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STUDIES ON THE BIOLUMINESCENCE OF *RENILLA RENIFORMIS*

I. REQUIREMENTS FOR LUMINESCENCE IN EXTRACTS AND CHARACTERISTICS OF THE SYSTEM*

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SUMMARY

A method is described for the preparation of luminescent extracts from the sea pansy, *Renilla reniformis*. Light production requires the presence of *Renilla* luciferase, an adenine-containing nucleotide (either ATP, ADP, or AMP), *Renilla* luciferin, and oxygen. The reaction is specific for adenine-containing nucleotides and *Renilla* luciferin. The luminescent reaction was shown to involve a nucleotide-dependent oxidation of *Renilla* luciferin.

Various factors that affect the system were studied. These include the effect of pH, enzyme concentration, temperature, inhibitors, nucleotide concentration, and luciferin concentration.

The evidence presented indicates that ATP is involved indirectly in this sytem by functioning as a generating system for ADP and AMP via an ATPase reaction.

Abbreviations used: AMP, ADP, and ATP are the mono-, di- and triphosphates of adenosine respectively; CMP, CDP and CTP are the mono-, di- and triphosphates of cytidine respectively; UMP, UDP, and UTP are the mono-, di- and triphosphates of uridine respectively; GMP, GDP, and GTP are the mono-, di- and triphosphates of guanosine respectively; GSH, reduced glutathione; PCMB, *p*-chloromercuribenzoate; ATPase, adenosine-5′-triphosphatase; FMN, $FMNH_2$, flavin mononucleotide and dihydroflavin mononucleotide respectively; FAD, flavin adenine dinucleotide.

* This is contribution No. 18 from the University of Georgia Marine Institute, Sapelo Island, Georgia.

INTRODUCTION

Although many thousands of bioluminescent species exist throughout the animal kingdom[1], our knowledge of the biochemistry of these light reactions is scanty. Studies on the identity of factors required for the luminescence of extracts of luminous bacteria, the firefly, and the luminous ostrocod, *Cypridina*, have been reported in recent years[2-3]. Data on one additional luminescent system, the luminous fungi, has recently been reported[4]. With the exception of the four systems mentioned above, the nature of the components involved in other bioluminescent systems has not been established. This report is the first of a series of studies that deals with the luminescence system of the sea pansy, *Renilla reniformis*, and represents the first description of luminescence in extracts of this organism. The requirements for luminescence in extracts are presented as well as additional characteristics of the system. A preliminary report of a portion of this work has appeared[5].

MATERIALS AND METHODS

The purine and pyrimidine nucleotides and the adenosine used in these experiments were obtained from the Sigma Chemical Company.

Preparation of crude extracts

Freshly collected sea pansies were placed in a dark, running sea water aquarium for 4 to 8 h. Since these animals have a diurnal rhythm of luminescence[1], this procedure was necessary to rejuvinate the luminescence system. Approximately 50 animals at a time were destemmed and dropped into a cold mortar containing 10 ml saturated ammonium sulfate, pH 7.5. They were cut into small pieces with scissors and finally ground with sand for 30 min. A total of 200 ml of saturated ammonium sulfate was gradually added with stirring and the suspension centrifuged at 12,000 $\times$ *g* for 10 min. The precipitate was carefully drained to remove excess ammonium sulfate, and then extracted by grinding in a mortar with a total of 50 ml of 0.05 *M* potassium phosphate buffer, pH 7.5, containing 0.001 *M* GSH(phosphate–GSH buffer). The extract was centrifuged for 10 min at 12,000 $\times$ *g* and the resulting supernatant centrifuged for 30 min at 140,000 $\times$ *g*. The latter centrifugation yields a clear, amber colored supernate which contains the activity and will be referred to as the crude extract. The activity of the extract was preserved by storing at —20°. The grinding, centrifugation and extraction procedures were done at 0–3°.

For the experiments reported here, unless otherwise indicated, the crude extract was precipitated once with saturated ammonium sulfate, pH 7.5 (to 75 % saturation), and the precipitated protein redissolved in phosphate–GSH buffer.

Treatment of the animals with ammonium sulfate is an essential feature of the extraction process. Grinding rejuvinated organisms with sand in the presence of phosphate–GSH buffer without ammonium sulfate treatment does not yield an active extract. Ammonium sulfate produces at least two observable effects: firstly, it allows a greater amount of protein to be extracted; and secondly, it acts as an anesthetic thus preventing an excessive luminescent response during grinding, which would result in oxidation of *Renilla* luciferin.

Preparation of crude Renilla *luciferin*

The once extracted *Renilla* residue was reextracted with phosphate–GSH buffer in the same manner as described for preparation of the crude extract except that centrifugation at 140,000 × *g* was not required. This extract was heated at 100° for 1.5 min, cooled to room temperature, and the denatured protein removed by centrifugation. The supernate contained *Renilla* luciferin which can be stored at —15° for weeks without significant loss of activity.

Assay system

Unless otherwise specified, the luminescence assay system consisted of the following additions: potassium phosphate buffer, pH 7.5 (50 μmoles); GSH(2 μmoles); ATP, ADP, or AMP(5 μmoles); *Renilla* luciferin (0.2 ml); enzyme (0.5 mg protein); water to 1.5 ml. In the experiments reported, ADP was used in preference to ATP since the latter compound functions indirectly in this system. All reactions were carried out at 30° unless otherwise stated.

Light measurements

Light from this luminescence system was measured by a dry-ice cooled 1P21 photomultiplier made by RCA. Approx. 960 V was applied across the photomultiplier by a Victoreen, model 683, DC power supply. The output of the photomultiplier was channeled into a Keithley, model 410, micro-microammeter and the output of the latter channeled into a Varian, model G-10, recorder. This system is quite sensitive and allows continuous recording of luminescence with time.

Temperature effects

The effect of temperature on luminescence was studied over the temperature range of 5–40°. The procedure followed is the same as that followed by STREHLER AND CORMIER during similar studies on the bacterial luminescence system[12] and tends to eliminate systematic errors due to enzyme denaturation or transient changes of other types.

RESULTS

Characteristics of Renilla *luciferase*

Crude extracts from *R. reniformis* give a relatively weak luminescence that is stimulated approximately 10–30 fold (depending upon the extract) on the addition of either ATP, ADP, or AMP. These results are presented in Table I. Enzyme preparations can be obtained, by repeated precipitations with saturated ammonium sulfate, that are stimulated 100 fold upon the addition of adenine-containing nucleotides. Adenosine is inactive in this system, as well as the monophosphates and tri-phosphates of guanosine, cytidine and uridine. The diphosphates of guanosine and cytidine are also inactive although some activity was exhibited by UDP. Whether the latter activity is due to contamination with adenine-containing nucleotides is not certain, although on the basis of concentration curves (Fig. 1), 1 % contamination with any one, or a combination, of the adenine-containing nucleotides would have been sufficient to account for the light observed. Furthermore, since UTP and UMP are inactive, it appears unlikely that uridine-containing nucleotides are active in this system. Thus,

luminescence in extracts of *R. reniformis* is dependent upon the presence of adenine-containing nucleotides and is specific for the adenine moiety, at least in the initial step of the reaction. The enzyme responsible for light emission in these extracts will be referred to as *Renilla* luciferase.

TABLE I

EFFECT OF NUCLEOTIDES ON THE LUMINESCENCE OF *R. reniformis* EXTRACTS

Conditions: Same as described in the MATERIALS AND METHODS section. 5 μmoles of each nucleotide or nucleoside were added as indicated.

Expt.	*Addition*	*Relative light intensity*
I	None	16
	ATP	445
	ADP	390
	AMP	440
	Adenosine	16
	GTP, CTP, or UTP	16 ± 1
	GMP, CMP, or UMP	16 ± 1
II	None	20
	ADP	450
	GDP	23
	CDP	36
	UDP	122

Since light emission in the firefly requires a primary activation by ATP[14], and, since the *Renilla* system responds to either ATP, ADP or AMP, there appeared to be a good possibility that the two systems were similar with respect to nucleotide requirements. The purity of the ADP and AMP was therefore checked for contamination with ATP. The AMP used in these experiments did not contain ATP as determined by ATP analysis using the firefly technique[2,6]. The sensitive light detecting apparatus used in these experiments readily allowed the detection of 10^{-3} μg ATP/ml. Likewise, insufficient amounts of ATP were found in the ADP preparations to account for the light observed. Furthermore, various commercial sources of ADP and AMP were found to be equally effective. In addition, the AMP used was not contaminated with ADP, which was measured by coupling adenylate kinase to the firefly system. The activities exhibited by AMP and ADP, therefore, were not due to contamination with ATP.

The possibility existed, however, that the AMP and ADP activities could be attributed to phosphorylations in the crude extract leading to the formation of ATP. This possibility can be ruled out since AMP and ADP are equally effective in a phosphate-free system as well as in the presence of 2,4-dinitrophenol (10^{-2} *M*, final concentration). The method of FISKE AND SUBBAROW[7] was used to determine the absence of inorganic phosphate in these experiments. In addition, adenylate kinase could not be demonstrated in the crude extracts since, under anaerobic or aerobic conditions, no ATP formation could be detected from ADP using the firefly assay. Thus, the AMP and ADP activities are not due to contamination with ATP or to their conversion to ATP.

At this point it is significant to note that crude extracts contain a phosphatase(s)

that causes the liberation of Pi from ATP and ADP. In one experiment, for example, it was found that a preparation containing 1.5 mg protein/ml caused the release of 0.5 and 1.0 μmole of Pi/10 min from ADP and ATP respectively under the experimental conditions given in MATERIALS AND METHODS. Under conditions that support luminescence then, these extracts apparently convert ATP to ADP, whereas ADP is apparently converted to AMP. That these products are actually formed via phosphatase action was confirmed by separation and identification of the nucleotides on formate-charged Dowex-1 columns, as described by HURLBERT *et al.*[8]. The fact that ATP can be broken down to ADP and AMP in the crude extracts, whereas AMP or ADP are not converted to ATP, suggest that ATP functions as a generating system for ADP and AMP and that one (or both) of the latter two nucleotides is directly responsible for driving the light reaction. This suggestion is supported by additional data included later in the text.

The effect of nucleotide concentration on the luminescence of *Renilla* extracts is illustrated in Fig. 1. The data in Fig. 1 show that ATP is more effective than ADP or AMP at the lower concentrations, and that the response obtained with ADP is somewhat less than that with AMP. The higher efficiency of ATP at lower concentrations is also shown by calculation of the apparent Michaelis constants, which, for the ATP reaction, is approximately one-fifth that of the ADP or AMP stimulated reactions. Since generating systems are frequently more efficient than substrate levels of a reactant, the higher efficiency of ATP could be explained on the basis that it acts as a generating system for ADP and AMP.

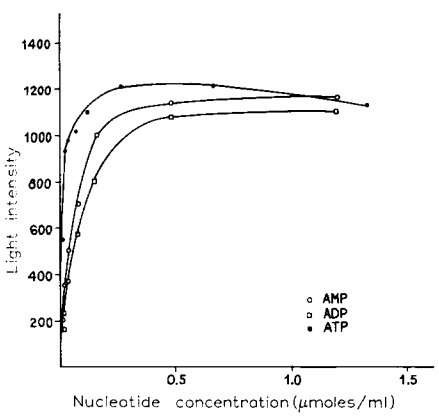

Fig. 1. Effect of nucleotide concentration on the luminescence of *Renilla reniformis* extracts. Conditions are the same as described under MATERIALS AND METHODS except that the nucleotides were added as indicated.

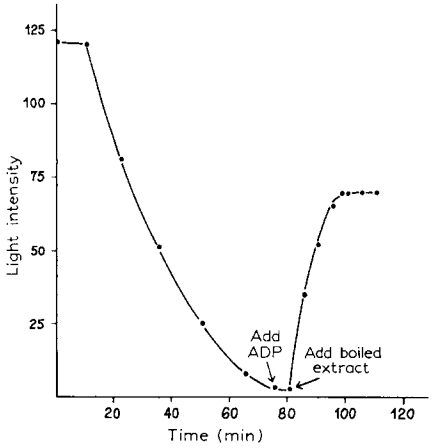

Fig. 2. Effect of boiled extract on luminescence after luminescence decay. Conditions are the same as described under MATERIALS AND METHODS with the exception that ADP and boiled extract were added as indicated.

A requirement for metal ions could not be demonstrated regardless of the nucleotide added. This was true even after repeated ammonium sulfate precipitations followed by dialysis. The lack of a metal requirement in the case of ATP is surprising if the ATP effect is to be explained by ATPase activity. It is not unusual, however, to have difficulty showing metal requirements for this type reaction in relatively crude extracts.

Since other bioluminescence systems require an oxidizable substrate, termed luciferin, for luminescence, an attempt was made to demonstrate the necessity for luciferin in this system. The requirement for an additional factor became apparent by studying luminescence decay as a function of time. Fig. 2 shows that, at the end of approximately 70 min, the luminescence rate decays to a negligible value that is not affected by the addition of nucleotides. Although ADP is added in this case, ATP and AMP are also without effect. Nucleotides are required, however, to bring about the luminescence decay. For example, an extract incubated 70 min in the absence of nucleotides responds to nucleotide addition in a manner analogous to non-incubated extracts. The addition of boiled extracts promotes a rapid return to a steady state value, which depends upon the amount of boiled extract added. Thus, the decay of luminescence is brought about as the result of a nucleotide-dependent utilization of some unknown factor required for luminescence. The factor in the boiled extract, which was been found to be dialyzable, will be referred to as *Renilla* luciferin by analogy to other bioluminescence systems.

Oxygen, as well as nucleotides, is required for luciferin utilization, since a decrease in luminescence activity does not occur by incubation of the complete system under anaerobic conditions. The effect of oxygen on the luminescence of *Renilla* extracts is illustrated in Fig. 3. Apparently, normal oxygen tension is not sufficient to saturate this system, since flushing with oxygen causes an increase in the steady state luminescence rate whereas flushing with air does not. The light is essentially abolished in 2 min by flushing with nitrogen, and the signal is returned by subsequently flushing with oxygen. The somewhat lower steady state value following the second oxygen flushing is due to some enzyme inactivation as a result of agitation during the flushing process.

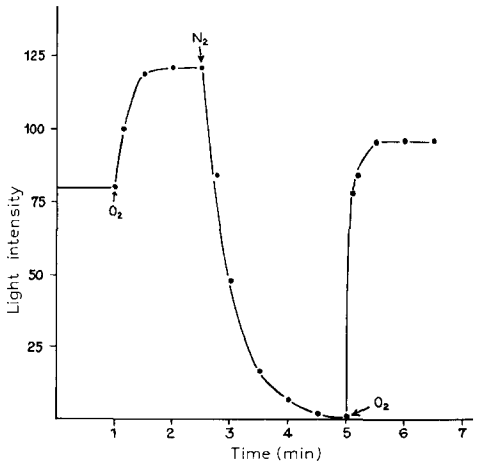

Fig. 3. Effect of oxygen on luminescence. Conditions are the same as described under MATERIALS AND METHODS except that the system was flushed alternately with oxygen and nitrogen as indicated.

Thus the luminescent reaction involves a nucleotide-dependent oxidation of luciferin. Indeed, some preliminary observations on the properties of luciferin indicate that it is oxidizable under certain conditions. For example, luciferin activity is

destroyed by heating at 100° for 1 min in the presence of 0.06 *N* HCl and oxygen, but is stable at this temperature and HCl concentration providing the system is kept anaerobic.

An enzyme system whose luminescence has been allowed to decay to a negligible level in the presence of nucleotide can be treated with ammonium sulfate to precipitate the enzyme and the latter (resolved enzyme) used as an assay for the unknown factor. When this is done, the effect of increasing concentrations of boiled extract on the light intensity can be studied. The results in Fig. 4 show that 80-fold stimulations of luminescence were observed upon the addition of sufficient amounts of crude luciferin.

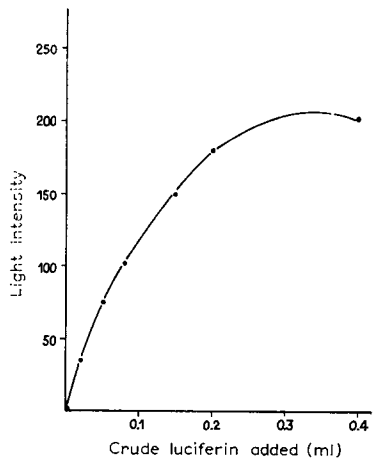

Fig. 4. Effect of luciferin concentration on luminescence. Conditions are the same as described under MATERIALS AND METHODS except that luciferin was added as indicated.

TABLE II

EFFECT OF VARIOUS LUCIFERIN PREPARATIONS ON THE LUMINESCENCE OF *Renilla* EXTRACTS

Conditions: Same as described under MATERIALS AND METHODS except that the source of luciferin was that listed in the table, and the enzyme preparation was a resolved enzyme of the type used to obtain the data in Fig. 4.

Source of luciferin preparation added	*Light intensity*
None	0
Renilla	60
Firefly	0
Cypridina	0
Bacterial ($FMNH_2$)	0
Fungal	0

No increase above background is observed in the absence of added nucleotide. The apparent saturation at higher levels may be due to inhibitors of luminescence in the boiled extract. Luciferin cannot be replaced by divalent cations such as magnesium or manganese and many others that were tested. No luciferin activity could be demonstrated by the addition of long chain aldehydes which are involved in bacterial luminescence, yeast concentrates, or of crude extracts from beef or rat liver, heart, kidney or spleen. After each of these additions, the luminescence system would still respond to the addition of boiled *Renilla* extract. Thus *Renilla* luciferin cannot be demonstrated in a variety of naturally occurring materials. Similar observations have also been made in this laboratory for fungal and firefly luciferins.

Luciferin preparations from a number of bioluminescent systems were tested for activity in the *Renilla* luminescence system. Table II shows that firefly*, *Cypridina*, bacterial, or fungal luciferin will not replace *Renilla* luciferin. All luciferin preparations were shown to be active in their respective luminescence systems prior to testing them with *Renilla* luciferase. Likewise, *Renilla* luciferin will not react in any of the

* The author wishes to express his appreciation for the generous gifts of firefly and *Cypridina* luciferins by Drs. W. D. McELROY and E. N. HARVEY respectively.

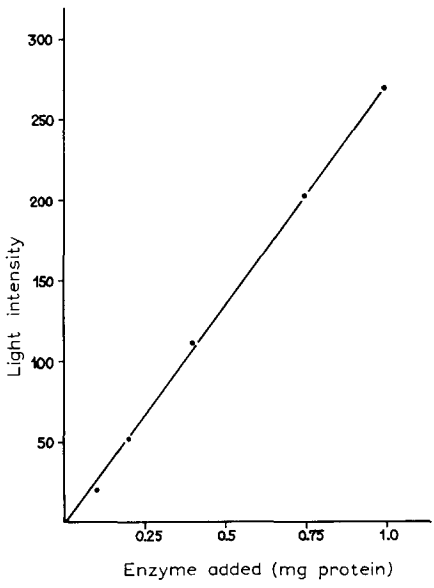

Fig. 5.

Fig. 5. Effect of enzyme concentration on luminescence. Conditions are the same as described under MATERIALS AND METHODS except that the enzyme was added as indicated.

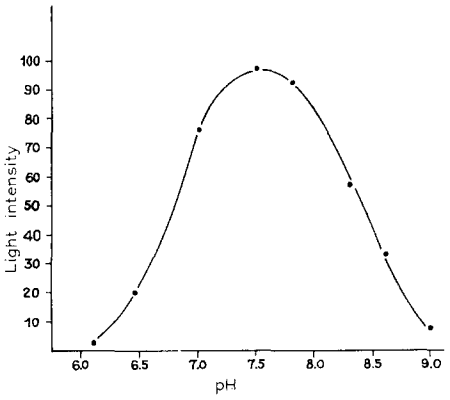

Fig. 6.

Fig. 6. Effect of pH on luminescence. The final pH was varied over the range indicated and the conditions are the same as described under MATERIALS AND METHODS.

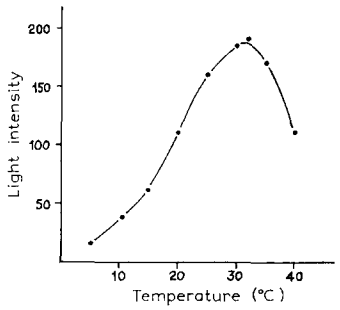

Fig. 7.

Fig. 7. Effect of temperature on luminescence. The temperature was varied as indicated. The procedure followed and the conditions used are the same as that described under MATERIALS AND METHODS.

above mentioned luminescence systems. *Renilla* luciferin is specific, therefore, for *Renilla* luciferase.

Fig. 5 illustrates the effect of luciferase concentration on luminescence. A typical straight line response is obtained with increasing enzyme concentration.

The response of this luminescence system to pH is shown in Fig. 6. The activity declines on the acid side of neutrality, but shows considerable activity over a relatively wide range on the alkaline side with an optimum at pH 7.5.

Fig. 7 illustrates the effect of temperature on luminescence. Under these experimental conditions, the optimum for this reaction is 32°.

Although the spectral energy distribution of the light from this luminescent reaction has not been determined, the luminescence is visible to the 5 min dark-adapted eye, and some visual observations have been made. The greenish-blue color of the light in extracts appears to the eye to be the same color as that emitted from the intact animal.

Inhibitor studies

A number of well known inhibitors of other bioluminescent systems, as well as classical enzyme inhibitors, were tested for their inhibitory action on *Renilla* luminescence. Table III shows that potent inhibitors of bacterial luminescence such as menadione and cytochrome *c*[2,9] do not inhibit luminescence of *Renilla* extracts, indicating that these compounds do not oxidize *Renilla* luciferin as they do bacterial luciferin ($FMNH_2$).

Fluoride is a partial inhibitor at high concentrations, but only when ATP is added. For example, when ADP is used, fluoride does not inhibit luminescence as shown in Table III. Similar results were obtained with AMP. In the presence of ATP, however, a 33 % inhibition is observed. Under the same conditions, a 34 % inhibition

TABLE III

EFFECT OF INHIBITORS ON *Renilla* LUMINESCENCE

Conditions: Same as listed under MATERIALS AND METHODS. Inhibitors added as indicated.

Addition	*Final concentration (M)*	*Per cent inhibition*
NaF	10^{-1}	0
Menadione	10^{-4}	0
Cytochrome c	10^{-5}	0
KCN	10^{-3}	0
NaN_3	10^{-3}	0
FMN	10^{-5}	17
FAD	10^{-5}	8
Na_3ASO_4	10^{-3}	0
Versene	10^{-3}	58
	10^{-2}	63
Sodium pyrophosphate	10^{-3}	0
	$3 \cdot 10^{-2}$	10
PCMB	10^{-4}	93
N-ethylmaleimide	10^{-4}	31
$CuSO_4$	10^{-4}	100
	10^{-5}	85
$AgNO_3$	10^{-4}	100
	10^{-5}	63

of ATPase activity by fluoride was observed. These observations support the suggestion that ATP is involved indirectly in this system via an ATPase reaction.

The respiratory inhibitors cyanide and azide are non-inhibitory at the concentrations tested. FMN and FAD are slightly inhibitory at 10^{-5} *M*, whereas arsenate does not inhibit.

It has been shown, in the case of firefly luminescence, that inorganic pyrophosphatase can adversely affect the steady state luminescence[11]. Likewise, inorganic pyrophosphate can either inhibit or stimulate luminescence depending upon whether it is added prior to or after ATP. These results have been explained in terms of a primary ATP-dependent activation reaction that results in the formation of luciferyl adenylate and pyrophosphate[10]. As seen from Table III, inorganic pyrophosphate has little or no effect on *Renilla* luminescence. This is true regardless of the order in which pyrophosphate is added. Furthermore, the addition of inorganic pyrophosphatase (300–600 units) has no effect on luminescence of this system. These observa-

tions are in direct contrast to those made by McELROY *et al.*[11] on firefly luminescence, and indicate a difference in the mechanism of ATP action in the two systems. In addition, it would be difficult to explain the stimulation of *Renilla* luminescence by AMP on the basis of identical mechanisms.

A requirement for free sulfhydryl groups in the luminescent system is indicated by the inhibitory action of PCMB, N-ethylmaleimide, and copper and silver salts as shown in Table III. The inhibition produced by PCMB can be partially reversed (to 60 % of the initial response) by the addition of 4 μmoles of GSH. In addition, GSH has been found to help maintain luciferase activity during initial purification steps.

Regardless of the nucleotide used in the assay system, a large number of metal ions have no effect on the activity of the crude extract at 10^{-3} M concentrations. These include magnesium, manganese, nickel, calcium, cobalt, zinc, molybdenum, and iron. Although metal ions have no effect on the crude extract, chelating agents such as Versene are potent inhibitors as seen in Table III. Regardless of the nucleotide used in the test system, the inhibitory action of Versene requires approximately 15 min incubation with the test system to reach a maximum level. The addition of magnesium or manganese ions, in amounts in excess of that of Versene, does not reverse the inhibition. Thus, Versene inhibition cannot be explained simply in terms of binding one of these metal ions.

In the absence of phosphate, zinc ion is a potent inhibitor of luminescence. Under these conditions, for example, luminescence is completely inhibited at $2 \cdot 10^{-3}$ M zinc ion. The lack of zinc inhibition in phosphate buffer is due to the fact that an insoluble zinc phosphate forms at the pH used in the assay system.

Although the luminous bacteria and sea pansies discussed here are both marine organisms, the properties of the enzymes involved in the two luminescent systems differ considerably in their response to salt. For example, salts such as NaCl and KCl inhibit the luminescence system of *Renilla*. The inhibition increases linearly with increasing salt concentration, 50 % inhibition being reached at approximately $2 \cdot 10^{-1}$ M salt. The luminescence of extracts of luminous bacteria, on the other hand, is stimulated by increasing salt concentrations, reaching a maximum steady state at approximately $1.7 \cdot 10^{-1}$ M salt[13].

DISCUSSION

Although ATP is quite active in supporting luminescence in crude extracts of *R. reniformis*, the data presented suggest that ATP functions indirectly via an ATPase reaction. The products of this reaction are ADP and AMP, and one of these must be responsible for supporting luminescence in this system. This suggestion is based on a number of observations. Firstly, the system is specific for adenine-containing nucleotides. Secondly, the AMP and ADP used were not contaminated with ATP nor are they converted to ATP by phosphorylation reactions. In addition, the lack of an adenylate kinase in the extracts ruled out the formation of ATP from ADP. Thirdly, the crude extracts contain ATPase activity that rapidly converts ATP to ADP and AMP. Thus it appears that ATP functions simply as a generating system for ADP and/or AMP.

Other supporting data for the indirect role of ATP in this system involves studies with inhibitors. For example, fluoride will partially inhibit ATP-stimulated lumi-

nescence, but has no effect on the ADP or AMP driven reactions. The fact that the crude extracts contain an ATPase that is also partially inhibited by fluoride is consistent with the view that ATP functions as a generating system for ADP and AMP via an ATPase reaction.

Recent studies on the purification of *Renilla* luciferase in this laboratory have shown that as the ATPase activity is removed, the luminescence response to ATP drops accordingly, while the response to ADP or AMP is unaffected. In addition, recent studies with partially purified fractions show much faster kinetics for ADP and AMP as compared to ATP. These recent observations leave no doubt that ATP plays an indirect role in *Renilla* luminescence. Further studies on the purification of *Renilla* luciferase, and on the mechanism of nucleotide action in this system, are in progress.

The above considerations indicate a distinct difference in the biochemical mechanisms involved in firefly and sea pansy bioluminescence. Whereas firefly luminescence requires a primary activation by ATP, sea pansy luminescence is stimulated indirectly by ATP. The data indicate the direct participation of ADP or AMP, rather than ATP, in the luminescence reaction of the sea pansy.

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