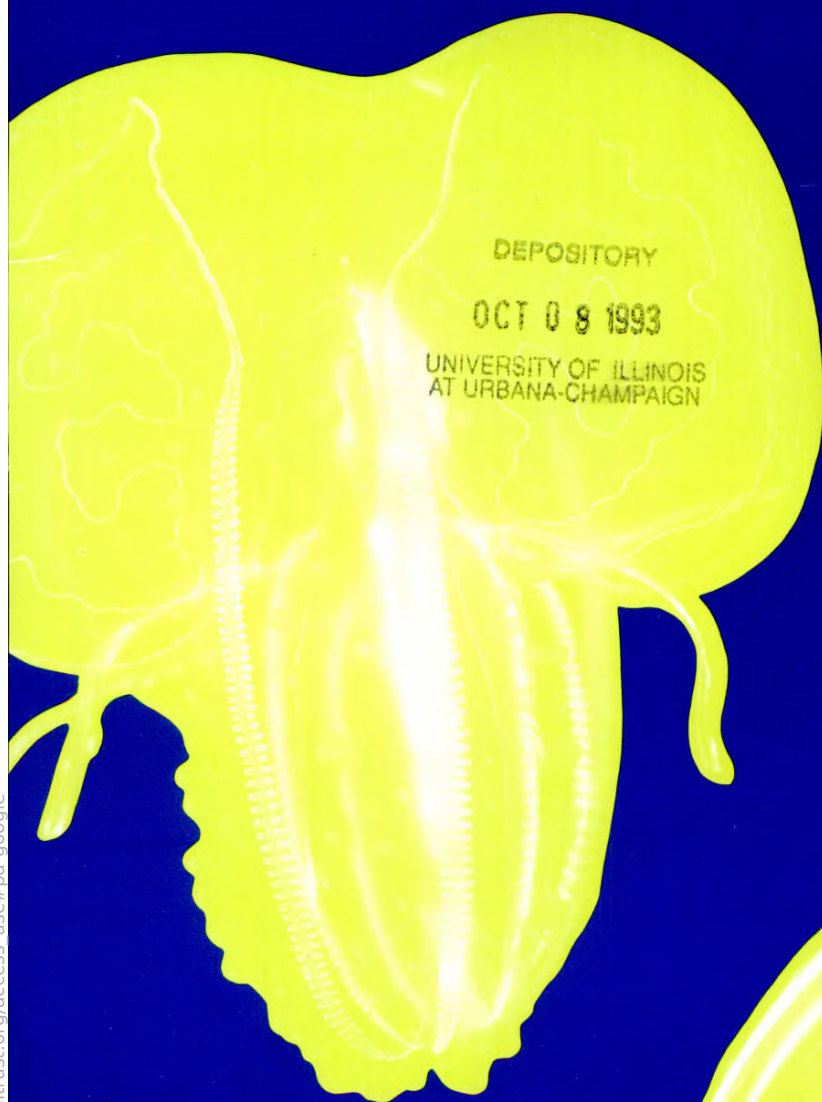


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**Bioluminescence
In the Sea**

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The Hawaiian squid Euprymna scolopes posses a specialized light organ (4 lobes of light organ in center of picture) to culture bioluminescent bacteria (Vibrio fischeri). Through the use of a shutter covering the light organ, the squid is able to flash the light generated by the luminescent bacteria in behavioral displays. (Photo by W. Ormerod, University of Southern California)

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These marine invertebrates are in the phylum Ctenophora of which nearly all members are brightly luminescent. These specimens, *Leucothora pulchra*, display their bioluminescence in convoluted filaments called comb plates that are along the axis of the animal and gastrovascular canals that run along the surface of the body wall. They hunt in surface waters, capturing prey with sticky oral tentacles that are periodically brought to the mouth. Some ctenophores are so prevalent seasonally that they can be significant predators on juvenile fish. (Photo by S.H.D. Haddock, University of California, Santa Barbara).

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Bioluminescence: The Fascination, Phenomenon and Fundamentals

*Randall S. Alberte
Office of Naval Research*

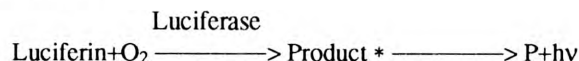
Biologically produced light is a phenomenon that has escaped notice by very few. Many of us still hold fond childhood memories of watching, chasing and often collecting “lightning bugs” (fireflies) in the early evening. Though this experience is undoubtedly widespread in the world, fewer individuals have observed the rather spectacular and dramatic bioluminescence displays in marine environments. These displays are typically visualized in waves breaking on the shore, in the wakes of boats, and by swimmers due to their own swimming motions. In some cases and at certain times of the year or the lunar cycle, entire bays or estuaries take on a glow or show bright light events. Indeed, the diversity of organisms responsible for the sources of light in the sea is extensive and much greater than that found for terrestrial species (Table 1). Some 700 genera representing 16 phyla are known to possess bioluminescent species^{1,2,3}.

The myths surrounding bioluminescence have been a part of many ancient cultures and certainly dominate the lore of

early seafarers. Scientists turned their attention to the phenomenon of bioluminescence in the late 1800s, and, subsequently we have learned a great deal about species diversity of the sources, the basic chemistry of bioluminescence, the physiology and behaviors coupled to light production, and, more recently, both the molecular biology and genetic regulation of this process and the oceanographic implications of bioluminescence. As summarized in the articles that follow, the study of bioluminescence is still in its infancy. Though our understanding of the chemical details of bioluminescence, a specific case of chemiluminescence, are advanced, (see Seliger, this issue) many questions related to the biology and natural mechanisms of stimulation remain unresolved.

Though not true for all bioluminescent systems, many share some common features at that level of the chemical reactions leading to light production. Luciferase, the enzyme that catalyzes the light generation reactions, is an oxygenase, and as such requires molecular oxygen (O₂) as a substrate. The

molecular weight of luciferases ranges from about 20,000 to 420,000 Daltons and most likely differ in details of the catalytic reactions depending on the source of the enzyme. In addition, luciferin (a class of compounds that includes aliphatic aldehydes, tetrapyrroles, imidazopyrazines, etc (see ref. 1) serves as a substrate for all bioluminescent reactions. The generalized reaction is,



where P* is the product in an electronically excited state which is sufficiently energetic to release a photon (hν) and yield the production in the ground state (P).

The light produced by the diversity of known bioluminescent organisms can range in spectral quality from wavelengths of about 410 nm to about 560 nm, covering most of the blue, all of the green and a part of the yellow portion of the visible spectrum (400-700 nm). Most marine species, however, generate blue wavelength bioluminescence^{1,2}.

The cellular distributions, sites of synthesis or storage, modes of acquisition, coupling to other metabolic processes, and means of regulating luciferase and luciferin levels are obscure or poorly understood in most species, especially the marine invertebrate phyla. For example, in some photosynthetic dinoflagellates (unicellular algae), the capacity for significant bioluminescence exists only at night and is localized in a specific subcellular organelle (see Hastings, this issue), while in some fish species, bioluminescence is only acquired through a symbiotic association with luminescent bacteria (see Nealson, this issue). In other fishes, like the midshipman fish, the capacity for bioluminescence is strictly dependent upon ingestion of bioluminescent organisms for a source of luciferin.⁴ In most of these cases the light produced is visualized as flashes which vary in length and intensity. The flashes can be induced by mechanical stimulation and/or controlled by neuronal innervation. In sharp contrast to this situation, which is characteristic of most eukaryotic phyla, luminescent bacteria (prokaryotes) produce light continuously, but at very low levels (ie., 2-5 orders-of-magnitude lower than dinoflagellates on a cell basis).

In some marine and freshwater invertebrates, luciferase and luciferin are packaged and stored. Under specific circumstances, these packages are expelled from the body cavity generating a light signal free in the water column. In cases where animals actively swim away from their bioluminescent displays (ie. many copepods), this behavior likely reduces predation by confusing potential predators (see Case, this issue). In other invertebrates (ie., Ostracods), this behavioral strategy, which can be highly elaborate and species-specific, is used in courtship displays to attract mates.⁵ In both of these cases, luciferin and luciferase are stored and perhaps synthesized in specialized light organs. However, light organs in certain fishes and some squid are quite different. They are

specialized not for the storage and packaging of the bioluminescence machinery, but for the culture and maintenance of luminescent bacteria in a symbiotic association (see Nealson, this issue). In these animals, the light flashes observed result from the animal's use of a shutter to control the light signal which is generated continuously by the symbiotic bacteria in the light organ. The bioluminescent signaling observed in nature may be involved in species communication, courtship behaviors or mate recognition, or predator-prey interactions.

The tremendous diversity of bioluminescent organisms confounds our ability to understand the evolution of this trait (see Seliger, this issue; ref. 3). There are no clear systematic or phylogenetic pattern to the distribution of luminescent species among the phyla in which they occur. This observation has led to the speculation that this capacity had to have multiple origins during evolution. Most investigators would agree that bioluminescence is probably not a primitive trait (See Seliger, Nealson and Hastings, this issue). Clearly its biological role in mid- and deep-water species would argue that bioluminescence capacity could not have evolved much before the development of the sensory systems employed to detect the sources (see Case).

Molecular biological tools can now be used to address the evolutionary patterns that could have generated the modern distribution of bioluminescent capacity. Recent applications of this approach have revealed that at least in certain symbiotic luminescent bacteria found in fish, the bacterial symbiont species co-evolved with its host fish species and are of recent origins compared to their free-living luminescent counterparts.⁶ It is clear that these approaches will doubtless provide new insights into the evolution and ecological significance of one of the most unique, spectacular and yet mysterious biological processes.

Our ability to isolate the genes for bioluminescence from certain bacteria (termed lux genes) and to move them into non-luminescent species (ie. bacteria, plants, animal tissue culture cells, etc.), has provided an invaluable means to study a vast number of biological processes that were previously intractable. For example, Silverman and colleagues⁷ were among the first to manipulate lux genes so that they could report, through light production, the "sensing" of a surface by a fouling bacterium into which the lux genes were moved. The use of the lux genes as a "reporter" for a variety of sensing or detection functions is just beginning to be exploited (See Nealson, this issue). For example, Sayler and colleagues⁸ have used the lux genes as a reporter for the detection of trace toxicants in the environment. The potential future uses and applications of this capability for food quality screening, water quality assessment, detection of race chemical toxicants and exploitation in a broad range of biosensors is enormous.

There remain many unanswered questions about the bioluminescence phenomenon. Future research in this field

ranging from molecular studies to ecological and populational investigations will greatly expand our knowledge and understanding of biological light production, in general, and specifically in the sea. It has been the use of our basic understanding of light production and the characteristic of light signals from a number of marine sources studied in the laboratory that lead to the design and development of the current state-of-the-art bathyphotometers, instruments for the quantification of oceanic bioluminescence (see Case, this issue). Despite the infancy in our understanding of this process, several significant advances have been made in assessing bioluminescence in the oceans (see Case, this issue), elucidating its role in predator-prey interactions, reproductive behaviors and exploiting the molecular genetic aspects of this system.

The following articles briefly summarize our current state of knowledge of the bioluminescence process, the properties of luminescence in bacteria and dinoflagellates, the current detection technologies for oceanic assessments and the ecological significance and evolutionary features of this widespread and fascinating phenomenon in the sea. The Office of Naval Research has been the principal in the support of basic research in this area, and in conjunction with the operational Navy pioneered the development and deployment of ocean assessment technologies.

Table 1.

*Distribution of bioluminescence in marine, freshwater and terrestrial environments. Common names are used where possible. *Indicates that bioluminescence arises from luminescent bacterial symbionts.*

Marine Systems	Terrestrial Systems
Bacteria	Bacteria
	Fungi (mushrooms)
Algae	
Dinoflagellates	
Invertebrates	Invertebrates
Coelenterates (jellies)	
Annelids (worms)	Annelids (worms)
Arthropods (crustaceans)	Arthropods (insects)
Molluscs* (squid, octopus)	
Bryozoans	
Echinoderms	
Urochordates* (sea squirts)	
Vertebrates	
Pices* (fish)	
	Freshwater Systems
	Molluscs

References

1. Herring, P.J. 1987. The systematic distribution of bioluminescence in living organisms. *J. Biolum. Chemilum.* 1:147-167.
2. Herring, P.J., Campbell, A.K., Whitefield, M. and Maddock, L. (Eds.) 1990. *Light and life in the sea*. Cambridge Univ. Press, Cambridge, 357 p.
3. Hastings, J.W. 1983. Biological diversity, chemical mechanisms and the evolutionary origins of bioluminescent systems. *J. Molec. Evol.* 19:309-321.
4. Mesinger, A.F. and Case, J.F. 1991. Bioluminescence maintenance in juvenile *Porich tys notatus*. *Biol. Bull.* 181:181-188.
5. Moran, J.G. and Cohen, A.C. 1991. Bioluminescent displays, courtship, and reproduction in Ostracodes. In: *Crustacean sexual biology* (R.T. Bauer and J.W. Martin, eds.). Columbia Univ. Press, NY, pp 1-16.
6. Wolfe, C.J. and Haygood, M.G. 1991. Restriction fragment length polymorphism analysis reveals high levels of genetic divergence among the light organ symbionts of flashing fish. *Biol. Bull.* 181:135-143.
7. McCarter, L., Hilmen, M., and Silverman, M. 1988. Flagellar dynamometer controls swarmer cell differentiation of *V. parahaemolyticus*. *Cell* 54:345-351.
8. King, J.M.H., DiGrazia, P.M., Applegate, B., Burlage, R., Sanseverino, J., Dunbar, P., Larimer, F. and Sayler, G.S. 1990. Rapid and sensitive bioluminescent reporter technology for naphthalene exposure and biodegradation. *Science* 248:778-781.

Bioluminescence: Excited States Under Cover of Darkness

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Introduction

Bioluminescence is an efficient enzyme-catalyzed chemiluminescence. It involves the production of excited states of molecules by chemical reactions which are likely selected and optimized for the function of inter- and intra-species communication in the dark. In all species studied the function appears to have been optimized not only for the maximum efficiency for producing chemi-excited electronic states^{1,2,3} but for the most efficient utilization of this light in nature. This is in complete analogy with optimizations for visual communication among daylight-active species. The spectral distributions (colors) of the bioluminescence maximally overlap the visual spectral sensitivities of the intended receivers^{4,5}. In addition light organs have reflecting and focussing properties, and photophores (light-emitting organs) have species-specific spatial patterns for recognition. Further, irradiance levels of external ambient sunlight or biological clocks entrained to natural photoperiods determine the times of initiation of bioluminescence signals. (See article by Hastings.)

Bioluminescence is directed, triggered in response to external stimuli, i.e., female firefly flash in response to male signals, or in the dinoflagellates, membrane deformation re-

sults from raptorial predators. Brightnesses of signals, their temporal patterns (flash sequences or glows) and the behavioral responses to bioluminescence are species-specific. All of these represent the addition of a biological source of light, chemiluminescence, to the armamentarium of intra- and inter-species communication already served by olfaction (chemical pheromones) and vision in ambient sunlight (to detect shape, motion and visible color patterns achieved by pigment distributions).

In light-limited environments, bioluminescence provides the visual stimulus for both the approach and the final contact. Pheromones, tactile stimuli and vision function at close quarters in low ambient light, presumably to confirm the identity of the species. Bioluminescence is instantaneously transmitted, leaving no trace for predators to follow, permitting digital and species-specific repetitive signalling by individuals moving rapidly over large distances in crowded environments. The light may be used for passive camouflage as in fish species that adjust the diffuse brightness of their ventral surfaces to mimic the intensities of downwelling light irradiance, appropriately named "light to hide by"⁶ in that they cast no "shadow" to a predator below. It may be used for species-specific patterns of luminous photophores by which males of fish species recog-

nize their potential mates or other species, or for the emission of "attracting" light from organs within or dangling in front of, the open mouths of predator fish species. It may be for species-specific digital flash patterns exchanged between male and female fireflies, or for the mimicking of these patterns by predator firefly species, or for the emission of "light-shock" flashes or the expelling of luminous slime to confuse predators.

Occurrence of Bioluminescence in Nature

A distinction should be made between the occurrence of bioluminescence and the occurrence of bioluminescent species^{7,8}. Bioluminescent organisms emit light for the function of intra-species signalling, for the confusion of predators, or to attract prey. Therefore the timing of the emission and the amount of light that they emit is carefully regulated. This is particularly valid when the organisms occupy and migrate vertically within the upper 200 m of the ocean. Presumably during daylight hours, sunlight will interfere with detection of bioluminescence. Therefore, optimization of energy efficiency for bioluminescent displays will select for regulation mechanisms for the inhibition of bioluminescence during daylight. The behavior of fireflies might be used as an example. The larvae are bioluminescent, with distinct light organs. They can be "irritated" to produce a glow, implying that the glow is not constant. They can be located at night by their glow, but only those that happen to glow contribute to our statistical sample; we are not certain of the fraction of the total population these represent. They do appear to glow for a time when they emerge from the ground at night. These glows are much less bright than the flashes used for longer range signalling in the adults, again a selection for the optimum balance between efficiency for detection and energy conservation. Our measurements indicate that an individual firefly enters into its adult state in summer with all of the luciferin and luciferase it will need to carry out flash signals to find a mate during its brief ca. 1-2 week adult existence. Many fireflies such as the common North American species *Photinus pyralis*, carry out active flashing activity for ca. 1 hr each twilight. Other species flash less frequently all night long.

A similar picture holds for marine bioluminescence. Although a dinoflagellate can be stimulated (by creating high turbulence under laboratory conditions) to emit all of its bioluminescent potential within a few seconds, this very seldom happens in nature. If a dinoflagellate is not disturbed, i.e., by turbulence produced by a fish swimming nearby or by a copepod predator attempting to grasp it, it will emit negligible bioluminescence. Many long nights of observation in bioluminescent bays have confirmed this fact. The dinoflagellate, although exquisitely sensitive at night to membrane deforma-

tion triggering stimuli, by bubbles rising to the surface, by copepod predators, by fish swimming, by gentle raindrops, is essentially non-luminescent. Even in dense laboratory culture the spontaneous photoperiodic bioluminescence of *Gonyaulax polyedra*, originally reported by Beatrice Sweeney and J.W. Hastings, comprises less than 10^{-4} of the stimutable bioluminescence. They were fortunate in observing this aspect of a biological clock mechanism because the spontaneous bioluminescences are even lower in intensity in the *Pyrodinium* and *Pyrocystis* species which also do not show significant rhythmicity. Bioluminescent signalling for mate location should have seasonal periodicities and may be involved in schooling, while light-shock signals or luminous secretions should not occur unless stimulated by predation activity. Only light emission "to hide by" or for attracting prey might be present more consistently, although these too should vary with ambient sunlight intensities and with prey availability. Generally, therefore, "spontaneous" bioluminescence in surface waters will be restricted to mating displays, predator avoidance flashes and the anthropogenic turbulence introduced by surface ships, submarines and the detecting devices deployed by scientists attempting to measure the bioluminescence.

Bioluminescence is exhibited by relatively few terrestrial species (see Alberte, Table 1). These include the ubiquitous fireflies (Coleoptera), springtails (Collembola), termites (Isoptera), lantern flies (Hemiptera), some fungi (Basidiomycetes), earthworms (Oligochaeta), some marine worms (Polychaeta), millipedes (Diplopoda), centipedes (Chilopoda), a snail species (*Quantula striata*) and gnats or midges (Mycetophilidae), including the well known larva (glow worm) of the New Zealand-Australian fungus gnat (*Arachnocampa richardsae*). Bioluminescence appears to be a much more common phenomenon in marine organisms, occurring in dinoflagellates, coelenterates, ctenophores, tunicates, euphausiids, decapod shrimps, stomiatoid and myctophid fishes, and squid. With the latter two groups involving symbiosis with luminescent bacteria. Within the upper 200 m of the ocean the predominant **stimulable** bioluminescence is due to dinoflagellates. These are best known from the spectacular bioluminescent displays observed in tropical bioluminescent bays^{9,10} where the species *Pyrodinium bahamense* var. *bahamense* is retained year round at mean concentrations above 10^5 cells L^{-1} and forms blooms containing up to 10^7 cells L^{-1} . The organisms are concentrated enough to discolor the water, resulting in "red tides", and at night the stimutable bioluminescence in the wake of a boat is bright enough to read by.

Seasonally, following the relaxation of upwelling, other bioluminescent dinoflagellate species form dense, visibly colored surface patches or streaks (red tides) up to 10 km in length along continental shelves and in nearshore waters. In many geographical areas these red tides appear to be transported alongshore in the direction of the prevailing winds for hun-

dreds of kilometers. Seasonally, larger species may dominate the observed bioluminescence in surface waters. In nearshore waters, such as the Sea of Japan, large scale migration into surface waters occurs for spawning of the deep-sea bioluminescent squid species *Watasenia scintillans*, the "hotaru-ika" or firefly squid. In estuarine waters, ctenophores (possibly all species are bioluminescent) often dominate the stimutable bioluminescence. The swarming of euphausiids (Schizopoda) occurs in summer months in Antarctic waters (*Euphausia superba*) and in winter in Scottish waters (*Meganyctiphanes*). Vertically migrating luminous species are often associated with scattering layers (so called because in some regions high concentrations of diurnally migrating organisms produce the effect of a moving "layer" of water with significantly different sonar reflection properties). The correlation of scattering layers with bioluminescence is, however, not always significant because not all scattering layer species are bioluminescent. Bioluminescence has been reported in between 93-97% of individuals caught in mid-depth trawls (500 m depth).

Bioluminescent Reactions

The range of brightness of bioluminescence can be deduced from ecological and physiological considerations^{11,12,13,14,15,16}. From this it is possible to estimate the minimum proto-bioluminescence needed to have been emitted by an individual of an ancestral dark species. From bright sunlight to bright moonlight the irradiance on the surface of the earth and its oceans decreases by approximately six orders-of-magnitude. Within the 400-700 nm visible wavelength range for photosynthesis and vision, surface irradiances vary from 10^{17} to ca. 10^{11} photons $\text{cm}^{-2} \text{s}^{-1}$. In oligotrophic (clear) and eutrophic marine environments, surface irradiances are reduced by a factor of 100 at depths of 150 and less than 10 m, respectively. The threshold irradiance for phototaxis, e.g., a photic response in crustaceans as representative of potential detectors of bioluminescence, is less than 10^8 photons $\text{cm}^{-2} \text{s}^{-1}$ (of blue light). This latter intensity would, therefore, be the necessary minimum brightness for the detection of bioluminescence if it is to serve some signalling function. Flashing activity of nocturnal fireflies and the stimutable bioluminescence of some dinoflagellate species are significantly reduced in bright moonlight, implying that the organisms have undergone natural selection to turn off their bioluminescence when it becomes inefficiently detected, when ambient irradiances are comparable with the bioluminescence. Thus, ca. 10^{12} photons $\text{cm}^{-2} \text{s}^{-1}$ might be an upper limit for the brightness of bioluminescence. In nature bright bacterial colonies emit ca. 10^{12} photons $\text{cm}^{-2} \text{s}^{-1}$ continuously and are easily visible to the dark-adapted eye. Flashes from dinoflagellates, crustaceans, fireflies and fish contain between 10^8 - 10^{13} photons.

Almost all chemical oxidation reactions, particularly those involving molecular oxygen, release free energy during electron orbital rearrangements, and are therefore potentially chemiluminescent. The criterion for chemiluminescence in the visible region of wavelengths is that the free energy, F , released as the result of electronic orbital rearrangements must be localized to a single product molecule such that

$$|\Delta F| \geq hc / \lambda_{\max} \quad (1)$$

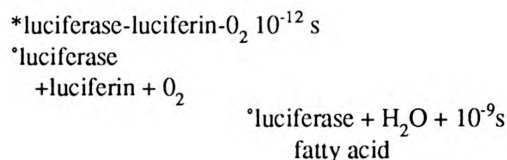
where h is Planck's constant, c is the velocity of light and $\lambda_{\max}$ is the long wavelength limit for the absorption manifold of the first excited state of the product molecule. For blue light-emitting marine organisms, this corresponds to $54 \text{ kcal mole}^{-1}$.

With electron photomultiplier tubes (PMTs) capable of detecting single photons, it has been possible to observe very weak chemiluminescences in the visible and ultraviolet portions of the spectrum from almost all oxidation reactions, even where the quantum yields, Q_{CL} , the ratio of photons emitted to molecules oxidized, are as low as 10^{-15} . The quantum yield of a chemiluminescent reaction producing a specific product molecule, i , is composed of three factors.

$$[Q_{\text{CL}}]_i = F_{\text{chem}} \cdot F_{\text{ex}} \cdot F_{\text{fl}} \quad (2)$$

where F_{chem} is the fraction of the oxidation pathways leading to the formation of a specific product molecule, i , F_{ex} is the fraction of the product molecules that are excited to vibrational levels of the first excited state manifold (equivalent to photo-excitation) and F_{fl} is the photo-fluorescence yield of the product molecule or the enzyme-product complex.

Bioluminescence is a special case of the efficient chemical production of light, where the individual factors in Eq (2) are high, approaching, as in the case of firefly bioluminescence, unity. In all bioluminescent species light is produced by a common enzyme reaction. A mixed function oxygenase (luciferase) catalyzes the oxidation of a substrate (luciferin) by molecular oxygen, producing an enzyme-product complex in an excited electronic state. This excited state relaxes rapidly (ca. 10^{-12} s) to the lowest level of the first excited state of the complex which then undergoes an efficient radiative transition (ca. 10^{-9} s) to the ground state as below.



The Chemiluminescent Reaction

The oxygenase reaction is common to all species. In the firefly system the luciferin, LH_2 , (D-2-[6'-hydroxy-2'-benzothiazolyl]-D²-thiazoline-5-dihydro-4-carboxylic acid) is activated to LH_2AMP via an adenylation reaction common to amino acid activation in protein synthesis and to fatty acid

activation in lipid synthesis, requiring adenosine triphosphate, (ATP). Only at this time will the ENZ-LH₂-AMP complex react with molecular oxygen, releasing AMP and forming a luciferin-4,4"-dioxetanone intermediate. This rapidly undergoes a stepwise decomposition, one oxygen atom remaining with the 4"-keto carbon releasing CO₂ and the other remaining with the 4-carbon of luciferin to form enzyme-bound decarboxy-keto-luciferin in an excited electronic state. In the jellyfish *Aequorea*, molecular oxygen is taken up as a hydroperoxide and held tightly bound to the luciferase-luciferin complex. The fact that bioluminescence of the extracted "aequorin" system could then be initiated by Ca⁺⁺ ions in the absence of molecular oxygen, i.e., when free molecular oxygen was removed from the medium, caused some initial confusion because it appeared that there might not be a common stoichiometry for bioluminescent reactions. When the jellyfish researchers finally felt compelled to abide by the law of conservation of energy to explain the production of excited states which emitted blue light, experiments on re-charging of spent aequorin protein and on the chemical composition of the oxidized luciferin were carried out which confirmed the requirement for molecular oxygen.

The bacterial bioluminescent system is an intermediate case of oxygen binding. Native luminous bacteria and the purified bacterial luciferase in the presence of the cofactor, reduced flavin mononucleotide, FMNH₂, will react with long chain aliphatic aldehydes (C₈-C₁₈) to release H⁺ + OH⁻ and the corresponding carboxylic acids at extremely low partial pressures of molecular oxygen. Fortunately, J.W. Hastings, whose laboratory has been instrumental in describing the complex biochemistry of the bacterial system, carried out his first postdoctoral studies in McElroy's laboratory, specifically to study the effects of oxygen partial pressures on bioluminescent systems, so that he was not easily misled about the requirement for oxygen. The comparative study of the chemiluminescent reaction in all of the other bioluminescent species studied¹, the positive inter-species cross reactions of luciferases and luciferins and the species-specificities of enzymes for colors of bioluminescence while utilizing identical substrates led to the conclusion that the oxygenase reaction and molecular oxygen were common to all bioluminescence mechanisms. These reactions oxidized hydrophobic molecules and provided sufficient free energy to leave enzyme-bound products in excited electronic states. Oxygenase reactions are common to all aerobic organisms, and are involved in hydroxylations, in lipid biosynthesis and metabolism, in degradation of amino acids, sugars, porphyrins, hormones, drugs and aromatic carcinogens. Lipids and aromatic molecules are hydrophobic and, therefore, hydroxylation reactions of these compounds are more readily carried out with molecular oxygen than with water. The key here is that in bioluminescent species the oxygenase reaction results in efficiently fluorescent electronically excited states.

The Luciferins

In all phyla studied the luciferin substrates of the bioluminescent species are characteristic of two functions for oxygenases, detoxification (of aliphatic aldehydes in bacteria, gastropods and earthworms) and degradation of fluorescent pigments (of porphyrin (bile) pigments in dinoflagellates and euphausiids and of pigments related to melanin), pyrazine derivatives; coelenterate-type luciferins in anthozoans (*Renilla*), hydrozoans (*Aequorea*), ctenophores (*Mnemiopsis*), mollusca (*Watasenia*), decapod shrimp (*Oplophorus*, *Systellaspis*) and some fish species, cypridina-type luciferins in ostracods (*Cypridina*) and other fish species (*Apogon*, *Porichthys*); benzothiazole derivative in fireflies (*Colopoptera*).

The oxidation of aldehydes to carboxylic acids in solution chemistry is not significantly chemiluminescent. In the bacteria, however, the enzyme-bound 4a-hydroxy-FMNH is strongly fluorescent and is the light emitter. Firefly bioluminescence is an example of direct chemiluminescence, the oxidized product molecule that furnishes the free energy for the excitation is itself fluorescent and the enzyme-product excited state is the emitter. Bacterial bioluminescence appears to be a sensitized chemiluminescence, in which the aldehyde oxidation furnishes the free energy for excitation which is trapped by the fluorescent enzyme-4a-hydroxy-flavin intermediate. In all cases chemiluminescence is the inverse of photosynthesis where reaction center chlorophyll *a* traps the excitation energy of photo-excited accessory pigments in order to do chemical work. The identification of common classes of luciferins involved in oxygenase reactions was another simplifying feature.

Origins of Bioluminescence

Bioluminescence must be relatively recent in evolution. It could serve a useful signalling function only after the evolution of vision or phototaxis. We start with several necessary (but not sufficient) conditions for the origin of the function of bioluminescence. Organisms must have already evolved mechanisms for the detection of dim light as well as photic responses to this light. An individual of an ancestral species, as a result of a mutation of its oxygenase which retained the chemical function but resulted in a fortuitously fluorescent enzyme-product complex, or exposure to a new metabolite or exposure to different environmental conditions, might produce a chemiluminescence concomitant to the metabolic reaction. If the chemiluminescence were emitted externally there might result a biological light source bright enough to be detected by another organism and to stimulate a photic response in the receiving organism. If the response to this photic signal either increased the efficiency of mate location, or attracted potential prey, or inhibited predation, or, as

in the case of saprophytic bacterial colonies, increased the probability of ingestion into the nutrient-rich gut of an omnivore, the reproductive success of the emitting individual might have increased over that of its non-chemiluminescent siblings.

Bioluminescent signals vary from steady glows to rapid flashes. It is possible to envision natural selection for optimizing the function of bioluminescence under light-limited conditions, the signalling patterns, the precise colors and the behavioral responses. But how in the world did the original "dark" ancestors of fireflies and luminous fish, indeed of all the present-day bioluminescent species, become bioluminescent? What was the first step that led to the "adaptation" of a chemiluminescent reaction in an individual of an ancestral non-bioluminescent species, resulting in a new species exhibiting the function of bioluminescence? How might a chance (enzyme or metabolite) mutation in an individual of this ancestral species, resulting in a chemiluminescent reaction, provide a reproductive advantage to the emitting individual that might select for the success of the individual's descendants, unless the chance chemiluminescent reaction resulted in a brightness and a spectral distribution of the chemiluminescence that were already sufficient to stimulate a "favorable" response by another organism? This requirement presupposes an already bright (efficient) chemiluminescence. There should be no useful function for incipient stages of chemiluminescence, i.e., mutations where only small amounts of light were emitted, insufficient for detection. This implies a minimum chemiluminescence brightness had to occur in all ancestors of bioluminescent species.

This dilemma is a molecular chemical analogue of the morphological dilemmas that Darwin's critics presented to his new theory. One of these was the paradox of "the evolution of incipient stages of useful structures". As stated by Mivart¹⁸, "...Natural Selection utterly fails to account for the conservation and development of the minute and rudimentary beginnings, the slight and infinitesimal [and functionless] commencements of structures, however useful those structures may afterward become." For example, how could natural selection account for the "evolution" of mimicry, or structures such as limbs on previously limbless vertebrates, or a bird or insect wing, when an incipient pigment spot or bump on an individual of an ancestral species could serve no useful function?

Mivart's reasoned objections to Darwin's evolutionary mechanism were that functions and species characteristics were proposed to have evolved solely by "infinitesimal, random changes". Mivart quotes Huxley's suspicion, a forerunner of recent saltation theory, that Nature "does make considerable jumps in the way of variation now and then, and that these saltations give rise to some of the gaps which appear to exist in the series of known forms." What he did not propose was that the combination of "saltation" and "infinitesimal random changes" may have produced an almost infinite variety of species compared to that tiny fraction of the total that remain

now, those that have persisted in the ensuing intense predation and competition for ecological microniches and that continue to optimize their functions in constantly changing ecological gradients².

E.N. Harvey, the American leader of the study of the biochemistry of bioluminescence, raised a dilemma which is a refinement of Darwin's. Harvey commented upon the apparently random distribution of bioluminescence among the phyla. He likened it to the throwing of wet sand upon a list of all species written upon a blackboard; bioluminescence occurred wherever a grain of sand stuck to a species³. While this did not answer the question of the origin of bioluminescence, it served to emphasize Mivart's questions about randomness in evolutionary design. The elements for the answer to Harvey's dilemma were provided by the studies of W.D. McElroy, and a succession of McElroy's students and co-workers, J. Buck, J. W. Hastings, M.J. Cormier, E.H. White, M.A. DeLuca, J. Lee and the author of this article. It has combined biochemistry, physical chemistry, spectroscopy and has often required that the various bioluminescent species be studied in their natural habitats. In attempting to study origins it is important that the present-day "perfection" of the signalling function of bioluminescence (optimizations of the myriad of biochemical regulatory processes, colors and brightnesses, intra- and inter-species interactions, etc.) be separated from the initial step, the production of efficient chemiluminescence.

Darwin answered his critics by enunciating, five editions later, the principle of "functional change in structural continuity"⁴, essentially the multifunctional utility of structures. He proposed that structures evolving for one function by natural selection might at some later stage of the evolutionary development of the species, reach a size or shape where the structure became fortuitously useful for an altogether unrelated new function, which at that time increased the survival or reproduction of the organism in its ecosystem. In the case of an insect wing this structure, whatever might have been the original selection process in its evolution, would be called a "proto-wing"^{5,6,7}. Subsequent to this apparent "saltation", i.e., a jump to useful wings, natural selection could again be invoked to explain the further evolution of these proto-wings (now functionally useful for flight) into the different and highly specialized wings characteristic of gliding and flight in present-day species of birds and insects. This beautiful conception, that useful new functions could arise (fortuitously and at a later time) from structures evolved for other functions, not only avoided the requirement for extrapolating structure and function simultaneously to incipient stages of structures but introduced the element of chance into function and natural selection.

Bioluminescence is the biochemical analogue of Darwin's functional change in structural continuity. It is proposed that the original chemiluminescent reaction, proto-bioluminescence, arose independently in individuals of all

non-bioluminescent ancestral species whose enzymes were already evolved for the significant function of metabolic degradation utilizing molecular oxygen^{8,9,10,11}. Three alternatives for this first step of efficient concomitant chemiluminescence were possible. First, a point mutation of an oxygenase modified the binding of an oxidized product to the enzyme, producing a fortuitously fluorescent enzyme-bound product (concomitant chemiluminescence). Second, individuals evolving a variety of porphyrin or melanin pigments¹² for other functions, produced a fortuitously fluorescent metabolite whose oxidative degradation gave rise to a concomitant chemiluminescence. Third, in the bioluminescent bacteria changes in environmental conditions such as hypoxia selected for (individuals with) oxygenases with flavin cofactors that could oxidize toxic aldehyde metabolites to carboxylic acids at low partial pressures of oxygen¹³. Those (individuals with) aldehyde oxidations utilizing reduced flavin mononucleotide, FMNH₂, as a cofactor produced fortuitously concomitant chemiluminescence. Their selection for a new "species" was initially an ecological selection, ability to utilize molecular oxygen under hypoxia, permitting the colonization of niches where metabolism could occur under both oxygen-rich and hypoxic conditions. Only then might the same concomitantly chemiluminescent oxygenation become a "proto-bioluminescence," and only for colonial, saprophytic species, since a single bacterium would not be sufficiently bright to be detected. Symbiosis in specially-evolved light organs could come later.

Now we are in a position to answer Harvey's dilemma by listing the necessary conditions for proto-bioluminescence.

1. Highly exergonic oxidations and high fluorescence efficiency products.

Reactions of essential oxygenases and molecular oxygen with oxidizable hydrophobic metabolites, aldehydes or fluorescent pigment molecules, are sufficiently exergonic to produce electronically excited states and are energetically capable of producing direct or sensitized chemiluminescence in the visible range of wavelengths. The direct chemiluminescences appear to proceed through dioxetane intermediates, an efficient chemiluminescent pathway. Therefore, a mutation of an oxygenase or of a pigment synthesizing pathway which resulted in concomitant direct or sensitized chemiluminescence while still retaining chemical function could produce a proto-bioluminescence. In the bacteria it is proposed that a mutation to utilize the FMNH₂ cofactor rather than the more usual reduced flavin adenine dinucleotide, FADH₂, resulted in an environmental selection for the former cofactor.

2. The requirement for emission of chemiluminescence external to the organism would be satisfied by oxidation of pigment molecules, as these would be involved with the coloration of the organism and would generally be located on the outside of the organism. The secretion of toxic or disagreeable slime for chemical protection would also be an efficient

vehicle for a fortuitous proto-bioluminescence. In small organisms, translucency would permit proto-bioluminescence to be emitted.

3. Presence in the ecosystem of a "suitable" nearby recipient of the photic stimulus.

The spectral distribution of the proto-bioluminescence must overlap the visual spectral sensitivity, and the brightness of the emission must be such that the illumination of the receiver is significantly above its threshold for photic response. Most importantly this photic response must enhance the survival, or the reproductive success of the emitter and its descendants. It is possible that the mutation that gives rise to a bright concomitant chemiluminescence in [1] and [2] above, in an individual of the non-bioluminescent ancestral species, results in a slightly less efficient metabolic reaction than the same metabolic reaction in its non-chemiluminescent siblings. In the absence of requirement [3] the mutation would provide no selective advantage, would probably be selected against and eventually be lost.

The simultaneous interaction of requirements [1] and [2] and [3], the combined probabilities of rare biochemical, physiological and ecosystem interaction events, provide a retrospective explanation for the apparent randomness and rareness of bioluminescence among the phyla observed by Harvey. The requirements for high efficiency, visible wavelength chemiluminescence reduce the pathways available for proto-bioluminescence to oxygenases. The ecological interaction requirement is very stringent. The explanation of why only some species of a genus are bioluminescent and closely related species are non-bioluminescent requires examination of their ecosystems and their interactions.

It is admittedly difficult in the bacteria to explain the distribution of bioluminescence among the genera and species. Bioluminescence might however, correlate with dinoflagellate species whose major predators are raptorial feeders and not with those whose major predators are filter-feeding fish or larvae. These latter species might at some time in the past have produced proto-bioluminescence without any accruing ecological advantage in predator inhibition. Conversely the line of descent of a species of non-bioluminescent dinoflagellate may have been through a common bioluminescent ancestor and, in the absence of raptorial predators, further random mutation resulting in the loss of concomitant chemiluminescence in this species would not be selected against. Some slight evidence for this argument can be inferred from the fact that when captured individuals from natural samples of the dinoflagellate species *Gonyaulax tamarensis* var. *excavata* and var. *tamarensis* were cultured some clones were non-bioluminescent¹³. The fact that almost all mid-depth organisms appear to be bioluminescent is an argument that the hierarchical taxonomic species list from which Harvey posed his dilemma may not be the proper frame of reference for bioluminescence. Perhaps if the list were re-arranged to rep-

resent types of ecological and behavioral interactions it might result in a strong positive correlation between those species whose interactions favored proto-bioluminescence and the appearance of bioluminescence.

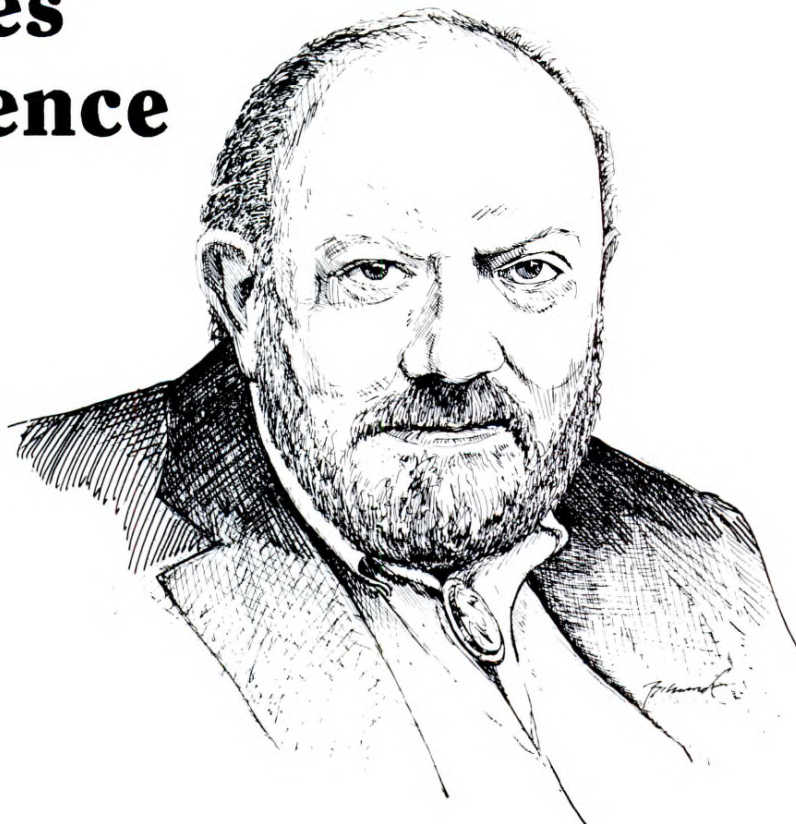
Biography

See "Profiles in Science" on page 12.

References

1. Seliger, H.H. and McElroy, W.D. 1959. Biochem. Biophys. Res. Commun. 1, 21-24
2. Seliger, H.H. and McElroy W.D. 1960. Arch. Biochem. Biophys. 88, 136-141
3. Lee, J. 1972. Biochem. 11, 3350-3359
4. Seliger, H.H., Lall, A.B., Lloyd, J.E. and Biggley, W.H. 1982. Photochem. Photobiol. 36, 673-680
5. Seliger, H.H., Lall, A.B., Loyd, J.E. and Biggley, W.H. 1982. Photochem. Photobiol 36, 681-688
6. Hastings, J.W. 1971. Science 173, 1016-1017
7. Harvey, E.N. 1952. Bioluminescence Academic Press, New York 649 pp
8. Herring, P.J. Ed. 1978. Bioluminescence in Action Academic Press, New York, 570 pp
9. Seliger, H.H., Carpenter, J.H., Loftus, M. and McElroy, W.D. 1970. Limnol. and Oceanogr. 15, 234-245
10. Seliger, H.H., Carpenter, J.H., Loftus, M., Biggley, W.H. and McElroy, W.D. 1970. Limnol. and Oceanogr. 16, 608-622
11. Johnson, F.H. and Haneda, Y. Eds. 1966. Bioluminescence in Progress Princeton University Press, Princeton, NJ
12. Cormier, M.J., Hercules, D.M. and Lee, J. Eds. 1973. Chemiluminescence and Bioluminescence Plenum Press, New York, 515 pp.
13. DeLuca, M.A. Ed. 1978. Bioluminescence and Chemiluminescence Academic Press, New York, 653 pp.
14. DeLuca, M.A. and McElroy, W.D. Eds. 1981. Bioluminescence and Chemiluminescence Academic Press, New York, 782 pp. 15. Burr, J.G. Ed. 1985. Chemiluminescence and Bioluminescence Marcel Dekker, New York
16. DeLuca, M.A. and McElroy, W.D. Eds. 1986. Bioluminescence and Chemiluminescence Academic Press, New York, 649 pp.
17. See Chapters 1 through 6 of reference 8
18. Mivart, St. G.J. 1871. On The Genesis of Species D. Appleton & Co. New York, 35 pp.

Profiles in Science



Howard H. Seliger

Professor Howard H. Seliger has been in the Biology Department of The Johns Hopkins University since 1958. He holds joint appointments in the McCollum Pratt Institute, the School of Hygiene and Public Health, the Chesapeake Bay Institute and is Chairman of the Committee on Radiation Control.

After 10 years as a nuclear physicist at the National Bureau of Standards, he came to Hopkins in 1958 on a Guggenheim Fellowship. He wanted to study the physics of the bioluminescence of fireflies, his boyhood passion, with the biochemist W.D. McElroy, who was a student of E. Newton Harvey a pioneer in the field of bioluminescence. The fellowship was for a year, but he made bioluminescence his life's work. Professor Seliger made the first determination that the quantum yield of firefly bioluminescence was essentially unity. He extended his studies to the ecology and evolutionary significance of two magnificent bioluminescent phenomena in nature, fireflies and marine dinoflagellates.

In 1960 the Office of Naval Research funded the first study by Seliger and McElroy on the famous bioluminescent bay, Oyster Bay, Jamaica, West Indies. This resulted in the first

analysis and demonstration of how a bioluminescent bay works, the interactions of physical hydrography and organism physiology that result in the species-specific dominance of bioluminescent dinoflagellates. Recently these mechanisms on different spatial scales have been applied to bioluminescent red tides in coastal upwelling areas as different as Maine and Borneo.

His studies of the mechanisms of the chemical production of excited states due to metabolic chemiluminescence paved the way for prediction and demonstration of the presence and detoxification of carcinogenic polycyclic aromatic hydrocarbons. He has published mechanisms for the fortuitous biochemical origin and evolution of bioluminescence, a mathematical model for the optimization of the colors of firefly bioluminescence and visual spectral sensitivity, and a quantitative study of the emission of stimutable bioluminescence by dinoflagellates as an inhibitor of copepod predation.

Professor Seliger has published over 250 papers in scientific journals and meeting proceedings. He is a fellow of the American Physical Society and founding member and past president of the American Society for Photobiology.

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Bacterial Bioluminescence: Three Decades of Enlightenment

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University of Wisconsin, Milwaukee

Introduction

During the past three decades a great deal has been learned about various aspects of the marine luminous bacteria. The 1960's featured major advances in the understanding of the biochemistry of the luminous system; bacterial luciferase, the enzyme responsible for catalyzing light emission, was purified, its subunits were characterized, and the intermediates in the reaction proposed. All luminous bacteria were shown to possess similar enzymatic machinery which was unique for the prokaryotes, and distinct from all other luciferases then known. While the mechanistic details of this early work have changed somewhat, the general ideas of how bacterial light emission is achieved have stood the test of time. In the 1970's, taxonomic schemes for the identification of the various luminous bacterial species were developed, making it possible to study the distribution and abundance of luminous bacteria with a degree of accuracy and rapidity previously impossible. In addition, major advances in understanding the regulation of the luminous system were made through studies of the physiology of luminous bacteria; autoinduction was described and a model proposed, catabolite repression was shown, and regulation by oxygen, osmolarity, and iron were demonstrated. The 1980's featured use of the techniques and approaches of molecular genetics, which lead to the cloning of several bacterial luciferases, comprehensive studies of the enzymes and initial elucidation of the control systems at the genetic level. And what about the 1990's? It is easy to predict that the use of

molecular approaches will lead to major advances in our understanding of both the evolution of the *lux* system and the ecology of the luminous bacteria, especially with regard to the many different symbioses in which they participate. An admittedly opinionated history of the past three decades is presented here, citing several reviews that contain the details of the many excellent works in this field.

The Biochemical Decade

The sixties might be called the decade of biochemistry for bacterial luminescence; major advances were made in the understanding of the biochemistry of the bacterial luminescent system, and the stage was set for continued discoveries. Bacterial luciferase, a soluble enzyme responsible for catalyzing light emission, was purified and shown to be an α - β dimer of approximately 80,000 MW. The two subunits of approximately equal size were separated after denaturation in urea, and recombined to yield active enzyme. The substrates for the light emitting reaction were identified as reduced flavin mononucleotide (FMNH₂) and long chain aldehyde (RCHO), and various schemes were postulated to explain the molecular mechanism of light emission^{2,7,9,11,20} (see Seliger article in this issue). It became clear from this work that the bacterial luminescent system was biochemically distinct from the luminescent systems of eukaryotic organisms, and that at the level of enzyme properties and substrate requirements, probably all bacterial luciferases were similar.

Knowledge of the biochemical properties of the bacterial luminescent system continued to be amassed in the 1970's and 80's. Advances included the identification of the binding sites for both aldehyde and FMN on the α subunit, and the demonstration of the *in vitro* activity of the aldehyde recycling enzymes (fatty acid reductase system). Several different flavin oxidoreductases from various luminous bacteria were purified and studied, revealing that many different mechanisms and properties existed within this group of organisms. It also became clear that accessory proteins were involved in the light emitting reaction in some strains. These accessory proteins showed different emission spectra: the blue fluorescent proteins (BFPs) shift the wavelength of emission towards the blue, while the yellow fluorescent proteins (YFPs) shift the wavelength of emission towards the red.

Extensive use of chemically-generated mutants was made during this time. Studies yielded support of the notion that the α -subunit contained the catalytic sites, and confirmed the absolute requirement for the β -subunit for luciferase activity, probably for the structural stability of the complex. Fully functional hybrid luciferases were generated between α and β

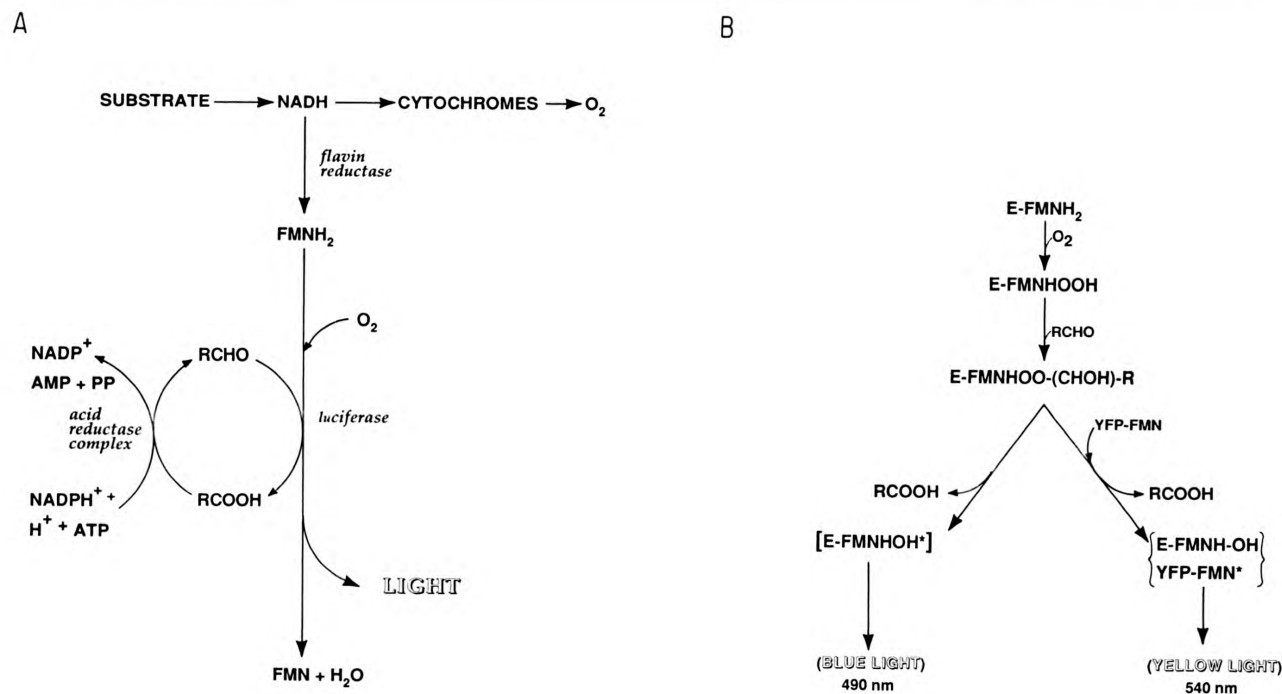
subunits from different bacterial species, suggesting that the luciferases were closely related, a suggestion that was further supported by amino acid sequence data from purified luciferases from two different bacteria. Amino acid sequence data also implied that the two subunits of luciferase shared a common origin, presumably arising via a gene duplication of one to form the other. The biochemistry of the bacterial luciferase system is summarized in Figure 1, depicting the various enzymes involved, and some of the postulated intermediates in the light emitting reaction, and the relationship of the luminescence pathway to cellular metabolism.

A Decade of Systematics, Physiology and Regulation

The 1970's saw some major changes in our views of the luminous bacteria, the first and foremost being our ability to identify the organisms. Prior to the 1970's, the taxonomy of these organisms was confused, and it was difficult to definitely identify a new isolate. The work of Reichelt and Baumann¹⁸

Figure 1.

Pathway and intermediates leading to light emission by the luminous bacteria. (A) The relationship between cell metabolism and the bioluminescent pathway, showing the major reactions involved in the light emitting pathway, and presenting the overall stoichiometry of light emission. (B) The alternative pathway that may occur if another protein such as the yellow fluorescent protein (YFP) is also involved.



presented a workable taxonomic scheme that made identification of the major luminous groups routine, and while the names they proposed for many of the luminous species have not all stood the test of time (Table 1), the methods they developed for distinguishing between the various groups are still used extensively^{1,14}. Table 1 summarizes some of the past and present species names for the luminous bacteria.

The importance of a workable taxonomic scheme cannot be overestimated. With the ability to easily identify the luminous species, many workers proceeded to examine a variety

of environments for the presence, types, and abundances of different luminous species. It became clear that the ecology of the luminous bacteria was complex, with species changing in abundance as a function of temperature, depth, radiation, season, and other unknown parameters. The importance of symbiosis in the biology of the luminous bacteria also became apparent, with some symbioses being examined in detail, as demonstrated in Figure 2. In addition, based on cultural studies, several different symbioses were shown to be species specific with regard to the bacterial symbionts, and models of interactions between host and symbiotic bacteria were proposed based on studies of host structures and symbiotic physiologies were proposed^{10,15,16}. A representation of some of the complexities in habitats occupied by various species is shown in Figure 3, in which the planktonic or free-living forms are represented in relation to the many other niches. The importance of associations with higher organisms, whether it be as loosely associated gut symbionts, parasites, or species specific light organ symbionts is obvious from such a presentation, and is perhaps one of the major insights that appeared during the decade of the 1970's¹⁷.

The 1970's was also the time of intensive work in the area of bacterial physiology, specifically with regard to the study of factors that regulate light emission. Again, the ability to distinguish between various species proved to be important, and physiological differences that might have appeared random or even contradictory, when viewed in a taxonomic/ecological perspective, could be often traced to physiological differences between organisms.

Probably all species of marine luminous bacteria have members that regulate luminescence via the mechanism of autoinduction. According to this mechanism, first reported in

Figure 2.

Monocentris japonicus, a bioluminescent fish light organ structure. (A) Line drawing of fish and ventral view, showing location of light organ (l.o.) on lower surface of jaw. (B) Scanning electron micrograph of dorsal surface of light organ. Arrows indicate emissary ducts via which bacteria emerge from large dermal papillae (Bar = 0.2 mm). (C) Light micrograph of a sagittal section of the lower jaw. (m = melanocytes; t = tubules with bacteria; b = mandibular bone; d = dermal layer; p = dermal papillae). Arrow points to emissary duct (Bar = 50 mm). (E) Transmission electron micrograph of tubule containing luminous bacteria. (lm = light staining mitochondria; dm = dense-staining mitochondria; e = erythrocyte; n = nucleus of epithelial cell; t = tubule lumen with numerous cells of luminous bacterial symbionts) (Bar = 1 mm).

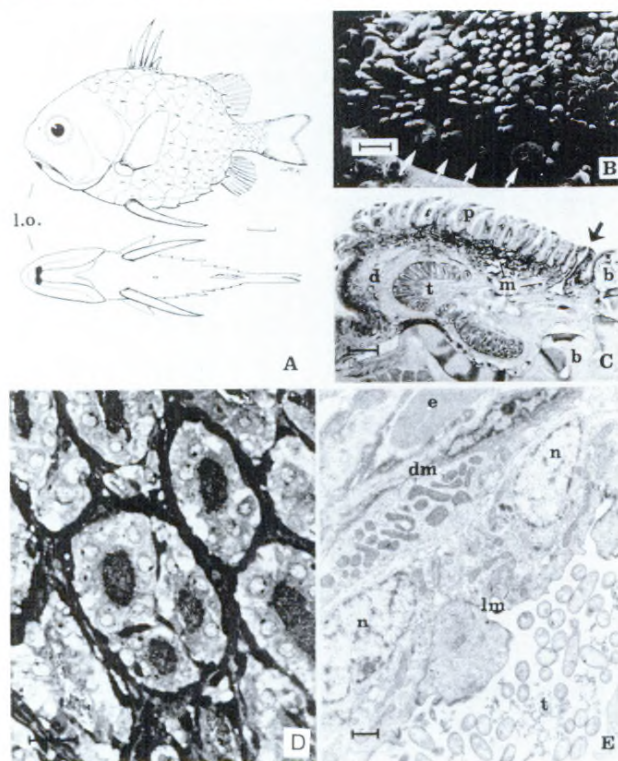


Table I. Known Species of Luminous Bacteria

Species Designation	Previous Designations
Marine Bacteria	
<i>Vibrio harveyi</i>	<i>Achromobacter</i> ^{1, 17} <i>Beneckea</i> ^{1, 18}
<i>Vibrio splendida</i>	<i>Beneckea</i> ¹⁸
<i>Vibrio orientalis</i>	<i>Beneckea</i> ¹⁸
<i>Vibrio fischeri</i>	<i>Photobacterium</i> ¹
<i>Vibrio logei</i>	<i>Photobacterium</i> ¹
<i>Photobacterium phosphoreum</i>	
<i>Photobacterium leiognathi</i>	
<i>Shewanella hanedai</i>	<i>Alteromonas</i> ^{1, 17}
<i>Vibrio vulnificus</i>	<i>Beneckea</i> ^{1, 17}
Non-Marine Bacteria	
<i>Vibrio cholerae</i> ¹⁷	
Soil Bacteria	
<i>Xenorhabdus luminescens</i> ¹⁷	

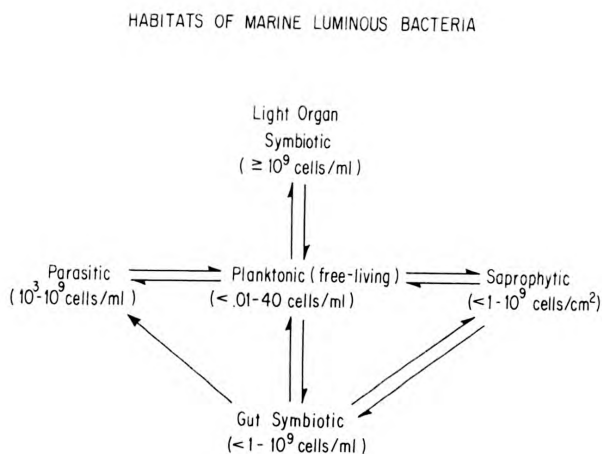
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Figure 3.

Habitats of marine luminous bacteria. The planktonic habitat is thought to be influenced by, and possibly to influence, all of the other habitats.



the early 1970's^{5,13,17}, the bacteria continuously produce a small, freely diffusible molecule that accumulates in the growth medium. When it reaches a critical concentration (approximately 10^{-9} M for *Vibrio fischeri*) it acts as the specific inducer of the luminescent system. The first autoinducer purified was that of *V. fischeri*, shown to be oxohexanoyl-homoserine lactone⁵; Fig. 4). This molecule is structurally similar to the inducer for *V. harveyi* (Fig. 4) although both compounds are species specific, inducing only members of their own species¹². It has been proposed that this mechanism affords the bacteria with a "spatial sensing ability" since they induce the luminescent system only when confined under conditions in which the autoinducer can accumulate^{5,13}; Fig. 4). The advantage, it is reasoned, is that free-living or planktonic forms will not waste energy emitting light, and thus have a better survival capacity in the nutrient limiting environment of the open sea. Chemostat studies with *V. fischeri* cells held at different densities supported the notion that the autoinducer model was correct, and involved an extracellular phase for the autoinducer.

When bacteria are presented with two nutrients of different richness, they often utilize the richer nutrient first; one mechanism for accomplishing this is via cyclic-AMP (cAMP) mediated catabolite repression. For reasons not entirely clear, rich carbon sources also inhibit luminescence in some species, and this inhibition can be reversed by cAMP, implying catabolite repression of luminescence. The physiology of this repression of luminescence was studied in *V. fischeri* and *V. harveyi*, has now been studied in detail at the molecular level for *V. fischeri*⁴.

Oxygen regulation was studied for four species of luminous bacteria and found to be very different between the four. While all species show growth limitation under low oxygen levels, some species decrease luminescence, some are not affected, and others are notably stimulated for luminescence^{7,17}. Such differences have been proposed to be of major importance in the ecology of the various groups of luminous bacteria, especially in terms of the various symbiotic niches they inhabit, many of which may utilize oxygen concentration to limit growth while maximizing luminescence activity.

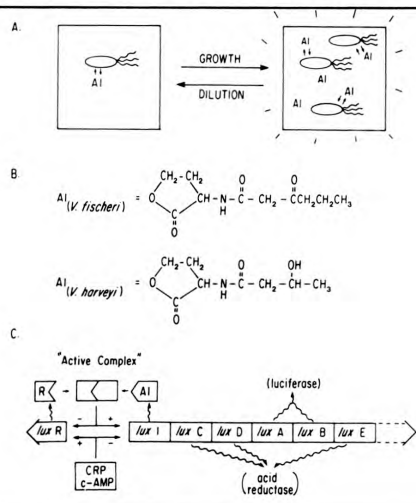
Similarly, salt concentration and osmolarity were found to regulate the luminescence activity of different species of luminous bacteria in different ways. While some species show only small effects of changes in osmolarity on the specific activity of luminescence, others show large effects: notably, *P. phosphoreum* shows maximal luminescence at salt concentrations greater than that of seawater, while *P. leiognathi*, a closely related species, shows maximal luminescence at salt concentrations below those characteristic of seawater. As with the effects of oxygen on luminescence, the regulation by osmolarity has been proposed to be of importance in the establishment and maintenance of viable luminescent symbiotic relationships.

A final regulatory factor involved with the luminous bacteria is that of iron inhibition of the luminous system. Iron represses the synthesis of the luminescent system in all marine bacteria tested. The rationale for iron regulation is not known, but the message is clear: when iron limitation of growth occurs, luminescence is not inhibited, resulting in very high specific activity of the luminescent system. As with other regulatory factors, it is tempting to speculate that this regulatory feature could be advantageously used by a host fish or squid harboring a luminous bacterial symbiont, but there is no evidence yet available to support such a contention.

While a great deal of progress has been made in elucidating the factors that control luminescence, huge gaps in our understanding still exist. Regulation has been intensively studied in only a few of the species. Furthermore, attempts to relate regulatory mechanisms and patterns to the ecology of the various groups have not been systematically made. Herein lies a true challenge for the future, one which may be pursued in earnest using not only the tools of physiology, but those of

Figure 4.

Autoinduction Mechanism. A. Depiction of cells of *V. fischeri* at low cell density in which they are dark and at high density, at which they are bright. According to the model, continuous production of autoinducer (AI) occurs during growth of bacteria. AI, which is freely diffusible accumulates in the growth medium, and when it reaches a sufficient concentration (about 10^{-9} M for *V. fischeri*) acts as a species specific inducer for the lux operon. B. Chemical structures of the AIs from *V. fischeri* and *V. harveyi*. C. Proposed mechanism of action of AI, indicating a requirement for the luxR gene product, suggesting a role for cAMP, and showing that the gene responsible for AI production (*luxI*) is the first gene in the lux operon induced by AI (e.g. AI induces its own synthesis) (see ref. 12).



molecular genetics, which are now available and being utilized for detailed studies of the luminous bacteria.

Enter Molecular Genetics: Definition of the *lux* System

In the decade of the 1980's, while studies of biochemistry, physiology and ecology continued, a revolution occurred - a revolution that has changed the way that we view the luminous bacteria and the way that we can approach many different kinds of questions concerning them. Beginning in the early 1980's, the Office of Naval Research began intensive support of molecular approaches to ocean sciences. Some of the initial research conducted by students at Scripps Institution of Oceanography, in conjunction with scientists from the Agou-ron Institute successfully cloned the luminescence (*lux*) genes from luminous bacteria, and expressed these genes in *E. coli*^{3,6}. The *lux* genes from *V. fischeri* that encoded the functions required to make *E. coli* luminescent were shown to be linked in a fashion shown diagrammatically in Figure 5. While there are differences known between *lux* operons from different sys-

tems, the five structural genes shown in Figure 5 appear to be present in all species so far examined (*lux* operons have been cloned from at least 10 different organisms!), and to be sufficient to initiate light emission in *E. coli*.

The advent of molecular genetics approaches, and specifically, the cloning of the *lux* genes has led to rapid advances in several areas. The first of which has to do with studies of the regulation of luminescence. Shortly after the *lux* system was cloned in *V. fischeri*, it was possible to locate the regulatory genes, one of which (*luxI*) coded for the enzyme responsible for autoinducer synthesis, and another (*luxR*) which encoded a regulatory (R) protein needed for response to the autoinducer (AI). In a very short time, mutants were produced on the cloned DNA, and a model was constructed at the molecular level to explain the mechanism of autoinduction. As the genes have been sequenced and studied in detail, the mechanism has become more complex, but in essence, what was proposed on the basis of physiological analyses and chemostat experiments remains as shown in Figure 4.

The sequencing of the *lux* genes has proceeded rapidly, especially in comparison to the direct protein sequencing of the luciferase itself. From the nucleotide sequences, it has been possible to ascertain complete amino acid sequences for both *luxA* and *luxB* for several different luciferases from different bacterial luminous species. Both gene and luciferase sequence data support the notion that luciferase has evolved only once, probably via a gene duplication, and that it has been reasonably highly conserved. Furthermore, using site-directed mutagenesis, it has been possible to ask very specific questions about the effect of substituting specific amino acids in the proposed active site of the α subunit of luciferase. This ability to create custom-made altered enzymes, should make it possible to systematically test models of enzyme catalysis of light emission. Such detailed analyses would have been impossible before the advent of these molecular approaches^{11,12}.

Whither Goeth The Nineties: Future Directions

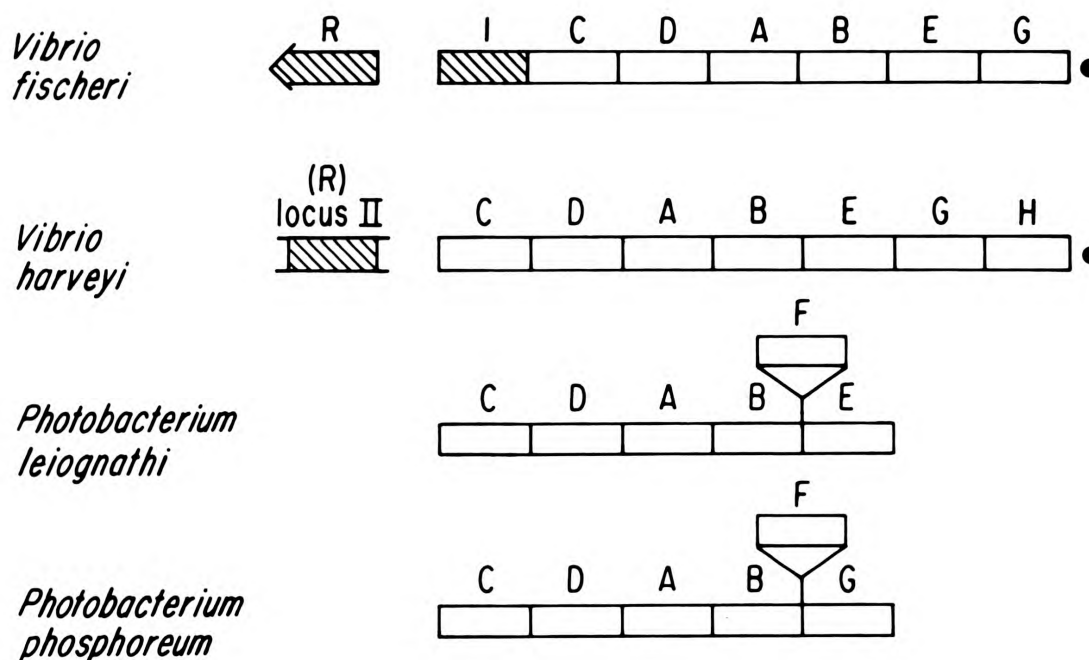
There is little doubt that the decade of the 1990's will feature molecular approaches to answer many of the questions that still linger in the field of bacterial bioluminescence. With regard to biochemistry and the mechanisms of light emission, one may expect that further refinements of mutants will be used to answer detailed questions about the precise mechanism of light emission.

As for regulation and physiology, the approaches offer much. It is expected that further molecular studies of *V. fischeri* will yield insights into just how autoinduction functions at the molecular level. It is hoped that similar detailed investigations will follow in the other species of luminous bacteria, those for which regulation by oxygen, iron and catabolite repression

Figure 5.

Genes and gene products known for four different species of marine luminous bacteria. This diagram shows the five structural genes (*luxA* through *luxE*) that are present in all species so far examined, as well as regulatory genes (*luxI* and *luxR*) known in two species, and genes of unknown function (*luxF*, *luxG*, *luxH*). Filled circles indicate transcriptional stop signals where they have been identified.

lux GENES AND GENE PRODUCTS OF SOME LUMINOUS BACTERIA



STRUCTURAL GENES: *lux A* α subunit } Bacterial Luciferase
lux B β subunit }
lux C reductase } Fatty Acid Reductase
lux D transferase }
lux E synthetase }
lux F }
lux G } homologous genes of unknown function
lux H }

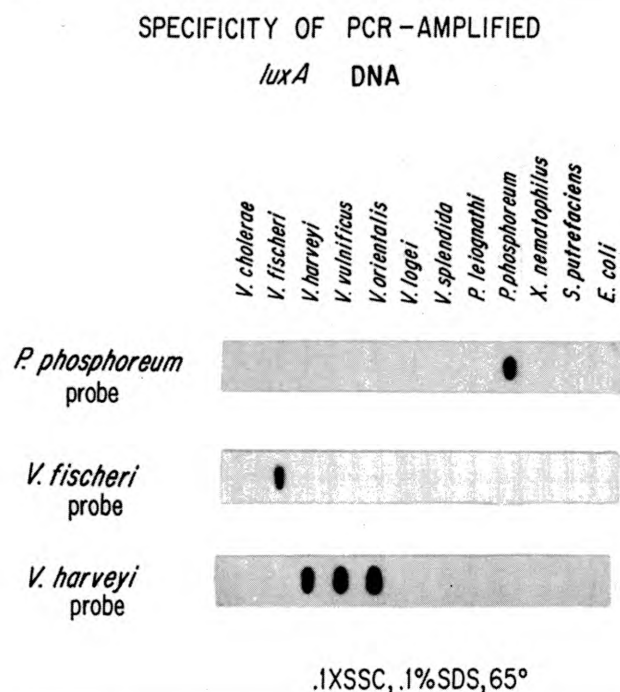
REGULATORY GENES: *lux I* Protein needed for autoinducer synthesis
lux R Positive regulatory protein

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Figure 6.

Specificity of PCR-amplified *luxA* DNA. Five mg samples of DNAs from the species indicated at the top were loaded onto slot blots and hybridized with ³²P-labeled *luxA* probes from *P. phosphoreum*, *V. fischeri* and *V. harveyi*. Washes were done in 0.1 X SSC-0.1% SDS at 65° C. The first two probes are species specific, while the *V. harveyi* probe detected three closely related *Vibrio* species.



show differences that may be related to the niches they inhabit. Unfortunately, while the structural genes of the various groups are reasonably well characterized (Fig. 5), the regulatory systems are virtually uninvestigated at the molecular level, with the exception of some studies of *V. harveyi*. Comparative study of the regulatory systems, especially if coupled with careful physiological work, should lead to major insights involving the marine luminous bacteria.

Other advances can be expected in the area of the ecology of the luminous bacteria. DNA sequences specific for certain genes can be used to identify the presence of these genes in natural populations (e.g. as hybridization "probes"). Using this approach, the cloned *luxA* gene was recently used as a specific probe to identify luminous bacterial isolates¹⁹. Using (polymerase chain reaction) PCR-amplified *luxA* genes from four species of luminous bacteria, it was possible to show that under conditions of high hybridization stringency they could be used to identify the species from which they were obtained. Using hybridization conditions in which the probes were less species specific (low stringencies) these same probes were useful as

general *lux* probes, to identify any bacteria containing a gene sequence similar to *luxA*. An example of the use of such approaches is seen in Figure 6; it takes taxonomy of the luminous bacteria to a new level of speed and ease: what took weeks to months can now be done overnight using species-specific hybridization probes. It is anticipated that such approaches will be major tools of ecologists in this field and others in the ensuing years.

Molecular approaches have included more than just analyses and use of the cloned *lux* genes. Sequence studies of 16S rRNA genes of the major groups of luminous bacteria have led to the realization that this group of organisms are closely related to each other, and that the ability to luminesce is confined to a remarkably clustered group of bacteria. Furthermore, considering the group of enterobacteria to which they are closely related, it may be speculated that these bacteria are rather "young" evolutionarily, and thus, that the *lux* system may be a rather recent invention. It is tempting to speculate that the bacterial *lux* system, and in fact the luminous bacteria are a group whose evolution has closely paralleled the evolution of higher organisms, specifically those that utilize and respond to visible light in the oceans. This is an area that will be possible to study using sequence analyses of *lux* genes in comparison to sequence analyses of rRNA genes, and it seems likely that major statements will be possible to make concerning not only the phylogeny of the luminous bacteria, but of their evolution and radiation as luminous species, and of the evolution and distribution of the *lux* genes and other genes containing similar sequences.

One area of particular interest with regard to the above question is that of the study of symbioses in which the various luminous bacteria participate. While many of these can be studied by routine methods, others can not because it has not been possible to culture the light organ symbionts. In such cases, molecular genetic approaches offer particular advantages not previously available. For instance, Haygood and Cohn⁸, isolated and studied the *lux* genes from a non-culturable symbiont of the flashlight fish, *Anomalops katoptron*. From these studies it was possible to identify the gene as a bacterial luciferase, and ultimately it should be possible to relate it, via sequence homology, to the known *luxA* genes. Similar analyses, using 16S rRNA genes will allow one to identify the bacteria containing the *lux* genes, thus allowing one to study the evolution of these symbioses, even in the absence of culturability of the symbionts. Since the various symbioses of the luminous bacteria range from loosely associated gut symbionts to obligate intracellular associations, such approaches should add immensely to our understanding of the biology of luminous bacteria.

As a final note, it should be mentioned that a great deal of interest is now focused on the use of cloned *lux* genes as reporter genes for a variety of purposes. The genes have been expressed in a variety of prokaryotic and eukaryotic hosts¹²,

and have been used for such varied purposes as measuring the expression of single genes inside cells, to detecting specific molecules or metabolites in the environment.

Biography

Professor Nealson received his bachelor of science degree in biochemistry in 1965, and a doctorate in microbiology in 1969, both from the University of Chicago. After postdoctoral work for two years with Dr. J.W. Hastings at Harvard University, he took a position at Scripps Institution of Oceanography in 1973, where he was promoted to the rank of Full Professor in 1984. During this time he received a Guggenheim Fellowship for Sabbatical Leave, and spent one year at the University of Washington in Seattle. In 1985 he moved to the University of Wisconsin in Milwaukee, where he holds the Shaw Distinguished Professor position at the Center for Great Lakes Studies. Dr. Nealson has worked in the field of bioluminescence for more than 20 years, concentrating mainly on the area of bacterial bioluminescence. The studies have included work in physiology, biochemistry, ecology, and more recently, in molecular biology. Current work involves the development of molecular probe methods for the examination of natural populations of luminous bacteria, and physiological and genetic studies of the regulation of luminescence in the bacterial group *Xenorhabdus luminescens*, an insect pathogenic soil bacterium.

Reference

1. Baumann, P., Baumann, L., Woolkalis, M. and Bang, S. 1983. Evolutionary relationships in *Vibrio* and *Photobacterium*. A basis for a natural classification. *Annu. Rev. Microbiol.* 37:363-398.
2. Campbell, A.K. 1989. Living light: biochemistry, function and biomedical applications. *Essays Biochem.* 24:41-76.
3. Cohn, D., Ogden, R., Abelson, J., Baldwin, T., Nealson, K., Simon, M. and A. Mileham, 1983. Cloning of the *Vibrio harveyi* luciferase gene: use of a synthetic oligonucleotide probe. *Proc. Nat. Acad. Sci. U.S.A.* 80:120-123.
4. Dunlap, P. 1989. Regulation of luminescence by cyclic AMP in *cya*-like and *crp*-like mutants of *Vibrio fischeri*. *J. Bacteriol.* 171:1199-1202.
5. Eberhard, A., Burlingame, A., Eberhard, C., Kenyon, G., Nealson, K. and Oppenheimer, N. 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochem.* 20:2444-2449.
6. Engebrecht, J., Nealson, K., and Silverman, M., 1983. Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. *Cell* 32:773-781.
7. Hastings, J.W., Potrikus, C., Gupta, S., Kurfurst, M., and Makemson, J. 1985. *Adv. Microbial. Phys.* 26:235-291.
8. Haygood, M.G. and Cohn, D. 1986. Luciferase genes cloned from the unculturable luminous bacteroid symbiont of the Caribbean flashlight fish, *Kryptophanaron alfredi*. *Gene* 45:203-209.
9. Lee, J. 1985. The mechanism of bacterial bioluminescence. pp. 401-437. In: J.G. Burr (Ed.) *Chemiluminescence and Bioluminescence*. Marcel Dekker, Inc. N.Y.
10. Leisman, G., Cohn, D. and Nealson, K.H., 1980. Bacterial origin of luminescence in marine animals. *Science* 208:1271-1273.
11. Meighen, E.A. 1988. Enzymes and genes from the lux operons of bioluminescent bacteria. *Ann. Rev. Microbiol.* 42:151-176.
12. Meighen, E.A. 1991. Molecular Biology of bacterial bioluminescence. *Microbiol. Rev.* 55:123-142.
13. Nealson, K., 1977. Autoinduction of bacterial luciferase: occurrence, mechanism and significance. *Arch. Microbiol.* 112:73-79.
14. Nealson, K.H. 1978. Isolation, identification, and manipulation of luminous bacteria. *Methods Enzym.* 57:153-156.
15. Nealson, K.H. 1979. Alternative strategies of symbiosis of marine luminous fishes harboring light-emitting bacteria. *Trends Biochem. Sci.* 4:105-110.
16. Nealson, K.H., Cohn, D., Leisman, G. and Tebo, B., 1981. Coevolution of luminous bacteria and their eukaryotic hosts. *Ann. N.Y. Acad. Sci.* 361:76-91.
17. Nealson, K.H. and Hastings, J.W., 1991. The luminous bacteria. Chp. 24 In: *The Prokaryotes*. Springer-Verlag, Berlin.
18. Reichelt, J. and Baumann, P., 1973. Taxonomy of the marine luminous bacteria. *Arch. Mikrobiol.* 94:283-330.
19. Wimpee, C., Nadeau, T. and Nealson, K. 1991. Development of species-specific hybridization probes for marine luminous bacteria by using in vitro DNA amplification. *Appl. Environ. Microbiol.* 57:1319-1324.
20. Ziegler, M.M. and Baldwin, T.O. . 1981. Biochemistry of bacterial bioluminescence. *Curr. Topics Bioenerg.* 12:65-113.

Dinoflagellates: Cell Biochemistry and its Regulation of the Millisecond and 24 Hour Time Scale

J. Woodland Hastings
Harvard University

Introduction

Awak'd before the rushing prow,
The mimic fires of ocean glow,
Those lightnings of the wave;
Wild sparkles crest the broken tides,
And flashing round the vessel's sides
With elfish lustre lave;
While far behind, their livid light
To the dark billows of the night
A blooming splendor gave

Sir Walter Scott
The Lord of the Isles

Flushing a toilet at night aboard a ship that uses a sea water system for the purpose can be a flashing experience! The brilliant "phosphorescence" – more properly bioluminescence – is due to small organisms, commonly dinoflagellates, that

are mechanically stimulated by the water movement. They may emit enough light to read by, and in any event it may come as a shock to someone who does not expect it.

Dinoflagellates often occur in blooms, where a single species predominates, as in the "phosphorescent" bays in Puerto Rico and Jamaica, where high populations of *Pyrodinium bahamense* persist throughout the year¹. Along the U.S. West Coast, from Santa Barbara southward, *Gonyaulax polyedra* is the organism responsible for many of the blooms, the so – called red tides². These often involve the formation of distinct red (fluorescent) patches, aggregations in concentrations up to 10^6 or 10^7 cells per liter, 100 to 1000-times greater than the population density in the water outside the patch. At such times, organisms can be harvested directly from the ocean by filtration and then used for biochemical analyses. Collecting at night, one sees the undulating luminescent pattern left behind by fleeing fish, darting away as the boat approaches. World War II aviators based on carriers in the South Pacific

tell of the ease with which they could relocate their ship after a mission: a luminescent wake is sometimes seen for many miles as a consequence of the persistent turbulence which stimulates the luminescence of the cells. A submarine or a diver under water may be similarly located by the luminescent track.

More poetic prose is found in the record of the “Challenger”³, as she passed southeast of the Cape Verde Islands:

“On the night of the 14th the sea was most gloriously phosphorescent to a degree unrivalled in our experience. A fresh wind was blowing and every wave and wavelet, as far as one could see from the ship on all sides to the distant horizon flashed brightly as they broke while above the horizon hung a faint but visible white light. Astern of the ship, deep down where the keel cut the water, glowed a broad band of blue, emerald green light, from which came streaming up, or floated to the surface, myriads of yellow sparks which glittered and sparkled against the brilliant cloud below until both mingled and died out astern far away in our wake. Ahead of the ship, where the old bluff bows of the “Challenger” went ploughing and churning through the sea, there was enough light to read the smallest print with ease. It was as if the “Milky Way” as seen through a telescope, “scattered in millions like glittering dust” had dropped down into the ocean, and we were sailing through it.”

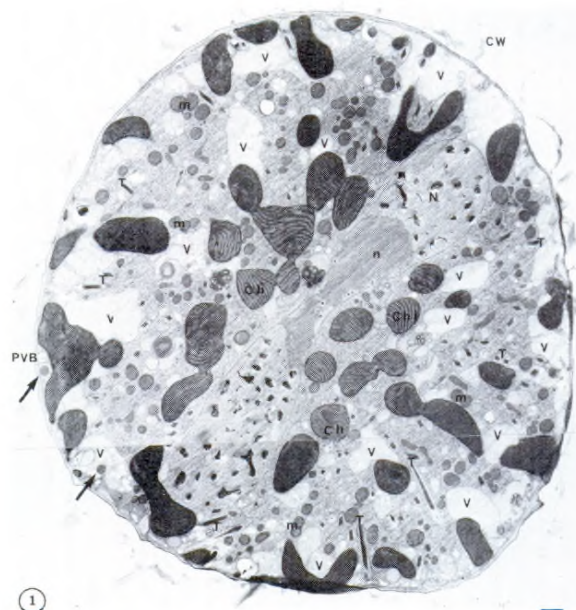
History

For many years the origin and nature of the “phosphorescence” of the ocean was debated⁴. Descartes thought that when a wave hits an obstacle, the agitation imparted to the particles of salt caused them to separate from the particles of water and to “generate sparks” similar to those emitted by flint. Over the years, many other ideas and variations thereupon were put forward, including theories involving electricity, phosphorus and putrefaction. Although many serious students were concerned with the question, including Robert Boyle, J.J. D. de Mairan and Benjamin Franklin, it is significant that none of the great microscopists of the period (e.g., Malpighi, Van Leeuwenhoek, Hooke and Grew) recorded any interest or observations concerning the phenomenon. Thus, it was not until the late part of the eighteenth century that luminescence of the sea was definitely attributed to living organisms, and even then dispute continued, both as to whether or not all cases could be so explained, and how living organisms are capable of emitting light.

The first definitive demonstration that dinoflagellates are luminous is attributed to G.A. Michaelis in 1830; the fact that these dinoflagellates are clearly responsible for the sparkling luminescence of the sea can be credited to him and to C.G. Ehrenberg.

Figure 1.

Electron microscopic section of a *Gonyaulax polyedra* cell illustrating the large nucleus (N), chloroplasts (Ch), trichocysts (T), mitochondria (m), polyvesicular bodies (PVB) and the extensively branching vacuolar (V) system. The mature light emitting organelles (scintillons, arrows) protrude into vacuoles (from ref. 18). Bar = 1 micron.



Biology

Dinoflagellates represent an important group of organisms in the marine environment in terms of numbers and diversity as well as their physiological, ecological significance. They commonly occur as free-living, photosynthetic, marine unicells, but also include endosymbiotic, parasitic, heterotrophic and freshwater taxa.

Thus, many (but not all) dinoflagellates are photosynthetic, and some species are responsible for the production of a potent neurotoxin (e.g., saxitoxin) that may be accumulated by shellfish and pose a threat to public health^{5,6}. Not all dinoflagellates are bioluminescent: no freshwater species are bioluminescent and only a fraction of the marine species are capable of emitting light⁷.

The feature of dinoflagellates that most strikingly distinguishes them from other eukaryotes is the nucleus and its chromosomal organization; indeed, the higher-order structuring of the DNA is an enigma^{8,9}. The nucleus is large (Fig. 1) and contains many (>100) chromosomes. They remain condensed throughout interphase and are largely devoid of associated protein and lack the nucleosome structure of other

eukaryotes. The chromosome fibrils, by appearance and dimensions, resemble procaryotic DNA, and as much as 70% of the thymine may be replaced by 5-hydroxymethyluracil¹⁰, previously thought to occur only in chloroplast and bacteriophage genomes. For still unknown reasons, the actual content of DNA is extremely high, and may be as much as (or in some species, fifty-times more than) that present in the human nucleus. During division, a conventional mitotic spindle is absent, and the nuclear envelope remains intact throughout. Cytoplasmic invaginations of the envelope are accompanied by microtubular elements, some of which attach to electron-dense pads positioned within the nuclear envelope adjacent to the point of chromosome attachment, thereby mediating chromosome segregation.

Functions of light emission: an indirect alarm or a direct diversion?

Two different proposals for the function of bioluminescence in dinoflagellates have been advanced. Burkenroad¹¹ speculated that dinoflagellate luminescence, stimulated by predators, might alert the predators of those predators, and thus indirectly protect the dinoflagellates. In contrast to this "burglar alarm" hypothesis, other authors have argued that the effect might be a direct one. For example, dinoflagellate flashing caused by the mechanical stimulation from an approaching predator might startle or otherwise divert the predator¹². This idea has been supported by experiments, including ones in which it was found that predation rates on luminous *Gonyaulax polyedra* by copepods were less at the phase of peak luminescence^{13,14,15}.

Figure 2.

Spontaneous light emission as a function of time (8 sec record) from a culture of 32,000 *Gonyaulax* cells, showing the steady background glow with several superimposed flashes (durations, d msec; Temperature, 23° C) (from ref. 20).

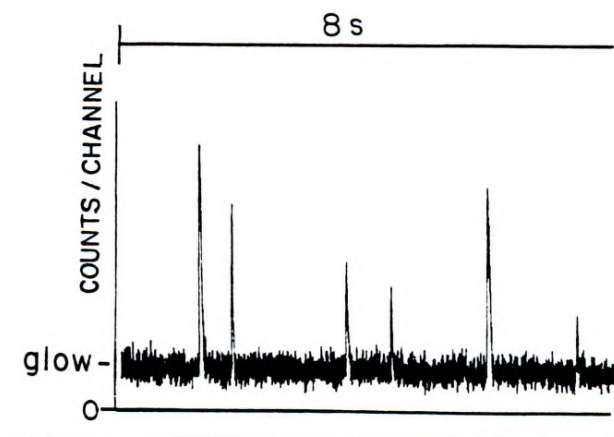
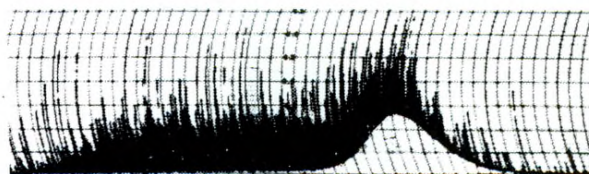


Figure 3.

Spontaneous light emission (ordinate) of a culture (~ 40,000 cells) of *Gonyaulax* at 23° C recorded over a longer time span (abscissa, 1 division = 20 min) showing the circadian rise and fall of the background glow as well as the rhythm in the frequency of the superimposed flashes (not time-resolved). This pattern is repeated about every 24 hours (see Fig. 10a)



In Vivo luminescence: bright flashes and a very dim glow.

The luminescence of an individual dinoflagellate cell commonly occurs upon stimulation and is visualized as a discrete flash, with the emission of 10^7 to 10^{10} photons and lasting only a fraction of a second (Fig. 2). The environmental signal engendering a flash is believed to be mechanical, generating a change in shear or acceleration (and possibly pressure) at the surface of the cell¹⁶, which is transduced via an unknown receptor mechanism. Eckert¹⁷ showed with *Noctiluca* that stimulation yields slow, graded negative potentials across the excitable vacuolar membrane that reflect the strength of the stimulus, ultimately initiating an action potential that travels along the vacuolar membrane at a velocity of about 6 cm s^{-1} , triggering luminescence as it goes. Luminescence arises from discrete subcellular regions, called microsources, now identified as specific luminescent organelles called scintillons^{18,19}.

Luminescence also occurs spontaneously. This is manifested mostly by occasional flashes, which occur mostly during the night phase, as can be seen from Figure 3. On the average, a given cell only emits one or two flashes per day²⁰. But the question as to why cells flash spontaneously remains unanswered. The possibility that such flashes are caused by cell-cell encounters, or by collisions with the walls of the vial, is not supported by experiments^{21,22}.

There is also a spontaneous but very dim steady glow, which occurs for only a few hours each day, rising and falling near the end of the night. The glow is definitely not the result of many micro-flashes integrated in time to give a smooth intensity. Cell divisions, when they occur, take place at about the same time, so it had been suggested that the luminescent glow might accompany division. However, non-dividing cells give an unattenuated glow²³, so this idea must be rejected. It may be that light emission accompanies the breakdown of the luminescent organelle, which occurs at the end of every night, controlled by the cellular circadian clock²⁴.

Figure 4.

Comparison of the kinetics of the *in vitro* light emissions from the soluble (supernatant) fraction (top panel, right) and the sedimentable (particulate) fraction (lower panel, right). In both cases the reactions are initiated by a rapid shift of the pH from about 8 to 6, and in both cases the straight lines represent plots of the data on a log scale. In the case of the soluble system, the kinetics are a function of concentration, whereas in the case of the particulate system, they are not. Each scintillon is a flashing unit with a fixed luciferase concentration (from refs. 31,32).

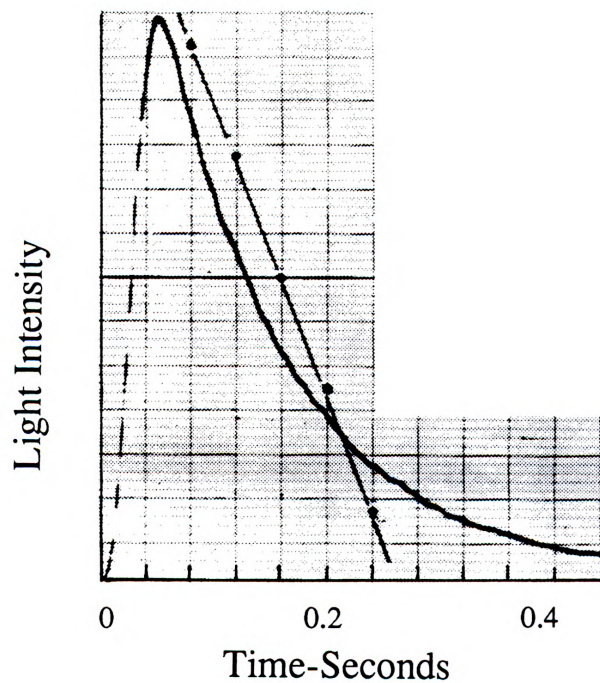
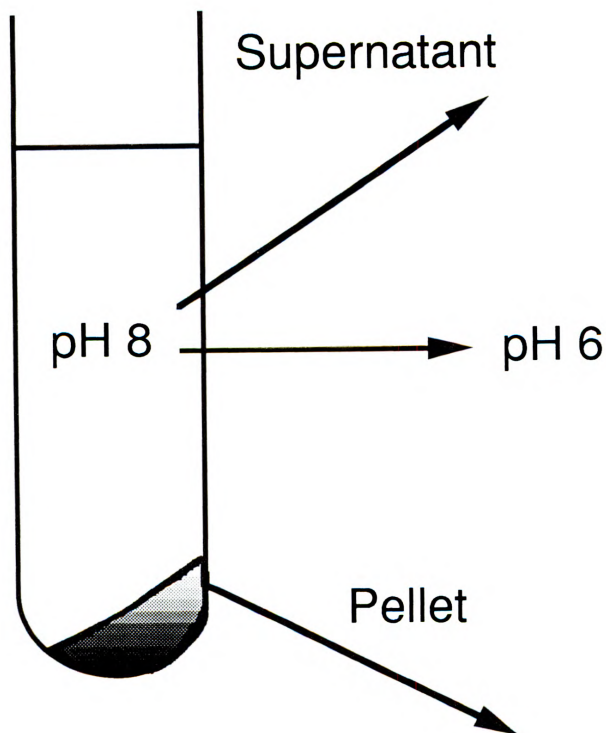
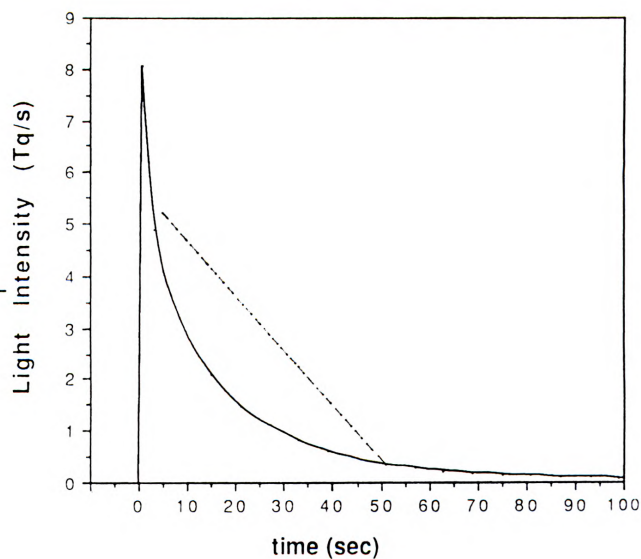


Figure 5.

Scheme illustrating the postulated steps that occur in the pH-triggered bioluminescence. The luciferase, inactive at pH 8, becomes active at pH 6. The luciferin binding protein (LBP) forms a tight complex with luciferin (LH₂) at pH 8 but releases it at pH 6, so that it is free to bind luciferase and be oxidized to form an electronically excited product, thus emitting light.

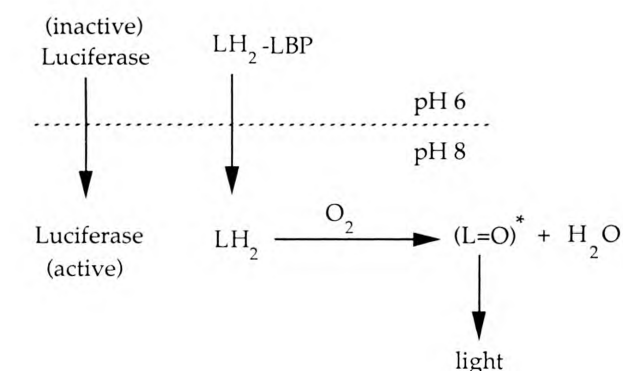
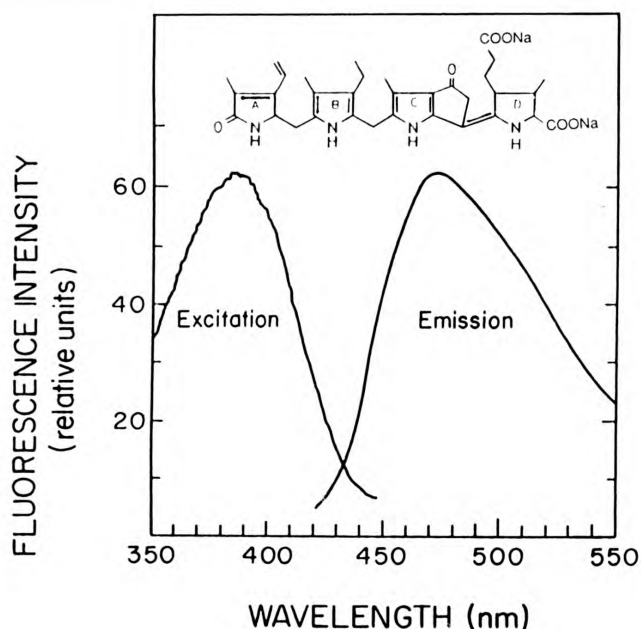


Figure 6.

Corrected excitation and emission spectra of partially purified dinoflagellate luciferin (from ref. 35). The structure of the luciferin is shown at the top (from ref. 25).



In Vitro luminescence: the biochemical components and the organelle

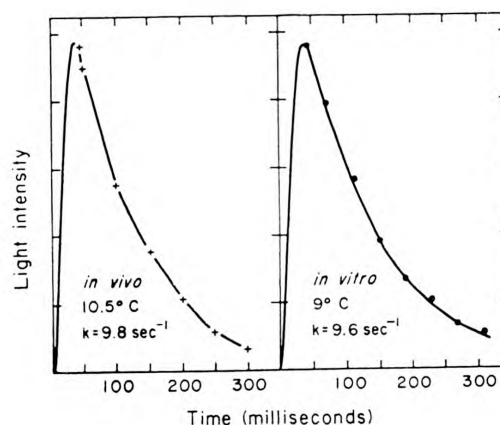
The active biochemical components of the light emitting reaction can be isolated by extraction at pH 8 of cells harvested during the night time or night phase of the circadian cycle. But these components occur in both the soluble and particulate fractions (Fig. 4); in both cases oxygen is required and light emission occurs simply upon shifting the pH to 6. In the supernatant the emission continues for minutes, this being dependent upon the concentration of the extract.

Biochemical reactions usually involve both an enzyme and a substrate. When cell extracts were subjected to gel filtration (separating the components by molecular sizes), only a single active fraction was found. This fraction proved to contain two proteins, the *Gonyaulax* enzyme luciferase (140 kD), and a lower molecular weight protein that binds the substrate, dinoflagellate luciferin. The kinetics of the reaction depend on the luciferase concentration.

The initiation of the reaction by a pH shift-down is a clue to the mechanism of the reaction (Fig. 5) and, as will be seen below, to the nature of its cellular control. At pH 8, the substrate (luciferin) is tightly bound to its binding protein, so that it is prevented from associating with the enzyme. The enzyme itself is also inactive at pH 8, giving a double assurance that the reaction will not occur. A 1% "leak" could

Figure 7.

Comparison of *in vivo* and *in vitro* flashes of *Gonyaulax*. The *in vivo* flash was recorded from a culture placed in front of a phototube, stimulated mechanically by tapping lightly on the housing. The *in vitro* flash was obtained by mixing, in a stopped-flow apparatus, a partially purified particulate fraction (scintillons) suspended in assay buffer (pH 8.2) from one syringe and 0.03 N acetic acid from a second syringe (from ref. 33).



presumably be tolerated in many biochemical reactions; a 1% light leak might be seen!

The structure of the luciferin, a linear tetrapyrrole somewhat similar to the chromophore of phycobiliproteins (Fig. 6), has recently been elucidated²⁵. The luciferin is subject to rapid nonenzymatic autoxidation in air; it has absorption maxima at 245 and 390 nm, and a high quantum yield of fluorescence which peaks at 474 nm²⁶. Paradoxically, the fluorescence emission spectrum of the luciferin before reaction (Fig. 6) corresponds to that of the bioluminescence, yet the oxidation of the luciferin must precede the formation of the emitter.

As mentioned above, the pellet of the centrifuged cell extract (Fig. 4) also has bioluminescence activity, which is also initiated simply by shifting the pH of resuspended particles. In this case, however, the light emission occurs as a flash, kinetically very similar to that of the intact cell (Fig. 7), and kinetically independent of the concentration of particles. The particles responsible are identified with the luminous organelles, the scintillons. The activity may be purified by sucrose isodensity gradient centrifugation, where it bands at a density of 1.23 gm mL⁻¹.

Although living cells flash repeatedly upon repeated stimulation, isolated scintillons emit only a single flash following

a pH jump. Discharged scintillons, however, may be "recharged" in vitro by readjusting the pH back to 8 and incubating with free (not protein-bound) luciferin (isolated separately); a second pH jump yields a second flash similar to the first.

Cell Biology

The relationship between the in vitro soluble and scintillon activities, and how an action potential propagated along the vacuolar membrane might trigger a flash, seemed something of an enigma. How could an organelle be responsive to a membrane event? The question was largely resolved with the identification of the ultrastructural disposition of the scintillon, and its association with the vacuolar membrane¹⁸. This was accomplished by immunogold labeling (Fig. 8), using antibodies raised against the two proteins involved in the bioluminescence reaction^{18,19,24}. The scintillon represents a new type of organelle, an electron dense region of the cytoplasmic compartment which in its mature state protrudes into the acidic vacuole like a balloon, with its narrow neck retaining the cytoplasmic association.

Figure 8.

EM section of a *Gonyaulax* cell showing gold labeling of scintillons with two antibodies, one against luciferase (5 nm gold particles) and the other against the luciferin-binding protein (LBP) (10 nm) Bar = 0.1 μ m (from ref. 19).

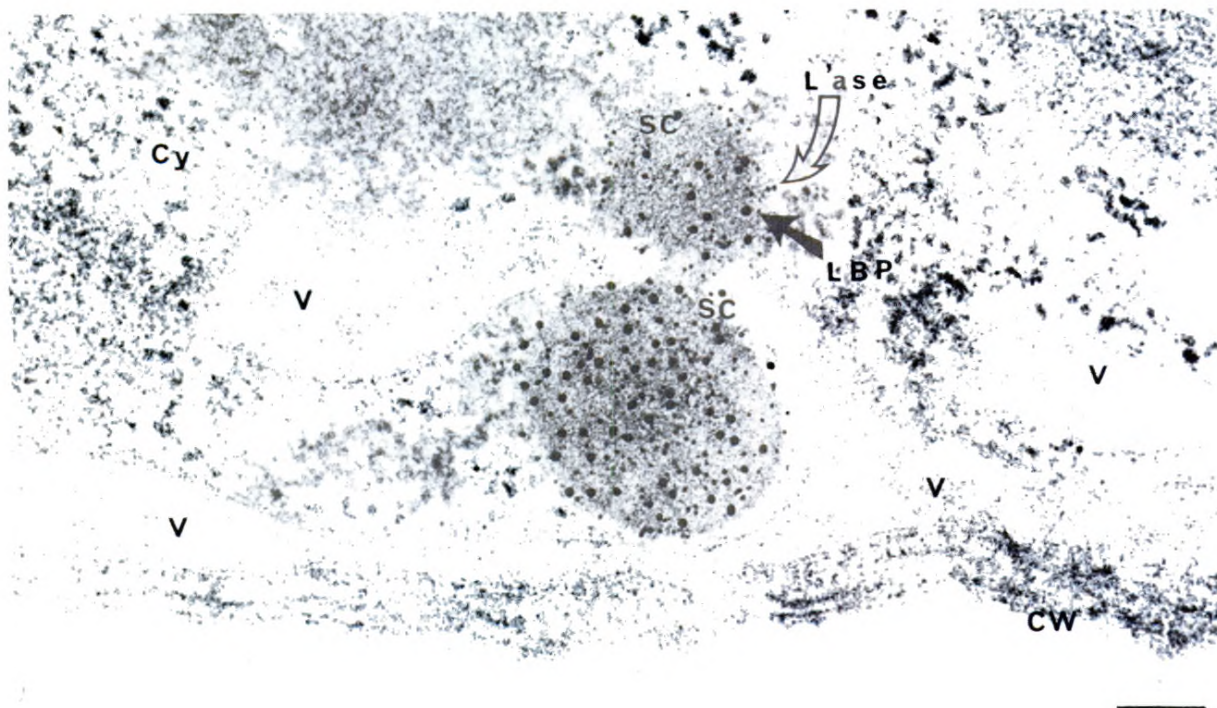
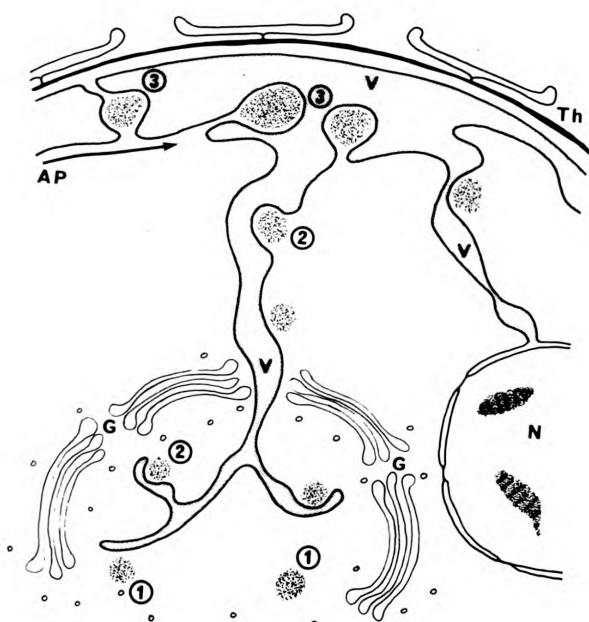


Figure 9.

Schematic representation of the postulated steps in the formation and differentiation of the scintillons and of the excitation – bioluminescence coupling. Pre-scintillons appear as free cytoplasmic condensations in the Golgi (G) area (1), contact the vacuolar membrane (2), and migrate towards the periphery of the cell. In the final stage of the development, mature scintillons (3) protrude from the cytoplasmic compartment (white area) into the vacuolar space (V, stippled). A flash is postulated to be generated by a tonoplast action potential (AP); as it spreads along the vacuolar membrane it opens voltage gated proton channels. The proton influx from the acidic vacuole to the dense body of the scintillon thus triggers the flash. (N) nucleus, (TH) thecal plates (from ref. 18).

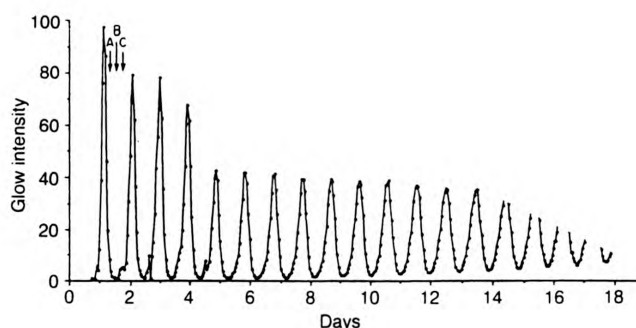


The *in vivo* flash finds its explanation in the topological configuration of the scintillon. In the model shown (Fig. 9), a conducted action potential (AP) of the vacuolar membrane opens voltage gated proton channels as it sweeps over the scintillon, allowing H^+ ions from the acidic vacuole to enter and trigger the flash. The ATP-dependent proton translocating pumps in the vacuolar membrane around the scintillons are postulated to function in restoring and maintaining the alkaline pH of scintillons in between flashes.

The fact that the scintillons are in the cytoplasmic compartment is consistent with the fact that both luciferase and the luciferin-binding protein (LPB) are soluble, not membrane associated proteins. The fact that these proteins are found in the supernatant of cell extracts is accounted for by postulating that some organelles are ruptured during extraction; others break and reseal at the neck, generating vesicles that constitute the active *in vitro* scintillons.

Figure 10a.

The circadian rhythm of the steady glow of bioluminescence of *Gonyaulax*. Measurements were made about once per hour over a 19 day time span, with a culture grown in a 24 hr light – dark cycle (12/12; L/D) and then transferred at zero time to constant temperature ($19^\circ C$) and dim white light (except for 6 min dark every hour during measurements). Other cellular processes show similar rhythmicity, but may peak at different times, as indicated by arrows (A, cell division; B, photosynthesis and cell aggregation; C, luminescent flashing frequency).



Circadian Control

Control of light emission in dinoflagellates occurs at the millisecond time scale for the flashes, as described above. But its control also involves a daily cycle, a rhythm of luminescence in which cells emit light by night but not by day. This rhythm continues even when the cells are maintained under constant conditions, indicative of control by a circadian biological clock (Fig. 10). This circadian control is mirrored in virtually every aspect of the luminescent system, and is also exerted over many other cellular processes. The cells exhibit luciferin fluorescence by night, but very little by day, reflecting changes in their luciferin content. We have shown by immunological detection methods that both luciferase and luciferin-binding protein are present in extracts made during the night phase, but are absent in day – phase extracts^{27,28,36}. Indeed, immunolabeling studies have shown that the organelles themselves are abundant by night, and almost absent during the day²⁴.

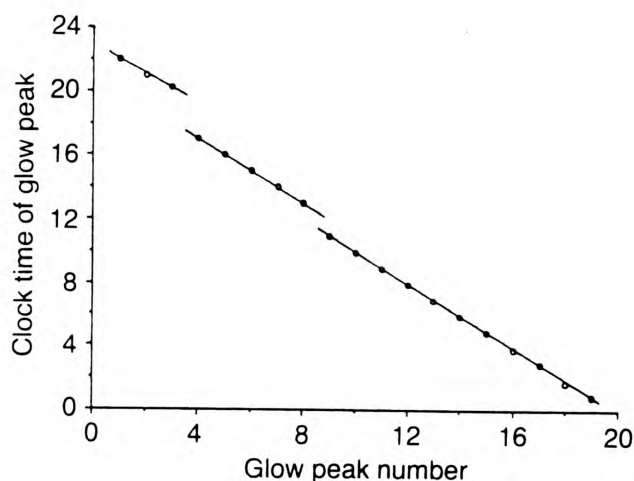
How does the biological clock regulate this rhythm? Is it a control of synthesis or degradation – or perhaps both? Pulse labeling with ^{35}S -methionine showed a 3 to 5 hr burst of synthesis of luciferin-binding protein during the early night phase (Fig. 11; 28). Degradation is also restricted to a rather short period of time, at the end of the night phase.

What turns synthesis on and off, and how does it relate to the cellular clock? Is it the level of mRNA which varies over the circadian cycle? For the luminescent system, apparently not. Northern blots (for detection of specific mRNAs) using a cDNA probe for the luciferin-binding protein indicate that the amounts of transcript for luciferin-binding protein are constant (Fig. 11).

Given the fact that the mRNA level for luciferin-binding protein is constant, it is inferred that translational control is involved in regulating the daily changes in its synthesis and cellular level. Translational control appears to be involved in the synthesis of a number of other *Gonyaulax* proteins²⁹, but many details are not yet understood. The bioluminescence system, where there is a clear biochemical correlate of a robust circadian rhythm, provides an excellent tool for the investigation of such regulatory processes.

Figure 10b.

The period of the circadian rhythm, though not exactly 24 hours, may be very precise, as shown by a plot of the times of day at which sequential peaks occur. While the best fit for all the points gives a period of 22.8 ± 0.6 hr, far better fits are obtained by considering three different segments, with unexplained phase jumps between days 3 and 4, and 8 and 9. Depending on the physical and chemical environment, the circadian period may vary considerably, ranging from 19 to 27 hr (from ref. 34).

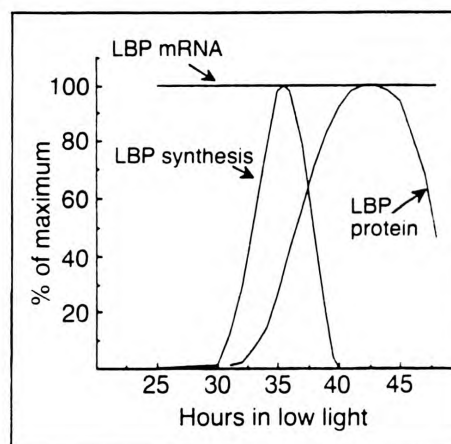


Biography

J. Woodland Hastings is the Paul C. Mangelsdorf Professor of Natural Sciences at Harvard University and has been a principal investigator of the Office of Naval Research for 38

Figure 11.

Circadian rhythm in the amount of extractable luciferin-binding protein parallels the *in vivo* bioluminescence. Its synthesis occurs earlier, over a period of several hours during late subjective day and early night. However, messenger RNA levels for LBP, as determined from northern blots, remain constant (from ref. 34).



years. He is recognized for pioneering work in the isolation and biochemical characterization of both the firefly and bacterial luciferase reactions. In 1955 at Scripps Institution of Oceanography, he initiated studies on the biochemistry and circadian rhythmicity of the marine dinoflagellate *Gonyaulax polyedra*.

Professor Hastings has served as a member and chairman of numerous advisory committees for the National Academy of Sciences, National Institutes of Health, National Sciences Foundations, and the National Aeronautics and Space Administration. He was a John Simon Guggenheim Fellow and visiting professor at Rockefeller University; a National Institutes of Health Fellow in Paris and a NATO Senior Fellow. In 1979, he was a recipient of the Alexander Von Humboldt prize and a visiting professor at the University of Konstanz, Germany, and a Yamada Foundation Fellow serving as visiting professor at the National Institutes of basic biology in Okazaki, Japan.

References

1. Seliger, H.H., Carpenter, J.H., Loftus, M., Biggley, W.H. and McElroy, W.D. 1971. Bioluminescence and phytoplankton successions in Bahia Fosforescente, Puerto Rico. *Limnol. Oceanogr.* 16:608 – 622.
2. Sweeney, B.M. 1975. Red tides I have known. In: Proceedings of the First International Conference on Toxic

- Dinoflagellate Blooms. (V.R. LoCicero, ed.), pp. 225 – 234, The Massachusetts Science and Technology Foundation, Wakefield, MA.
3. Thomson, C.W. 1877. The Voyage of the Challenger 2:85.
4. Harvey, E.N. 1957. A History of Luminescence. American Philosophical Society. Philadelphia, PA.
5. Anderson, D. M., White, A. W., Baden, D. G., Eds. 1985. Toxic Dinoflagellates; Elsevier/North Holland; New York.
6. Anderson, D.M., Dulis, D.M., Sullivan, J.J., Hall, S and Lee, C. 1990. Idynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. Mar. Biol. 104:511 – 524.
7. Sweeney, B.M. 1963. Bioluminescent dinoflagellates. Biol. Bull. 125:177 – 181.
8. Spector, D.L. 1984. (Ed.) Dinoflagellate nuclei. In: Dinoflagellates, pp. 107 – 184, Academic Press, NY.
9. Taylor, F.J.R. 1987. The Biology of Dinoflagellates. Bot. Monographs 21. Blackwell Scientific Publishers, Palo Alto, CA.
10. Rae, P.M.M. 1976. Hydroxymethyluracil in eukaryotic DNA: A natural feature of the Pyrrhophyta (dinoflagellates) Science 194:1062 – 1064.
11. Burkenroad, M.D. 1943. A possible function of bioluminescence. J. Mar. Res. 5:161 – 164.
12. Morin, J.G. 1983. Coastal bioluminescence: Patterns and Functions Bull. Mar. Sci. 33:787 – 817.
13. Esaias, W.E. and Curl, H.C., Jr. 1972. Effect of dinoflagellate bioluminescence on copepod ingestion rates. Limnol. Oceanogr. R. 17:901 – 906.
14. White, H.H. 1979. Effects of dinoflagellate bioluminescence on the ingestion rates of herbivorous zooplankton. J. Exp. Mar. Biol. Ecol. 36:217 – 224.
15. Buskey, E.J., Mills, L. and Swift E. 1983. Dinoflagellate luminescence effects on copepod behavior. Limnol. Oceanogr. 28:575 – 579.
16. Anderson, D.M, Nosenchuck, D.M., Reynolds, G.T. and Walton, A.J. 1988. Mechanical stimulation of bioluminescence in the dinoflagellate *Gonyaulax polyedra* Stein. J. Exp. Mar. Biol. Ecol. 122:277 – 288.
17. Eckert, R. 1966. Bioelectric control of bioluminescence in the dinoflagellate *Noctiluca*. Science 147:1140 – 1145.
18. Nicolas, M-T., Nicolas, G., Johnson, C.H., Bassot, J.-M. and Hastings, J.W. 1987. Characterization of the bioluminescent organelles in *Gonyaulax polyedra* (dinoflagellates) after fast – freeze freeze fixation and antiluciferase immunogold staining. J. Cell Biol. 105:723 – 735.
19. Nicolas, M-T., Morse, D. Bassot, J.-M., and Hastings, J.W. 1991. Colocalization of luciferin binding protein and luciferase to the scintillons of *Gonyaulax polyedra* revealed by immunolabeling after fast-freeze fixation. Protoplasma 160:159-166.
20. Hastings, J.W. and Krasnow, R. 1981. Temporal regulation in the individual *Gonyaulax* cell. In: International Cell Biology 1980/1981 (H.G. Schweiger, ed.), pp. 815-822, Springer-Verlag, Berlin.
21. Krasnow, R., Dunlap, J. Taylor, W., Hastings, J.W., Vetterling, W., and Gooch, V.D. 1980. Circadian spontaneous bioluminescent glow and flashing of *Gonyaulax polyedra*. J. c. Physiol. 138:19-26.
22. Krasnow, R., Dunlap, J., Taylor, W., Hastings, J.W., Vetterling, W., and Haas, E. 1981. Measurements of *Gonyaulax* bioluminescence, including that of single cells. In: Bioluminescence: Current Perspectives, (K.H. Nealson, ed.), pp. 52-63, Burgess Publ Co., Minneapolis, MN.
23. Homma, K. and Hastings, J.W. 1988. Filtration – selection of cell cycle-synchronized *Gonyaulax polyedra*: quantized generation times. J. Biol. Rhythms 3:49-58.
24. Fritz, L., Morse, D. and Hastings, J.W. 1990. The circadian bioluminescence rhythm of *Gonyaulax* is related to daily variations in the number of light emitting organelles. J. Cell Science 95:321-328.
25. Nakamura, H., Kishi, Y., Shimomura, O., Morse, D., and Hastings, J.W. 1989. Structures of dinoflagellate luciferin and its enzymatic and non-enzymatic air-oxidation products. J. Am. Chem. Soc. 111:7607-7611.
26. Dunlap, J. and Hastings, J.W. 1981. Biochemistry of dinoflagellate bioluminescence: The purification and characterization of dinoflagellate luciferin from *Pyrocystis lunula*. Biochemistry 20:983-989.
27. Johnson, C.H., Roeber, J., and Hastings, J.W. 1984. Circadian changes in enzyme concentration account for rhythm of enzyme activity in *Gonyaulax*. Science 223:1428-1430.
28. Morse, D., Milos, P.M., Roux, E., and Hastings, J.W. 1989. Circadian regulation of the synthesis of substrate binding protein in the *Gonyaulax* bioluminescent system involves translational control. Proc. Natl. Acad. Sci. USA 86:172-176.
29. Milos, P., Morse, D. and Hastings, J.W. 1990. Circadian control over synthesis of many *Gonyaulax* proteins is at the translational level. Naturwissenschaften 77:87-89.
30. Hastings, J.W. 1960. Biochemical aspects of rhythms: Phase – shifting by chemicals. Cold Spring Harbor Symposia on Quantitative Biology 25:131-144.
31. DeSa, R.J., Hastings, J.W. and Vatter, A.E. 1963. Luminescent “crystalline” particles: An organized subcellular bioluminescent system. Science 141:1269-1270.
32. Morse, D., Pappenheimer, A.M and Hastings, J.W. 1989. Role of a luciferin binding protein in the circadian bioluminescent reaction of *G. polyedra*. J. Biol. Chem. 264: 11822-11826.
33. Hastings, J.W., Vergin, M., and DeSa, R. 1966. Scintillons: The biochemistry of dinoflagellate bioluminescence. In: Bioluminescence in Progress (F.H.

Johnson and Y. Haneda, eds.), pp. 301 – 329 (Princeton University Press.

34. Morse, D., Fritz, L. and Hastings, J.W. 1990. What is the clock? Translational regulation of circadian bioluminescence. *Trends in Biochem. Sci.* 15:262-265.
35. Njus, D.L. 1975. Ph.D. Thesis. Harvard University. The Control of bioluminescence in *Gonyaulax polyedra*.
36. Johnson, C.H., Imoue, S., Flint, A. and Hastings, J.W. 1985. Compartmentation of algal bioluminescence: autofluorescence of bioluminescent particles in the dino-flagellate *Gonyaulax* as studied with image-intensified video microscopy and flow cytometry. *J Cell Biol.* 100:1435-1446.

Assessment of Marine Bioluminescence

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Introduction

Seafarers are unavoidably aware of bioluminescence. Often beautiful, as when porpoises at a ship's bow streak through luminescent dinoflagellates, outlining their bodies in light and leaving behind persistent glowing tracks, bioluminescence is nearly always noticeable in bow waves, wakes and in breaking waves. Thus, it has implications for many types of naval operations.

The expression of bioluminescence in the sea is a function of processes and events from populational to molecular levels. This biological property is, in turn, modulated by the physical and chemical states of the ocean. Consequently, bioluminescence ranks among the most complex of oceanic phenomena.

Bioluminescence occurs in all marine environments, frequently to a phenomenal extent, and is found as well in a variety of terrestrial and to a vanishingly small degree in freshwater environments. It is far more prevalent in the ocean

than on land, occurring in a remarkable variety of organisms representing the majority of marine phyla, from bacteria to fish¹; see Alberte Table I). It has been demonstrated to play many biological roles ranging from predator-prey interactions, through camouflage, to reproductive and social interactions². One speculates that this list, although extensive, is far from inclusive for so pervasive an oceanic phenomenon.

As investigation of marine bioluminescence has progressed there has been a valuable interplay of pure and applied studies, much of which has been supported by the Navy. Indeed, initiation of the modern phase of mapping oceanic bioluminescence unexpectedly resulted from a study by George L. Clarke³ of light transmission to depth using an early bathyphotometer equipped with a photomultiplier (PMT) light detector (Fig. 1A). When lowered to the limit of detectable solar light, Clarke's instrument continued to register brief pulses of light. These were soon attributed to luminescent organisms stimulated by motion of the detector.

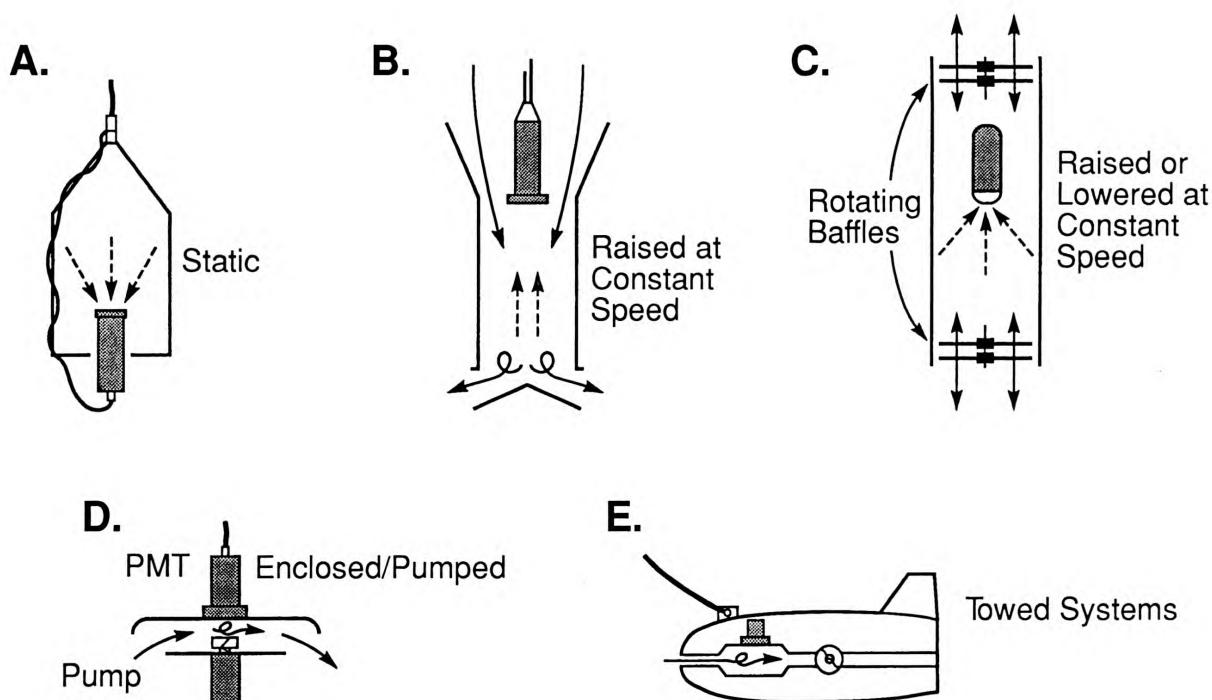
Evolution of Bioluminescence Assessment Methods

As quantitative devices, instruments such as Clarke's suffered from sensitivity to ship-induced motion through the suspending cable and, having an unrestricted field of view, their light measurements lacked volumetric reference. The most valuable further developments of bioluminescence bathyphotometers have emphasized instruments that deliver hydro-mechanical excitation in a repeatable fashion to a known volume of water. These have the greatest potential for rapidly sampling the vertical distribution of bioluminescence in the sea, or, as towed instruments, mapping bioluminescence on large spatial scales. An excellent and extensively used suite of instruments of this type is described by Ondercin^{4, 28}. In such instruments, luminescence is triggered by turbulence created by steady movement of the bathyphotometer (Fig. 1 B,C) or by a pump impeller (Fig. 1 D,E).

Relatively little consideration was given to how effectively early instruments sampled the variety of luminescent species present, to the duration of the luminescent signal relative to the time organisms occupied the detector chamber, and, finally, to the relationship between hydro-mechanical stimulus properties and light output. These design limitations originated, in part, from the early assumption that the dominant component of luminescence in the photic zone was from dinoflagellates, single celled, poorly or nonmotile auto- or heterotrophic phytoplankton (See Hastings). However, zooplankton, or animal, luminescence, primarily from crustaceans and gelatinous forms such as coelenterates and ctenophores (see cover), may be orders of magnitude brighter and of far longer duration than dinoflagellate luminescence. Moreover, the locomotor and sensory abilities of zooplankton raise the possibility of evasion of capture by slowly pumped bathyphotometers. Zooplankton are also generally rarer than dinoflagellates and, thus, are more difficult to sample rigorously with instruments that process small volumes of water.

Figure 1.

Various bioluminescence bathyphotometers. (A) Clarke's,³ (B) An early sounding bathyphotometer used by Gitelson. Raised at constant speed, water is entrained by upper funnel and luminescence is primarily triggered by turbulent flow at exit baffle. (C) A recent modification of the Gitelson apparatus equipped with entry and exit baffles which also provide excitation as water is entrained by raising or lowering³⁰. (D). Generic sketch of a low volume enclosed and pumped bathyphotometer in which excitation is provided by pump impeller, detector chamber volume about 50 ml with indeterminate flow path. (E) Generic towed system with excitation provided by entry baffle and flow provided either by forward motion or pump downstream from detector chamber^{31, 32}



Consequently many types of pumped bathyphotometers do not fully characterize bioluminescence potential in certain environments. In particular, they may underestimate the contribution of zooplankton⁵.

HIDEX Bioluminescence Bathyphotometers

Uncertainty about the ability of traditional bathyphotometers to provide comprehensive data on the variety of bioluminescent sources and signals in the sea led, at the request of the Oceanographer of the Navy, to the convening in 1981 of a panel of University and Navy researchers to recommend improvements in marine bioluminescence research and, especially, bathyphotometry. To implement one element of these recommendations the Bioluminescence Group at The University of California at Santa Barbara (UCSB) undertook

development of a new type of bioluminescence bathyphotometer.

Design studies were conducted with cultured dinoflagellates of known bioluminescence potential in a full-scale transparent plastic prototype. Laser velocimetry was used to evaluate hydrodynamic excitation stress and low light level imaging (ISIT, intensified silicon intensified target, video) and photomultiplier tube (PMT) measurements were used to assess luminescence induction as a function of excitation. The first prototype instrument was successfully tested at sea in early 1987⁶. The U.S. Naval Oceanographic Office representatives contributed important recommendations for design changes to facilitate operation in heavy seas. These were implemented along with improvements in detector systems and data handling. The first operational instrument incorporating these changes was accepted by Navy on a cruise in June 1990. A similar instrument was provided to Naval Oceanographic and Atmospheric Research Laboratory in December 1990. A third instrument at UCSB is currently being employed in ongoing

Figure 2.

Schematic of HIDEX detector assembly. The detector chamber is 130 x 12 cm diameter. Within the entry, 100 microsecond pulsed infrared strobes provide stop-motion back or side lighting for video camera. The freely rotating baffle blocks ambient light with minimum premature excitation. The excitation grid marks entry to detector chamber. The event counter detects luminescent organisms at up to 100 s^{-1} and shares light with the bioluminescence spectrometer. In the white detector chamber 8 zone array pairs conduct light to eight R647 Hamamatsu PMTs. Total light is detected by 78 fiber optics leading to a single R268 Hamamatsu PMT. At the end of the detector chamber water is extracted past the flowmeter by the pump and exhausted to the outside through a radial diffuser which also serves as a light baffle.

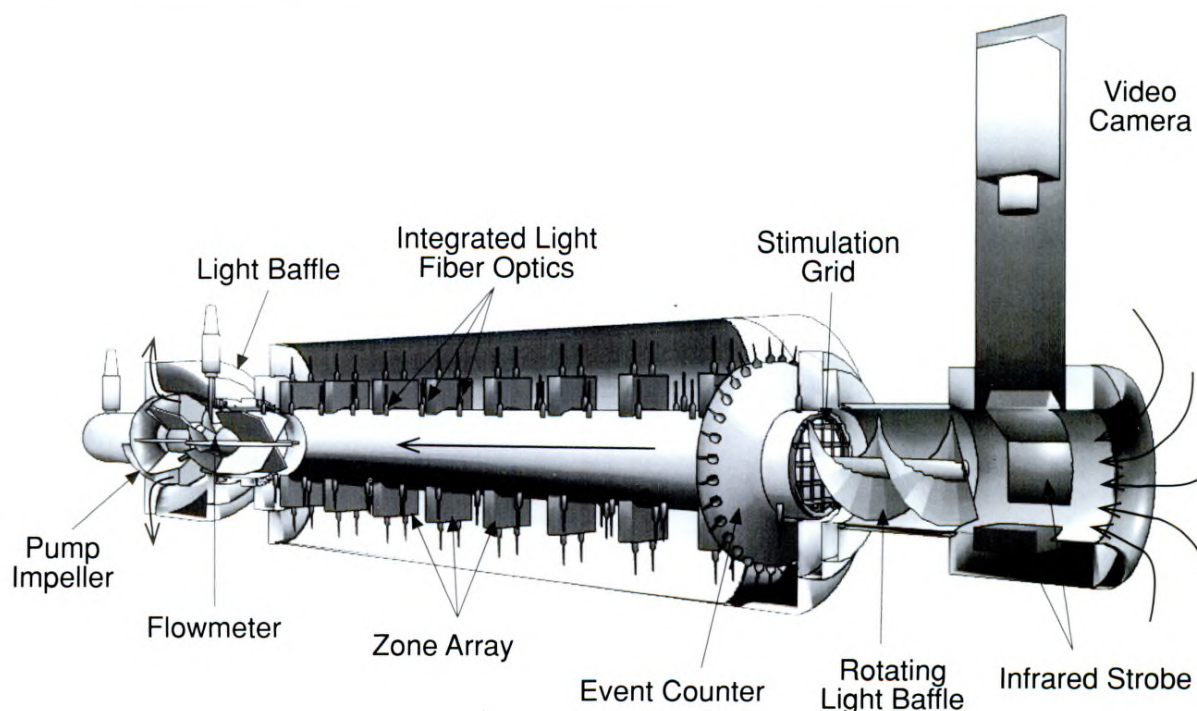
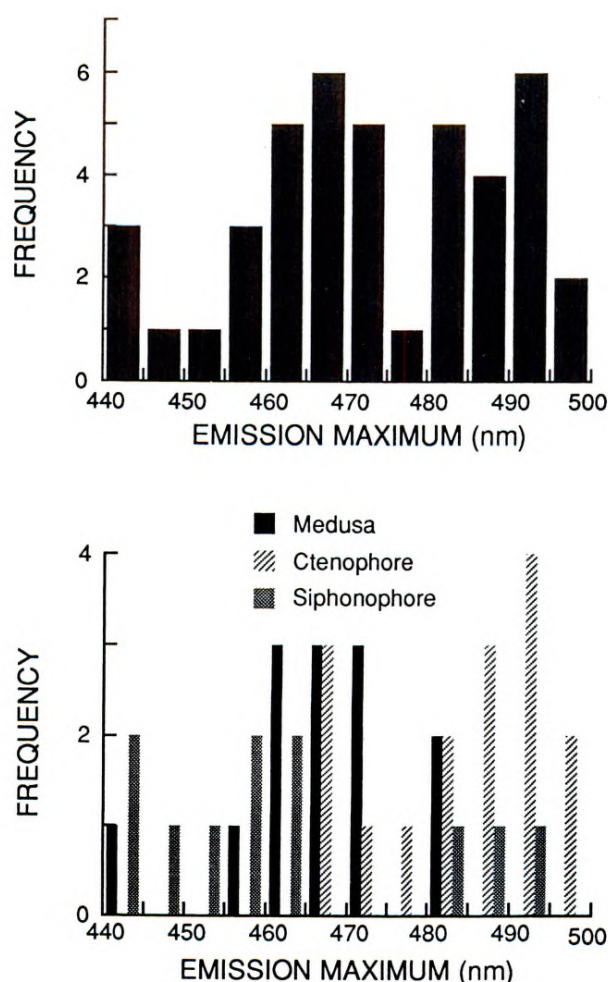


Figure 3.

Examples of variation of color of bioluminescence among marine species. Upper plot shows frequency of emission peaks as distributed among all species tested. Lower plot shows how emission maxima are distributed among three major groups of gelatinous zooplankton. From unpublished data collected in the Bahamas in 1989 by M. I. Latz and S.H.D. Haddock.



basic research and serves as a test bed for technical improvements.⁷

Characterization of the New Design Bathyphotometers

The fundamental properties of these instruments are emphasized by their acronym: HIDEX, originating from High Intake Defined Excitation (Fig. 2). Luminescence is excited

by a hydrodynamically and bioassay characterized coarse grid at the chamber entry. Further excitation is minimal during flow through the chamber. Therefore, the total light response can be related to the magnitude of the grid induced turbulence. Bioluminescence is measured during flow through a 130 x 12 cm tube which is long enough to sample most of the bioluminescence triggered by the grid at typical flow velocities.

Light is sampled in several ways to derive maximal information. Total light emitted during passage through the white-surfaced detector tube is sampled by a single PMT receiving light from an array of fiber optics viewing the interior of the entire 16 L volume detector tube. A short distance downstream from the excitation grid, luminescent particles are counted and their instantaneous individual radiances are measured by another photomultiplier viewing a narrow cross section of the chamber through a circumferential light guide. Finally, a set of eight equally spaced PMTs viewing narrow cross-sections along the length of the detector tube assess the kinetics of the populational flash. Information on flash kinetics guides computer controlled adjustment of flow through the chamber so as to capture the populational flash appropriately within its volume. This ensures that the total light signal accurately represents as much of the entire flash as is practical. Flash kinetics also help to identify the general types of organisms emitting light^{8,9}.

The profiler is operated, both in descending and ascending mode, at selected flow rates of from 16 to 35 L s⁻¹. This is higher than the rates attained by other pumped vertical profilers (ranging from 0.2 to 1.1 L s⁻¹) and serves to improve sampling statistics even when profiling is conducted rapidly in sparsely populated water. During a standard HIDEX profile, 15 min are required to reach 200 m during which over 100 L are sampled per vertical meter traversed. The high flow rate and the large diameter detector chamber inlet facilitate capture of strongly swimming organisms. For animals capable of escape velocities up to 10 cm s⁻¹ the capture volume ahead of the entry is about 4.5 L. The infrared-strobed video camera monitoring the entry of the UCSB instrument confirms capture of large crustaceans and occasionally fish.

Effects of ship's vertical movement on flow through the horizontally deployed chamber are minimized by flowmeter regulated pump control. A problem with enclosed volume bathyphotometers is attaining adequate ambient light baffling of the entry without prematurely exciting entrained organisms. In HIDEX this is managed by a detection chamber inlet shaped to effect transition to maximal flow with smooth acceleration and by a freely rotating helical light baffle immediately before the stimulus grid. Quantified ISIT video evaluation of the baffle installed in the transparent prototype shows that less than 5 % of maximal excitation results from the baffle itself.

The standard HIDEX profilers are equipped with CTDs (instrument packages measuring conductivity, temperature and depth), transmissometers measuring absorption and scat-

tering of light at red and blue wavelengths and fluorometers for quantifying algae such as dinoflagellates by their chlorophyll content. An intensified CCD-based spectrometer was developed for the UCSB instrument by C. Moore (UCSB Free Electron Laser Laboratory). It collects light from the bioluminescent particle counter and can register the populational bioluminescence spectrum as well as the spectra of individual brightly luminescent organisms when flow rate is low¹⁰. It is expected to contribute to identification of the dominant forms of organisms processed in the bathyphotometer, and also to provide in situ source spectra for radiative transfer calculations. This is desirable since bioluminescent spectra vary among species across a wide spectral range when measured in the laboratory (Fig. 3) and, thus, may be expected to vary in situ according to local species composition¹¹. A shipboard 386-based computer communicating with a second computer on the HIDEX is used for system control.

Communication and power is provided by a load-bearing seven-conductor cable and dedicated winch. The shipboard computer manages realtime data display, analysis and archiving during operations. During profiles a plot is maintained of all measured parameters as a function of depth updated every 0.5 s. There is a 100 Hz display of the total light signal and a 500 Hz display of the luminous particle counter signal. A bar graph display tracks the signal from each kinetic array detector. System housekeeping data are continuously displayed. After a profile all data are immediately available for statistical analysis.

The UCSB group has developed a self-contained HIDEX variant suitable for mooring deployment called MOORDEX (Fig. 4). It was recently used for the first time on a deep-sea mooring near Iceland as part of a current ONR oceanography program. Unattended, this instrument registers luminescent particle counts and measures ambient light, total bioluminescence, flow rate, temperature and depth, pumping 10 L s^{-1} in 2 min bursts, eight- times per day for approximately 4 months. Besides data storage on hard disc, reduced data is stored in a solid state memory hardened to survive mooring failure. Completing the HIDEX development program, a system for towing is being constructed at UCSB and is scheduled for use by the Navy starting in 1992. An instrument with a HIDEX-type detector chamber has been used successfully in the arctic¹².

Deployments of HIDEX Vertical Bioluminescence Profilers

Over the past several years the three HIDEX vertical profilers and the prototype have been deployed on 10 cruises in the N. Pacific, Gulf Stream, Sargasso Sea, Bahamas, N. Central Atlantic, Vestfjord (Norway) and the Alboran Sea. The

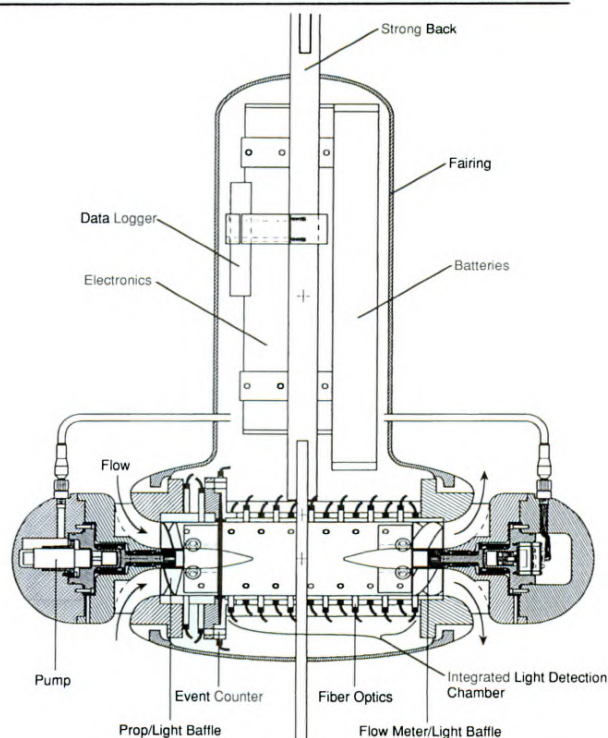
following are examples of work conducted in two characteristic bioluminescence regimes.

Phytoplankton Dominated Bioluminescence

Vestfjord, a large, arctic Norwegian fjord has dense populations of bioluminescent dinoflagellates, both photosynthetic and heterotrophic. In late summer these dominate the luminescent plankton. In August, 1990, contemporaneous shipboard investigations and HIDEX profiles were carried out during dawn and dusk transitions in the daily cycle of luminescence¹³. Shipboard laboratory measurements on fresh samples of the dominant local photosynthetic and heterotrophic dinoflagellates exposed to the local light cycle indicated for both types, that the shift between luminescent and non-luminescent states paralleled in time and intensity those detected by repeated profiles with HIDEX. A dusk HIDEX time series (Fig. 5) of seven profiles at half-hour intervals showed nearly

Figure 4.

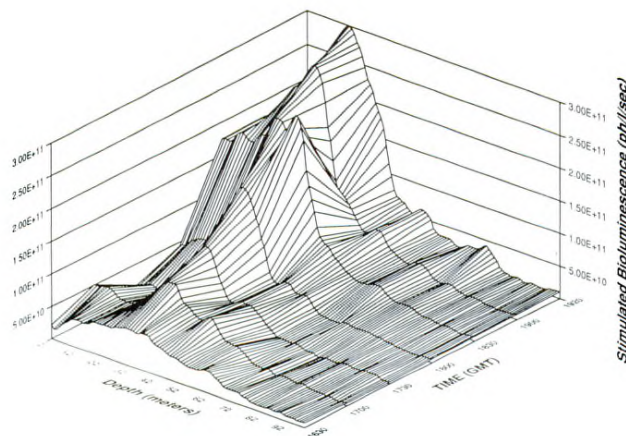
MOORDEX, a HIDEX-type bathyphotometer for unattended operation on moorings or drifting buoys, shown in longitudinal section. While the detection scheme is the same as in HIDEX, with luminescent particle counter and total light measurement, power restrictions dictate an impeller excitation mechanism, a 500 msec residence time and 10 L s^{-1} flow rate. Height of the shroud is 1 m.



Original from

Figure 5.

HIDEX dusk profile series from Vestfjord showing only the total stimulated light signal as a function of depth and time.



an 8-fold increase in stimuable light at about 25 m depth. The profile series in conjunction with net casts also eliminated the alternative hypothesis that vertical migration of luminescent organisms from deeper water, might have produced the 25 m peak, a common occurrence in other situations. Since little increment in bioluminescence was noted deeper than 35 m, the dusk increase in bioluminescence must have occurred in place and could only be explained by daily cycling in bioluminescence potential of a persistent shallow zone of dinoflagellates. Indeed, laboratory studies (see Hastings) have shown that there is circadian regulation of bioluminescence in some species of dinoflagellates and that in these species significant bioluminescence capability exists only during the night period (see also refs. 14 and 15).

Mixed Phytoplankton and Zooplankton Bioluminescence

The Alboran Sea, has been noted for intense luminescence by various observers. Donaldson (1989) made extensive measurements throughout much of the Mediterranean using a laboratory photometer to measure water samples from the condenser flow of the USS John F. Kennedy. The brightest signals in that survey, 10^9 photons $L^{-1} s^{-1}$, were from the Alboran Sea, while the remainder of the very large Mediterranean sample set was in the range of 10^7 to 10^8 photons $L^{-1} s^{-1}$. These are substantial intensities considering that the tested samples must have undergone premeasurement excitation in the condenser flow and from other unavoidable manipulations.

In the Spring of 1991 in the Alboran Sea, two HIDEX profilers were used in a two-ship survey with the Johnson-Sea-Link submersible. The bioluminescent environment was com-

plex with unusually high concentrations of brightly luminescent *Noctiluca*, a large heterotrophic dinoflagellate, in the upper few meters. Crustaceans (copepods and euphausiids) were in large numbers at deeper levels together with gelatinous zooplankton. Immediately after a HIDEX profile, the submersible conducted short transects at depths of interest revealed by the HIDEX. Luminescence was evaluated on the submersible transects by low light level video of a coarse screen mounted at the front of the submersible, as referenced below.

An example of data collected by the two methods is shown in Figure 6. The HIDEX profile shows intense luminescence very near the surface, so bright that the interior of the submersible was lit enough for reading. Stimulated light then drops rapidly 3 orders-of-magnitude and levels off at around 40 m to intensities that are still high. The ISIT video images at 12 m also show intense luminescence. The video tape reveals short duration flashes almost exclusively. The HIDEX kinetics plot at position 1 in Fig. 6 is confirmatory, showing short duration flashes decaying to half maximum in about 0.3 sec. This value is characteristic of dinoflagellate flashes under laboratory conditions¹⁷. The increase in flash duration appearing on the kinetics plot from positions 1 through 2 to 3 is matched by a marked change in the 90 m video image. At that depth, screen excited luminescence is dominated by large, amorphous shaped glows produced by long persistent luminescence from copepods and euphausiids along with a diminished dinoflagellate signal. This shift from dinoflagellate to crustacean dominated luminescence continues to greater depths where the video image reveals rare, bright luminescences of long persistence almost exclusively from copepods and euphausiids and rarer gelatinous zooplankton. Much of the signal is from highly persistent secreted luminescent material discharged when animals strike the screen. The high variability in the kinetics trace from position 3 through 4 and beyond is indicative of bright but increasingly rare luminescent sources. Finally, the HIDEX fluorescence signal confirms the presence of nannoplankton in near surface water.

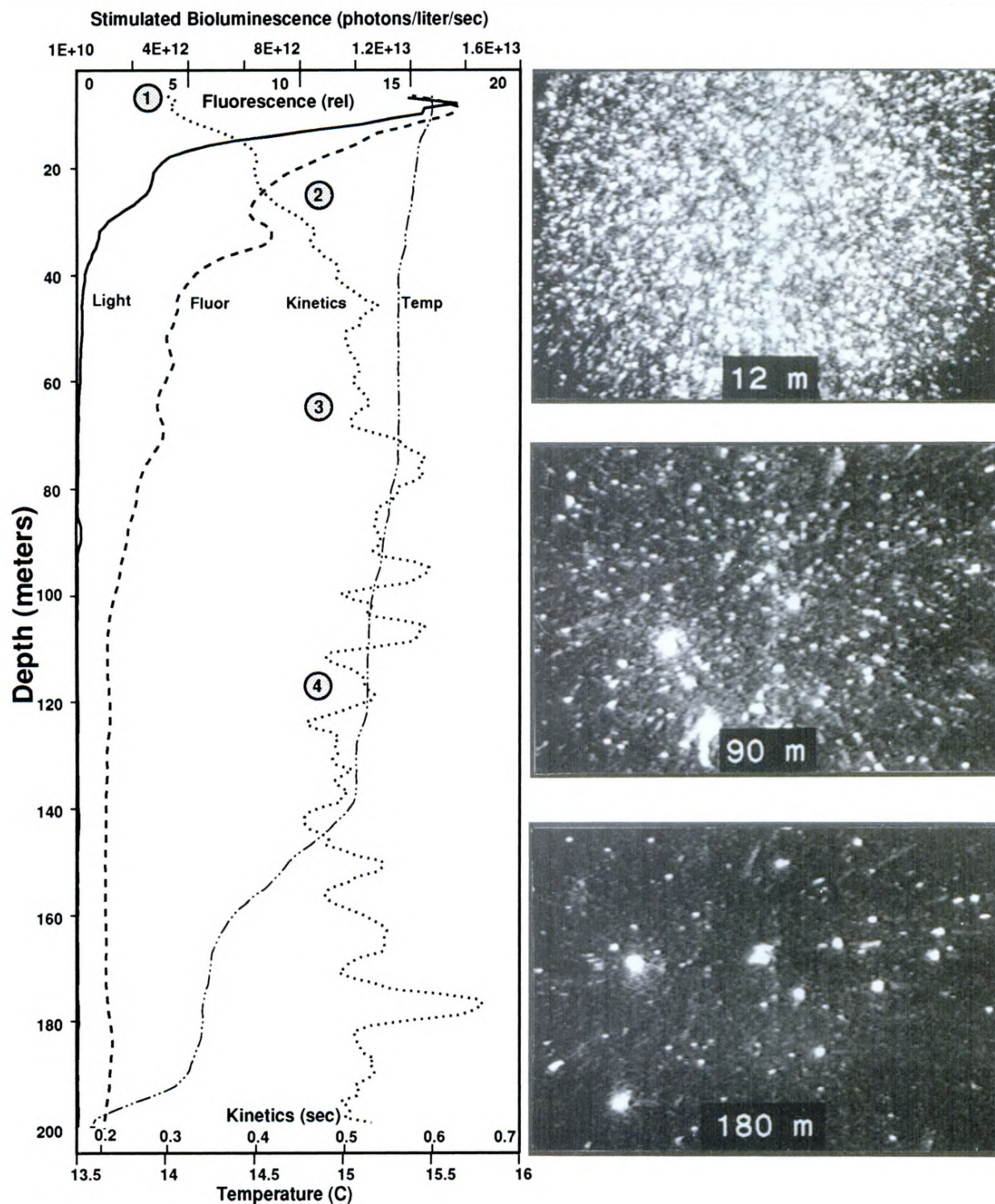
Other Methods of Assessing Oceanic Bioluminescence

Tests on Collected Organisms

In parallel with bioluminescence bathyphotometry in the sea, there has been a long-term research effort to identify bioluminescent sources and to assess their luminescent properties. Such information is essential to an understanding of the populational basis of luminescence which, in turn, is essential to developing predictive capability. Organisms may be collected by quantitative net tows, by net capture of organisms in the exhaust of low pumping volume bathyphotometers, or directly by plankton pumps. Enumeration of such material provides information on population structure. These data to-

Figure 6.

Alboran Sea joint HIDEX and Johnson-Sea-Link bioluminescence profile. Width of video field is 1.0 m. (See text).



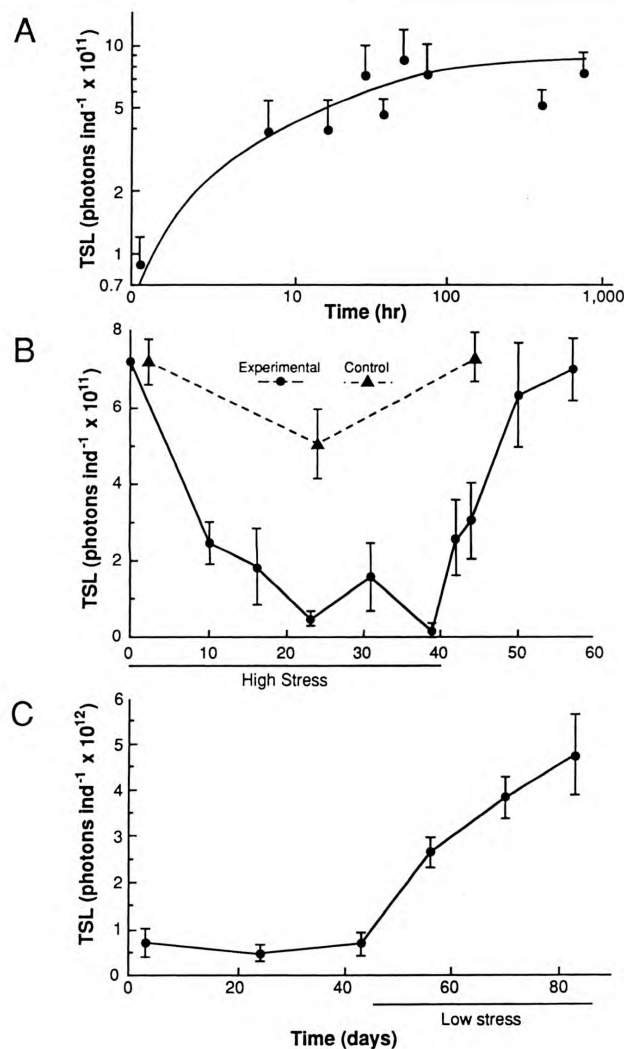
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Figure 7.

Stress effect on bioluminescence potential in a copepod crustacean, *Gaussia princeps*. (A) In this plot zero denotes time of capture by a deep trawl with a thermally protected, closing cod end. The first 100 hr were on shipboard. (B) Under shore laboratory conditions with standardized high and low stress environments.



gether with measurement of total stimuable light of identified specimens measured in shipboard photometers are used to construct bioluminescence light budgets. These are estimates of *in situ* bioluminescence potential derived from the estimated numbers of bioluminescent organisms present multiplied by the laboratory measured bioluminescence potential of each species. Our several laboratories have made substantial improvements in this methodology by using integrating sphere quantum counters in conjunction with fast spectrometers

to precisely characterize bioluminescence emissions from many varieties of luminescent organisms. Such data, however, is of uncertain value as a basis for construction of bioluminescence light budgets, even when low values presumably due to capture stress are discarded¹⁸.

This pessimism comes from the view that the *in situ* bioluminescence stress level of many, perhaps most organisms can only be determined by *in situ* measurement. It is recognized that the bioluminescence potential of captured specimens is strongly influenced by recovery conditions between capture and measurement^{19, 20}. The recovery time and environment available on shipboard is likely to be inadequate except, perhaps, for dinoflagellates and some other very small organisms. This is illustrated by a recent investigation on *Gaussia princeps*, a large (5 mm long) and brilliantly luminescent copepod which has the great advantage of surviving in good condition indefinitely under shore marine laboratory conditions²¹. It is, therefore, a model organism for investigation of bioluminescence potential as influenced by capture stress and stress derived from conditions of laboratory maintenance. Figure 7A shows nearly an eight-fold increase in the light response of *Gaussia* to a standard mechanical stress during the first 1000 h (42 days) after net capture^{20, 22}. Moreover, shore laboratory tests continued over the ensuing 140 days show that luminescence potential, not unexpectedly, depends on the physical stress levels experienced under culture (Fig. 7B). Remarkably, when stress on control animals is further reduced to an absolute minimum from that experienced in the standard laboratory maintenance regime, their bioluminescence potential rises markedly and continues to rise after 20 days on the minimal stress regime (Fig. 7C). If this copepod species can be considered typical of zooplankton, the bioluminescence potential of organisms *in situ* may be considerably at variance with measurements made on captured specimens irrespective of how they may be treated after capture. This is because it is logical to expect that *in situ* luminescence potential varies with environmental conditions such as turbulence and predator encounters. Consequently, restoration to *in situ* bioluminescence capacity after capture cannot be guaranteed by any period of recovery before testing since the natural level of excitation, being unknown, cannot be reestablished in the laboratory.

Bioluminescence Measurement From Research Submersibles

Research submersibles have the capacity to utilize the advantages of both bathyphotometry and single organism analysis, with the considerable advantage that these *in situ* methods avoid the problems of capture stress. While submersibles are not practical for continuous and large scale bioluminescence survey work, they are an extremely valuable means of verification for other methods and provide information that would otherwise be unavailable regarding *in situ*

excitability and fine scale resolution of local distributions of organisms, as exemplified in the Alboran Sea study.

Bioluminescence has been a constant motif in the history of research submersibles. William Beebe described bioluminescence during his pioneer bathysphere descents as "pyrotechnic fireworks"²³. His enthusiastic descriptions accompanied by dramatic paintings of luminescent animals in his popular writings undoubtedly marked many "tyro" marine biologists for life. Piccard and Dietz²⁴, observed from the bathyscaph Trieste "luminescent animalcules in very large numbers". Also working from the Trieste, Rechnitzer (1962) as described by Boden and Kampa²⁵, observed bioluminescence in terms of "the number of stars that can be seen in the heavens on a clear, dark night". On dives in the Straights of Messina using the mesoscaph Forel, Baguet et al.²⁶ made the first quantitative bioluminescence measurements from submersibles. They used a PMT to measure downwelling irradiance, and noted a visually diffuse luminescence, which they believed to be bacterial. Similar phenomena have been observed repeatedly since then at many locations but without identification of the causal organisms. Losee et al.²⁷ used both an open field and a pumped bathyphotometer on the Johnson-Sea-Link and Alvin submersibles to make the first vertical profiles of bioluminescence from a submersible. Widder et al.²⁸ deployed an ISIT video camera on the single person submersible Deep Rover to record both stimulated and unstimulated bioluminescence in Monterey Canyon and then quantified the video data by computer image analysis. More recently this procedure has been adapted for use on the Johnson-Sea-Link submersible in combination with an open field photomultiplier tube calibrated for irradiance (Widder, unpublished).

Measurements of bioluminescence from research submersibles have two major advantages over measurements made with bathyphotometers lowered from ships. First, undesirable premature stimulation of organisms is reduced because a submersible in neutral buoyancy is largely motionless relative to surrounding water. A submersible is, therefore, an ideal platform for investigations on the extent of background, or natural, bioluminescence in the ocean.

Investigations of bioluminescence in Monterey Canyon²⁸ and similar investigations in the Gulf of Maine, off the Bahamas and in the Mediterranean Sea have failed to detect spontaneous emission (Widder, unpublished). This supports the hypothesis that the majority of background bioluminescence in the ocean is a consequence of encounters between organisms and, where encounters are rare, natural bioluminescence will be rare, even in regions of high levels of stimutable bioluminescence¹². Similar results are obtained at much greater depths when open field detectors are uncoupled from ship motion, as demonstrated in test deployments of components of the prototype DUMAND neutrino detector array off Hawaii²⁹. Bottom anchored, open-field detectors at about

4500 m detected less luminescence than free-falling or ship tethered instruments.

The second advantage of the research submersible as a measurement platform is the ability to identify organisms responsible for stimutable bioluminescence. In addition, it is possible to make simultaneous, quantified luminescence measurements on individuals and on the populational sample stimulated by the grid pushed ahead of the submersible. This methodology is, indeed, perhaps the only one available for coping with the fragile and often very large (lengths up to several meters) gelatinous organisms which are destroyed by nets and pumps, but which represent some of the brightest sources of light in the ocean²⁸. From submersibles it is often possible to identify the dominant light producers by relating an organism to a particular display. More recently it has become possible to measure the intensity and kinetics of identified zooplankton bioluminescence *in situ* using a radiometer mounted on the Johnson-Sea-Link submersible to evaluate luminescence of single organisms *in situ* after non-stimulatory isolation in a suction stimulatory screen device (Widder, unpublished).

Problems Remaining

Marine scientists typically have considered bioluminescence to be an interesting curiosity with little relevance to the central problems of marine ecosystems. While it has been a matter of endless fascination to molecular biologists, physiologists and behaviorists, investigation of bioluminescence has never become part of the routine of oceanographic research. When it has been studied at sea, it has usually been on those rare instances when bioluminescence has been a research focus of a cruise. This neglect seems unfortunate because the prevalence of oceanic bioluminescence argues that it must play a large role in the oceans. The roles of bioluminescence thus far defined seem almost trivial in view of this fact.

In large part this perceived neglect may be an example of a problem waiting for adequate technology. Marine bioluminescence survey work has been so limited that correlations of bioluminescence with oceanic conditions are insufficient to support any but the most elementary hypotheses concerning its roles in marine life. Indeed, one might as well derive an empirical transfer function between bioluminescence and biomass and be done with it as far as present knowledge is concerned. This solution, however, neglects the possibility that bioluminescence may itself have a role in population dynamics beyond those now understood. It may have a more general role in regulation of marine populations, especially in that dominant fraction of the world's inhabitable volume in which bioluminescence is the only light and, thereby, the only indicator of the state of the local environment outside the limited spatial coverage of the chemoreceptors and mechanoreceptors of the inhabitants.

Perhaps the instrumentation and methods described here point out a new path in marine biology in which new methods and apparatus will permit testing hypotheses in which bioluminescence is seen as playing a regulatory role in population dynamics along with the more traditional driving forces. Technology is now available or conceptually plausible to explore larger questions such as bioluminescence potential as a regulatory signal in optimal exploitation of biological carrying capacity in oceanic communities.

Biography

James F. Case is Professor of Biology and Associate Vice Chancellor for Research at the University of California, Santa Barbara. He received the PhD from Johns Hopkins University. His research interests are in invertebrate sensory physiology and in bioluminescence from the physiological and behavioral points of view, with emphasis at present in assessing the role of bioluminescence in marine population dynamics and in developing instrumentation to facilitate investigations of marine bioluminescence. His research on bioluminescence has been conducted on expeditions to New Guinea, Malaysia and Indonesia and on many research cruises.

References

- Herring, P.J. 1987. Systematic distribution of bioluminescence in living organisms. *J. Bioluminescence and Chemiluminescence* 1:147-163.
- Buck, J.B. 1978. Functions and evolutions of bioluminescence, pp 419-460 in Herring, P ed *Bioluminescence in Action*. Academic Press.
- Clarke, G.L. and Wertheim, G.K. 1956. Measurements of illumination at great depths and at night in the Atlantic by means of a new bathyphotometer. *Deep-Sea Res.* 3:189-205.
- Ondercin, D.G. 1989. The spatial characterization of bio-optics in the Sargasso Sea. Johns Hopkins APL Tech. Dig. 10:45-53.
- Swift, E., Biggley, W.H., Verity, P.G. and Brown, D.T. 1983. Zooplankton are major sources of epipelagic bioluminescence in the Southern Sargasso Sea. *Bull. Mar. Sci.* 33:855-863.
- Widder, E.A., Case, J.F., Bernstein, S.A., MacIntyre, S., Lowenstine, M.R. and Cook, D.P. 1993. A new large volume bioluminescence bathyphotometer with defined turbulence excitation. *Deep-Sea Res.* 40:607-627.
- Case, J.F. 1991. Marine bioluminescence: New approaches and results. Symposium on Bioluminescence, Chronobiology and Photosynthesis. *Photochemistry and Photobiology* 53:47s-49s.
- Nealson, K.H., Arneson, A.C. and Huber, M.E. 1986. Identification of marine organisms using kinetic and spectral properties of their bioluminescence. *Mar. Biol.* 91:77-83
- Latz, M.I. 1992. Bioluminescence signatures of the plankton of the North Atlantic. *Eos* 73:263.
- Case, J.F., Neilson, D.J., Bernstein, S.A., Haddock, S. and Moore, C. 1992. Plankton investigations using *in situ* bioluminescence spectra. *Eos* 73:262.
- Widder, E.A., Latz, M. and Case, J.F. 1983. Marine bioluminescent spectra measured with an optical multi-channel detection system. *Biol. Bull.* 165:791-810.
- Buskey, E.J. and Swift, E. 1990. An encounter model to predict natural planktonic bioluminescence. *Limnol. Oceanogr.* 35: 7 1469-1485.
- Lapota, D., Young, D.K., Bernstein, S.A., Geiger, M.L., Huddell, H.D. and Case, J.F. 1992. Diel bioluminescence in heterotrophic and photosynthetic marine dinoflagellates in an arctic fjord. *J. Mar. Biol. Assn. U.K.* 72:733-744.
- Widder, E.A. and Case, J.F. 1982a. Luminescent microsource activity in bioluminescence of the dinoflagellate, *Pyrocystis fusiformis*. *J. Comp. Physiol.* 145:517-527.
- Widder, E.A. and Case, J.F. 1982b. Distribution of sub-cellular bioluminescent microsources in a dinoflagellate, *Pyrocystis fusiformis*, *Biol. Bull.* 162:423-448.
- Donaldson, T.Q. 1989. USS Kennedy Conducts Bioluminescence Study in the Mediterranean. *Naval Oceanography Command News*, August, 1989: 3-4.
- Hastings, J.W. and Sweeney, B.M. 1957. The luminescent reaction in extracts of the marine dinoflagellate *Gonyaulax polyedra*. *J. Cell. Comp. Physiol.* 49:209-225.
- Batchelder, H.P. and Swift, E. 1988. Bioluminescent potential and variability in some Sargasso Sea planktonic halocyprid ostracods. *J. Crustacean Biol.* 8:520-523.
- Latz, M.I., Frank, T.M., Bowlby, M.R., Widder, E.A. and Case, J.F. 1987. Variability in flash characteristics of a bioluminescent copepod. *Biol. Bull.* 173: 489-503.
- Latz, M.I., Bowlby, M.R. and Case, J.F. 1989. Recovery and stimulation of copepod bioluminescence. *Jour. exp. mar. Biol.* 136:1-22.
- Barnes, A.T. and Case, J.F. 1972. Bioluminescence in the mesopelagic copepod *Gaussia princeps* (T. Scott). *J. exp. mar. Biol. Ecol.* 8:53-71.
- Bowlby, M.R. and Case, J.F. 1991. Flash kinetics and spatial patterns of bioluminescence in the copepod *Gaussia princeps*. *Marine Biol.* 110:329-336.
- Beebe, W.M. 1934. "Half Mile Down" Harcourt, Brace and Company, New York.

24. Piccard, J. and Dietz, R.S. 1957. Oceanographic observations by the bathyscaph Trieste (1953-1956). *Deep-Sea Res.* 4:221-229.
25. Boden, B.P. and Kampa, E.M. 1964. Planktonic bioluminescence. *Oceanogr. Mar. Biol. Ann. Rev.* 2:341-371.
26. Baguet, F., Piccard, J., Christophe, B. and Marechal, G. 1983. Bioluminescence and luminescent fish in the Strait of Messina from the mesoscaph "Forel". *Mar. Biol.* 74:221-229.
27. Losee, J., Lapota, D. and Lieberman, S.H. 1985. Bioluminescence: A new tool for oceanography, pp 211-234, In *Mapping strategies in chemical oceanography*, (A. Zirino ed.) *Advances in Chemistry Series*, 209, American Chemical Society.
28. Widder, E.A., Bernstein, S.A., Bracher, D.F., Case, J.F., Reisenbichler, K.R., Torres, J.J., and Robison, B.H. 1989. Bioluminescence in the Monterey Submarine Canyon: Image analysis of video recordings from a midwater submersible.
29. Webster, M.S., Roos, C.E., Roberts, A., Okada, A., Ohashi, Y., O'Connor, D., Mitiguy, R., Matsuno, S., March, R., Learned J.G., Karl, D., Clem, J., Blackinton, G., Bradner, H. and Babson, J. 1991. Mechanical stimulation of bioluminescence in the deep Pacific Ocean. *Deep-Sea Res.* 38: 201-217.
30. Gitelson, II and Levin, L.A. 1989. Bioluminescence in Oceanology. *J. Bioluminescence and Chemiluminescence* 4:555- 562.
31. Aiken, A. and Kelley, J. 1984. A solid state sensor for mapping and profiling stimulated bioluminescence in the marine environment. *Continental Shelf Res.* 3:455-464.
32. Nelson, C.V. 1985. Bio-optical measurement platforms and sensors. *Johns Hopkins University Applied Physics Laboratory STD-R-1160*, 44pp.

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