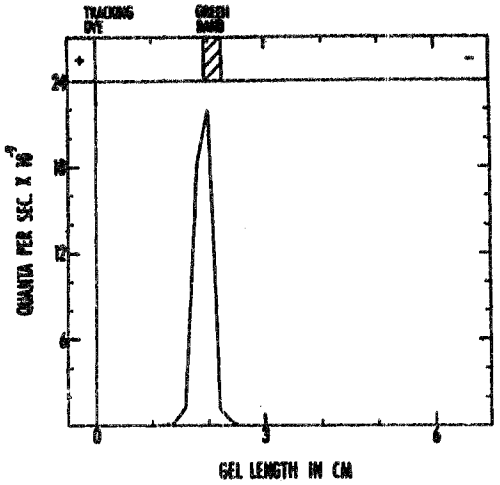


FIGURE 2: Banding of binding protein activity on polyacrylamide gels. Electrophoresis was from negative to positive. The upper insert shows the position of the visible band prior to cutting the gel into 2-mm sections.

binding protein with a gradient of NaCl (0.005–1.0 M). Preparations from both *R. mülleri* and *R. reinformis* were used in the remaining experiments with essentially the same results with either one.

Samples of purified binding protein were electrophoresed on 10% polyacrylamide gels. Following electrophoresis a single, yellow-green band was visible which was found to correspond to the major band of protein as revealed by Coomassie Blue staining. A second gel was sliced into 2-mm sections followed by an assay of each section for binding protein. Figure 2 shows that the yellow-green band on the gel corresponds approximately to the single band of binding protein activity. The slight shift in the activity distribution was probably due to gel contraction during slicing. The precise correspondence between the yellow-green band and activity was also noted during visual observation of the position of binding protein activity. The absorption spectrum of a binding protein preparation was taken to determine the source of the yellow-green color (Figure 3). Figure 3 also shows that the absorption spectrum of binding protein corresponded closely with the absorption spectrum of synthetic luciferin dissolved in absolute methanol (Hori *et al.*, 1973). The fluorescence spectrum of binding protein was also similar

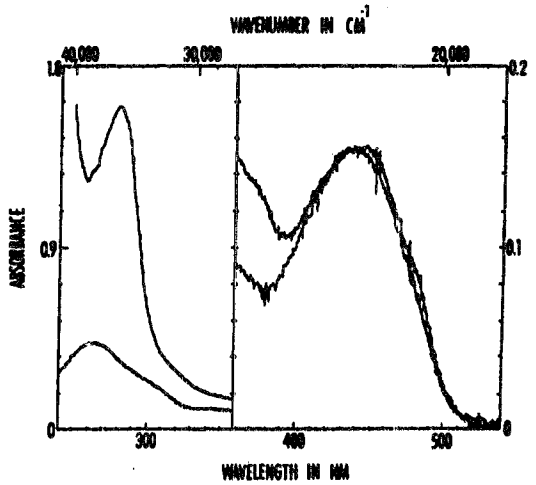


FIGURE 3: Adsorption of *Renilla reniformis* purified binding protein. The upper curve is an 11-scan average adsorption spectrum of binding protein. The lower curve is the adsorption spectrum of synthetic luciferin in methanol.

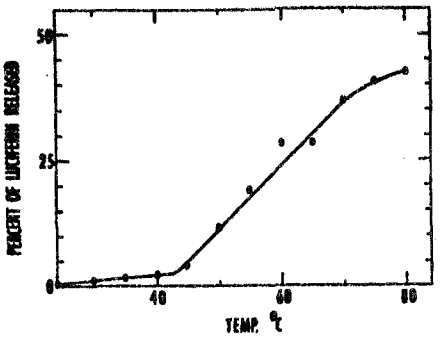


FIGURE 4: Release of luciferin from binding protein by heating. Samples were heated at designated temperatures for 1 min followed by cooling and assay for free and bound luciferin.

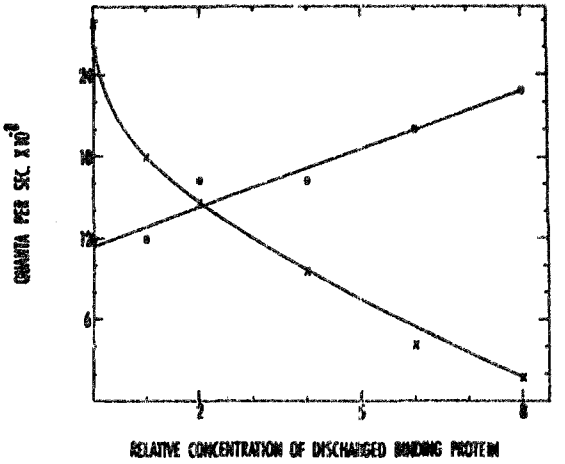


FIGURE 5: Inhibition of the reaction of luciferase with synthetic luciferin by discharged binding protein. The reaction was initiated by addition of luciferin (×) followed, after 5 sec, by the addition of calcium ions (○).

to that of synthetic luciferin in methanol. Furthermore, binding protein, in the presence of luciferase, produces a calcium-induced bioluminescence whose emission spectrum is identical with that of a typical luciferase–luciferin reaction. Taken together these data suggest that the yellow-green color associated with the binding protein is due to protein-bound luciferin.

In fact, luciferin can be released from binding protein by heating at temperatures above 45° (Figure 4). Samples were treated for 1 min at the designated temperatures (Figure 4) followed by cooling. The samples were assayed immediately for free luciferin activity, *i.e.*, activity not dependent upon the presence of calcium. This was followed by assay for calcium-induced luminescence. Below 80° all the light intensity could be accounted for. At higher temperatures luciferin was rapidly autoxidized leading to erroneous measurements. However, boiling a solution of binding protein under a helium atmosphere for 2 min did release 60% of the bound luciferin activity. Similar experiments done with *Aequorea* photoprotein resulted in inactivation by heating without liberating any luciferin-like activity.

Luciferin chemiluminesces in solvents such as dimethylformamide and dimethyl sulfoxide (Hori *et al.*, 1973). The luciferin bound to the luciferin binding protein should exhibit similar behavior. If the binding protein is injected into dimethyl sulfoxide a chemiluminescence typical of *Renilla* luciferin (Hori *et al.*, 1973) is observed. *Aequorea* photoprotein, on the other hand, produces only a short flash of light in dimethyl sulfoxide rather than the long-term chemiluminescence of luciferin.

The removal of luciferin from the binding protein upon the addition of calcium ion was attempted but was hindered by the fact that concentrated solutions of binding protein tended to precipitate when calcium ions were added. Dialysis of binding protein solutions against calcium ions not only caused precipitation but also failed to remove all of the bound luciferin. Even dialysis against luciferase and calcium ions failed to discharge the binding protein completely.

The problem could be overcome by flowing the binding protein through a G-25 Sephadex column, equilibrated with calcium ions. The luciferin was completely removed from the binding protein by this procedure. This discharged binding protein was found to be an inhibitor of the reaction of luciferase with synthetic luciferin (Figure 5). Inhibition was presumably by removal of luciferin from the reaction mixture by the discharged binding protein. The subsequent addition of of calcium ions partially relieved the inhibition as would be expected (Figure 5). The discharged binding protein could be recharged to 68% of its original activity with synthetic luciferin. The recharging was done with a large excess of luciferin and the unbound luciferin was removed on G-25 Sephadex as described under Experimental Methods. The recharged binding protein had the same properties as native binding protein.

The characteristics of *Renilla* luciferin binding protein and *Aequorea* photoprotein are compared in Table I. In *Renilla* the luciferase and binding protein, along with the green fluorescent protein, are packaged within a subcellular particle, the lumisome. There are a number of differences between the characteristics of the reaction of *Renilla* binding protein plus luciferase and the reaction of *Aequorea* photoprotein but the reaction of the Anthozoan lumisome appears very similar to that of photoproteins. Since the chemical reaction involved in producing light in both *Renilla* and *Aequorea* appears to be the same (Hori *et al.*, 1973) then the basic difference between the two systems remains one of enzymology.

Discussion

The light producing reaction of *Renilla* bioluminescence involves three distinct proteins. The first of these is the luciferin binding protein which sequesters the supply of luciferin in a stable state until calcium ions are made available. This is a mechanism which could allow the organism to control the timing and amount of luminescence by controlling calcium ion access to the binding protein. This type of binding protein probably exists among a number of the Anthozoans since lumisomes prepared from them have properties similar to those found in *Renilla* and since the kinetics of the soluble calcium-induced bioluminescent activity of the Anthozoans, but not the Hydrozoans, is similar to that found in *Renilla* (Cormier *et al.*, 1973; Anderson and Cormier, 1973b). The luciferin, once it is released, is oxidized by a second protein, luciferase, producing the monoanion of oxyluciferin in the electronic excited state (Hori *et al.*, 1973). Finally, this excitation energy is transferred from the oxyluciferin monoanion to a third protein, the green fluorescent protein which emits green light. In the Hydrozoans, the same end result occurs with apparently only two proteins participating; the photoprotein and the green fluorescence protein. In this case the photoprotein is viewed as playing a dual role. It binds a *Renilla*-like luciferin (Hori *et al.*, 1973), thus acting like a

TABLE I: Comparison of *Aequorea* Photoprotein with *Renilla* Binding Protein and Lumisomes.

	Aequorin	*Renilla* Binding Protein[a]	Lumisome
A. Requirements for light reaction			
Oxygen	Not required[c]	Required	Required
Luciferase	Not required	Required	Not required[b]
Calcium	Required[c]	Required	Required
B. Effect on kinetics of			
Oxygen concentration	None[c]	Rate limiting	Rate limiting
Luciferase concentration	None	Rate limiting	None[b]
Aequorin, binding protein, or lumisome concentration	None[c]	Rate limiting	None
C. Emission λ_{max}	466 nm[c]	490 nm	509 nm
D. Emission, whole organism λ_{max}	509 nm	509 nm	509 nm
E. Chemiluminescence in dimethyl sulfoxide; length of reaction	<1 sec	>10 sec	
F. Effect of heating	Loss of act.[c]	Release of luciferin	

[a] All binding protein assays required added luciferase. The same concentration was used in each assay unless otherwise noted. [b] Initial intensity and rate of decay. [c] These data on aequorin are taken from the work of Drs. Shimomura and Johnson (1969, 1972, 1973).

binding protein, and it converts this luciferin in the presence of calcium ion to a *Renilla*-like oxyluciferin (Hori *et al.*, 1973; Shimomura and Johnson, 1973), thus also acting like a luciferase. It is likely that *in vivo Aequorea* packages the photoprotein and green fluorescent protein together into *Renilla*-like lumisomes since such lumisomes have been found to occur in other Hydrozoans (Anderson and Cormier, 1973b).

The key to controlling *Renilla* bioluminescence is the lumisome where the proteins involved in producing bioluminescence are packaged. It is obvious from Table I that the characteristics of the soluble system, *i.e.*, luciferase plus binding protein, are not the same as those of the lumisome system whose properties resemble those of a photoprotein. The lumisome, therefore, plays an important role in organizing the proteins involved in *Renilla* bioluminescence as well as controlling calcium access to these proteins.

Interestingly the *Renilla* bioluminescent system has striking similarities to those found in the dinoflagellate, *Gonyaulax polyedra*. In *Gonyaulax* the bioluminescent system is packaged in subcellular particles, termed scintillons (DeSa *et al.*, 1963; Fuller *et al.*, 1972), which recently have been shown to be membrane-bound vesicles similar to *Renilla* lumisomes (Fogel *et al.*, 1972). Furthermore, the bioluminescent system in *Gonyaulax* consists of a luciferin binding protein and a separate luciferase (Fogel and Hastings, 1971). The *Gonyaulax* binding protein releases its luciferin in response to an influx of hydrogen ions while the *Renilla* binding protein releases its luciferin in response to an influx of calcium ions.

Bioluminescence in *Renilla* is apparently controlled by a nerve network, *i.e.*, bioluminescence is triggered by a nerve impulse. In other systems calcium ion is known to play a direct role during the early phase of synaptic and neuromuscular transmission (Katz and Miledi, 1965; Katz, 1966). The calcium triggering of the luciferin binding protein of *Renilla* is the logical choice of mechanism for linking the nerve impulse to the bioluminescent system. Thus the *Renilla* luciferin binding protein, which could also be viewed as a calcium binding protein, may serve as a model for events which occur during the calcium-dependent release of neurotransmitters by a nerve impulse.

Acknowledgments

We thank Drs. F. H. Johnson and O. Shimomura, Princeton University, for a gift of aequorin and Dr. Kazuo Hori for supplying *Renilla* synthetic luciferin.

References

Anderson, J. M., and Cormier, M. J. (1973a), *in* Chemiluminescence and Bioluminescence, Cormier, M. J., Hercules, D. M., and Lee, J., Ed., New York, N. Y., Plenum, p 387.

Anderson, J. M., and Cormier, M. J. (1973b), *J. Biol. Chem. 248*, 2937.

Cormier, M. J., Hori, K., and Karkhanis, Y. D. (1970a), *Biochemistry 9*, 1184.

Cormier, M. J., Hori, K., Karkhanis, Y. D., Anderson, J. M., Wampler, J. E., Morin, J. G., and Hastings, J. W. (1973), *J. Cell. Physiol. 81*, 291.

Cormier, M. J., Karkhanis, Y. D., and Hori, K. (1970b), *Biochem. Biophys. Res. Commun. 38*, 962.

Cormier, M. J., and Totter, J. R. (1968), *Photophysiology 4*, 315.

DeLuca, M., Dempsey, M. E., Hori, K., Wampler, J. E., and Cormier, M. J. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1658.

DeSa, R. J. (1972), *in* Computers in Chemical and Biochemical Research, Klopefenstein, C., and Wilkins, C., Ed., New York, N. Y., Academic Press.

DeSa, R. J., Hastings, J. W., and Vatter, A. E. (1963), *Science 141*, 1269.

DeSa, R. J., and Wampler, J. E. (1973), *Appl. Spectrosc. 27*, 279.

Fogel, M., and Hastings, J. W. (1971), *Arch. Biochem. Biophys. 142*, 310.

Fogel, M., Schmitter, R. E., and Hastings, J. W. (1972), *J. Cell. Sci. 11*, 305.

Fuller, C. W., Kreiss, P., and Seliger, H. H. (1972), *Science 177*, 884.

Gabriel, O. (1971), *Methods Enzymol. 22*, 565.

Hastings, J. W., and Morin, J. G. (1969), *Biochem. Biophys. Res. Commun. 37*, 493.

Hofstee, B. H. J. (1973), *Anal. Biochem. 52*, 430.

Hori, K., and Cormier, M. J. (1973), *Proc. Nat. Aci. Sci. U. S. 70*, 102.

Hori, K., Nakano, Y., and Cormier, M. J. (1972), *Biochim. Biophys. Acta 256*, 638.
Hori, K., Wampler, J. E., Matthews, J. C., and Cormier, M. J. (1973), *Biochemistry 12*, 4463.
Johnson, F. H., Shimomura, O., Saiga, Y., Gershman, L. C., Reynolds, G. T., and Waters, J. R. (1962), *J. Cell. Comp. Physiol. 60*, 85.
Karkhanis, Y. D., and Cormier, M. J. (1971), *Biochemistry 10*, 317.
Katz, B. (1966), Nerve, Muscle and Synapse, New York, N. Y., McGraw-Hill.
Katz, G., and Miledi, R. (1965), *Proc. Roy. Soc., Ser. B 161*, 496.
Morin, J. G., and Hastings, J. W. (1971a), *J. Cell. Physiol. 77*, 305.
Morin, J. G., and Hastings, J. W. (1971b), *J. Cell. Physiol. 77*, 313.
Shimomura, O., and Johnson, F. H. (1969), *Biochemistry 8*, 3991.
Shimomura, O., and Johnson, F. H. (1972), *Biochemistry 11*, 1602.
Shimomura, O., and Johnson, F. H. (1973), *In* Chemiluminescence and Bioluminescence, Cormier, M. J., Hercules, D. M., and Lee, J., Ed., New York, N. Y., Plenum Press, p 337.
Shimomura, O., Johnson, F. H., and Saiga, Y. (1962), *J. Cell. Comp. Physiol. 59*, 223.
Shimomura, O., Johnson, F. H., and Saiga, Y. (1963a), *J. Cell. Comp. Physiol. 62*, 1.
Shimomura, O., Johnson, F. H., and Saiga, Y. (1963b), *J. Cell. Comp. Physiol. 62*, 9.
Wampler, J. E., and DeSa, R. J. (1971), *Appl. Spectrosc. 25*, 623.
Wampler, J. E., Hori, K., Lee, J. W., and Cormier, M. J. (1971), *Biochemistry 10*, 2903.
Wampler, J. E., Karkhanis, Y. D., Hori, K., and Cormier, M. J. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 1133.

A Special Form of Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerase from Oocytes of *Xenopus laevis*. Isolation and Characterization†

John Wilhelm, Dino Dina, and Marco Crippa*

ABSTRACT: DNA-dependent RNA polymerase activity has been extracted in low salt from *Xenopus laevis* ovaries. The readily soluble enzyme has been purified 200-fold by DEAE-cellulose, phosphocellulose, and Sepharose 6B chromatography. The purified enzyme shows general elution and enzymatic properties similar to form A (I) from other tissues but can be distinguished by two main criteria: it is almost completely inhibited by high levels of α-amanitin and transcribes preferentially native DNA. An analysis of the activities in liver and kidney nuclei has shown that they contain only the known A (I) and B (II) forms of the enzyme, leading to the conclusion that the enzyme described below is probably a special form localized in the ovary. By microdissection techniques it has been found that this enzyme is localized in the nucleus of the oocytes and it is therefore called "oocyte-soluble polymerase." Germinal vesicles of stage 6 oocytes contain a large amount of RNA polymerase, which has been calculated to be roughly 10^5 times larger than a liver cell. In addition to a small amount of form B (II) polymerase (10–15% stage 6 oocytes contain predominanty the special oocyte soluble polymerase, whereas form A (I), if present, must represent less than 5% of the total RNA polymerase activity. This large amount of enzyme exists at a time when the oocyte nucleus also contains the amplified rDNA cistrons, and the possibility that the polymerase is involved in rDNA transcription is discussed.

During oogenesis the amphibian oocyte accumulates large quantities of different RNA species for later use during early embryonic development. Several biochemical events connected with the synthesis of the different RNA species are now well established.

An interesting situation exists in fully grown *Xenopus laevis* oocytes which contain a large store of ribosomes. The synthesis of the ribosomal RNA (rRNA)[1] components (28, 18, and 5 S) during oogenesis is achieved by different and rather intriguing processes.

The *Xenopus* diploid genome has at least 54,000 5S RNA genes (Brown and Weber, 1968) which are active during the entire period of oogenesis (Mairy and Denis, 1971). On the contrary, there are only 900 rRNA genes per diploid genome. The oocyte, however, selectively replicates its rDNA complement and then transcribes the amplified genes very actively but only in a temporally limited period.

We were interested in studying the properties of the RNA polymerases[1] operating inside of the oocyte during these events. In eukaryotic systems different forms of RNA polymerase have been separated (Roeder and Rutter, 1969; Kedinger *et al.*, 1970; Jacob *et al.*, 1970) and purified (Chambon *et al.*, 1970; Blatti *et al.*, 1970; Weaver *et al.*, 1971; Chesterton and Butterworth, 1971a,c; Gissinger and

† From the Department of Animal Biology, University of Geneva, 1224 Geneva, Switzerland. *Received July 23, 1973.* Supported by Grant No. 37541-72 from the Swiss National Fund and a special fellowship from the Emil Barell Foundation of Hoffmann-La Roche to J. W.

[1] Abbreviations used are: DEAE, diethylaminoethyl; RNA polymerase, nucleotide triphosphate:RNA nucleotidyltransferase (EC 2.7.7.6); EDTA, ethylenediaminetetraacetate; rRNA, ribosomal ribonucleic acid.