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CHEMISTRY AND CONTROL OF LUMINESCENCE IN MARINE ORGANISMS

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ABSTRACT

Bioluminescence occurs in very many different marine organisms. The reaction is an enzyme (luciferase) mediated chemiluminescence in which a substrate (luciferin) is oxidized by molecular oxygen; energy thereby available is conserved in the form of a molecule in an electronically excited (singlet) state which subsequently emits light. The luciferins and luciferases utilized in different organisms may be very different, but all known luciferases may be classed as oxygenases, involving peroxides (in some cases ring peroxides) as intermediates; in some cases electron transfer has been postulated in the terminal steps leading to excitation. The cellular control of bioluminescent reactions involves special mechanisms such as the sequestration of substrate by a binding protein or the accumulation of a reaction intermediate, and a triggering step, as for example by H^+ or Ca^{++}.

Although bioluminescence is not confined to the marine environment, the preponderance of luminous species do in fact occur in the sea (Harvey, 1952; Lynch, 1981). Why this is so has had no satisfactory explanation, either in terms of evolutionary or ecological factors, and there appear to be no truly unique biological features of marine luminous systems as such. There also seem to be no important distinctions between marine and nonmarine systems with respect to the chemical reactions responsible for light emission. All are similar in that they involve the oxidation of a substrate by molecular oxygen and result in the formation of a product molecule in an electronically excited state sufficiently energetic to result in the emission of a photon. Such bioluminescent reactions are also characterized by a high chemiluminescent quantum yield.

It is interesting, however, that systems from different organisms may differ completely with regard to the structure of the substrate molecule (generically, luciferins) being oxidized as well as the nature of the enzyme (generically, luciferases) responsible for catalyzing the reaction (Hastings and Wilson, 1976). In some phyla, such as the coelenterates, all luminous organisms appear to be similar biochemically, while there is diversity in others (arthropods and molluscs, for example). Altogether, it has been estimated that present day luminescent systems are derived from as many as 30 different evolutionarily independent origins (Hastings, in press). This conclusion was based perforce upon the structure of the luciferin molecules and luciferase properties, rather than on sematides, namely molecules that carry the information of the genes or a transcript thereof (Zuckerkandl and Pauling, 1965). An important task at hand is to obtain information concerning DNA or protein sequences of relevant molecules in order to establish with some certainty the possible evolutionary relationships amongst the different luminous systems and amino acid sequences of the apparently diverse luciferases which, however, may all be classified as oxygenases.

In this brief overview we will mainly confine ourselves to four marine systems: bacteria, dinoflagellates, coelenterates and the crustacean *Vargula* (formerly *Cypridina*; Poulsen, 1962).

These groups serve to illustrate the equally diverse character of the control of light emission (Table 1). In two cases there is no real control at the biochemical level, namely the continuously emitting bacteria and the crustacean *Vargula*; in

Table 1. Control in bioluminescent reactions

Bacteria
Photobacterium: light is continuous (respiratory pathway)
Crustacean (Cypridina)
Vargula: extracellular mixing of enzyme and substrate
Dinoflagellates
Gonyaulax: release of bound substrate by pH change
Coelenterates
Renilla: release of bound substrate by Ca^{++}; activate presubstrate
Aequorea: trigger enzyme intermediate (Ca^{++})

the latter the luciferin and luciferase are formed in separate glands and reacted by squirting together into the sea water. A direct biochemical control of the reaction is achieved in the bioluminescent system of the dinoflagellate *Gonyaulax* by sequestering the substrate (luciferin) with the aid of a special substrate binding protein, thereby keeping it away from the enzyme (luciferase). Its release is then triggered by a pH change. A similar mechanism, involving release triggered by Ca^{++}, occurs in the coelenterate *Renilla.* Some mechanisms for control at the biochemical level involve a chemical alteration of the substrate prior to its reaction with oxygen. This was first elucidated in the firefly system, but is also known to occur in *Renilla.* Such a mechanism may not be directly involved in the control of light emission in vivo.

In certain coelenterates, and probably in members of other groups as well, a rather sophisticated precharging mechanism appears to be involved directly in the occurrence of flashing. In *Aequorea,* for example, an enzyme substrate intermediate is accumulated (the peroxy-luciferin) and then triggered by Ca^{++} to continue to the light emitting stage. Thus the first oxidative steps occur and then the reaction is held up at that point in the catalytic cycle. Its continuation and the burst of photons occurs upon reaction of the intermediate with the triggering substance.

Bacteria

Although luminous bacteria are found ubiquitously as planktonic forms in the oceans, they also occur in various associations with higher organisms, including symbioses in special light organs and as intestinal flora (Hastings and Nealson, 1981). Light emission is evidently of functional importance in these cases and the high chemiluminescent quantum yield of the reaction, variously estimated between 0.1 and 0.3 (Hastings and Nealson, 1977), or even higher (Karl and Nealson, 1980) attests to a positive selection for efficient and bright emission.

The light emitting pathway in bacteria occurs as a biochemical shunt of the electron transport pathway, taking electrons from reduced pyridine nucleotide via luciferase directly to oxygen. Indeed, luciferase may have a true respiratory function under certain conditions, such as low oxygen or low iron, where respiration via the cytochrome pathway is impaired (Nealson and Hastings, 1977; 1979; Makemson and Hastings, 1982). For such a biochemical function, light emission as such is not essential, and may be totally irrelevant.

The biochemical character of bacterial luciferase explains the continuous emission of the luminescence; in association with higher organisms control may be exerted by the host, for example by means of a mechanical shutter.

Biochemically, bacterial luciferase is an external flavin monooxygenase (Hastings and Presswood, 1978). FMN is reduced by a flavin reductase (step 1), while luciferase catalyzes the mixed function oxidation of $FMNH_2$ and long chain aldehyde (RCHO), in steps 2 and 3, with the emission in step 4.

$$NAD(P)H + H^+ + FMN \rightarrow NAD(P) + FMNH_2 \quad (1)$$
$$FMNH_2 + O_2 \rightarrow FMNH\text{-}OOH \quad (2)$$
$$FMNH\text{-}OOH + RCHO \rightarrow (FMNH\text{-}OH)^* + RCOOH \quad (3)$$
$$(FMNH\text{-}OH)^* \rightarrow 0.1\ h\nu + FMN + H_2O \quad (4)$$

In this system we isolated the intermediate and characterized it as the luciferase-bound flavin peroxide whose further reaction with aldehyde results in light emission (Hastings et al., 1973; Hastings and Balny, 1975). The relatively long lifetime of this intermediate (seconds at 20°, hours at 0°, days at subzero temperatures) allowed me and my colleagues to purify it chromatographically. At first we utilized cryoenzymological techniques, with chromatography and spectral measurements at −30°, where the intermediate is completely stable; we subsequently carried out the procedures at 0° (Becvar et al., 1978). The absorption spectrum of this peroxyintermediate, peaking at about 372 nm, differs from both reduced and oxidized FMN, and decays spontaneously to the latter with a half-life at 0° of about 1 hour. The intermediate gives bioluminescence simply upon the addition of aldehyde; oxygen is not required. Spectral comparison with an authentic 4a-hydroperoxyflavin (Kemal and Bruice, 1976) as well as ^{13}C NMR studies (Ghisla et al., 1978) provided evidence that the luciferase-bound compound is a 4a-hydroperoxyflavin.

A key to the structure of the emitter in this bioluminescence is the fluorescence emission of the flavin peroxy intermediate, which is centered at about 490 nm and corresponds closely to the bioluminescence emission spectrum (Balny and Hastings, 1975); FMN itself fluoresces at 530 nm. The peroxyflavin cannot itself be the emitter (aldehyde has not yet reacted), but a 4a-substituted flavin (the 4a-hydroxy compound, for example; Fig. 1) should have a similar emission spectrum. The postulate that such a species is the emitter (Hastings and Wilson, 1976; Hastings and Nealson, 1977) has been supported by model studies (Bruice, 1982) and by studies of the fluorescence emission spectra of luciferase-flavin-peroxy compounds prepared with analogues whose fluorescence and bioluminescence emission spectra differed from FMN itself (Hastings et al., 1981; Kurfurst et al., 1982a).

In at least some species of bacteria the color of the light may be shifted by virtue of energy transfer to a second chromophore which is associated with a different protein. The emission of a blue shifted bioluminescence in *P. phosphoreum* has been attributed to a blue-fluorescent protein with lumazine as the prosthetic group (Gast and Lee, 1978; Koka and Lee, 1979), while a remarkable yellow emitting strain of *P. fischeri* (Ruby and Nealson, 1977) has been shown to possess a protein with a tightly bound oxidized flavin emitting in the yellow (Leisman and Nealson, 1982).

Figure 1 provides a scheme depicting the pathways and intermediates currently known or postulated in the bacterial luciferase reaction. The recently demonstrated (Kurfurst et al., 1982b) luciferase-bound neutral flavin semiquinone radical is shown at the top. As such, the pure luciferase-bound radical is inactive for light emission either with or without aldehyde, and is not in (relevantly rapid) equilibrium with the luciferase 4a-peroxyflavin. However, it will react with the superoxide ion ($O_2^{\cdot -}$) and long chain aldehyde to produce bioluminescence, a reaction that proceeds via the 4a-peroxyflavin intermediate (Kurfurst et al., 1983).

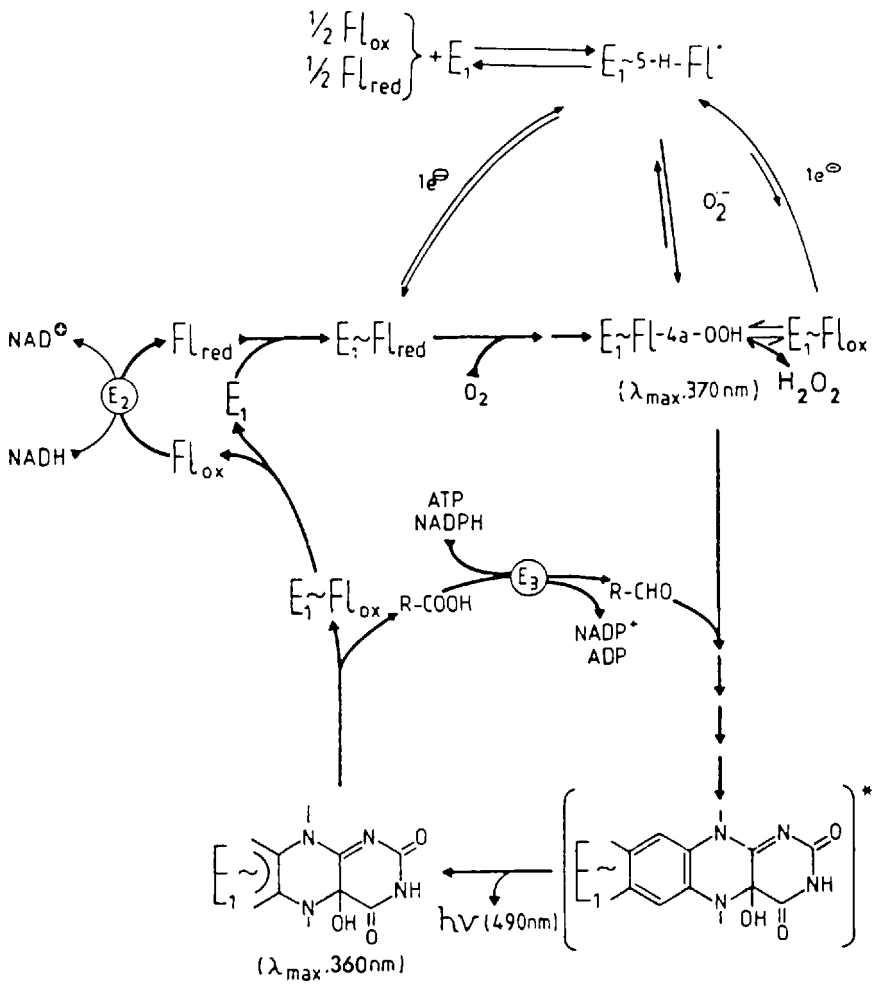

Figure 1. General scheme representing the catalytic pathway of bacterial luciferase and the possible modes of formation and decay of the blue radical. The steps leading to light emission start with the formation of $FMNH_2$ from FMN and NAD(P)H catalyzed by a separate enzyme (FMN reductase, E_2) Luciferase (E_1)-bound $FMNH_2$ reacts with O_2 to form the intermediate flavin peroxide, which then reacts via several steps with long-chain aldehyde to form an excited species (designated as the flavin 4a-pseudobase). The fatty acid which is formed is then released and reconverted to aldehyde by a third enzyme (E_3). The formation of the radical is shown at the top. The steps involved in this reaction are generally slow and the radical thus does not appear to be in a significant equilibrium with the catalytically important flavin peroxide (from Kurfürst et al., 1982b).

Dinoflagellates

A second and biochemically quite different luminescent system is found in dinoflagellates (Hastings, 1978). These are unicellular phytoplanktonic organisms responsible for both red tides and phosphorescence in the ocean; the light is characteristically emitted as a brief (0.1 sec) bright (10^8–10^{10} quanta) flash upon stimulation (Krasnow et al., 1981). Some evidence concerning the subcellular localization of the emitting sites is available in different species (Eckert, 1966;

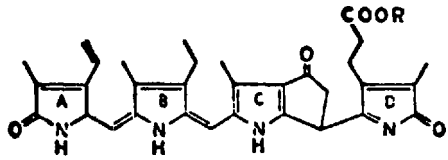

Figure 2. Dinoflagellate luciferin (from Dunlap et al., 1981).

Reynolds et al., 1966; Widder and Case, 1982), and particles termed scintillons, capable of light emission in vitro have been isolated and partially characterized from *Gonyaulax polyedra* (Fogel and Hastings, 1972; Fogel et al., 1972). In addition, an active soluble system is found in extracts, including a luciferase, a luciferin, and a novel substrate-binding protein which serves to sequester the luciferin and function in the control of flashing by releasing it following stimulation (Fogel and Hastings, 1971).

The luciferin molecule in the dinoflagellate *Pyrocystis* has recently been shown to be a tetrapyrrole, a bile pigment structurally related to chlorophyll a or c (Fig. 2), differing from ordinary bile pigments which are derived from and therefore structurally related to heme (Dunlap et al., 1981; Dunlap and Hastings, 1981). A cross-reacting but not identical substance is active in the luminescent system of euphausid shrimp (Shimomura, 1980; Dunlap et al., 1980), where it may be obtained nutritionally.

The active molecule is highly reduced; exposure to even traces of oxygen for a few minutes results in a non-luminescent oxidation with concomitant loss of luciferin activity. The luciferase catalyzed reaction itself requires molecular oxygen and may very well also involve a peroxide intermediate. A curious fact is that the active luciferin is highly fluorescent with an emission spectrum that is essentially identical to that of the bioluminescence. However, the luciferase-catalyzed oxidation of luciferin must *precede* the formation of the excited state, and at the same time must alter the chemical structure of the luciferin. This poses a dilemma, since the product (oxidized luciferin) is itself not fluorescent and cannot as such be the emitter. One possibility is that the energy of the primary excited state is transferred to an unoxidized luciferin molecule which then emits. A more likely explanation is that the emitter is a transient intermediate which, though altered, retains structural components of the original luciferin molecule that make its emission properties very similar. For example, the first oxidative steps releasing the energy for the population of the excited state could involve a part of the lucifern molecule not in conjugation with the chromophore, and the energy could be transferred to the (intact) chromophore portion and emitted as light.

The key to the biochemical control of luminescence in the dinoflagellate system appears to be pH (Krieger and Hastings, 1968); at an alkaline pH the luciferase is completely inactive, and the binding substrate protein avidly binds luciferin (Fogel and Hastings, 1971). The converse is true at an acid pH. Thus the soluble fraction of extracts made at pH 8 emits no light; but upon shifting to pH 6.3 there is light emission lasting several minutes (depending on luciferase concentration).

The pH activity curve is therefore, in its simplest description, a combination of the effect of pH on the activity of luciferase and the liberation of sequestered luciferin. There is yet another effect. In extracts made at pH 6 an endogenous protease breaks down the native luciferase molecule (MW 130,000) to a smaller

(MW 35,000) but still active fragment. It is active also at pH 8 so long as one adds free luciferin, readily obtained by dissociating it from the binding protein by heat (Krieger et al., 1974).

In extracts made at pH 8 the several soluble elements just described occur in the supernatant. But there is also activity in the pellet, due to particles (scintillons), which contain luciferase, luciferin and some kind of luciferin binding capacity (Fogel and Hastings, 1972; Henry and Hastings, 1974). As with the soluble elements, the key to activity is pH. If the pelleted material is resuspended at pH 8 and then rapidly mixed with acid to a final (and optimal) pH of 5.7, a flash occurs which is very similar kinetically to the flash of the living cell. It seems likely that the in vitro scintillons derive from in vivo physiologically active structures and do not simply represent some ad hoc assembly of soluble elements released during cell homogenization. Although flashes have been obtained from the soluble system at high luciferase concentrations (Fogel and Hastings, 1972), attempts to demonstrate such possible artifactual activities have failed. Conversely, the molecular elements associated with scintillons are not in a (relevantly) rapid equilibrium with their soluble counterparts; the flash kinetics are independent of, and intensities proportional to, dilution of scintillon preparations over a range of nearly 10^7 (DeSa and Hastings, 1968).

The sedimentation constant of scintillons is about 10,000 S (corresponding to a molecular weight of about 10^9) and their buoyant density about 1.23 gm ml^{-1}; this latter property allows them to be easily separated from chloroplasts and mitochondria by sucrose isodensity gradient centrifugation.

Living cells flash repeatedly upon repeated stimulation, and scintillons can also be recharged after a flash by readjusting the pH to 8 and adding free luciferin. The recharging takes some time (up to 30 min, depending on luciferin concentration); the recharged scintillons band like native particles in a sucrose isodensity gradient, carrying the bound luciferin and leaving unbound luciferin at the top of the gradient (Fogel and Hastings, 1972).

Our molecular model for flashing in vivo proposes that luciferase is bound as an integral membrane protein adjacent to luciferin binding protein molecules, these associated with the membrane as peripheral protein (Hastings, 1978). Stimulation results in the rapid movement of protons, causing a large but transient pH change in the compartment into which the luciferase faces; since both proteins have a sharp pH profile, substrate molecules are released from the binding protein and acted upon by the activated luciferase molecules. The ensemble provides a flash derived from the concerted reaction of many or all luciferase molecules operating, in effect, in synchrony. Excess luciferin that may be released by the binding protein and not utilized is recaptured as the pH is restored and is available for a subsequent flash. Oxidized luciferin diffuses away and is replaced by fresh luciferin, possibly coming from a soluble reservoir.

The dinoflagellate bioluminescence reaction constitutes an elegant model system for some unusual biochemical control mechanisms, including proton activated control. Enzymes are conventionally thought of in terms of their catalytic function, especially their ability to process large quantities of substrate in a short period of time. While not denying the catalytic role of *Gonyaulax* luciferase in one sense, a quite different and novel function of luciferase in our model is that it is enlisted in a laser-like burst of activity which all units engaged in a synchrony. Such a function may be uniquely relevant for a flash reaction, since the brightness of an emission is not dependent on the total number of photons emitted but on the instantaneous rate of reaction (Henry and Hastings, 1974).

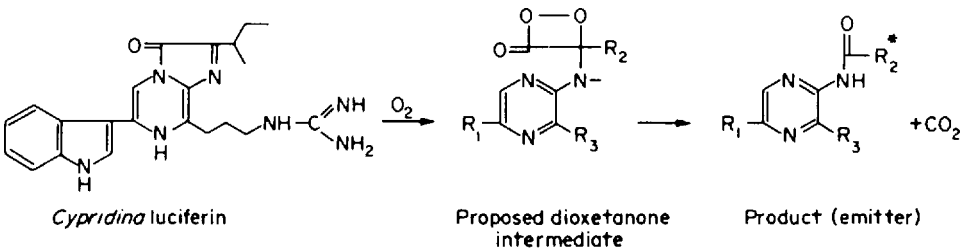

Figure 3. Cypridina luciferin and postulated pathway, intermediates and products (from Hastings and Wilson, 1976).

Vargula (=*Cypridina*)

At the other end of a control-sophistication spectrum is the regulation of light emission in the ostracod crustacean *Vargula,* where luciferin and luciferase are contained in separate glands and reacted by squirting together in the sea water (Harvey, 1952). The light is believed to serve as a diversion or decoy, allowing the animal to escape while its would be predator concerns itself with an investigation of the luminescence.

The chemistry of the reaction is also relatively simple; the reaction involves only enzyme and substrate, with no adventitious biochemical control elements. The structure of the luciferin (Fig. 3) was elucidated some years ago (Kishi et al., 1966); its reaction with oxygen, catalyzed by cypridina luciferase, was one of the first to be implicated in a hypothetical mechanism (McCapra and Richardson, 1964; McCapra, 1968) involving the formation of a dioxetanone intermediate. The subsequent cleavage of this species generates an electronically excited carbonyl product (the emitter) and CO_2; the latter is indeed produced in the reaction (Stone, 1968). The existence of such an intermediate has been confirmed by ^{18}O tracer studies (Shimomura and Johnson, 1971; 1973; 1979). The breakdown of this cyclic peroxide and the concomitant formation of an excited state has recently been proposed to involve an electron exchange mechanism, which may be applicable to bioluminescent (and chemiluminescent) reactions more generally (Koo and Schuster, 1977; 1978; Koo et al., 1978).

Cypridina luciferin is also known to be utilized in the luminescence system of certain fish, who may obtain it nutritionally like a vitamin. Thus, naturally nonluminous midshipmen fish develop full luminescence following ingestion of luminous *Vargula* or by the intraperitoneal injection of purified or synthetic cypridina luciferin (Tsuji et al., 1972; Warner and Case, 1980).

Coelenterates

The luciferin from coelenterates (Fig. 4) is structurally quite similar to the cypridina molecule, containing the same imidazopyrazine skeleton, but with different substituents. This luciferin, referred to as coelenterazine by Shimomura et al. (1980), is especially notable for its widespread phylogenetic distribution and its involvement in many different types of bioluminescence systems, including hydroids, colonial anenomies and jellyfish (Cnidaria), comb jellies (Ctenophora), squid (Mollusca), shrimp (Arthropoda) and fish (Chordata). In some cases its occurrence may be accounted for nutritionally; in others it may be a genetic property of the species.

In some species (e.g., *Renilla*) the luciferin may occur as a precursor molecule,

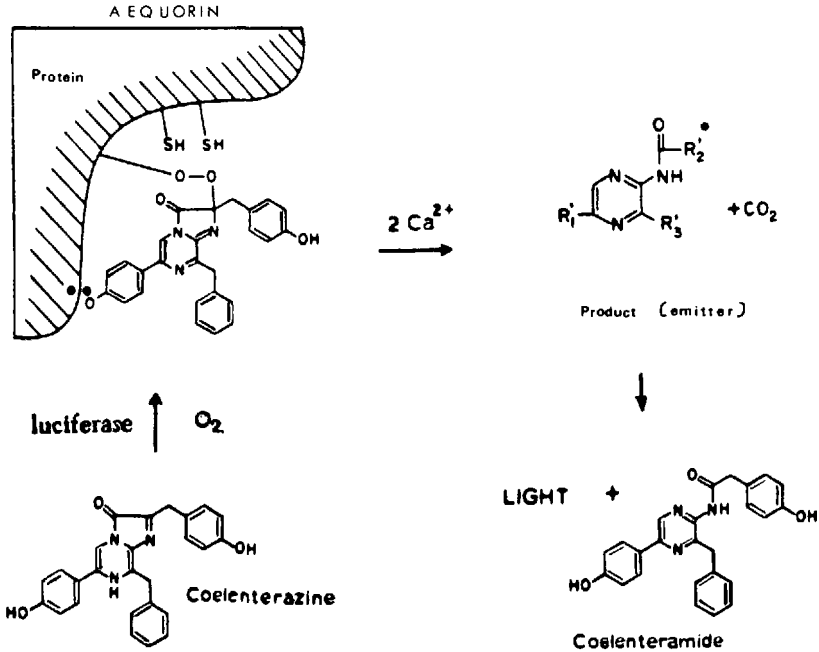

Figure 4. Postulated catalytic cycle of the aequorin reaction starting with coelenterate luciferin (coelenterazine) and luciferase (spent aequorin protein).

namely the sulfated form of the active luciferin. This pro-luciferin may be a storage form of the molecule; it is converted to the active form by reaction with diphosphoadenosine (Cormier and Totter, 1964). The active form may also be sequestered by binding to a specific Ca^{++} sensitive substrate binding protein. The latter reaction, rather than the former, is probably involved in the control of flashing.

The enzymatic intermediates in the light emitting reaction of coelenterazine have been found to be similar to those proposed for cypridina (Shimomura, 1982) with, however, the addition of a luciferase-mediated calcium control in some cases. At first the coelenterate system seemed to represent an exception to the rule that all light emitting reactions require molecular oxygen: a photoprotein, termed aequorin, was isolated from the jellyfish *Aequorea* and shown to emit light simply upon the addition of calcium in the complete absence of oxygen (Shimomura et al., 1962). This can now be explained in terms of the occurrence of a stable luciferase-coelenterazine peroxide intermediate (isolatable as aequorin) which can be triggered by calcium to continue to the light emitting stage. This postulate (Hastings and Gibson, 1963; Hastings and Morin, 1969) was supported by the in vitro production of aequorin; this was achieved by reacting spent photoprotein (=aequorin luciferase) with coelenterazine in the (obligatory) presence of oxygen (Fig. 4; Shimomura and Johnson, 1975). Aequorin may thus be viewed as analogous to the reduced flavin peroxide intermediate in the bacterial luciferase reaction discussed above, whose emission may be triggered by aldehyde, also without oxygen (Hastings and Gibson, 1963). A membrane action potential probably triggers the intracellular release of calcium in the *Aequorea* system as in the dinoflagellate system with H^+.

Discussion

The several marine luminous systems described provide some view of the common features of bioluminescent reactions, but, at the same time, illustrate the impressive diversity with regard to chemistry, control and function. The different systems utilize a number of structurally different substrates (luciferins), including flavin, aldehyde, tetrapyrrole and molecules with an imidazopyrazine skeleton. However, all reactions require molecular oxygen and their (quite different) luciferases may be classed as oxygenases. Future work may be directed to the elucidation of the biochemistry of the 20 or more systems where the structure of the luciferin and the character of the luciferase are still not known. More than that, knowledge of evolutionary origins and possible relationships of luminous systems should give important insights concerning the basic nature and ecology of light emission in biological systems.

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