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SURVEY OF BIOLUMINESCENCE RESEARCH PERTINENT TO EXPLOSIVES DETE--ETC(U)
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SURVEY OF BIOLUMINESCENCE RESEARCH
PERTINENT TO EXPLOSIVES DETECTION

November 1976

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This report presents a literature review of aspects of bioluminescence pertinent to explosives detection including the origins of the bioluminescent property, its generation, growth, and the effects of environmental parameters such as temperature, pressure, pH, and previous history. The structure of the bioluminescent species is discussed including the form contributory to the bioluminescent phenomenon; spectroscopic and chemical observations are included.

An appendix detailing MERADCOM's efforts in bioluminescent detection is included which illustrates the sensitivity and specificity of the specific micro-organisms developed for TNT vapor detection. The responses to various chemicals both like and unlike TNT and ways and means of increasing the sensitivity and specificity were part of the MERADCOM efforts. The cost of the MERADCOM effort from 1970 to 1974 is included.

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SURVEY OF BIOLUMINESCENCE RESEARCH PERTINENT TO EXPLOSIVES DETECTION

I. INTRODUCTION

1. **Background.** The luminescence of living things is commonly known as bioluminescence and is best known in the firefly, glowworm, and many small creatures of the sea. The ability to glow gives rise to the phosphorescence of the sea. A fairly large number of groups of organisms in both plant and animal kingdoms are luminous, and they are scattered in a rather haphazard way on the evolutionary tree of life from the very simplest forms, mere single cells, to such complicated vertebrates as bony fishes.

Bioluminescence has been viewed as a special case of chemiluminescence. Chemiluminescence has been viewed as a reaction mechanism in which the energy from an exergonic chemical reaction is converted to light energy. The emission of light following its selective absorption is referred to as luminescence. It is a general term for the emission of light from a molecule following its excitation through the absorption of any form of energy.

The most interesting and, indeed, necessary aspect of bacterial luminescence is the low temperature at which it is produced. From the earliest observations of man, objects which gave off light without accompanying heat have excited his interest and comment. Aristotle wrote, "It is the nature of smooth things to shine in the dark; e.g., the heads of certain fishes and the juice of the cuttlefish."¹ We know today that he was referring to dead animals, all of which may light as a result of the growth of luminous bacteria on the flesh, which is excellent culture medium. Even prior to Aristotle, phosphorescence of the sea was compared to thunder and lightning. Lightning was thought to be a result of an explosion in the clouds, while a similar light attended the explosion when struck by an oar. Since then, many different and recurrent explanations have been advanced for this striking phenomenon as science has progressed. It is now certain that phosphorescence of the sea always comes from one or another species of luminous animals: some are so small they can be detected only with a microscope; some are large enough to be easily visible; many are of value for the chemical study of light production. Three organisms have been of particular value for chemical work: (1) A small marine ostracod crustacean, cypridina, (2) the firefly, and (3) luminous bacteria also found in the sea. All of these can be obtained in large quantities and prepared in various ways.

While many examples pertaining to specific species and reactions will be given, these are not intended to include all such observations but, rather, are intended

¹ Science, *American Scientist* 45, 372-378 (1957).

to illustrate the methods being discussed. To further delineate the scope of this review, several additional definitions are in order. Chemiluminescence is the emission of radiation from a chemi-excited species; chemi-excitation is a process by which the excited species are formed as a direct result of the formation of new chemical bonds. Analogously, bioluminescence is the emission of radiation from bio-excited species, and bio-excitation is the process by which the excited species are formed.

The biochemical pathway leading to the light emission in the sea pansy, *renilla reinformis*, is fairly well understood. Luciferyl sulfate, an inactive storage form of luciferin, is converted to luciferin by the enzyme luciferin sulfokinase. Luciferin is oxidized in the presence of oxygen and luciferase (*renilla*) to CO₂ and oxyluciferin in an electronically excited state. Return to the ground state of oxyluciferin results in the production of blue light. In addition, the structure determination and the chemical synthesis of a biologically active form of *renilla* luciferin has been accomplished by Hori and Cormier.²

A major problem in the study of bioluminescence has been in relating the in vitro reaction, above, to the in vivo bioluminescence. The in vitro reaction produces a blue emission (maximum = 490-nanometer band half width = 78 nanometers) which is in contrast to the in vivo green emission (maximum = 509-nanometer band half width = 20 nanometers). The green in vivo emission has been postulated to be due to the energy transfer from the electronic excited state of oxyluciferin to a second chromophore. A protein-bound chromophore has been isolated from *renilla* which exhibits fluorescence characteristics identical to the in vivo bioluminescence and, thus, must be in the in vivo emitter.

In the hydrozoan, *aequorea*, bioluminescence arises from the interaction of calcium ions and a protein, termed photoprotein luciferase, such as is found in *aequorea*. *Aequorea* does, however, contain luciferyl sulfate and a green fluorescent protein. A calcium-activated photoprotein has been extracted from *renilla*. The data, then, suggest that a common biological mechanism underlies the bioluminescence in coelenterates. The most recent finding of Hori and Cormier that a compound involved in the luminescence of *aequorea* photoprotein is an integral part of the structure of *renilla* luciferin reinforces this concept.

It is obvious from the above discussion that the individual components of the *renilla* bioluminescent system leading to light emission in vitro have been isolated and studied separately. The organization of these compounds into a biological system

² Kazuo Hori and Milton J. Cormier, "Structure and Synthesis of a Luciferin Active in the Bioluminescent Systems in the Sea Pansy (*Renilla*) and Certain other Bioluminescent Coelenterates," *Chemiluminescence and Bioluminescence*, Edited by M. J. Cormier, D. M. Hercules, and J. Lee, (Plenum Press: New York-London, 1973), pp. 361-368.

which explains the production and control of renilla in vivo bioluminescence remains to be determined. The transfer of energy from the excited state of oxyluciferin to the green-emitting chromophore must require protein-protein interaction between the two proteins involved. Furthermore, the existence of a calcium-activated protein in a cell requires protection of that protein from cellular supplies of calcium ion.

2. Experimental Observations. Some of the earlier findings of bacterial luminescence are as follows:

a. Bacterial luminescence is a respiratory phenomenon that has an absolute requirement for O₂, although luminescence is less sensitive to low O₂ than respiration (Eymers and Van Schouwenberg,³ 1937; Shoup,⁴ 1929).

b. The light respiration is essentially cyanide insensitive (Harvey,⁵ 1920), although various organic compounds, particularly naphthoquinones, are strongly inhibitory to it (Spruit and Schuiling,⁶ 1945; McElroy and Kipnis,⁷ 1947).

c. Ultraviolet light inhibits luminescence and shows a discrete "inactivation spectrum" (Gerrassen,⁸ 1915).

d. The light emitted is blue-green in color showing a band with a maximum at 500 nanometers for a number of species investigated (Spruit-Van de Burg,⁹ 1950).

e. Luminescence shows a temperature dependence similar to that of many respiratory processes (Johnson et al.,¹⁰ 1942).

f. Pressure-temperature studies show that luminescent systems behave as a typical protein enzyme; that is, bacterial luminescence is independent of oxygen pressure at pressures of oxygen above 10⁻¹⁰ atmospheres. Temperature studies indi-

³ J. G. Eymers and K. L. Van-Schouwenberg, "On the Luminescence of Bacteria," *Enzymologia*, *1*, 328-340 (1937).

⁴ C. S. Shoup, "The Respiration of Luminous Bacteria and the Effect of Oxygen Tension upon Oxygen Consumption," *J. Gen. Physiol.* *5*, 265-275 (1929).

⁵ E. N. Harvey, "Is the Luminescence of Cypridina an Oxidation?" *Am. J. Physiol.* *51*, 580-587 (1920).

⁶ C. J. P. Spruit and A. L. Schuiling, "On the Influence of Naphthoquinones on the Respiration and Light Emission of *Photobacterium Phosphoreum*," *Rec. Trav. Chim.* *64*, 220-228 (1945).

⁷ W. D. McElroy and D. M. Kipnis, "The Mechanism of Inhibition of Bioluminescence by Naphthoquinones," *J. Cellular and Camp. Physiol.* *30*, 359-380 (1947).

⁸ F. C. Gerretsen, "Die Einwirkung des Ultravioletten Lichtes auf Leuchtakterien," *Zbl. Bakt. (Abt. 2)* *44*, 660-661 (1915).

⁹ A. Spruit-Van de Berg, "Emission Spectra of Luminous Bacteria," *Biochim et Biophys Acta.* *5*, 175-178 (1950).

¹⁰ F. H. Johnson, D. E. S. Brown, and D. A. Marsland, "A Basic Mechanism in the Biological Effects of Temperature, Pressure and Narcotics," *Science* *95*, 200-203 (1942).

cate that an optimum temperature exists above and below which the intensity of luminescence decreases rapidly (Johnson,¹¹ 1947).

g. The yield of luminescence/O₂ consumed is about 1/100 to 1/1,000 (Van Schouwenberg and Eymers,¹² 1936).

h. Attempts to extract the active functionaries of the luminescent system, to isolate the identity of its components, and to establish the mechanism and kinetics of the light-giving substances have until very recently been unsuccessful and not capable of confirmation.

Many strictly chemical reactions have been observed to emit light. Some of those are:

- a. Hydration of calcium and barium oxides and of calcium chlorides.
- b. Oxidation in solution by hypohalogen (iodite, bromite, or chlorite), perhalogen, or acid permanganate as in the case of pyrogallol.
- c. Oxidation of organic compounds by heating in air or by heating with anhydrous alkali in air.
- d. Oxidation of higher alcohols, aldehydes, fatty acids, amides, polyphenols, sugars, urea, albumin, and a large variety of biological and organic compounds by ozone, oxygen, or hydrogen peroxide in alkaline or alcoholic potassium hydroxide solutions.
- e. Action of oxygen or the halogens on vapors of the alkali metals, on ammonia, on molten palmitic, and on stearic acids and their salts.
- f. Neutralization of alkaline oxides with concentrated acids.
- g. Cold flames: either vapor preheated to 260° C upon the admission of air gives a bluish, low-temperature flame. Stearic acid at 280° C; oleic acid, olive oil, and paraffin wax at 310° C; and sulfur at 180° C also give low-temperature, visible flames.
- h. Grignard reactions: the reactions between phenylmagnesium bromide or iodide and chloropicrin.

¹¹ F. H. Johnson, "Bacterial Luminescence," *Advances in Enzymol.* 7, 215-264 (1947).

¹² K. L. Van Schouwenberg and J. G. Eymers, "Quantum Relationship of Light-Emitting Process of Luminous Bacteria," *Nature* 138, 245 (1936).

- i. Action of halogen vapors on mercury.
- j. Reactions between sodium vapor and vapors of mercuric chloride, iodide and cyanide, cadmium iodide, hydrogen chloride, and potassium trichloride.
- k. Oxidation in air of phosphorous, phosphorous trichloride, and freshly cut surfaces of sodium and potassium at 70° C.
- l. Oxidation of unsaturated silicon compounds.
- m. Recombination of hydrogen atoms and oxygen molecules.
- n. Bioluminescence: enzymed catalyzed oxidation by oxygen of specific substrate molecules in biological systems.
- o. Anodic oxidation in electrolytic cells.
- p. Thermal decomposition reactions.

Since the production of light in chemical systems arises from many sources and parameters and since the origin of bacterial luminescence may be related to a chemical source, it is not possible to extrapolate from one luminescent system to another or from the behavior of one luminescent organ to that of another. However, since the emission of light by organisms is not restricted to any one group of animals or plants, one is led to believe that the cause of luminescence is a chemical reaction fundamental to all organisms. Still, the blue-green glow emitted by various species of luminous bacteria has been for a long time a recurrent object of curiosity among biologists, chemists, and physicists; more recently, possible applications of a practical nature have advanced the study and investigations of the phenomena. The biologist has shown interest in the evolution, selective advantage, and relation of the light production to other functions of organisms; the chemist concerns himself with the mechanism of excitation, the chemical identity of the reacting molecules and their products, and the kinetics of the reaction; while the physicist is primarily interested in the energetics and kinetics of the process and the effect of well-defined environmental variables on the light emission. Aspects of all three technologies and some of their effects on present-day knowledge of bioluminescence, including aspects of military applications, will be discussed in this report.

Before 1953,¹³ ¹⁴ little had been known about the chemical processes

¹³ B. L. Strehler, "Luminescence in Cell-Free Extracts of Luminous Bacteria and its Activation by DPN," J. Am. Chem. Soc. 75, 1264 (1953).

¹⁴ B. L. Strehler and M. J. Corimer, "Factors Affecting the Luminescence of Cell Free Extracts of the Luminous Bacterium *Achromobacter Fischeri*," Arch. Biochem. and Biophys 47, 16-33 (1953).

connected with the light production except to say that a substance, luciferin, appeared to be oxidized by dissolved oxygen in the presence of the enzyme, luciferase. Light production resulted from this catalytic oxidation. It was chemiluminescence. In the firefly, another compound, adenosinetriphosphate, appeared to be necessary. More recently, it has become clear that there are many different luciferins in different organisms and that these luciferins are totally different organic compounds. Within the last few years, (1961) the chemical structure of the luciferin complex has become known, a second type of luciferin has been isolated in the pure state, and both the luciferin and the luciferase of the firefly have been obtained in the crystalline form (Figure 1).

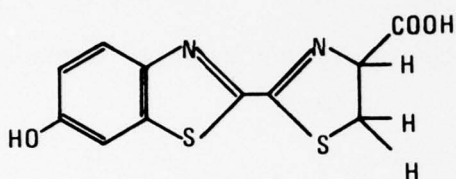


Figure 1. Firefly luciferin. (From E. H. White, in *Light & Life*, W. D. McElroy and B. Glass, Eds., Baltimore: The Johns Hopkins Press, 1961, p. 183.)

These discoveries have placed the chemistry of light production on a firm foundation and have made it possible to discuss the beginning and the evolution of bioluminescence in a logical and satisfactory manner. What follows immediately is a presentation on the latest views on the origin and mechanism of bacterial light emission.

Chemically, bacterial luciferin appears to be an aldehyde complex of dihydroflavin, mononucleotide (FMNH₂ for short) (Figure 2).

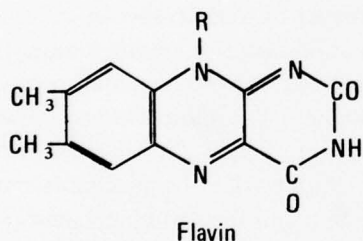
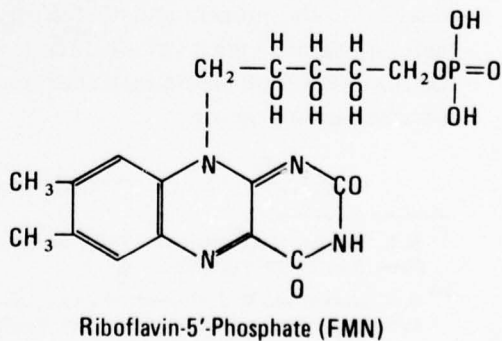
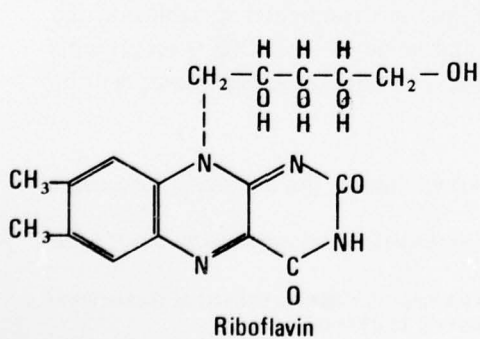


Figure 2. Derivation of FMNH₂.

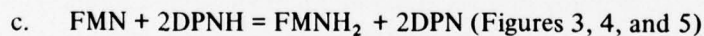


The compound is allied to riboflavin, which takes part in one of the important steps in oxidation of foodstuffs in the cell. Bacterial luciferase appears to belong to the group of flavin enzymes also important in cell respiration. The aldehyde must be a long chain containing more than six carbon atoms. If FMNH₂ and aldehyde in aqueous solutions are mixed with a cell-free luciferase solution extracted from luminous bacteria and containing oxygen, a bright flash of light will appear. The exact part played by the aldehyde is not fully understood; but, there is little doubt that during the luminescence FMNH₂, probably combined with the aldehyde, is oxidized to FMNH with the emission of light, while the aldehyde passes to an acid with intermediate peroxide formation.

One difference between luminescence in the plant kingdom (bacteria and fungi, the latter responsible for the light of rotten wood) and the animal kingdom has to do with the character of the light. Animals light only on stimulation; for example, mechanically when disturbed by agitation of the sea or by nerve action as in the flash of a firefly. Bacteria and fungi emit light all the time quite independent of any stimulation. Since bacteria luminesce continuously, there must be some way to keep the luminous chemical reaction going on in the bacterial cell. This is done by another compound, reduced diphosphopyridin nucleotide (DPNH for short), also involved in respiration. DPNH is important in reducing the FMN to FMNH₂, thus maintaining a steady supply of luciferin in the bacterium with constant emission of light. The reactions which proceed in a bacterial cell during the emission of light can be visualized from the following equations:



The long-chain aldehydeflavin mononucleotide complex



The second step is the light-emitting step, which is catalyzed by luciferase and probably takes place through the intermediate formation of a peroxide to account for energy represented by the blue light of the bacteria. The third step represents the reformation of dihydroflavin mononucleotide, which can again combine with new long-chain aldehyde and start the cycle again. This step is catalyzed by DPNH oxidase. When one mixes solutions of FMN, DPNH, aldehyde, and crude bacterial luciferase extract in water containing oxygen, it is possible to obtain a long-lasting light which completely mimics the steady bioluminescence of bacteria. At the present time, enzymes are the only substances which must be extracted from the living cell. All the other compounds necessary for luminescence can be synthesized.

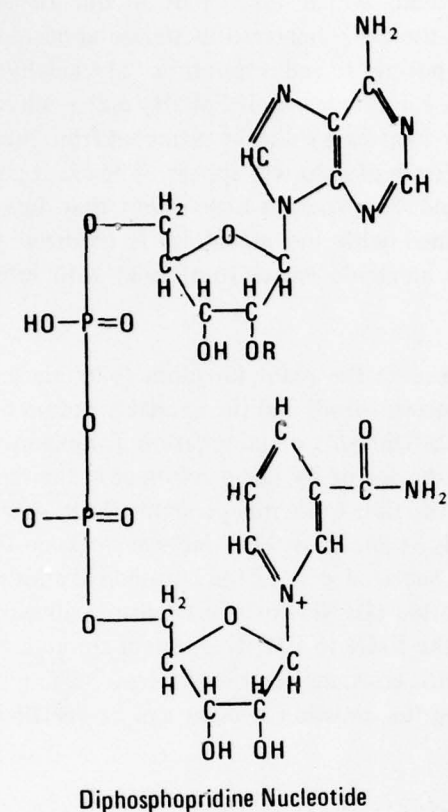


Figure 3. DPN (cozymase).

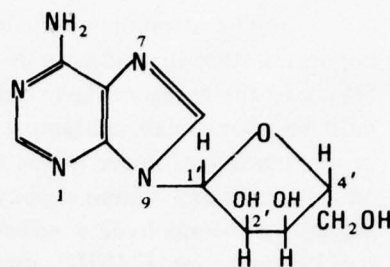


Figure 4. Adenosine.

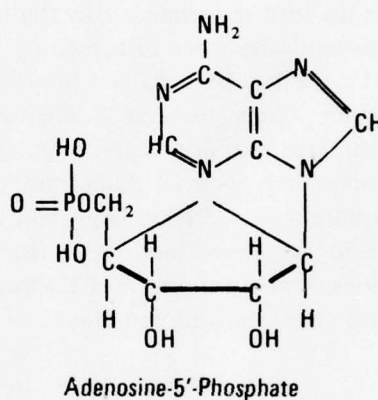


Figure 5. Adenosine 5'-monophosphoric acid (adenylic acid).

The chemistry of another luminous animal, the firefly, has been studied intensely by W. D. McElroy and his group at Johns Hopkins University in Baltimore. The actual fireflies used for experimentation were caught by small children who were paid 25 cents/100. The insects were then kept in a deep-freeze until ready for extraction. Literally tens of thousands have been used in this manner, and the city of Baltimore was known as the firefly capital of the world.

In the firefly, the mechanism of light production is quite different from that of bacteria. Luciferin, luciferase, adenosine triphosphate (ATP), magnesium ion, and oxygen are all necessary. Both luciferin and luciferase have been crystallized by the McElroy group, and the elementary composition of the firefly luciferin is known. The empirical formula is $C_{13} H_{12} N_2 S_2 O_3$. One thing is certain – the firefly luciferin is

quite different from the bacterial luciferin. Harvey and coworkers have also studied the marine ostracod crustacean, 1/8-inch long, with the complete scientific name *cypridina hilgendorffii*. This animal was unknown to the ancients except as they may have seen its luminescence in displays of the mysterious burnings of the sea. Cypridinas are caught along the coasts of Japan, dried quickly, and sent to Princeton in this dried condition. They are then ground in a mortar, and the ground powder is extracted by various solvents for chemical investigation. In contrast to the luminescent systems of bacteria and the firefly, in cypridina, only luciferin, luciferase, water, and oxygen are necessary for luminescence. Again, the luciferin and luciferase differ from the corresponding compounds in the firefly and luminous bacteria. Cypridina luciferin is yellow in color but after oxidation and light emission changes to a colorless oxidation product. By means of chromatography and countercurrent distribution techniques, it has been possible to separate a pure organic substance, luciferin – which emits a bright luminescence of blue color in the presence of purified luciferase – extracted from the same organism, cypridina. The luciferin of cypridina appears to be chromopolypeptid. Luciferase from no other animal except ostracods and no extracts from any non-luminous animal thus far tested can take the place of cypridina luciferase to produce light with cypridina luciferin. The latter can be oxidized with a faint luminescence in the presence of certain peroxides and other oxidants, but this light is weak when compared for intensity with that emitted in the presence of cypridina luciferase.

Cypridina luciferase oxidizes readily in the presence of dissolved oxygen, and the oxidation product can be reduced to luciferin again by various methods which add hydrogen to the oxyluciferin, provided the reduction is carried out without delay. Otherwise, irreversible oxidation products are formed. Harvey has noted as long ago as 1918 this reversibility and stressed the importance of bioluminescence. However, in the case of cypridina, unlike many other luminous animals, the light production takes place outside the cell after the secretion of the luminous substance into the seawater. Such behavior makes this reduction of the oxidation product unnecessary in the economy of the animal. Based on the firmly established chemical evidence of the last few years, it is not possible to state very definitely that the light production of living things is due to chemical reactions in the cells of the animals or plants and to actually write the chemical equations. It is a slow oxidation, or burning, like that of phosphorous; but phosphorous is not the compound which is burnt in living organisms, as was believed in the earliest part of the 19th century. Luciferins are organic, many of which are known to emit light when oxidized in the proper manner. We have seen that at least three kinds of luciferins exist; and the word must now be used as a class term – rather than that of a single, individual substance – in the same way that the word “vitamin” is used.

Organic chemists are familiar with the compound aminophthalic hydrazide, whose oxidation results in blue chemiluminescence. Dimethyldiacridinium nitrate,

another organic compound, emits a yellow light; and some methyl porphyrin compounds, a red chemiluminescence. All these colors are characteristic of one or the other luminous animal whose spectral emission corresponds closely to that of a particular organic chemical. Indeed, the famous "railroad worm" of South America has light of two colors — a row of greenish-yellow luminous along its sides and a red light on its head; hence, the name "railroad worm." When crawling on the ground, the "worm" really resembles a train. This "worm," 2 inches (5 centimeters) long, is actually the larva of a beetle. It is so rare that the nature of its luciferins is quite unknown.

How could the light-emitting process have arisen in living matter during the course of evolution? In particular, how could luminous animals have appeared so frequently at all levels of complexity as life evolved? It cannot be emphasized too strongly that many different genera of animals contain luminous species. Moreover, one species of a genus may be luminous, and a closely related species may be non-luminous. During the course of evolution, a photogen must have appeared, just as in the evolution of vision a photosensitive pigment was the first step in the development of the eye with its unbelievable complexities for forming an image and for adjusting to light intensities varying over a millionfold. Certain deep-sea animals such as the shrimp, squid, and other fish produce light in lantern-like structures, corresponding to those of the eye, possessing lenses and reflectors to form a beam of light. In one deep-sea squid, the color of the light from certain of its lanterns is variable, possibly controlled by color screens. This rare species, *lycoteuthis diadema*, discovered by the Valdivia deep-sea expedition in 1898, emits the patriotic colors — red, white, and blue.

The fact that one species may be luminous and a closely-related species non-luminous suggests that the photogen arose suddenly. It also appeared at many different times in many different groups. There appears to be no direct evolutionary path leading to more and more complex luminous organs. Many totally unrelated animals possess luminous "spots," groups of luminous cells on the surface of the body. This chance distribution suggests that some slight change, for example, a mutation in a chemical reaction necessary for vital functions of the cell in general, started the emission of light. Harvey in 1932 predicted that luminescence would be found to have evolved in connection with the respiratory chain of reactions necessary for the oxidation of foodstuffs. Later studies on luminous bacteria have amply confirmed this prediction since both bacterial luciferin (FMNH₂-aldehyde) and DPNH as well as bacterial luciferase are allied to cell respiratory substances and enzymes.

McElroy has recently discovered that there are dark strains of "luminous" bacteria which never luminesce because they lack the long-chain aldehyde necessary for the light producing system. If these dark strains are grown on bacteriological culture media, the colonies are dark; but, it is only necessary to blow the vapors of a long-

chain aldehyde over the culture to make it luminesce. What more striking experiment than this to illustrate the first beginnings of evolution of a light producing organism by adding a material necessary for a luminescent system! Other types of "dark" bacterial mutants lack luciferin and still other types lack luciferase.

Since it is well known that the intensity of chemiluminescence of an organic molecule depends greatly on the particular groups attached to the molecule, it is easy to imagine how a luminous mutant of an ordinary dark bacterium might arise. To a certain compound necessary for cell respiration there was added a particular "photophore" group which made it luminescent. This situation is quite similar to the development of color in plants, where it has been demonstrated that the addition of the proper chromophore group to an organic compound will produce a blue or red flower instead of a white blossom. Production of light could have arisen in this way at any stage in the evolution of various groups, thus accounting for the "spotty," or haphazard, occurrence of luminous species in the animal kingdom. The appearance of such mutations makes it far easier to visualize the beginning of a complicated organ. Darwin had trouble with the "first beginning" of a complex structure and in fact wrote "it is impossible to conceive by what steps these wondrous organs (light organs of insects) have been produced."¹⁵ Today, we are in a far better position to speculate. We can understand the first beginning of light production. In fact, recent discoveries concerning bacterial mutants suggest that a luminous mutant might suddenly appear in a well-known and much-studied bacterial form such as *Escherichia coli*. Why should not the various luminous bacteria isolated from fish actually be luminous mutants or saprophytic species, which are normally nonluminous? This possibility could and should be tested. Perhaps the goal of an investigation in this field should be the development and production of a luminous yeast cell or luminous amoeba.

If the principle of natural selection is to be followed and applied, it is necessary that the ability to luminesce be of some value to the organism. Admittedly, it is practically impossible to point out the use of light to a bacterium or to a fungus mycelium or to the myriads of simple but luminescent organisms which float about the surface of the sea, blown hither and thither by the winds. Only when the animal becomes complicated and develops a pattern of behavior can we say with certainty that the light is to attract the sexes (as in the firefly) or to keep a school of fish together in the dark depths of the ocean or for recognition of the species or to scare away predators or to attract food.

Together with our ability to designate a purpose or value for light, we find that instead of more luminous spots the luminous animals in question have frequently developed accessory techniques and structures for controlling or directing the light or

¹⁵ E. N. Harvey, *American Scientist* (autumn issue), Vol. 45, No. 4, Sept. 1957, pp. 372-378.

for concentrating the light into a beam by lenses and reflectors. Indeed, among deep-sea crustacea, squid, and fish, the most complicated lanterns have been developed — lanterns whose value is unquestioned for life in a sunless world. Can natural selection account for the evolution of highly complicated structures? It seems that it can. A lantern-like luminous organ is no different from an eye in reverse. If the eye with its perfection of physical optics has evolved from a pigment spot, so, also, the luminous organs must have developed from a luminous spot.

Whatever immediate steps have been lost, one is amazed at the perfection of the end results and tends to view with incredulity the fact of evolution. However, time is a powerful aid to progress. In the course of millions of years, it must have been possible by natural selection of small, useful mutations for even the most complex light producing devices to evolve and by quite separate evolutionary pathways for widespread distribution of light production throughout the animal kingdom.

II. SCIENTIFIC INVESTIGATIONS OF THE LUMINESCENT PROCESS

3. **General.** Intercellular luminescence, like all biological processes, exhibits an optimum temperature, that is, a temperature for maximum overall reaction rate. The actual temperature varies somewhat among different species, and within a single species it may be reversibly raised or lowered by physical or chemical changes in the environment of the cells, under given conditions. A reaction of fundamental importance in determining the optimum temperature and role played by the temperature activity curve is the reversible thermal denaturation of an essential enzyme. Qualitative evidence for this reaction resides in the ready reversibility by cooling and in the diminution in luminescent intensity during momentary exposures to temperatures well above the normal optimum. Analysis of quantitative data relating the amount of reversible diminution in intensity to various temperatures above the optimum indicates that a single reaction characterized by the high heat and entropy typical of protein denaturation is primarily responsible for thermal diminution. The rapidity of the changes in intensity on either heating or cooling is indicative of a mobile equilibrium.

The simple organisms such as bacteria, protozoa, coelenterates, ctenophores, and polychaetes are seen either to luminesce continuously or to light up only in response to external stimuli; and it is only in groups with well-developed nervous systems that photogeny becomes subject to the precise sort of regulation so well exemplified in the mating signals of fireflies. The ability to emit sharply delineated flashes of light does not depend upon a highly developed nervous system but upon an intercellular light productivity.

The complex chemistry of the living cell is engineered by thousands of different enzymes, each of which is a catalyst for a particular chemical reaction. It has

gradually become clear that the cell could not function if it were simply a little bag filled with enzymes in solution. Evidence also exists that the great majority and perhaps all of the intracellular enzymes function in an environment resembling a gel or while adsorbed at interfaces or in solid-state assemblages such as exist in mitochondria and other organelles of the cell. The architectural distribution of enzymes in the cell must be extremely precise; otherwise, the different enzymes, the substrates on which they act, the thousands of reaction products, and the wide variety of substances that inhibit special reactions would become mixed chaotically. However, time is a powerful aid to progress.

The chemist who views the phenomena of luminescence concerns himself with the mechanism of excitation and the chemical identity of the reacting molecules. The products of chemical reactions closely related to and contributory to the chemical aspects of bioluminescence are pressure, temperature, moisture, etc. Two aspects of the chemistry of bioluminescence present themselves immediately: the chemistry of *in vivo* luminescence and the chemistry of *in vitro* luminescence. The main difference between the two types is that in the *in vivo* study of luminescence, the luminescence is studied prior to disturbance of the bacterial organism, while *in vitro* luminescence concentrates on the study and investigation of selected extracts of the bacterial organism. *In vitro* luminescence is accordingly the study and analysis of chemiluminescence, while *in vivo* luminescence is the study and chemical analysis of bioluminescence. Parallel studies and reviews underway on enzymes and on enzyme catalyzed luminescent reactions are in this context studies of chemiluminescent reactions. The effort in this paper emphasizes bioluminescent reactions.

The intrinsic fascination of cold light shining from living organisms or, seemingly, from the sea or decaying wood or fish has inspired more than casual observation by renowned natural philosophers and scientists at least as far back as the first century. Such persons as Pasteur, Darwin, Faraday, and Davy wondered about the causes of such phenomena. Initially, the objectives have been completely uncontaminated with views toward practical application, and only rarely have unanticipated discoveries been turned into practical use such as the bioluminescent protein aequorin from the jellyfish; aequorin provides the most sensitive known specific chemical test for calcium or strontium. The bioluminescent protein from the marine worm chaetopterus can be used in perhaps the most sensitive specific test for ferrous iron, and bacterial luminescence offers a sensitive means of detecting contamination of the atmosphere inside space vehicles by jet fuels. More recently, a bacteria resembling photobacterium fischeri, found in the local waters near Los Angeles, has been extensively investigated as a biosensor for explosives. The investigations included the growth of a culture of luminescent micro-organisms on surfaces of suitable matrices and the mutagenic growth of cultures possessing the requisite specificity and sensitivity for explosive vapors in ultratrace amounts (1 ppb and less by weight).

The mechanism by which a biosensor generates a signal involves a change in the constantly emitted light level and is affected by the following parameters: (1) The nature and state of the reactants; (2) the nature and state of the products, (3) temperature, (4) pressure, (5) presence of catalysts, and (6) concentration of reactants. The luminescent response can be varied in at least five ways: quality (spectral emission); intensity; duration; spacial distribution; and frequency, or repetition. Control of one or more of these variables normally occurs by animals. Regulation of luminescence is related to neuro-effector control in general and involves control of four mechanisms: (1) Glandular secretion, (2) muscular contraction, (3) chromophore movement, and (4) photogeny.

A large share of work in the investigation of the phenomena of luminescence has been accomplished through the study of extracts obtained from luminous bacteria. In a study conducted by Strehler, reported in 1954, the spectral distribution of the luminescence of extracts and intact bacteria was compared and found to be identical (500 nanometers). Thus, the same reactions are occurring in both instances. The emitting molecule or molecular configuration is not identified. The broad fluorescent and chemiluminescent emission may be made up of a transition from two different excited states, and in certain instances only the most energetic one is formed. Temperature greatly affects the rate of luminescent reactions. This has been observed both *in vitro* and *in vivo* by F. Johnson.¹⁶ *In vitro*, the typical effect of temperature is illustrated by Figure 6, showing a fairly rapid rise in luminescence to a maximum followed by an equal decline indicating an optimum temperature. *In vivo* luminescence occurring in *A. fischeri* is illustrated in Figure 7, also illustrating a rapid rise to a maximum at optimum wavelength, followed by a decline. For *in vitro* *P. pyralis*, Figure 8 shows the variation of luminescence with temperature. The actual temperature varies somewhat among different species, and within the same species it may be reversibly raised or lowered by physical or chemical changes in the environment of the cell. Under given conditions, a reaction of fundamental importance in determining the optimum temperature and in part the thermal activity curve is the reversible thermal denaturation of an essential enzyme. Qualitative evidence for this reaction resides in the ready reversibility, by cooling, in the diminution in luminescent intensity during momentary exposure to temperatures well above the normal optimum. Analyses of quantitative data relating the amount of reversible diminution in luminescent intensity during momentary exposure to temperatures well above the normal optimum indicate that a single reaction characterized by high heat and entropy, typical of protein denaturation, is primarily responsible for the thermal diminution. The rapidity of the change in intensity on either heating or cooling is indicative of a mobile equilibrium. The simplest explanation is that an equilibrium exists between the native (active) and

¹⁶ "The Luminescence of Biological Systems," *Amer. Assoc. for the Advancement of Science (1955) Proceedings of the Conference on Luminescence* (Ed. by Frank Johnson), March 28 - April 2, 1954, p. 209.

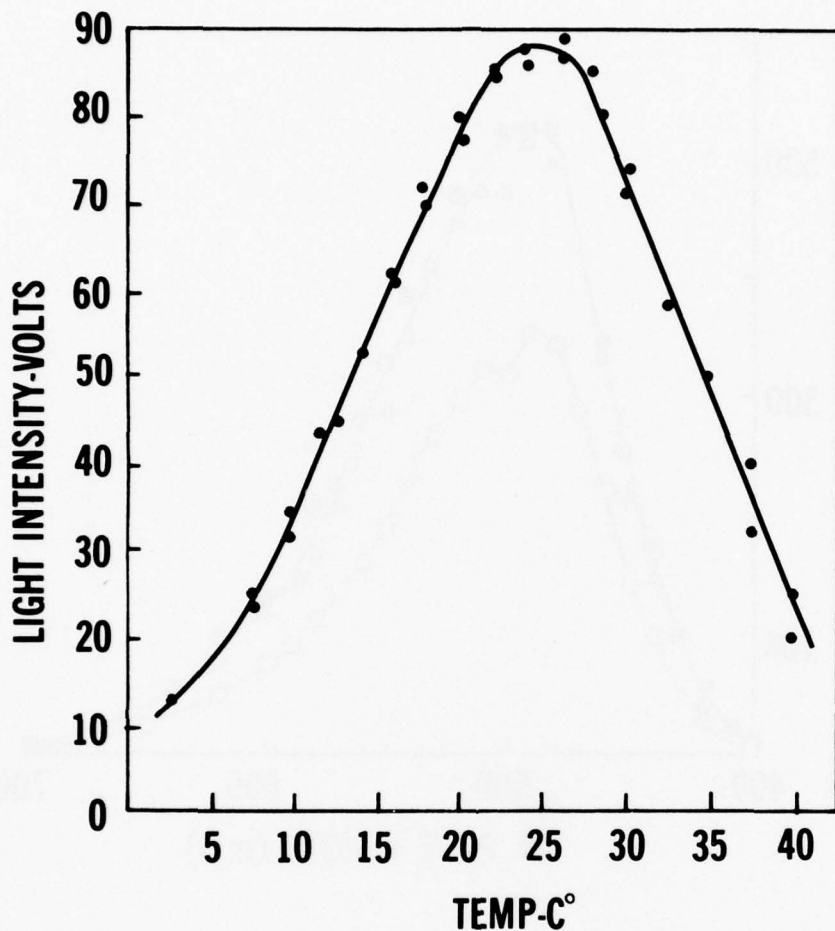


Figure 6. Effect of temperature on firefly luminescence. (From McElroy and Strehler, Arch. Biochem. & Biophysic 22, 420-433 (1949). Copyright 1955 by the American Association for the Advancement of Science.)

denatured (inactive) form of an enzyme essential to the overall process of light emission. Such an equilibrium coupled with the catalytic reaction of a limiting enzyme is sufficient to account for a major part of the temperature activity curve. Figure 9 represents the observed intensity (visual photometry) of the steady state luminescence in a suspension of *A. fischeri* cells during brief exposures to various temperatures above and below the optimum. The extremely high apparent activation energy observed cast some doubt on the meaning of this measurement (the presence of some artifact being indicated) since such high activation energy would hardly allow the reaction to proceed at significant rates.

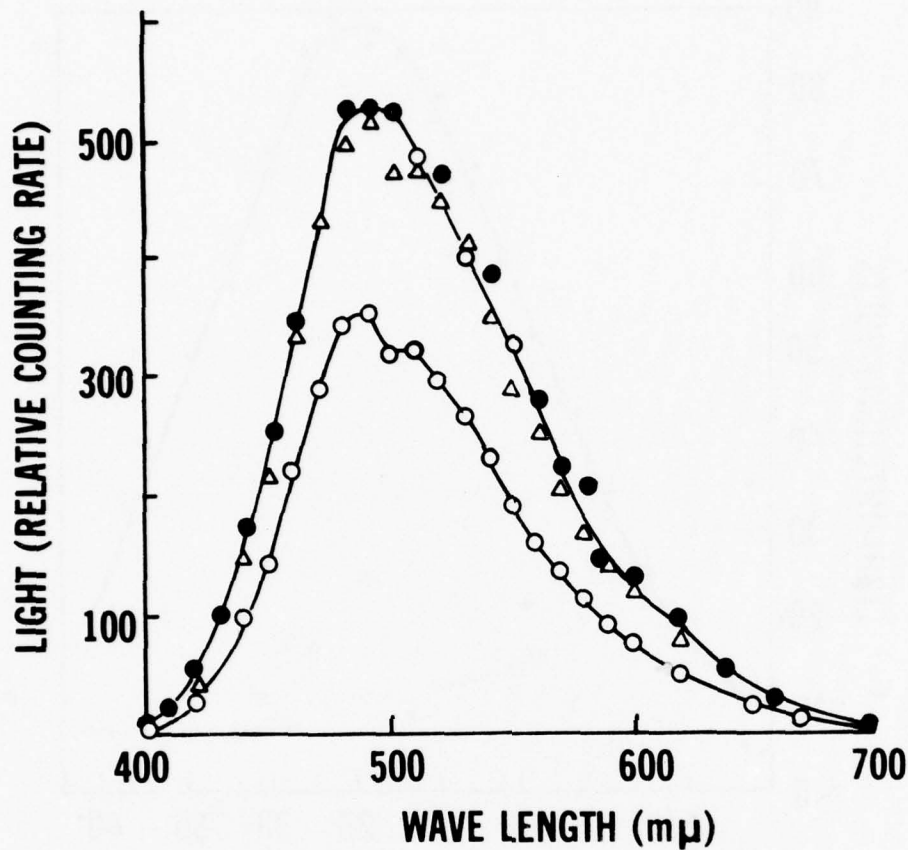


Figure 7. Emission spectra of intact *A. fischeri* and extracts obtained from *A. fischeri* (0.5-millimeter slit width Farrand quartz monochromator). ● Emission of bacteria. ○ Emission of extracts. △ Extract emission normalized to bacterial emission at 490 nanometers. The gross reading was not corrected for changes in dispersion of monochromator or sensitivity of photomultiplier. (From McElroy and Strehler, Arch. Biochem. & Biophysic 22, 420-433 (1949). Copyright 1955 by the American Association for the Advancement of Science.)

A large amount of effort has been expended during the years on the effects of pressure on the luminescence of intact luminous bacteria. Contributors to this effort were Johnson, Eyring, and their collaborators. When pressure is applied to either intact bacteria or luminous extracts obtained therefrom, there is an immediate increase in the level of luminescence to some higher level which slowly decays over a period of seconds or minutes to some lower state level. When the pressure is released, the exact converse effects occur – the luminescence drops instantly to a lower level and then slowly rises to the level of luminescence obtained prior to the application of

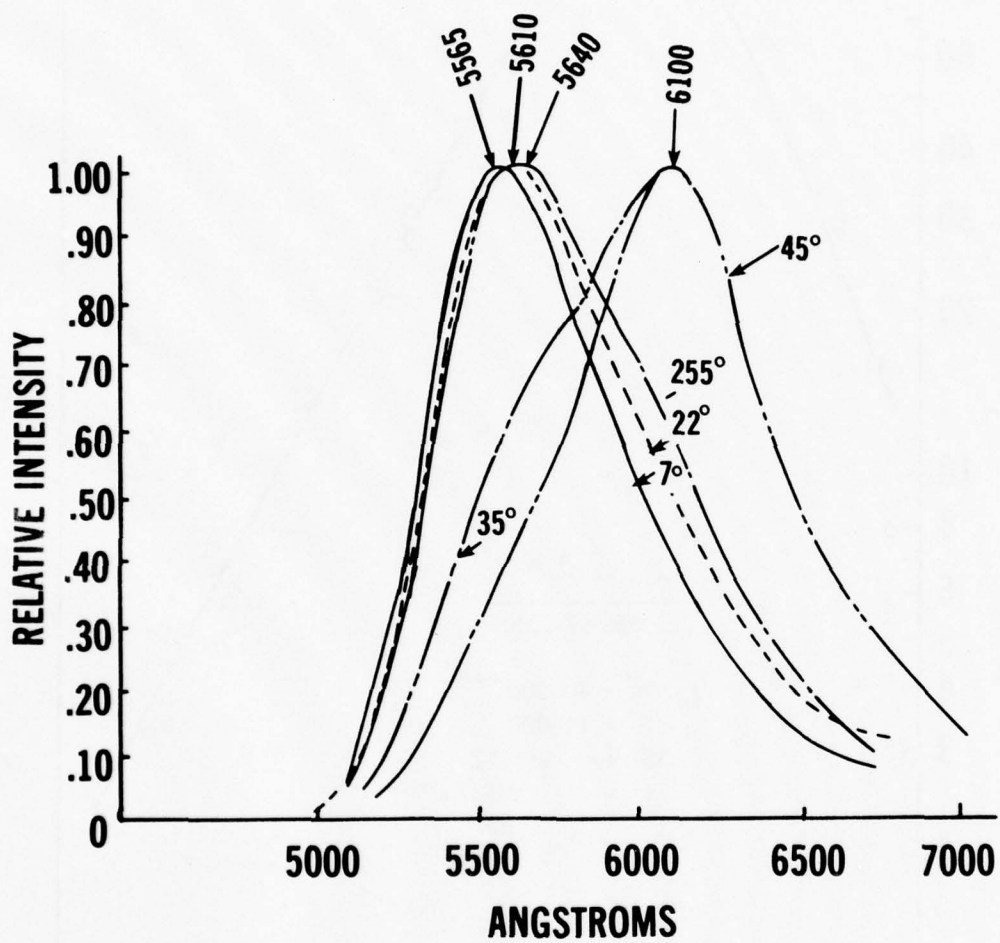


Figure 8. Variation with temperature on the in vitro bioluminescence emission spectrum of *P. pyralis*. Reactions measured at a pH of 7.6 in 0.025 M glycyl-glycine. (From "Bioluminescence in Progress," *Proceedings of the Luminescence Conference*, Ed. by Frank H. Johnson and Yata Haneda, Princeton University Press, Princeton, NJ (1966). Sponsored by the Japan Society for the Promotion of Science and by the National Science Foundation under the U.S.-Japan Cooperative Science Program, September 12-16, 1965, Hakone National Park, Kanayawa-Ken, Japan.)

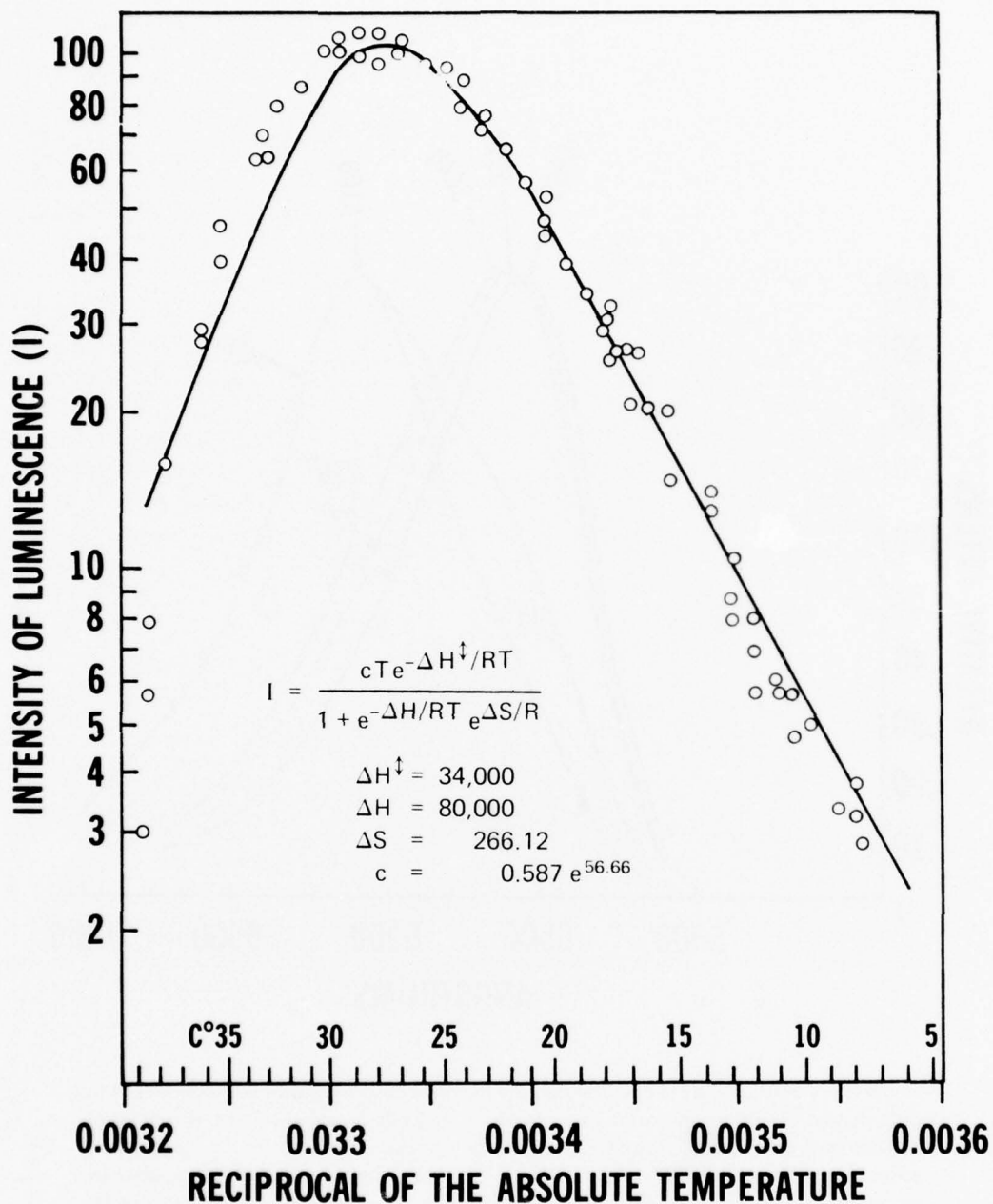


Figure 9. Influence of temperature on the intensity of luminescence in *A. fischeri*. The smooth curve was calculated in accordance with the equation and constants as given in the figure. (From Johnson, Eyring, and Polissar, 1954, courtesy of John Wiley & Sons; data of Johnson, Eyring, and Williams, 1942. Copyright 1955 by the American Association for the Advancement of Science.)

pressure. The most striking modifier of this action is the long-chain aldehyde. In the absence of long-chain aldehyde, the luminescence is virtually unaffected at suboptimal temperatures by the application or release of pressure. The effect of pressure on the steady-state level of luminescence becomes less as the temperature is raised toward the optimum, and at still higher temperatures the level increases rather than decreases under pressure. Over a temperature range of 0° C to 35° C, it is shown in Figure 10(a) that the steady-state luminescence of *P. phosphoreum* at low temperatures is reversibly decreased where the equilibrium constant is negligible. These results are recorded for a suspension of *P. phosphoreum*. In Figure 10(b), it is shown that the net observed effect of pressure at a given temperature depends upon the specific biological system involved.

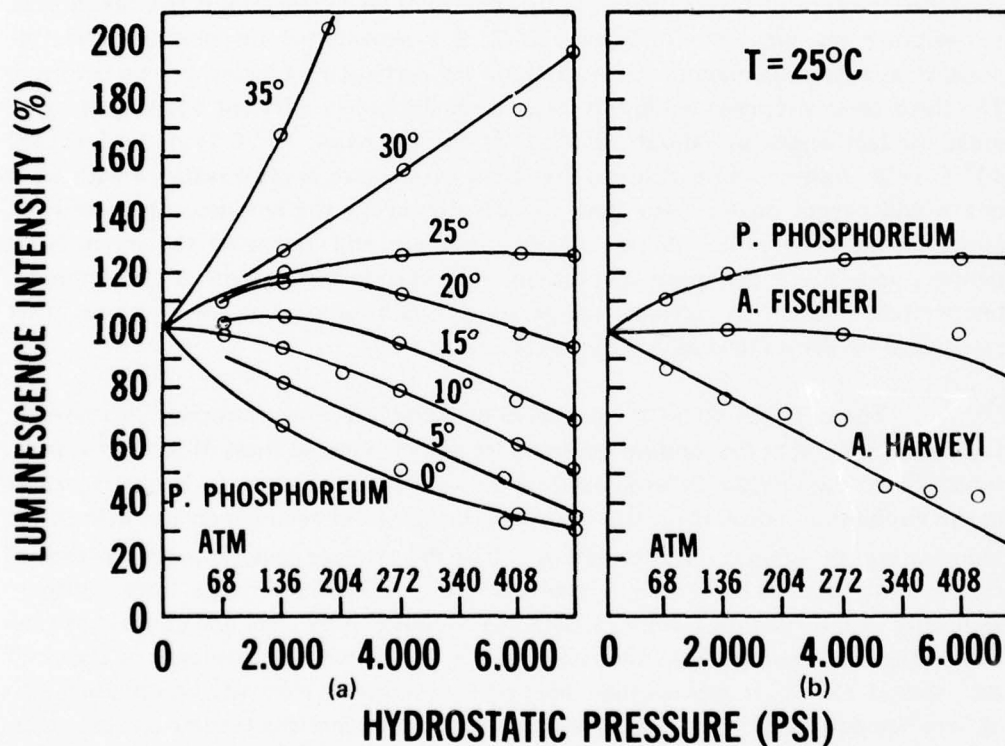


Figure 10. (a) Intensity of luminescence of *P. phosphoreum* as a function of pressure at different temperatures. The intensity at normal pressures is arbitrarily taken equal to 100 at each temperature in order to show the percent change in intensity with change in pressure. (b) Influence of increased pressure on the luminescence of three different species of bacteria at 25° C. (From Johnson, Eyring, and Polissar (1954), courtesy of John Wiley & Sons, Inc.; data of Brown, Johnson, and Marsland (1942). Copyright 1955 by the American Association for the Advancement of Science.)

There is yet no good data pertaining to pressure effects on well-defined purified enzyme systems for comparison with the luminescent data. The steady-state luminescence of *P. phosphoreum* at low temperatures where the equilibrium constant is negligible is reversibly reduced by increased pressure. The data indicates that the reaction leading to light emission proceeds with a net volume increase of activation amounting to about 50 cm³/mol at 0° C.

The observed effect of pressure on the steady-state level of luminescence becomes less as the temperature is raised toward the optimum, and at still higher temperatures the level will increase rather than decrease under pressure. The family of curves depicted in Figure 10(a) (taken from Eyring, Polissar, and Johnson, 1954) is similar to the type of curves shown for the tension of an auricle muscle as a function of temperature and pressure. In Figure 10(b), it is shown that the observed effect of pressure at a given temperature depends upon the particular biological system involved. The three species represented in this figure normally have a different temperature optimum for luminescence: about 20° C in *P. phosphoreum*, 26° C in *A. fischeri*, and 30° C in *A. harveyi*. In each case, the effect of pressure is in accordance with what one would expect on the basis that pressure diminishes the luminescent intensity at temperatures below that of the specific optimum and augments the intensity at temperatures above the specific optimum while having little effect at the optimum temperature itself. A pressure/temperature relationship such as this was first recognized by Brown in studies with muscle tissue.

The opposite effect of pressure at high and low temperatures as illustrated in Fig. 10(a) shows that the limiting reactions are not the same at these different temperatures. Since the increase in steady-state luminescence under pressure becomes greater as the equilibrium constant of the reversible denaturation becomes greater with rise in temperature, the simplest interpretation is that the pressure causes a partial reversal of the denaturation of the enzyme involved. The equilibrium change from native to reversibly denatured forms of the enzyme is accompanied by a volume increase of reaction. The data indicate that the value of ΔV in this reaction amounts to about 65 cm³/mol at 35° C. It appears that there are two reactions primarily limiting the overall luminescent reaction, viz, the catalytic reaction of a limiting enzyme and the reversible denaturation equilibrium. It shows in Figure 11 that the maximum intensity of luminescence remains practically the same at different pressures but the temperature at which the maximum occurs is higher under increased pressure than at atmospheric pressure. Frazer and Johnson report that highly purified trypsin with casein as a substrate undergoes a reversible thermal denaturation accompanied by a considerable volume increase of reaction.¹⁷

¹⁷ D. Frazer and F. H. Johnson, *J. Biol Chem* 190, 417-421 (1951).

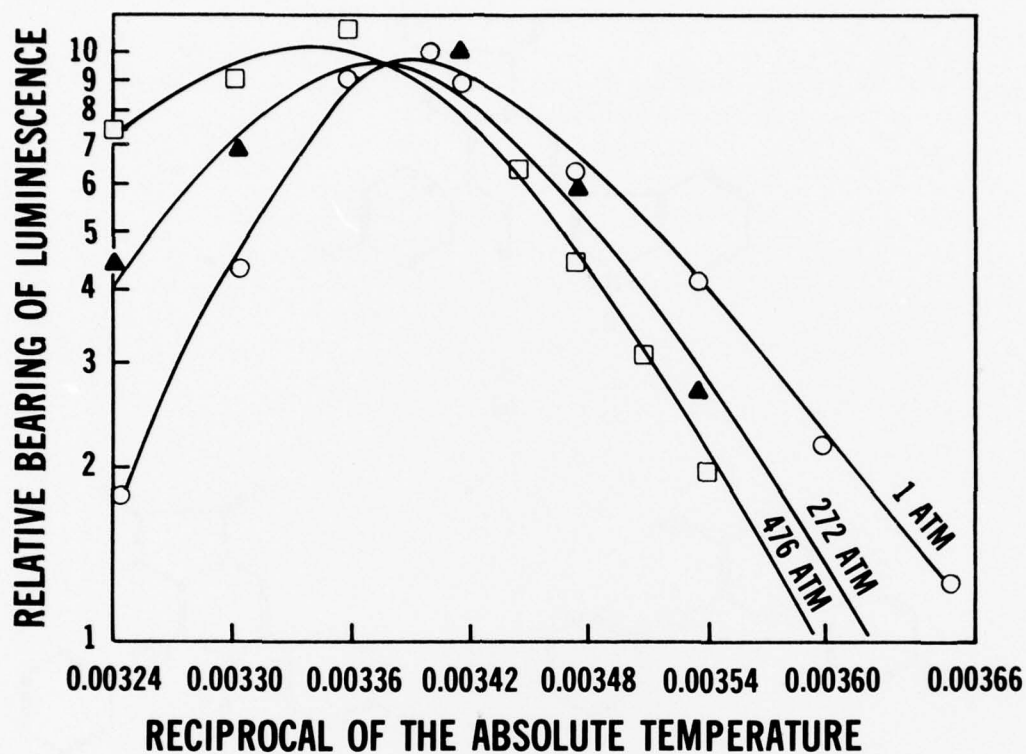


Figure 11. The brightness of luminescence in *P. phosphoreum* as a function of temperature at three different hydrostatic pressures. The points represent data from experiments by Brown, Johnson, and Marsland (1942). The smooth curves were calculated by Eyring and Magee (1942). (After Johnson, Eyring, and Polissar (John Wiley & Sons: 1954). Copyright 1955 by the American Association for the Advancement of Science.)

4. **Structure.** The elucidation of the chemical structure of luciferins is not an insurmountable problem. The difficulty arises from the tremendous effort required to obtain small amounts of purified material. For example, from about 1 ton of sea pansies (*renilla reniformis*), obtained by dredging the ocean bottom at depths of 10 to 20 meters, one can obtain about 1 milligram of pure renilla luciferin.

From about a half milligram of pure luciferin and the data obtained therefrom, structure I, Figure 12(a), was obtained for renilla luciferin. Because of the sample size and the fact that the compound is easily auto-oxidizable, it was impossible to obtain NMR data. This fact made it difficult to decide between alternate structures. Drs. Shimomura and Johnson of Princeton have made a number of observations which lead to the selection of a preference structure.

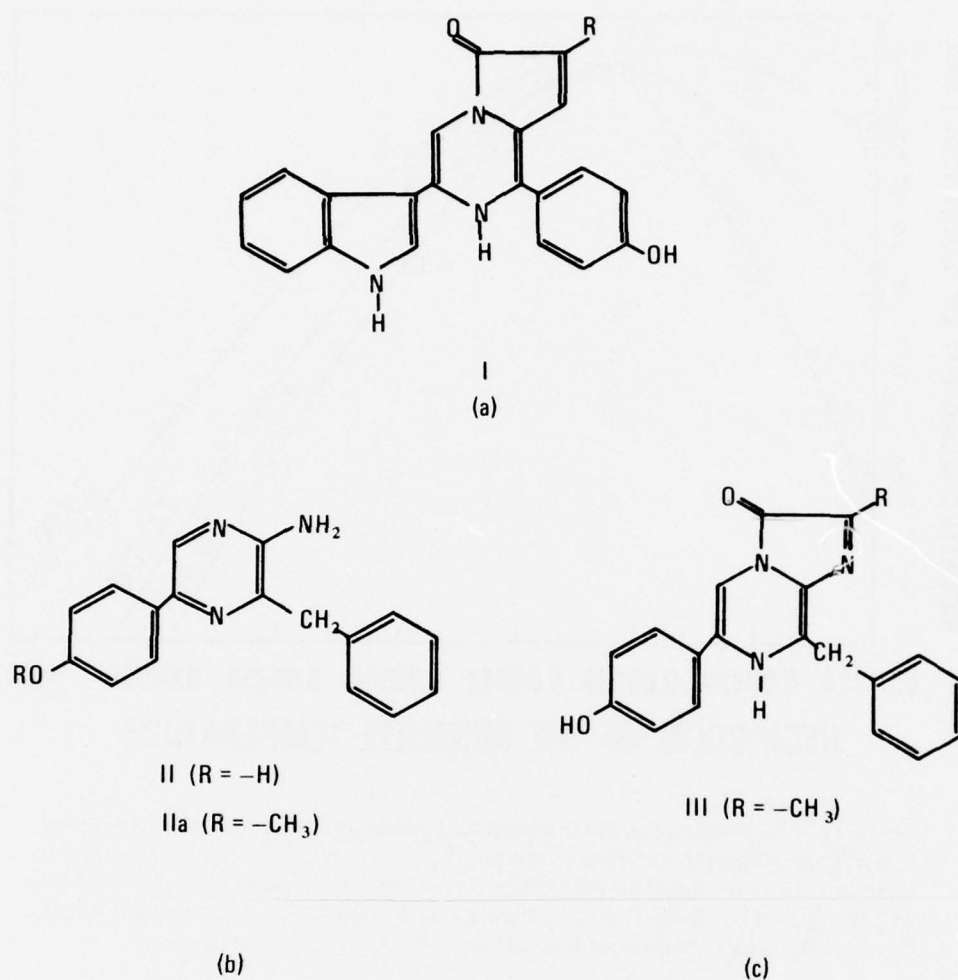


Figure 12. Possible structures of renilla luciferin. (From K. Huri and M. T. Cormier, "Structure and Synthesis of a Luciferin Active in the Bioluminescent Systems of the Sea Pansy (*Renilla*) and Certain Other Bioluminescent Coelesterates," 2nd International Conference on Chemiluminescence, University of Georgia (1972).)

A structure of AF 350 (structure II, Figure 12(b)) was recently reported,¹⁸ and this structure was confirmed by synthesis.¹⁹ It can be noted that the structure of Figure 12(a) with minor modifications allows the structure of AF 350 (structure II) to become an integral part. For example, a C₈H₇N fragment found in the mass spectrum

¹⁸ C. Shimomura and F. H. Johnson, *Biochemistry*, *11*, 1602-1608 (1972).

¹⁹ Y. Kishi, H. Tanino, and T. Goto, *Tetrahedron Letters*, *20*, 1609-1610 (1969).

could be explained either by an indole nucleus as shown in structure I or by a benzyl moiety as shown in structure III, Figure 12(c). Further, there are restrictions placed on structures involved in luminescent reactions. These restrictions are based in part on considerations of energy requirements for the creation of an electronically excited state. For these reasons and others already mentioned, the ring structure for renilla luciferin would involve fusing an imidazole ring to structure II, thus converting it to structure III. On the assumption that the R group of structure III would not significantly affect its biological activity, the compound was synthesized where R is methyl. Structure III was found to be biologically active. Additional properties of this material are described in later sections. (See pH effects and references by Hori and Cormier²⁰.)

Based on structure III and the mechanism of the luminescent reaction in renilla employing ¹⁸O, by DeLuca et al., a mechanism of the luminescent reaction consistent with the results and which adequately predicts the structure of the emitter (oxyluciferin) is shown in Figure 13. The hydrolysis of the peptide bond in oxyluciferin would lead to the formation of AF 350 (structure II).

Preliminary results on the chemiluminescence of structure III in organic solvents show that oxyluciferin is, indeed, the product of the chemiluminescence reaction and that emission occurs from the monoanion of oxyluciferin as shown in Figure 13.

5. Thermal Effects. The luminescence of *P. phosphoreum* as a function of temperature at three different pressures is shown in Figure 14. It is seen that the maximum intensity of luminescence remains practically the same at different pressures, but the temperature at which the maximum intensity occurs is higher under increased pressure than at atmospheric pressure. At temperatures above the normal optimum, the luminescent system is destroyed at rates that increase rapidly with rises in temperature; these rates generally have the characteristics of first-order reactions, and their increase with temperature resembles the first-order thermal decomposition of proteins. At a given temperature, the rate decreases with rises in pressure by an amount indicative of a volume increase of activation of about 70 cm³/mol. Thus, it appears not only that pressure acts to reverse the equilibrium change from native to denatured states of an essential enzyme but also that pressure retards the rate of thermal destruction. The influence of various hydrostatic pressures on the relative destruction rates of the luminescent system *P. phosphoreum* suspended in a phosphate buffered sodium chloride solution of neutral pH at 34° C is shown in Figure 14.

²⁰ Kazuo Hori and Milton J. Cormier, "Structure and Synthesis of a Luciferin Active in the Bioluminescent Systems of the Sea Pansy (Renilla) and Certain Other Bioluminescent Coelenterates," *Chemiluminescence and Bioluminescence*, Edited by J. J. Cormier, D. M. Hercules, and J. Lee (Plenum Press: New York-London, 1973).

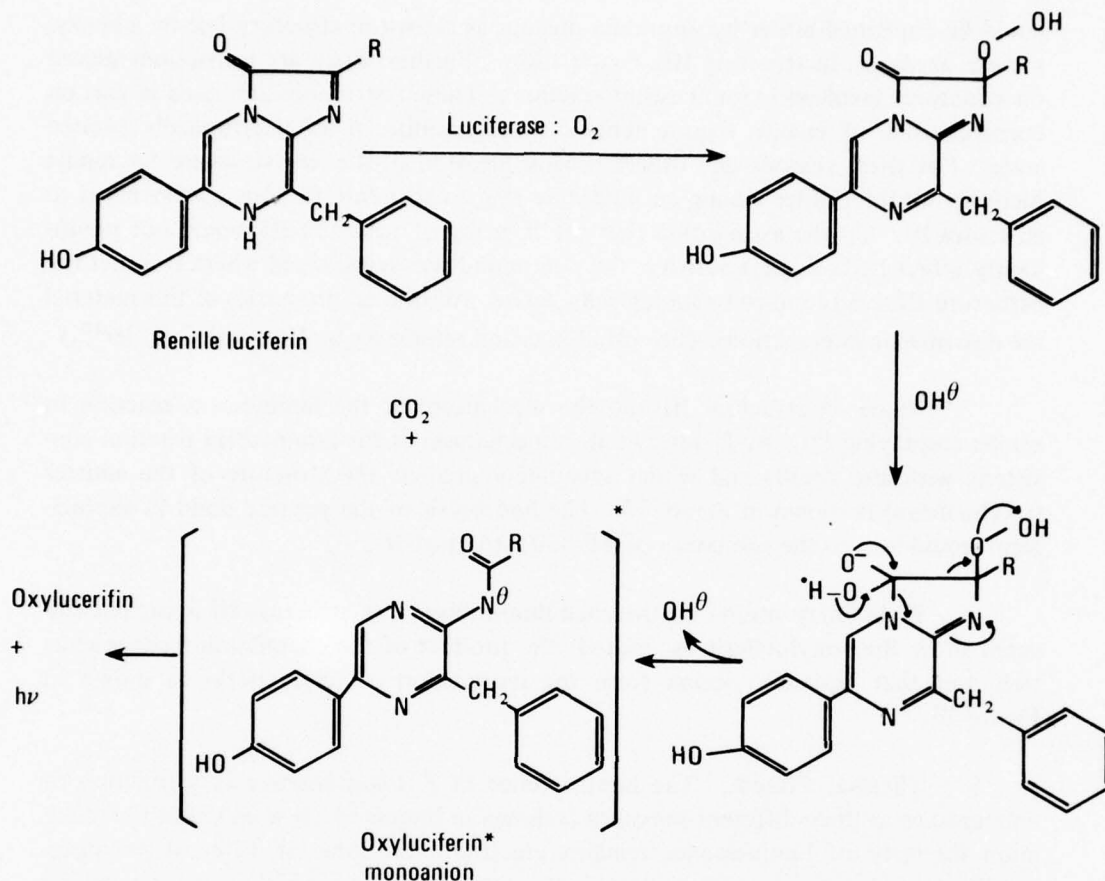


Figure 13. Mechanism of the renilla bioluminescent reaction.

Most of the work reported herein was performed on extracts of bacteria. There are two main reasons for the utilization of extracts: (1) Adequate amounts of the sample can be acquired at once for extended periods of laboratory investigation, and (2) there is an assurance of the reproducibility of the samples for these tests and evaluations. In Figure 15, a comparison is made of the emission spectra of intact *A. fischeri* and extracts obtained from the same batch of *A. fischeri*. The results show that within the experimental area the two spectra are identical. This finding along with other parallels between in vivo and in vitro luminescence make it almost certain that the same reactions are occurring under both conditions. It seems probable that flavin in association with luciferase and long-chain aldehyde is the light-emitting complex; the difference that exists between the emission maxima exhibited by riboflavin fluorescence and chemiluminescence on the one hand and the emission of the bacterial extracts on the other is a puzzling and, possibly, crucial point.

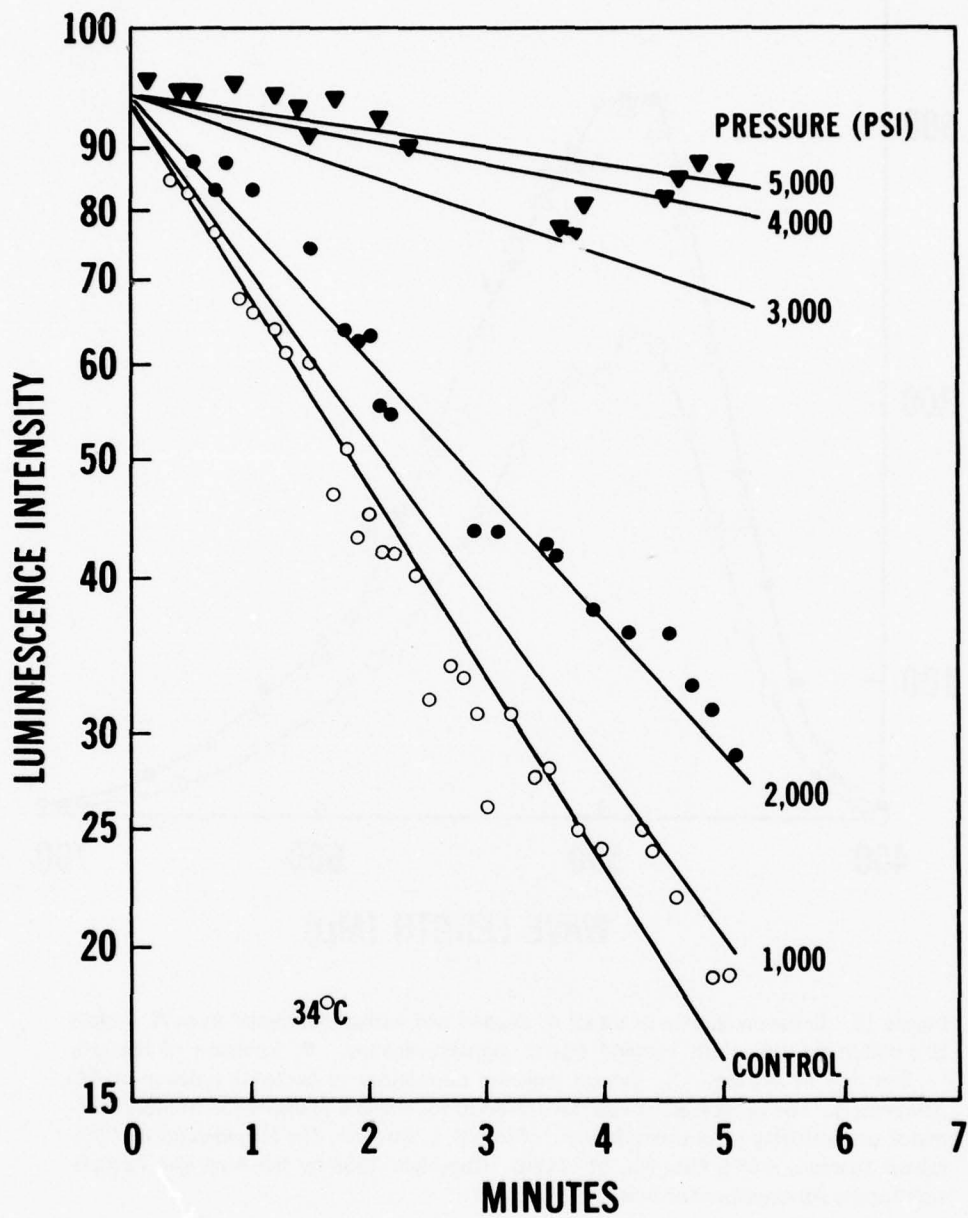


Figure 14. The influence of hydrostatic pressure on the relative rate of destruction of the luminescent system of *P. phosphoreum* suspended in phosphate-buffered sodium chloride of neutral pH at 34° C. (From Johnson, Eyring, and Polissar (courtesy of John Wiley & Sons: 1954); after Johnson et al., 1945. Copyright 1955 by the American Association for the Advancement of Science.)

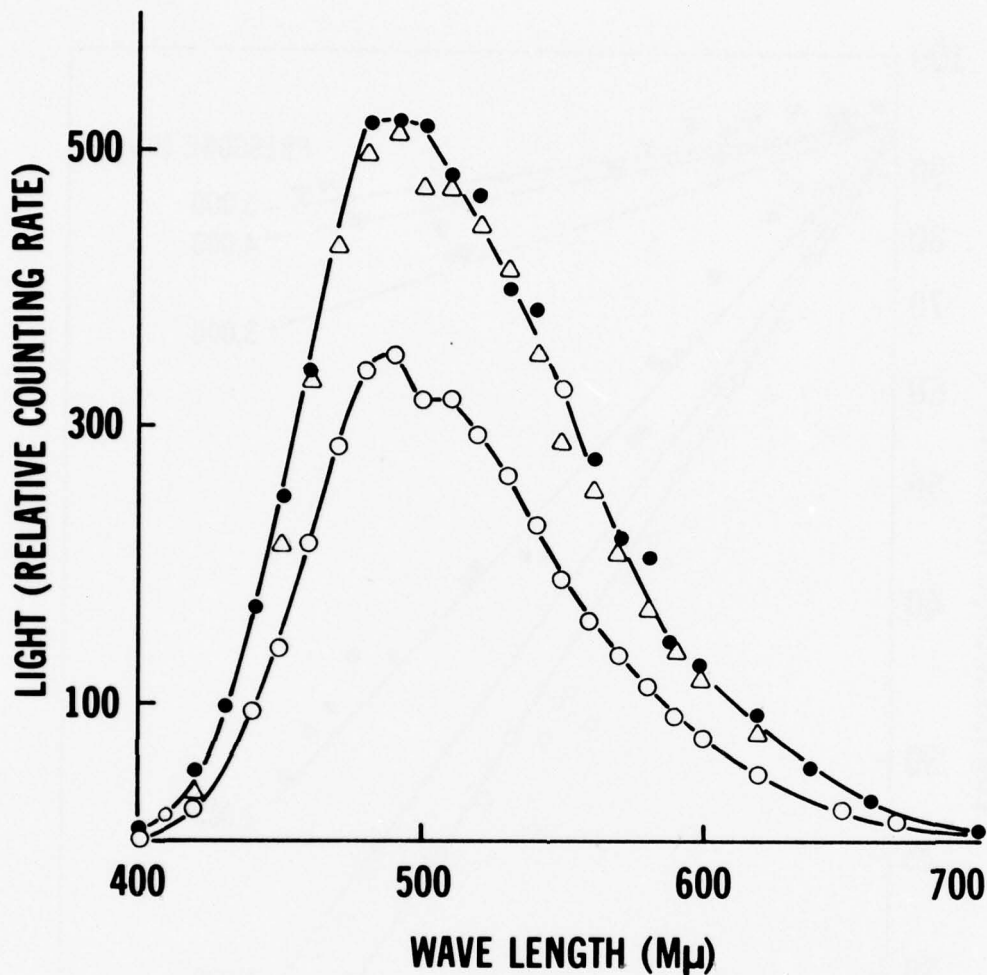


Figure 15. Emission spectra of intact *A. fischeri* and extracts obtained from *A. fischeri* (0.5-millimeter slit width Farrand quartz monochromator). ● Emission of bacteria. ○ Emission of extracts. △ Extract emission normalized to bacterial emission at 490 nanometers. The gross reading was not corrected for changes in dispersion of monochromator or sensitivity of photomultiplier. (From B. L. Strehler, *The Luminescence of Biological Systems*, AAAS Pub. No. 41 (1955). Copyright 1955 by the American Association for the Advancement of Science.)

6. pH Effects. Chemical activity greatly influences the activity of the luminescent specimens. The effect of acidity or pH on dialyzed bacterial extract is shown in Figure 16. The extract was dialyzed for 15 hours against distilled water at 0° C. Each vessel contained 50 micrograms of DPHN₂, 0.2 milliliter of enzyme, and 0.2

milliliter of NAH_2PD_4 (0.01M) titrated in turn to the desired pH with 1N NaOH (0.01M) at 23° C (total volume - 0.5 milliliter). The double optima may be due to the purified nature of the system, but duplication occurs when crude acetone extracts are used in the presence of DPNH_2 .

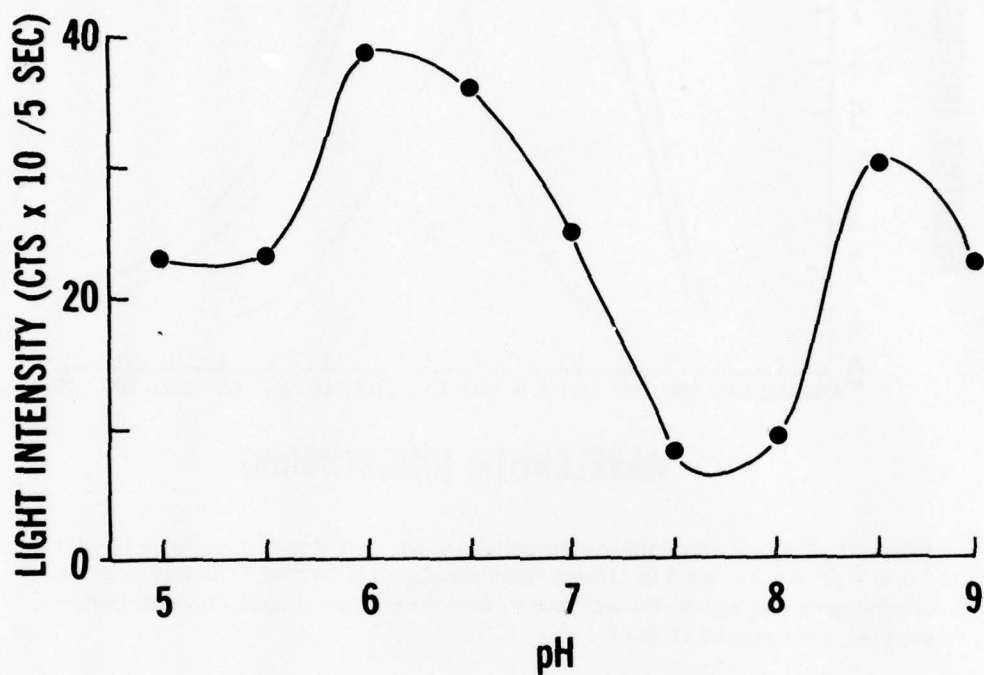


Figure 16. Effect of pH on dialyzed bacterial extract luminescence. (From B. L. Strehler, *The Luminescence of Biological Systems*, AAAS Pub. No. 41 (1955). Copyright 1955 by the American Association for the Advancement of Science.)

The effect of pH in the *in vitro* emission spectrum of *P. pyralis* luciferase is shown in Figure 17. It can be seen that as the pH of the *P. pyralis* extract is lowered, the intensity of the yellow-green (5,500- to 5,800-angstrom) bioluminescence decreases leaving a dull brick orange glow as observed by McElroy and Seliger. As can be seen, at neutral and alkaline pH, there is a single emission band in the yellow-green; at intermediate pH values, a red emission band appears at about 611 nanometers and at pH values below 5.5, the yellow-green emission is completely suppressed, and only the red band is evident. At acid pH, the number of light quanta emitted per luciferin molecule oxidized is markedly lower than 1 and indicates a predominantly dark reaction. However, at alkaline pH, although the rate of light emission is reduced to a fraction of the rate at a pH of 7.6, the quantum yield is essentially unity. Since the red emission is known to be due to the monoanion form of the product emitter, this suggests that

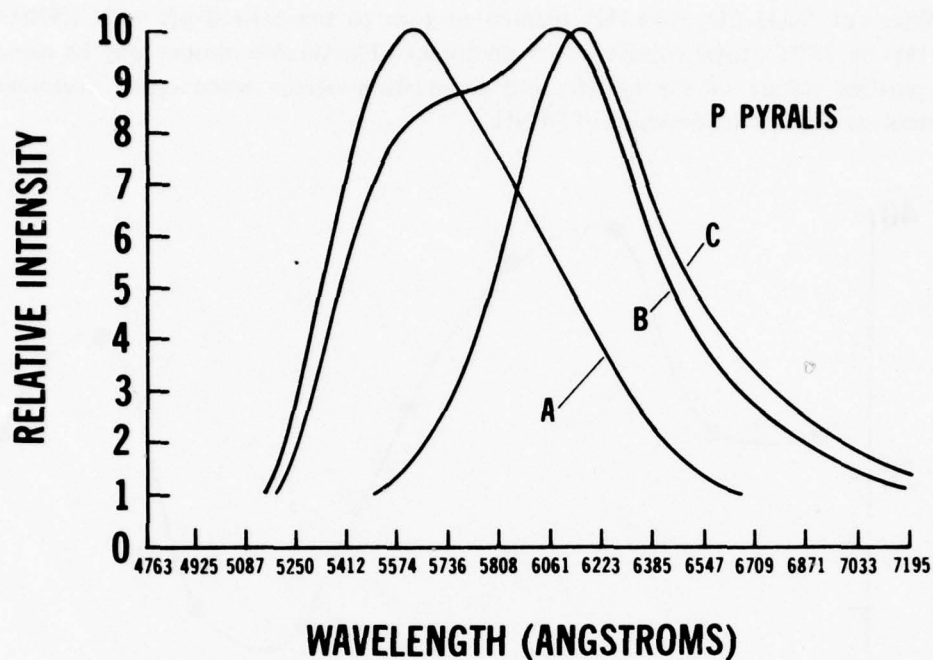


Figure 17. Effect of pH on the in vitro emission spectrum of *P. pyralis* luciferase (a – pH 7.6, b – pH 6.5, c – pH 5.0). (From "Bioluminescence in Progress," *Proceedings of the Luminescence Conference*, Ed. by Frank H. Johnson and Yata Haneda, Princeton University Press, Princeton, NJ (1966).)

the pH change must be affecting a group of the enzyme concerned with the abstraction of a proton from carbon 5; the pH for the appearance of red light is approximately 6.8. This suggests the strong possibility that a histidine residue in the enzyme is the active group responsible for the proton abstraction. Except for the partial denaturation of the enzyme in acidic buffer, the pH effect on the emission spectrum shift is completely reversible. A reversible red shift in emission spectra can be observed by increasing and then decreasing the temperature of the reaction by carrying out the reaction in 0.2M urea at normal pH values (7.6) in glycylglycine buffer or by adding small concentrations of Zn^{++}/Cd^{++} cations as chlorides.

7. Substrate Structure. The observations noted above are supportive of the idea that the color of the emitted light depends upon the nature of the binding of the reaction immediate to the enzyme. It seems likely, therefore, that a change in the structure of the substrate molecules (luciferin or adenosine triphosphate (ATP)) may alter the binding and, in turn, affect the color of the light. Unfortunately, it is not possible to change greatly the luciferin structure and still obtain a light-emitting sub-

strate. It turns out that the 6'-aminobenzthiazole compound is an active substrate, and in this case a red emission instead of the yellow-green is observed even at neutral pH. The emission at neutral and alkaline pH is red-peaking at 605 nanometers, very close to the bioluminescence emission of firefly luciferin at acid pH. More significantly, the color of the 6'-aminoluciferin bioluminescence is entirely independent of pH from below 6 to above 10 in exactly the range where native firefly luciferin shows the remarkable color shifts outlined above. Since phenols are stronger acids than anilines, this observation is supportive of the idea that it is the phenolate ion firefly luciferin that is involved in the normal yellow-green bioluminescence. The results further suggest that the amino group has a strong effect on the ability of the enzyme to abstract a proton from carbon 5.

Until recently, only ATP was shown to be active for the enzymatic reaction leading to light emission. Unidine Triphosphate (UTP), Cytidine 5'-triphosphate (CTP), and Adenosine 5'-phosphosulfate (ADP) and other pyrophosphates containing nucleotides were inactive. Recently, Leonard and his associates²¹ prepared an ATP with the ribose attached to the 3-position of the adenine ring (3-iso-ATP). This compound exhibits activity 10 to 15 percent of that normal to ATP in the light reaction. It is also observed that at a pH of 7.5 a significant portion of the light emitted is red when 3-iso-ATP is used. Thus, the nature (stereochemistry) of the nucleotide attachment to the enzyme is also of importance in determining the color of light.

Secrist, Barrio, and Leonard²² have prepared a modified ATP called E-ATP. The structural alterations are due to the addition of an aldehyde group which couples the 6-amino group of adenine to the number 2 nitrogen, thus making a four-membered ring at the position of the purine moiety. The E-ATP is completely inactive for initiating the light reaction. When E-ATP is used as a substrate to make LH₂-EATP, the latter is active for light emission and the emission is red instead of the usual yellow-green. The results indicate that the 6-amino group on the purine ring is essential for the activation reaction. This result is in agreement with the observation that ITP is ineffective as a substitute for ATP in the light reaction. In addition, the nature of the binding of the 6-amino group to the enzyme also influences the structure of the excited product in a manner which determines whether red or yellow-green is emitted.

The results from the E-ATP, E-AMP, and iso-ATP indicate that the binding of the adenylate to the enzyme induces changes in the enzyme structure that must be sustained during the subsequent decarboxylation that leads to the enzyme-product complex excited state. If this were not true, then the structure of the AMP should not affect the color of the light since it must be removed from LH₂ before the final creation of the excited state according to all proposed mechanisms.

²¹ N. J. Leonard *et al.*, *Science* 177, 279 (1972).

²² *Ibid.*

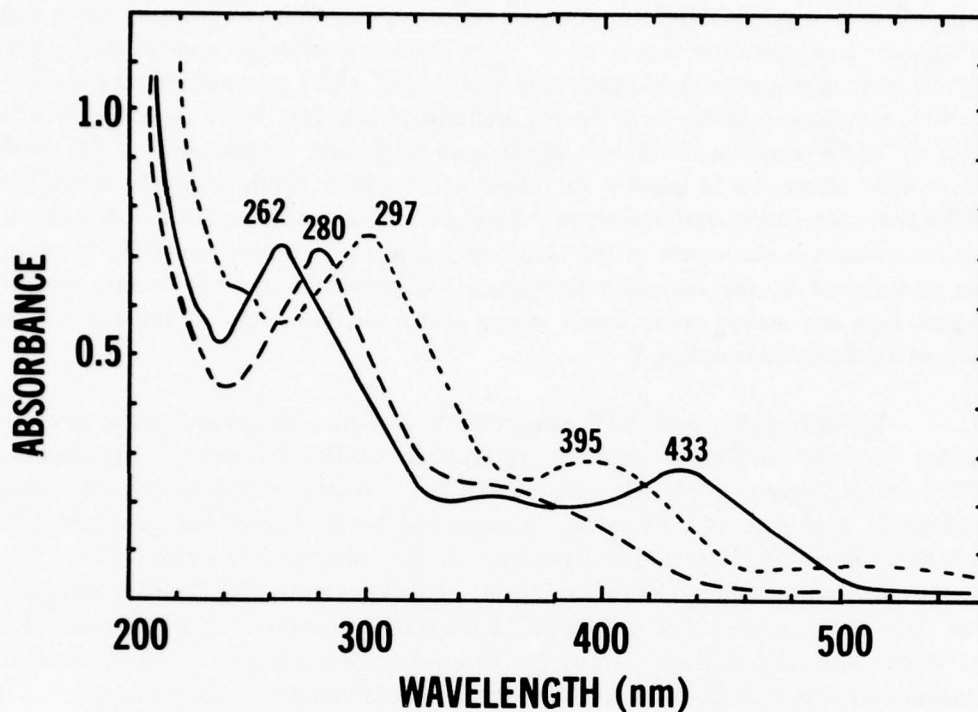


Figure 18. Absorption spectrum of methanol solutions of renilla luciferin as a function of pH. (Solid line, pH 7.0; dashed line, pH 1.0; dotted line, pH 11.0) (From *Chemiluminescence and Bioluminescence*, edited by M. J. Cormier, D. M. Hercules, and J. Lee (1973), p. 364.)

The absorption spectra of native renilla luciferin at different pH values is shown in Figure 18. At neutral pH and in methanol, the millimolar extinction coefficients at 265 and 433 nanometers were found to be 22.8 and 9.0, respectively. As shown in Figure 19, the absorption properties of synthetic luciferin (structure III, Figure 12(c)) are similar to those of the native compound. Identical pH shifts as noted in Figure 18 are also observed with synthetic luciferin. In addition, the two compounds have similar extinction coefficients for the two transitions listed above. Furthermore, both compounds exhibit identical yellow-green fluorescence ($\lambda_F = 558$ nanometers).

Both synthetic luciferin (structure III) and cypridina luciferin contain a pyrazine nucleus with a fused imidazole ring. The side chains, however, are considerably different. Side chains derived from tryptophan and arginine in cypridina luciferin²³ are replaced by those from tryosine and phenylalanine in structure III. Since

²³M. J. Cormier and J. R. Totter, *Ann. Rev. Biochem.*, 33 431 (1964).

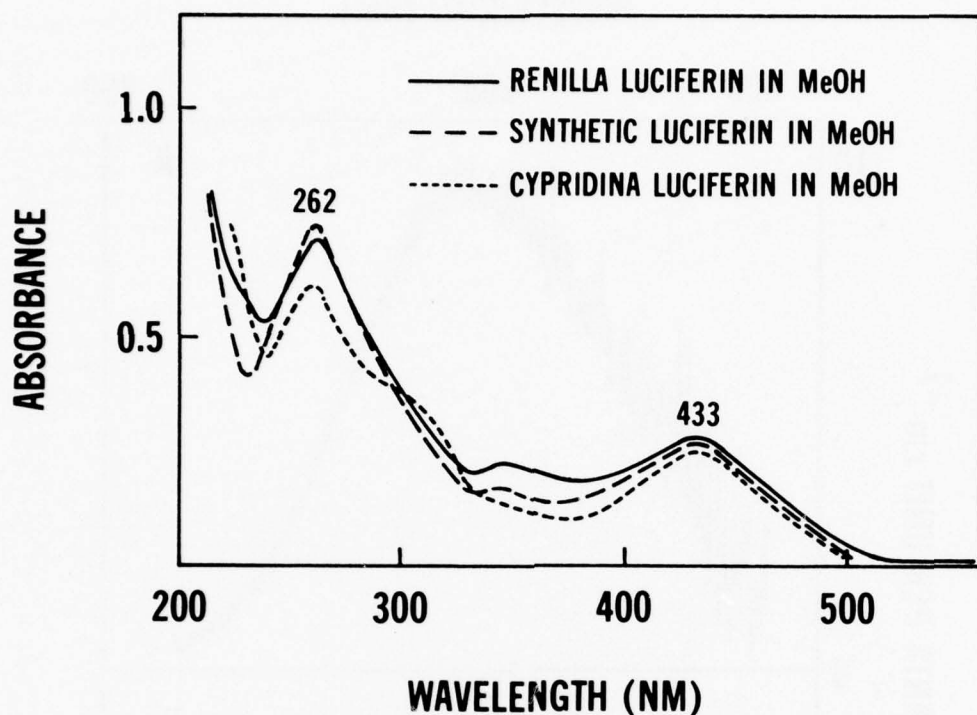


Figure 19. Comparison of absorption spectra of native renilla luciferin, synthetic luciferin, and cypridina luciferin. (From *Chemiluminescence and Bioluminescence*, edited by M. J. Cormier, D. M. Hercules, and J. Lee: New York, Plenum Press (1973), p. 365.)

the heterocyclic ring structure of both compounds is the same, it is not surprising that we noted similar striking similarities in their absorption properties (Figure 19). The fluorescence characteristic of both compounds as well as that of native luciferin are also similar.

The mass spectral pattern of structure III was found to be identical to that of native luciferin up to a mass of 331 mass units, which represents the molecular ion of structure III. Although the R group in native luciferin is bulky (197 mass units), it does not influence the spectral characteristic of the compound as judged by the absorption, fluorescence, and bioluminescence emission of native luciferin when compared with the synthetic compound.

Synthetic luciferin of structure III was found to be biologically active, while structure II was totally inactive. When structure III was added to renilla luciferase, light production occurred. When equal amounts of both native and synthetic luciferins were used, the kinetics of the corresponding bioluminescence were similar. Further, the color of bioluminescence was identical in both cases, as shown in Figure 20.

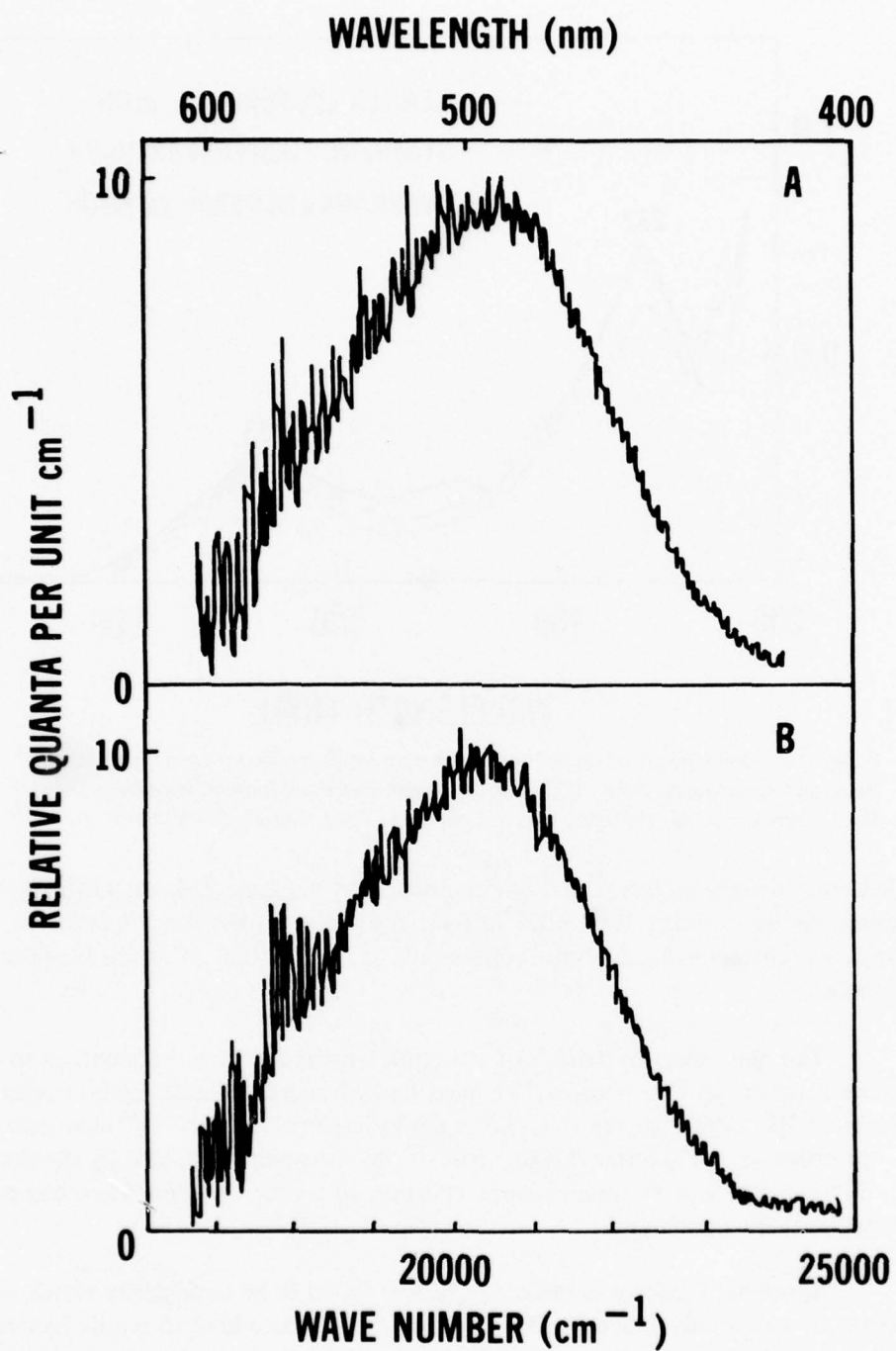
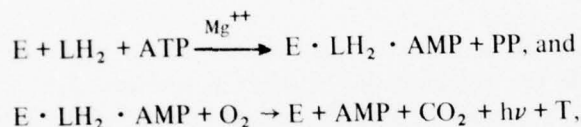


Figure 20. Comparison of the color of bioluminescence upon initiation of the reaction with synthetic luciferin (A) and native renilla luciferin (B). (From *Chemiluminescence and Bioluminescence*, edited by M. J. Cormier, D. M. Hercules, and J. Lee, New York: Plenum Press (1973), p. 36.)

By using the flash peak as a measure of initial intensity, structure III was found to be 10 percent as active as native luciferin in producing light with renilla luciferase. This difference in activities must reside in the R-group of luciferin. The methylated form of luciferin structure III was totally inactive in producing light with luciferase; upon demethylation, biological activity was restored.

8. **Problem Areas.** Every child has enjoyed the spectacle of flashing green-yellow lights from fireflies on warm summer nights; now, the chemical nature of this light production, known as bioluminescence, has enabled one to apply this phenomenon of cold light to many fields, and this includes the determination of the presence and quantity of bacteria, the detection of explosives, and scientific measurements in oceanography. Employing the basic investigations of Harvey (1965),²⁴ extensive and numerous investigations of luminescence displayed by organisms in nearly every phylum of the living world, McElroy and his associates (1969)²⁵ and subsequent workers extracted the light producing substances from fireflies and described the reaction mechanism and kinetics as follows: (Plant *et al*²⁶ 1968).



where:

E	=	firefly luciferase,
LH ₂	=	reduced luciferin,
ATP	=	Adenosine triphosphate,
AMP	=	Adenosine monophosphate,
PP	=	pyrophosphate,
T	=	Thiazolinone, and
hν	=	light (550 nanometers).

The amount of light produced is proportional to the reactants when each is limiting. A light measurement capability could be used as an assay method for any of the reactants. Since ATP is a metabolite significant in all energy exchanges with living cells, its assay has implications for many parameters that describe the biota; ATP is known to be present in all forms of life, so its measure could establish the presence or

²⁴ E. N. Harvey, *Living Light*, New York: Hadner Publishing Co., Inc. (1965).

²⁵ W. D. McElroy, H. H. Selinger, and E. H. White, *Photochem. - Photobiol.* 10 153-170 (1969).

²⁶ P. J. Plant, E. H. White, and W. D. McElroy, *Biochem. Biophys. Res. Comm.* 21 98-103 (1968).

absence of living things. Any element or stimulus that has an effect on life or on responses generated by living things can be measured quantitatively by a method that measures its effect on ATP. By purifying a luciferase extract and adding all the necessary chemicals except ATP in excess, an assay for ATP can be performed by measuring the amount of light produced when a sample containing ATP is introduced. By this means or a variation thereof, the effect of explosive vapors or other introduced substances can be determined. When an amount of a sample (TNT vapor for example) is exposed to a living luminescent organism, a measure of the resultant light indicates the amount of the sample. Within groups of luminescent organisms, their ATP content is relatively constant; then, the luciferase initiated light can be used to estimate the quantity of the sample. This method has been used to quantitate bacteria by NASA personnel.²⁷

NASA personnel developed procedures for the determination of life on other planets several years ago and, subsequently, evolved a program for applying these technological developments to public sector needs, particularly in the sector of health care delivery.

Many types of photometric instruments are in existence for quantiating light. Those suitable for discriminating changes in low-level light as emitted by the small amounts of ATP present in bacteria, picomoles (10^{-9} mole) of ATP, employ a sensitive photomultiplier (PM) tube as a detector and a d.c. amplifier or equivalent. These instruments are manufactured by several companies. Some investigators have assembled their own instruments from off-the-shelf amplifiers and photomultipliers. A very sensitive instrument has been produced that uses pulse counting. In some instances, the PM tube can be cooled to improve the signal-to-noise ratio. In one system, "Diogenes," the PM tube can be cooled to improve the signal-to-noise ratio in connection with an automatic injection system, and there is a selection of analog or digital readout.²⁷ With this instrument, 10^{-9} microgram of ATP produces a signal above the noise level. If there is a 1-1 molar reaction, this system allows the detection of 10^{-9} microgram of the corresponding affected sample. Because the light from unknown sample combinations may vary by several decades, an automatic electronic amplification switching capability or a digital readout in several digits is useful and eliminates the use of several samples of the luminescent reaction agent. Instruments that provide this capability are available from Dupont, Aimco, and Hewlett-Packard. Hewlett-Packard also makes a picoammeter with automatic ranging that can be coupled with a photomultiplier tube and its power supply.

In the laboratory environment, when reacting laboratory samples with ATP the light production upon injection of a sample containing ATP into a luciferase

²⁷ E. W. Chappelle and G. V. Levin, *The Design and Fabrication of an Instrument for the Detection of Adenosine Triphosphate (ATP)*, NASA CR 411 (1966).

environment rises to a maximum intensity then decays exponentially. Both the maximum intensity and the total output are proportional to the quantity of ATP added. Several types of display are accordingly suitable. When the proper display is used in conjunction with a capacitance circuit, the peak height can be sampled and a digital output produced and recorded. A rate function can be produced on a strip-chart recorder, and a peak height may be measured. Analog circuitry can be introduced, digitized, and printed to show the light output. Both the scintillation counter and a version of the JRB Co. ATP instrument provide an attachment for making this modification and, also, for reading the peak light.

9. Applications. Numerous areas of application are appropriate for using the luciferase assay for ATP itself as well as to measure organism levels. Some reactions are specific for ATP and can be performed in the presence of other compounds. It does not require isolation of ATP, so it can be measured in cell extracts and in body fluids. The ATP level is an indicator of metabolic changes in living organisms and, therefore, can serve as a monitor of cell integrity, genetic variants substrate utilization, attack by a virus, and cell growth. With subsequent research, these types of measurements could have wide applications in cancer research and immunology as well as in organ and tissue viability and transplant rejection.

When used as a measure of bacteria levels, ATP assay can be used for pollution monitoring in air and water supplies, such as drinking water, sewage treatment effluent, river/stream pollution levels, and industrial water supplies and effluents. When used in air pollution monitoring, it can serve as a trace-gas detector for explosives or for other effluvia associated with the production and processing of explosives; closed environmental monitoring could be performed for spacecraft, space stations, clean rooms, operating rooms, etc. Microbe levels could be measured in dried foods, cereals, spices, milk, beer, wine, liquors, canned and bottled foods, pharmaceuticals, cosmetics, ointments, creams, paints, gasolines, and oils. Agricultural uses include determination of fertility levels, spore viability, and sterility of soils, plants, and animals. Oceanographic monitoring can be performed to determine biomass and effects of pollutants. Medical applications include infection levels in blood, urine, cerebrospinal fluid wound excretion, joint fluid, and lung and pleural fluid. Evaluation of antibiotic levels in body fluids could be done using the luciferase assay methods.

Each of these areas and applications represents areas where more research is needed into the problem involved in making the luciferase assay applicable. These include sampling, sensitivity, and background. The advantages would be speed and specificity.

10. Kinetics and Thermodynamics. Reactions involving luminescent responses to stimuli are enzyme-catalyzed and involve a temperature optimum. The Arrhenius

equation predicts that an elementary reaction will speed up exponentially with temperature over the entire temperature range. Biological reactions showing the customary temperature must be complex. Bioluminescence reactions are typical of the oxidation reactions; and typical examples are the firefly, the glowworm, and various types of luminescent organisms in the sea. Take, for example, Cypridina and Renilla — about 40 of these creatures cover an average thumbnail. A cold extract of crushed cypridina luminesces for a while, then fades out. A hot extract gives no light but if cooled and added to the spent cold extract light is again emitted.

The cold extract when purified yields the enzyme, luciferase, with a molecular weight of about 70,000 as shown by Chase and Langridge.²⁸ Since the constituent amino acid has a molecular weight of about 100, the enzyme is a linear polymer of about 700 units coiled in rope-like fashion into a helix with slightly under four monomers to a turn. The enzyme is folded so that its polar groups extend out into the water and the hydrophobic groups are buried inside the coils making contact with adjacent hydrophobic groups.

If the temperature is raised, the enzyme molecule unfolds, losing its catalytic activity and is said to be denatured. From a hot extract, through purification, luciferin can be abstracted. Luciferin prepared from different species differs; for example, cypridina luciferin has the empirical formula $C_{12}H_{20}O_2N_2 \cdot 2HCl$, and the provisional formula given by Johnson²⁹ is shown in Figure 21.

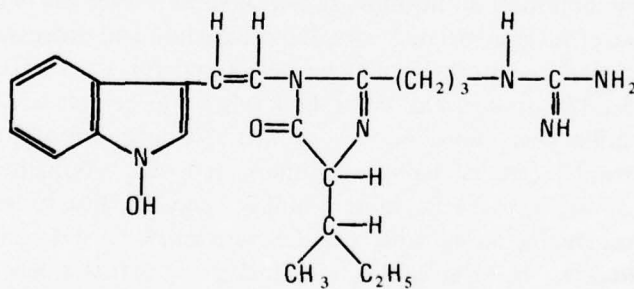


Figure 21. Luciferin.

²⁸ A. M. Chase and R. Langridge, "The Sedimentation Constant and Molecular Weight of Cypridina Luciferase," *Arch. Biochem. Biophys.* 88, 294-297 (1960).

²⁹ F. H. Johnson, *et al.*, *J. Cellular Comp. Physiol.* 60 85 (1962).

Many varieties of single cells luminesce. Exceptions are photobacterium phosphoreum, vibrio phosphorescence, and *A. fischeri* among others. In Figure 22, the effect of pressure and temperature on the luminescence of photobacterium phosphorescence is compared with theory. The results are good.

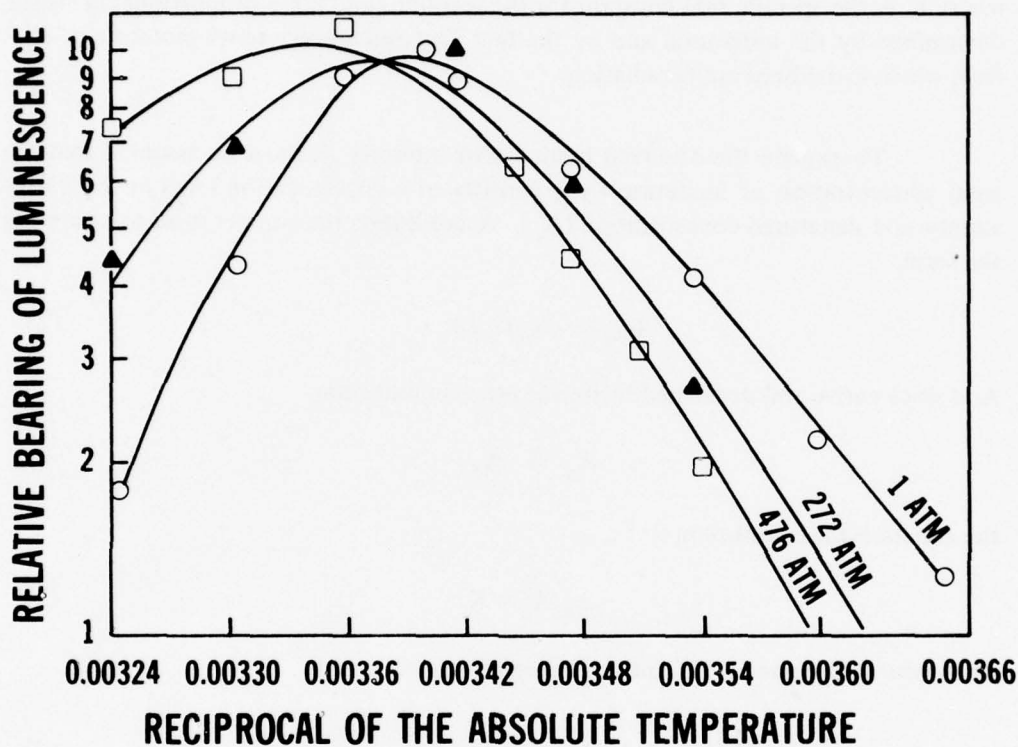


Figure 22. The brightness of luminescence in *P. phosphoreum* as a function of temperature at three different hydrostatic pressures. The points represent data from experiments by Brown, Johnson, and Marsland (1942). The smooth curves were calculated by Eyring and Magee (1942). (From Johnson, Eyring, and Polissar, *The Kinetic Basis of Molecular Biology*, New York: John Wiley & Sons (1954), p. 274. Copyright 1955 by the American Association for the Advancement of Science.)

A theory of luminescence can be developed. Since luminescence is an enzyme-catalyzed reaction involving the oxidation of luciferin LH_2 by the active luciferase A_n , it follows that the light intensity I is proportional to the concentration of reactants. This is true except for oxygen. Bacterial luminescence is independent of oxygen pressure at pressures of O_2 above about 10^{-10} atmospheres. At higher pressures, where luminescence is independent of oxygen, luciferin can acquire oxygen

molecules faster than it acquires the luciferin which is to be oxidized. This consideration leads to the equation:

$$I = bk' (A_n) (LH_2), \quad (1)$$

where k' is the specific rate constant for the reactions and b is a proportionality factor determined by the units used and by the fact that about every tenth molecule of luciferin which is oxidized emits radiation.

To explain the observed luminescent intensity, it must be assumed that the total concentration of luciferase (A_o) consists of a concentration (A_n) of the native variety and denatured concentration (A_d). Accordingly, the conservation equation has the form:

$$(A_o) = (A_n) + (A_d). \quad (2)$$

And since native and denatured luciferase are in equilibrium,



the equation for equilibrium is:

$$A_d/A_n = K. \quad (3)$$

Or, substituting equation (3) into equation (2) gives:

$$(A_o) = (A_n) + K (A_n); \text{ or } A_n = \frac{(A_o)}{1 + K}. \quad (4)$$

Substituting again, equation (4) into equation (1), gives:

$$I = \frac{bk' (A_o) (LH_2)}{1 + K}. \quad (5)$$

From previous work,³⁰ the equation for k' is:

$$k' = \kappa \frac{kT}{h} e^{-\frac{\Delta H_o^\ddagger}{RT}} e^{\frac{\Delta S^\ddagger}{R^0}} e^{-\frac{\overline{\Delta V}^\ddagger (p-1)}{RT}}, \quad (6)$$

³⁰ Henry Eyring and Edward Eyring, *Modern Chemical Kinetics*, Reinhold Publishing Co., New York and London (1963).

and

$$K = e^{-\frac{\Delta H_o}{RT}} e^{\frac{\Delta S_o}{R}} e^{-\frac{(p-1)\Delta\bar{V}}{RT}} \quad (7)$$

Substituting equations (6) and (7) into equation (5) gives:

$$I = \frac{a T e^{-\frac{\Delta H_o^\ddagger}{RT}} e^{-\frac{\Delta V^\ddagger (p-1)}{RT}}}{1 + e^{-\frac{\Delta H_o}{RT}} e^{\frac{\Delta S_o}{R}} e^{-\frac{(p-1)\Delta\bar{V}}{RT}}} \quad (8)$$

Here,

$$a = \left\{ (LH_2) (A_o) \kappa \frac{k}{h} e^{\frac{\Delta S_o^\ddagger}{R}} \right\}$$

is a constant which must be chosen to fit the data.

To obtain the agreement shown in Figure 22, the following values were found by Eyring and Magee:³¹

$$\Delta H_o^\ddagger = 17.22 \text{ kilocalories,}$$

$$\Delta H_o = 55.26 \text{ kilocalories,}$$

$$\Delta\bar{V}^\ddagger = 546.4 - 1.813T \text{ milliliters,}$$

$$\Delta\bar{V} = -922.8 + 3.206T \text{ milliliters, and}$$

$$\Delta S_o = 184 \text{ e}\mu.$$

A reasonable picture of the reaction leading to luminescence is that below the optimum temperature the enzyme is folded and in the native state. There must be two active sites on the enzyme; the oxygen must absorb on one site with the luciferin on a neighboring site. As the luciferin releases protons into the solution, the electrons are taken up by the oxygen with the aid of the enzyme. About 9 times out of 10 the outermost electrons are captured by the oxygen. The tenth time, an inner electron is removed; and the remaining outer electron drops into the empty level beneath, thus emitting a photon. When the enzyme unfolds due to heating or because of the presence of a denaturing agent the conformation shifts, making the enzyme ineffective as a catalyst.

³¹ Henry Eyring and J. L. Magee, "Applications of the Theory of Absolute Reaction Rates to Bacterial Luminescence," *J. Cellular Comp. Physiol.* 20, 169-177 (1942).

When narcotics such as alcohol, ethers, or ketones or explosives such as TNT or DNT are added to luminescent bacteria, their light is dimmed; all of these agents have a hydrophobic hydrocarbon portion joined to a hydrogen bond formation section. The dimming of the luminescence results from the inactivation of the luciferase as it forms hydrophobic bonds with about three molecules of an alcohol or other active agent; such bonds shift the enzyme equilibrium from the native to the denatured state by lowering the surface tension at the interface between the enzyme and solvent and, so, favoring the extended denatured state. This diminution of luminescence by a narcotic N results from the forming of an additional inactive enzyme species. The new species containing s narcotic molecules must be taken account of in the conservation equation thus:

$$(A_o) = (A_n) + (A_d) + (A_d N_s). \quad (9)$$

Further, we have:



for which the equilibrium constant is K_3 , and

$$A_d N_s = K_3 (A_d) (N)^3 = K_3 K (A_n) (N)^s. \quad (11)$$

Hence,

$$A_o = A_n + K (A_n) + K_3 K (A_n) (N)^s, \quad (12)$$

or

$$A_n = \frac{A_o}{1 + K + K_3 K (N)^3}. \quad (13)$$

The light intensity, I_n , in the presence of narcotic is, therefore,

$$I_n = \frac{bk' LH_2 (A_o)}{1 + K + K_3 K (N)^3}. \quad (14)$$

Thus,

$$I_n/I = \frac{1 + K + K_3 K (N)^3}{1 + K}. \quad (15)$$

Or,

$$\begin{aligned} \ln \left\{ \left(\frac{I_n}{I} - 1 \right) \left(\frac{1+K}{K} \right) \right\} &= \ln K_3 + S \ln(N) \\ &= \frac{\Delta H_3}{RT} + \frac{\Delta S_3}{R} + s \ln N \end{aligned} \quad (16)$$

When one plots the experimental quantity on the left of equation (16) against $\frac{1}{T}$ and against $\ln N$, one determines ΔH_3 and s , respectively. Finally, ΔS_3 is obtained from the remainder. For 0.4M in alcohol solutions containing photobacterium phosphoreum, Johnson *et al.*³² found $S = 2.7$, $\Delta H_3 = -37$ kilocalories, and $\Delta S_3 = -128$ e μ .

If photobacterium phosphoreum are inhibited by alcohol and then subjected to a few hundred atmospheres pressure, the light comes on just as it does when heated-denatured bacteria are subjected to hydrostatic pressure. Incidentally, much of the effect of alcohol and ether on people is caused by partial inactivation of the oxidative enzymes concerned with the respiration of the brain and, therefore, with consciousness. Salamanders and tadpoles can be made drunk with alcohol and can then be sobered instantly by applying hydrostatic pressure. However, when the pressure is released, they instantly sink back into a drunken stupor.

Intracellular luminescence, like all biological processes, exhibits an optimum temperature or temperatures for maximum overall reaction rate. The actual temperature varies somewhat for different species, and within a single species it may be reversibly raised or lowered by physical or chemical changes in the environment of the cells. Under given conditions, a reaction of fundamental importance in determining the optimum temperature and in part the temperature activity curve is the reversible thermal denaturation of an essential enzyme. Qualitative evidence for this reaction resides in ready reversibility, by cooling, of the diminution in luminescent intensity during momentary exposures to temperatures well above the normal optimum. Analyses of quantitative data relating the amount of reversible diminution in intensity to various temperatures above the optimum indicate that a single reaction characterized by the high heat and entropy typical of protein denaturation is primarily responsible for thermal diminution. The rapidity of the change in intensity on either heating or cooling is indicative of a mobile equilibrium. The simplest explanation is that equilibrium exists between the native (active) and denatured (inactive) form of an enzyme essential to the overall process of light emission. This equilibrium reaction together with the catalytic reaction of a limiting enzyme are sufficient to account for a

³² F. H. Johnson, *et al.*, *The Nature and Control of Reactions in Bioluminescence*, J. Gen. Physiol. 28 463-537 (1945).

major part of the temperature activity curve. If one designates the equilibrium constant for the reversible denaturation as K_1 and the specific rate constant of the catalytic reaction as k_1 , Figure 23 illustrates the observed intensity (by visual photometry) of steady-state luminescence in a suspension of *A. fischeri* cells during brief exposures to temperatures above and below the optimum. The solid line is a curve calculated in accordance with the equation and constants given in the figure, assuming only the two reactions with constants K_1 and k_1 referred to above. Although the theory is oversimplified, the curve fits the data within limits of experimental error except at the highest temperature, where destruction reactions with high temperature coefficients complicate the simplified picture. With some other species of bacteria, it has not proved possible to describe corresponding data with the same accuracy, showing again that the theory is oversimplified in not including additional reactions which apparently influence the quantitative variation of the overall process with temperature.

Among other processes, the simplified theory describes with considerable accuracy the rate of reproduction of *Escherichia Coli* as a function of temperature from somewhat above to well below the normal optimum of 37° C to 39° C under the conditions involved.³³ Bacteriostasis occurs at about 45° C, but growth is immediately resumed on cooling to 37° C. Analysis of the data indicates that more than one equilibrium reaction is involved in the reversible bacteriostasis at high temperature, although *most of the temperature-activity curve can be accounted for on the same basis as that of bacterial luminescence.*

III. SUMMARY

11. Discussion and Conclusions. This study of the molecular mechanism of the conversion of the energy of chemical bond formation into light still poses interesting questions. In bioluminescence, what role does the enzyme play, first, in catalyzing the attack by molecular oxygen and, second, in the emission of light? Is the stereospecificity of enzymes important only in choosing the proper substrate, or does the enzyme structure add something special not found in ordinary organic chemiluminescence? Does the enzyme itself participate in the emission of light? Can an excited state molecular species be represented as an enzyme complex (EP^\pm)? Is enzyme configuration important in bioluminescence, and might one be able to deduce information about relative enzyme configuration from bioluminescence measurements? Is there a direct analogy between the different values of λ_{\max} , the wavelength of peak visual sensitivity observed in certain species, say isolated from fish, and the different values of the wavelengths of peak intensity for different firefly species and for different luminous bacteria?

³³ F. H. Johnson and I. Lewin, "The Growth Rate of *E. Coli* in Relation to Temperature, Quinine and Coenzyme," *J. Cellular and Comp. Physiol.* 28, 47-75 (1946).

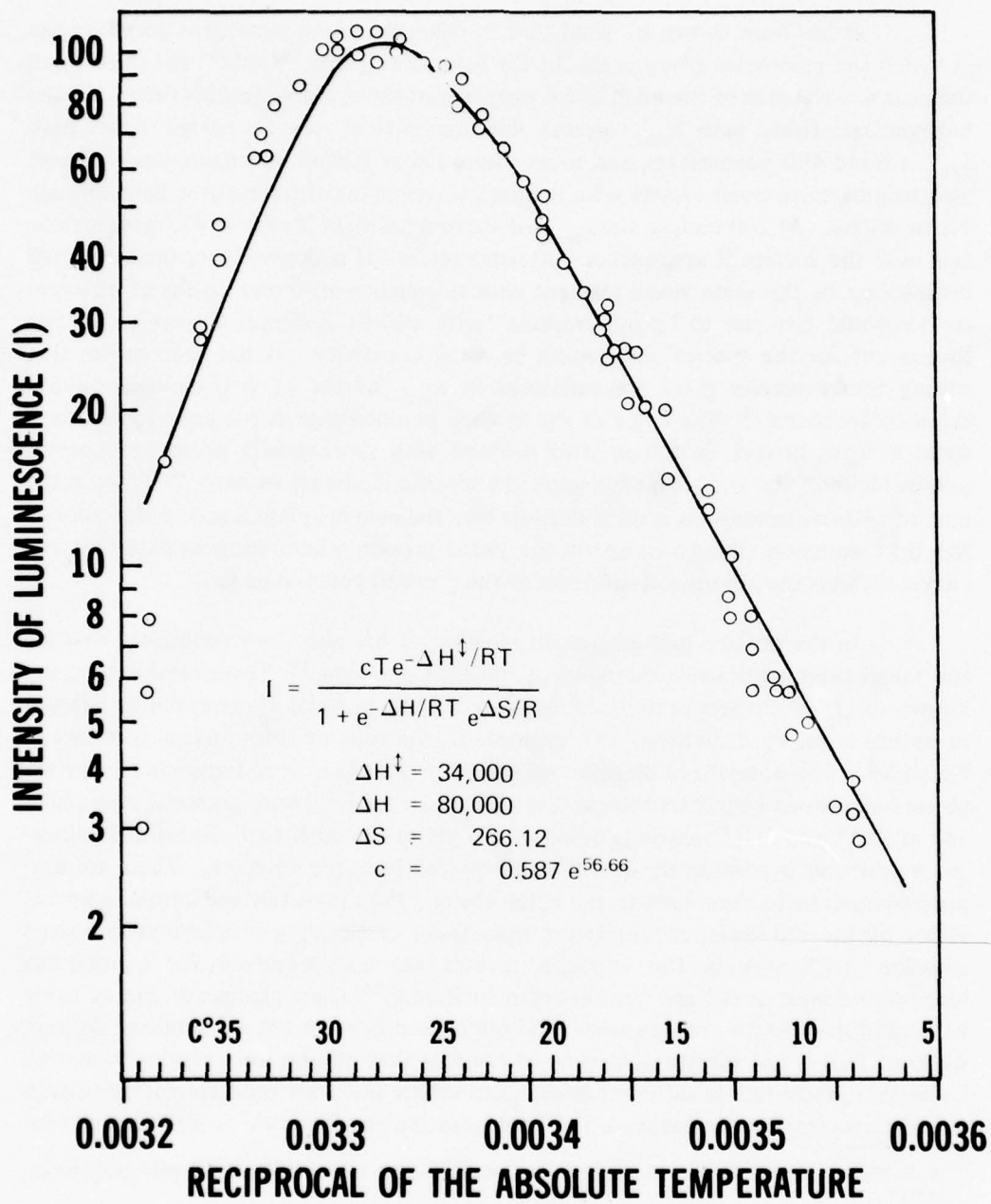


Figure 23. Influence of temperature on the intensity of luminescence in *A. fischeri*. The smooth curve was calculated in accordance with the equation and constants as given in the figure. (From Johnson, Eyring, and Polissar, *The Kinetic Basis of Molecular Biology*, New York: John Wiley & Sons (1954); data of Johnson, Eyring, and Williams (1942), p. 270. Copyright 1955 by the American Association for the Advancement of Science.)

It has been shown by Wald that in fishes the visual pigment is porphyropsin in which the prosthetic group is the 11-Cis form of retinene. Muntz³⁴ has shown that the peak wavelengths of the main visual pigments in fishes correlate with their habitats: bathypalagic fishes have λ_{\max} around 480 nanometers, surface palagic fishes have λ_{\max} around 490 nanometers, and rocky shore fishes around 500 nanometers. These wavelengths correspond closely with the peak wavelengths of transmitted light through ocean waters. At 200 meters, the λ_{\max} of transmitted light is around 475 nanometers, and near the surface it approaches 500 nanometers. It is conceivable, then, that the complexing of the same visual pigment with the slightly different opsins of different species could give rise to "porphyropsins" with slightly different absorption spectra to account for the species' differences in visual sensitivity. It has been shown that among firefly species there are variations in λ_{\max} of the in vivo bioluminescence emission spectrum.³⁵ The color of the in vitro luminescence is the same as that measured in vivo; further, in the in vitro reaction with synthetically prepared photinus pyralis luciferin the λ_{\max} depends upon the specific luciferase isolated.³⁶ Thus, in the case of bioluminescence, it is quite definite that the enzyme plays a role in the color of the light emission, the converse of the visual process where we postulate that the enzyme affects the absorption spectrum of the pigment complexed to it.

In the in vitro bioluminescent reaction, it has also been established that pH and metal cations influence the color of the light emission.³⁷ These same factors are known to effect changes in the configuration of other proteins as measured by changes in optical rotatory dispersion. An example of the type of color change is shown in Figure 24. The normalized emission spectra for *P. pyralis* in vitro bioluminescence are plotted at various concentrations and at pH's of 7.6 and 5.0 with no metal ions added and at Zn^{++} and Cd^{++} concentrations with a pH of 7.6 such that the normal yellow-green emission is completely absent and replaced by a red emission. These are normalized spectra to demonstrate the color change; the intensities and actual quantum yields of the red emission are lower than those of the very efficient yellow-green emission. Changes in the emission spectra are also observed for pyrophorus plagiophthalmus dorsal and ventral organ luciferase.³⁸ These changes do not coincide with photinus pyralis changes under the same conditions of pH or metal-ion concentration. This is not surprising in view of the fact that pyrophorus plagiophthalmus luciferases precipitate in different ammonium sulfate fractions and have entirely different disc electrophoretic patterns, the peak emission for *P. pyralis* is 502 nanometers.

³⁴ F. W. Muntz, *The Photosensitive Retinal Pigments of Marine and Euryhaline Teleost Fishes*, PhD Thesis, UCLA, (1957).

³⁵ H. H. Selinger and W. D. McElroy, *Arch. Biochem. Biophys.* 88, 136 (1964).

³⁶ H. H. Selinger and W. D. McElroy, *Proc. Nat'l. Acad. Sci. US* 52, 75 (1964).

³⁷ W. D. McElroy, H. H. Selinger, and M. Deluca, in V. Bryson and H. J. Vogel (eds.), *Evolving Genes and Proteins*, Academic Press, New York, (1965), p. 319.

³⁸ W. D. McElroy and H. H. Selinger, "Firefly Bioluminescence," in *Bioluminescence in Progress*, Frank Johnson and Yata Haneda, eds., Princeton University Press (1969).

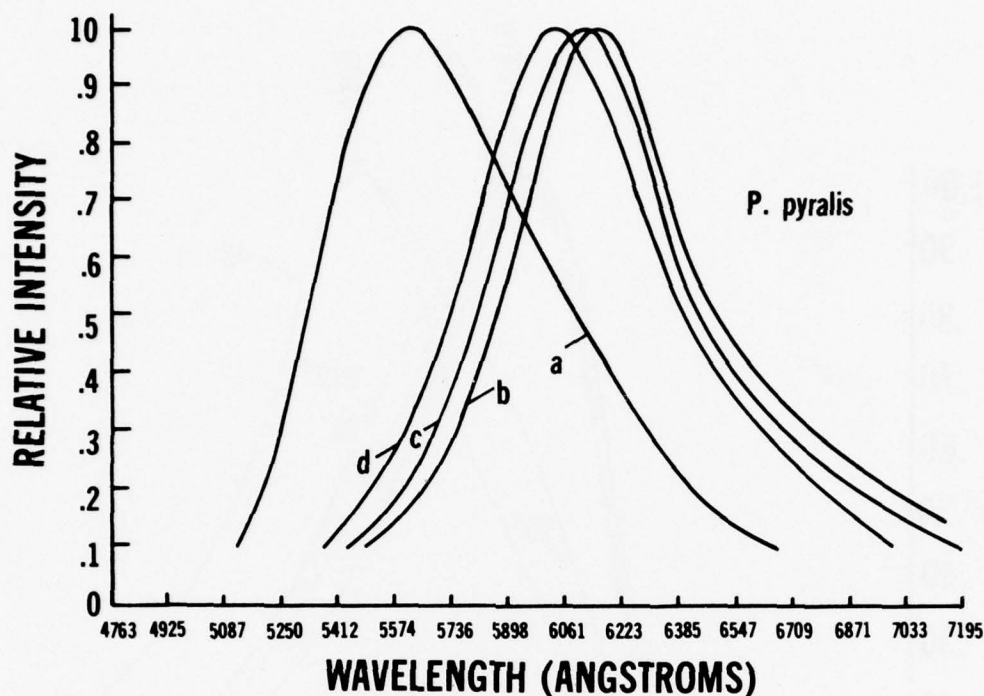


Figure 24. Maximum effects of pH, Zn^{++} , and Cd^{++} on the bioluminescence emission spectrum of *Photinus pyralis* in vitro reactions: (a) pH 7.6, no added metal ions; (b) pH 5.0, no added metal ions; (c) pH 7.6, 2.3×10^{-3} M Zn^{++} ; (d) pH 7.6, 1.2×10^{-2} M Cd^{++} . (From "Bioluminescence in Progress," *Proceedings of the Luminescence Conference*, Ed. by Frank H. Johnson and Yata Haneda, Princeton University Press, Princeton, NJ (1966).)

As an absolute minimum, the energy requirement for the light reaction is 57 kcal/mol. Since the color of light emitted in in vitro reactions can be altered and since other fireflies show different peak emissions, studies of the chemiluminescence reactions under various conditions are useful in the interpretation of color changes. Not only do the yellow-green and red emissions from *P. pyralis* show different pH optima but also they show different temperature optima.³⁹ In Figure 25, it is seen that the nature of the emission changes is the same as that observed for both pH and metal cations. There are two separately excited molecular species, one emitting in the yellow-green region and the other emitting in the red region. When the pH, the metal cation concentration, or the temperature is changed, there is not a gradual shift from a 5,565-angstrom peak to a 6,100-angstrom peak. Rather, the intensity of red emission is relatively constant while the yellow-green intensity (and quantum yield) decreases rapidly. The temperature at which the emission occurs, then, determines to a great extent the

³⁹ H. H. Selinger and W. D. McElroy, *Arch. Biochem. Biophys.* 88, 136 (1960).

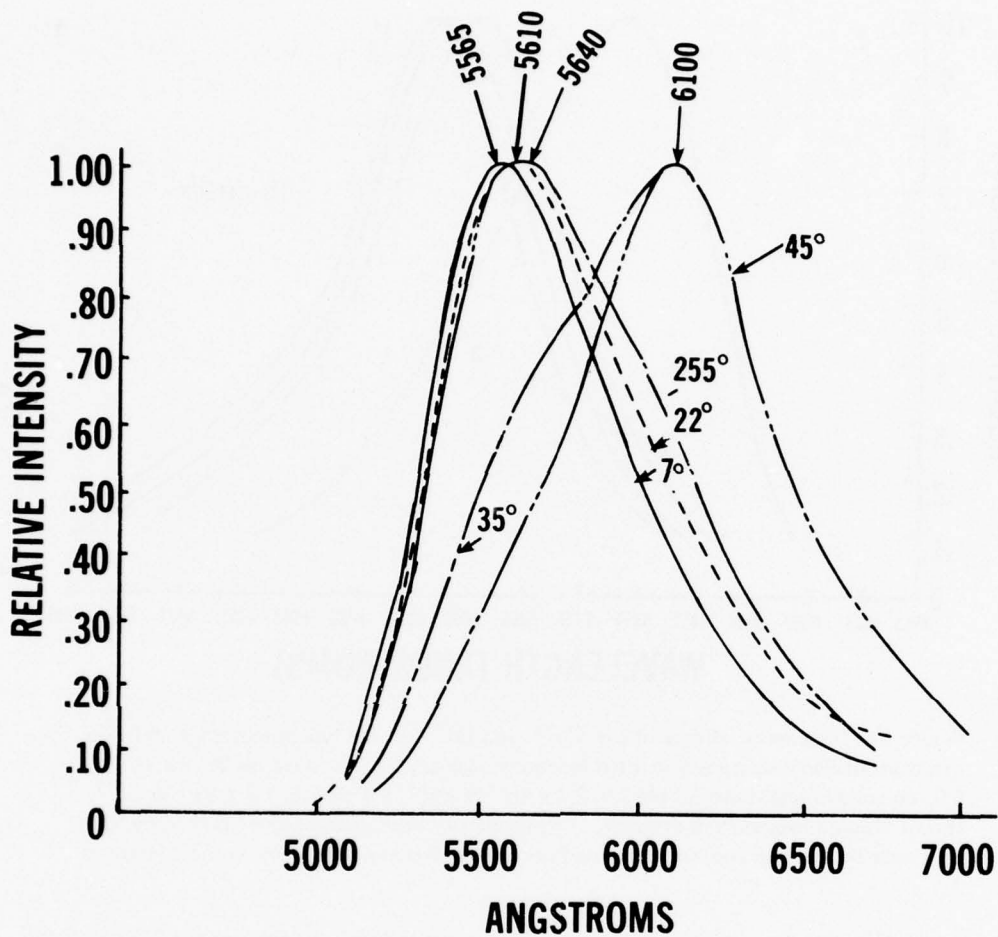


Figure 25. Variation with temperature of the in vitro luminescence emission spectrum of *P. pyralis*. Readings were taken at pH 7.6 in 0.025 M glycyl-glycine. (From H. H. Selinger and W. D. McElroy, in *Bioluminescence in Progress*, F. Johnson and Y. Haneda, eds., Princeton, NJ.: Princeton University Press (1960), p. 410.)

emission maxima. Selinger and McElroy⁴⁰ report that in the chemiluminescent oxidation of luminol in dimethylsulfoxide the fluorescence emission spectrum of completely reacted solution is identical with the chemiluminescence emission spectrum. White⁴¹ has demonstrated a recovery as aminophthalic acid of approximately 90 percent of the initial luminol oxidized. It appears that the luminol chemiluminescence is direct

⁴⁰ H. H. Selinger and W. D. McElroy, in *Bioluminescence in Progress*, F. Johnson and Y. Haneda, eds., Princeton, N. J.: Princeton University Press (1960).

⁴¹ F. H. White, in *Light and Life*, W. D. McElroy and B. Glass, eds., Baltimore: The Johns Hopkins Press, p. 183.

chemiluminescence; the chemistry of bioluminescence of firefly luciferin and luciferase appears to be more complicated. Fluorescence has not been observed corresponding to the observed bioluminescence in completely reacted solution. This negative result could indicate: (1) The product molecule on the enzyme is unstable subsequent to light emission and gives rise to one or more chemical products which are not fluorescent; and (2) the catalysis by the enzyme and the concomitant redistribution of charge of the enzyme-substrate and the subsequent excited-enzyme product allow a transient ionic configuration for the excited product molecule that has a high fluorescent yield. The subsequent relaxation of the enzyme configuration would then yield the product molecule in a nonfluorescent ionic configuration. Since the relaxation time for large enzyme molecules is on the order of 10^{-8} second, while the lifetimes of excited single states are of the order of 10^{-9} second, fluorescent emission from a transient configuration could occur with high probability.

For a long time, research on bioluminescence has been a prime example of pure science for which the chief incentive has been the wonder and delight it proffers. The initial objectives were always non-contaminated with views toward immediate practical application, and only rarely have unanticipated discoveries been turned to immediate practical use such as the currently well-known sensitive and specific assay method for ATP by luminescence of the firefly system. In principle, other luminescence systems could be turned to practical use, e.g.: the bioluminescent protein, acquerin, from the jellyfish provides the basis of the most sensitive known chemical test for calcium or strontium; the bioluminescent protein from the marine worm *Chaetopterus* can be used in perhaps the most sensitive known specific chemical test for ferrous iron; bacterial luminescence offers a sensitive means of detecting contamination of the atmosphere inside of space vehicles by jet fuels and for the detection of explosives by bioluminescent marine micro-organisms. Yet, by and large, the practical importance of bioluminescence is not immediately obvious; it is usually of unclear, if not seriously doubtful, significance even to the organism which possesses it. Partly because of this reason, relatively few investigators at any one period of time have devoted their major research efforts to problems pertaining to luminous organisms and the light they emit.

With reference to the more general significance, noteworthy is the use of bioluminescence as a "tool" for investigating the kinetic enzyme and other reactions of biological importance. For this purpose, luminescence has unique advantages, for it is the only living process in the world that has a natural visible indicator, viz, the intensity of emitted light in the rate-limiting step. The intensity is proportional to instantaneous velocity, the light intensity can be easily and accurately measured, and under appropriate conditions the same indicator can be used for the reaction either in living cells or in purified, cell-free extracts. Bacterial luminescence, in particular, has proved useful as such a tool and in fact has provided a key to the understanding of

certain aspects of the influence of temperature, hydrostatic pressure, narcotics, explosives, and some other chemical agents on the rate of biological processes.

The great majority of bioluminescent systems are chemically unidentified. In the biochemistry of luminescence, the problems which remain are manifold, challenging, and involve certain special difficulties – not the least of which is the difficulty of getting an adequate amount of raw material. The progress in research on bioluminescence through the last few years has not been gratifying, because for the most studied system, the firefly, the answer to the question “why does it flash?” has eluded explanation.

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APPENDIX

BIOLUMINESCENT DETECTION OF EXPLOSIVES

In 1970, MERADCOM became interested in developing a trace-gas detector for explosives employing bioluminescent properties. It was initially observed that when photobacteria furnished by the RPC Corporation were exposed to selected vapors in the laboratory, a change in the light level could be observed visually. The vapors exposed to the bacteria included methyl alcohol, xylene, chloroform, water, acetone, ether, dinitrotoluene, and trinitrotoluene. Each one of these materials caused some easily observable change in the light output when exposed to the bacteria except that in the case of TNT vapors it was necessary to heat the solid TNT to 100° C to observe a change. It was further observed that the observed responses were reproducible and that exposure to highly toxic vapors such as methanol and xylene did not impair the reproducibility of the responses. Out of these observations came the initial effort at MERADCOM for the development of a mine/explosives detector. A \$25,000 feasibility contract was awarded to the RPC Corporation of El Segundo, California, to commence the effort. The time allowed for completion of this initial feasibility study contract was 6 months.

A limited number of marine micro-organisms were selected and investigated. The investigation included selecting micro-organisms with increasing sensitivity to TNT vapors and developing nutrients for their growth, production, and retention. This study resulted in the development of some cultures that exhibited a change in light level when exposed to TNT vapors of ambient conditions. The apparent sensitivity of the cultures to TNT vapors had been increased.

The feasibility phase of this effort was successful in that the selection and quantitative evaluation of selected luminescent micro-organisms had produced specimens exhibiting increased sensitivities to explosives; thus, there was the possibility that it would be possible to develop a man-portable detector of concealed explosives and/or mines employing bioluminescent sensors.

The work at RPC Corporation continued by contract (dated 21 June 1971). In the extended effort, RPC Corporation was funded to continue to select, develop, and quantitatively evaluate luminescent micro-organisms. The emphasis in the strain development program was increased sensitivity, with the ultimate goal being the detection of explosives by their signature characteristics. Sensors were to be screened against the military explosives TNT, RDX, and Composition B. Engineering effort was introduced at this stage directed toward detector components, including the design of the requisite optics, electronics, and mechanical configuration of critical components.

Emphasizing the biosensor as the heart of the detector, the development of ancillary apparatus was initiated to avoid conditions when hardware development lagged behind the microbiological development. The additional effort extended the contract for 8 months at an increased cost of \$79,357.

The completed work after this effort showed additional progress; a breadboard model, six-channel photochemical sensor unit was constructed to speed the process of biosensor screening. The use of the short-pulse response of the biosensor through the use of a photomultiplier coupled with breadboard electronics was investigated as the detector circuitry and compared to a previous CdS photocell arrangement, new biosensors were prepared and developed from wild strains obtained through the procurement of new bacterial isolates from various marine sources, and mutant strains were obtained through increased mutagenic effort. New strains were evaluated (mutant and wild) for specificity and sensitivity to the Government-furnished military explosives, TNT, RDX, and Composition B. Investigations were made into the capability of preservation for long periods of time and freedom from the effects of interference. Nutritional parameters were studied, including the effects of pH, water vapor, and composition and their effects on stability, performance, and longevity. Also, provided by this effort were 12 lyophilized preparations of each superior biosensor culture with activation procedures for use in "in-house" laboratory investigations.

Additional effort was placed on the development of biosensors and related detector parameters by the addition of about \$100,000 in new effort. The additional effort added some 12 additional months to the contract. The increased effort provided for the continued development and refinement of biosensors through (a) isolation of new wild strains from the natural environment, (b) mutagenic effort to increase biosensor sensitivity to the explosive effluents of interest, and (c) preservation by lyophilization of the best strains developed. During the course of these investigations, technical instruction was furnished to Government personnel relative to initiation of in-house work. Optimization of biosensor performance continued; the biochemical variables, pH, nutritional requirements, and substrate gel were thoroughly investigated. The effect of carrier gas change was studied as were modifications of the biosensor unit toward enhancing vapor capture. A six-channel photochemical sensor unit (PSU) was improved, and a laboratory demonstration unit (LDU-1) was provided to the Government as part of this expanded effort. Calibration standards were prepared, and the laboratory calibration unit was furnished as part of a laboratory demonstration. A study was also initiated to determine the metabolic and chemical changes produced in the micro-organisms when in the presence of explosive vapors. Considerable progress was made in developing new strains, increasing sensitivities, and adapting a photochemical sensing unit to the observation of changes in light during the exposure to explosive vapors. A total of \$200,000 had been expended for the work at this time, and this period represented a major effort in the development of the explosive detector

employing bioluminescent micro-organisms.

At this point, hundreds of strains had been developed, the PSU had been improved, the sensitivity had been enhanced, and a voluminous amount of data had been collected. There seemed to persist, however, a lack of reproducibility whether from strain development or instrument development. The performance of the system in the presence of explosive vapors was not the same when exposure was made at the contractor's facility (West Coast) and at the Government facility (East Coast). Isolation of the causes of this discrepancy was elusive. The strains developed also showed a sensitivity to many chemicals including water vapor.

Additional effort was placed with the contractor through a \$155,000 addition to the contract. This additional effort was directed to developing and isolating potential strains; increasing sensitivity by means of mutagenesis; selecting and lyophilizing the best strains; optimizing the biosensor environment; and developing two-channel photo-sensor testing for specificity against impurities, functional groups, etc. The same type of technical difficulties were encountered in this effort. The parameters contributory to malfunctions in the system were never completely overcome. The final addition to this funded effort was \$48,000. All technical effort was completed in 2QFY73. Developed systems were delivered; these included a laboratory demonstration unit, a six-channel photochemical sensor unit (PSU), and lyophilized strains. The total effort was \$400,000 and about 3 years of experimental and technical effort.

Some indication of the lack of sensitivity and selectivity of the responses of typical bioluminescent systems is shown in Table 1.⁴² Threshold sensitivities for two instruments against EGDN and acetic anhydride are shown in Table 2. A value of 0.1 p/b (extrapolated) for EGDN at 10 percent scale sensitivity was observed on an ITI Model 58 explosive detector. A reading of 15 p/b at a 3:1 signal ratio was observed for EGDN, and a measurement of 24 p/b was observed for acetic anhydride, measured directly. In 1972, MERADCOM exposed a W. S. Bonita strain on gelatin and discovered a peak emission at 4,750 angstroms within the range 4,700 to 4,850 angstroms (Figure 26). Negative results were obtained on the response of RPC biosensors in the presence of explosives.⁴³ MERADCOM further reports no spectral shifts in the emitted light when the sensors are exposed to various trace gases (vapors of heptane, acetone, methanol, and benzaldehyde).

⁴² William A. Wall and Herbert M. Gage, *Evaluation of the PRC Dual-Channel Biosensor System and the ITI Model 58 Explosive Detector for the Detection of TNT and Other Compounds*, Technical Memorandum 74-14, U.S. Army Land Warfare Laboratory, Aberdeen Proving Ground, Maryland, May 1974.

⁴³ G. E. Spangler, and David Loental, *A Preliminary Spectroscopic Study of the RPC Bioluminescent Sensors*, Technical Memorandum 16, U.S. Army Mobility Equipment Research and Development Center, Fort Belvoir, Virginia, January 1972.

Table 1. RPC Bioluminescent Detector Test Results, Complete

Chemical Compounds of Interest	TNT	Dynamite		Acetic Acid	
	Negative Sensor	Negative Sensor	Positive Sensor	Negative Sensor	Positive Sensor
Drugs					
Cocaine	0	0	0	+	+
Heroin	+	0	+	-	+
Heroin (Street)	0	0	0	-, +	+
Marijuana	+	+	+	+	+
Morphine Sulphate	+	0	0	0	0
Explosives					
Black Powder	-	-	+, -	+	+
C-3	-, +	-, +	-, +	-, +	-, +
C-4	-, +	-, +	-	-	0
Comp B	-, +	-, +	+, -	+, -	+, -
Dynamite	-, +	-, +	+, -	-, +	-, +
Dynamite, Gelatin, 40%	-, +	-	+, -	+	-, +
Dynamite, Gelatin, 50%	-	-	+, -	+	-, +
Dynamite, Gelatin, 60%	-	-	+, -	-, +	-, +
Dynamite, Red Cross	-	-, +	+, -	-, +	-, +
Flex - X	0	+	+	+	+
Ethylene Glycol Di-Nitrate (EGDN)	-	-	+, -	+	-, +
Octol	-	+, -, +	+, -	-, +	-, +
PE-2	0	+, -	+	+	+
PEIN	0	+	+	0	0
Pentolite	+, -	+	+, -	+	+
PETN	0	+	+	+	+
PL-NP-10	0	+	+	+	+
RDX, Comp A-3	0	+	+	+	+
RDX (BRL)	+	+	+	+	+
SEMTEX-H	+, -	+	+	+	+
Tetryl	+, -	+	+	+	+
Tetryl, British	+	+	+	+	+
TNT (BRL)	-	+	+	+	+
TNT, Granular	-	+	+	+	+
Nitroglycerine on Lactose	-	-	+, -	+	+
Common Compounds					
Citrus Fruit, Grapefruit	+	0	+	+	+
Fertilizer, 10-8-7, Liquid	+	+	+	+	+
Gasoline (ARCO Regular) (Getty)	-, +	-, +	+, -	-, +	+, -
Heating Oil	+	+	+	+	+
Water, Distilled	+, -	+	+	+	+, -
Silicone Compound, MIL-S-8660B	-	-	+	+	+
Gun Related Compounds - Cleaners, Preservatives, Coatings					
Compound, Heavy	+	0	0	+	+
Corrosive Protective Compound (MIL-C-16130)	-, +	-	0	0	+
Cosmoline	0	0	0	+	+
Grease, Automotive & Artillery (MIL-C-10924)	0	0	0	+	+
Gun Oil	0	+	+	+	+
Gun Slick	0	0	0	+	+
Rust Inhibiting Grease, RIG Universal	-	0	0	+	+
Solvent, Nitro-Powder, Hoppe's	-, +	-, +	+, -	-, +	-, +
Acetic Acid (Glacial)	-	+, -	+, -	-, +	+
Acetic Anhydride	-, +	+, -	+, -	-, +	+, -
Acetone	+, -	0	+, -	+	+
Ammonium Hydroxide	-, +	-, +	-, +	-, +	-, +
Benzene	-, +	+, -	-, +	-, +	-, +
Citric Acid	0	0	0	+	+
Dimethyl Sulfoxide	-, +	-	+	+	+
Ether	+	+	+, -	-, +	-, +
Ethyl Alcohol	+, -	0	+	+	-, +
Pyruvic Acid	+	+, -	+, -	-, +	+

From William A. Wall and Herbert M. Gage, *Evaluation of the PRC Dual Channel Biosensor System and the ITI Model 58 Explosive Detector for the Detection of TNT and Other Compounds*, Technical Memorandum 74-14, U.S. Army Land Warfare Laboratory, Aberdeen Proving Ground, Maryland, May 1974.

+ Positive Deflection on Recorder
 - Negative Deflection on Recorder
 0 Insignificant Deflection
 X No Test

Table 2. Detector Response to Quantitative Tests for Additional Compounds of Interest

Detector	Compound Concentration and Response	Comments
Model 58 Explosive	0.1 p/b EGDN – 10% Scale Deflection, Range (x1)	Extrapolated
Model 16 Biosensor System (RPC)	15 p/b EGDN – 3:1 Signal to Noise	Measured Directly
	24 p/b Acetic Anhydride – 3:1 Signal to Noise	Measured Directly to Noise

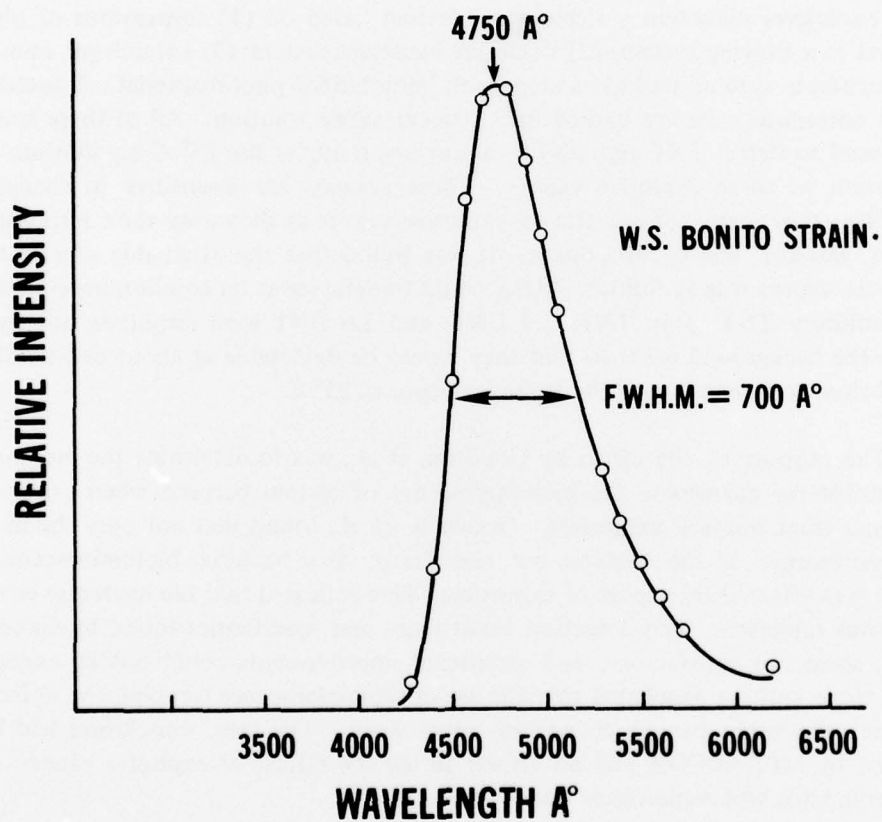


Figure 26. Spectral distribution of bioluminescent strain W. S. Bonita on gelatin (RPC Corporation), showing range of emission. (From G. F. Spangler and David Loental, *A Preliminary Spectroscopic Study of the RPC Bioluminescent Sensors*, Technical Memorandum 16, U.S. Army Mobility Equipment Research and Development Center, Fort Belvoir, VA, January 1972.)

All efforts to increase the sensitivity of the bioluminescent bacteria by selection of better strains, by creation of new strains via mutagenesis and by optimizing the nutritional, pH and temperature conditions had very limited success. The TNT detection sensitivity remained within the same order of magnitude as determined employing the RPC supplied instrument by the COR (Capt Hollis) at MERADCOM and was less than the sensitivities of commercially available explosive detectors. In addition, the light output of the bioluminescent bacteria was affected by the presence of many other substances. For these reasons, it was concluded that bioluminescent bacteria could not be developed into a sensing system useful for explosive detection and all efforts were terminated.

Goodson, Jacobs, and Wells⁴⁴ conducted an investigation directed toward the use of photobacteria and their enzyme systems in the detection of military explosives. Four explosives detection systems were devised based on (1) suspensions of photobacteria in a flowing system; (2) a cell-free luciferase system; (3) a starch-gel, immobilized luciferase system; and (4) a starch-gel, immobilized photobacterial cell system in which entrapped cells are bathed in 3 percent saline solution. All of these systems were used to detect TNT and DNT – about one-tenth of the 25° C equilibrium concentration of these explosive vapors. These systems are insensitive to changes in humidity; they were not specific to explosive vapors as shown by their responses to rubber, gasoline, and cyclohexanon. It was found that the attainable sensitivity to explosive vapors was as follows: RDX could be detected at its equilibrium concentration; military TNT, pure TNT, 2.4 DNT, and 2.6 DNT gave responses sufficiently above the background noise so that they would be detectable at about one-tenth the equilibrium concentration of the explosive vapor at 25° C.

The purpose of the effort by Goodson, et al., was to determine the mechanism responsible for changes in the bioluminescence of certain bacteria when exposed to effluents from military explosives. Goodson, et al., found that not only the in vivo bioluminescence of the bacteria but also the in vitro bacterial bioluminescence reaction was affected by vapors of explosives. This indicated that the luciferase enzyme itself was inhibited. The detection sensitivities and specificities found by Goodson, et al., were not satisfactory, and significant improvements could not be expected. Thus, these authors concluded that the use of bioluminescence for explosive detection was not promising enough to warrant more work. The same conclusion had been reached by MERADCOM and all efforts to use the effects of explosive vapors on in vivo or in vitro bioluminescence were terminated.

⁴⁴ L. Goodson, W. B. Jacobs, and F. E. Wells, *Mechanism of Bioluminescent Detection of Explosive Vapors (Report No. 19)*, Midwest Research Institute, for U.S. Army Mobility Equipment Research and Development Center, Fort Belvoir, Virginia, Final Report, July 1974.

GLOSSARY OF TERMS

Aerobically –	Requiring oxygen or not destroyed by oxygen.
ADP –	Adenosine 5'-diphosphate.
Aequorea –	Luminous tissues of bioluminescent jellyfish.
AMP –	Adenosine 5'-monophosphate.
Anaerobically –	Requiring absence of free oxygen or not destroyed by its absence.
Antigenic –	A substance which when introduced into animal tissue causes antibodies.
ATP –	Adenosine 5'-triphosphate.
CTP –	Cytidine 5'-triphosphate.
DPNH –	Diphosphopyridine nucleotide reduced form (cozymase).
FMN –	Riboflavin, Riboflavin 5'-phosphate.
FMNH ₂ –	Reduced flavin mononucleotide (flavin).
Gram-Negative –	Upon application of biological stain, will lose the stain to the decolorizer.
Gram-Positive –	Upon application of biological stain, will retain the stain.
LH ₂ –	Luciferine (general).
Luciferin –	Oxidizable substance supplying material capable of absorbing enough energy to emit in the visible region for those luminous organisms requiring dissolved molecular oxygen for luminescence.

Lyophilized – Freeze dried.

Parasitic Bacteria – Causing infection of various living animals.

Saprophytic Bacteria – Living on such dead matter as fish and meat.

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