BACTERIAL BIOLUMINESCENCE
LIGHT EMISSION IN THE MIXED FUNCTION OXIDATION OF REDUCED FLAVIN AND FATTY ALDEHYDE

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INTRODUCTION

Bacterial Luciferase

The in vitro reaction catalyzed by bacterial luciferase involves the mixed function oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long-chain aliphatic aldehyde (RCHO) by molecular oxygen, with the following overall stoichiometry and a chemiluminescent quantum yield of about 0.1:[1,2]

$$FMNH_2 + O_2 + RCHO \xrightarrow{\text{bacterial luciferase}} 0.1\ h\nu + FMN + RCOOH + H_2O \quad (1)$$

This luciferase can be classed as a monooxygenase of the mixed function or external type. The reaction proceeds in several steps involving enzyme intermediates, several of which have been isolated and/or characterized. Although luciferases from different species of luminous bacteria have certain differences in their structural features,[3,4] including one which is reported to be a membrane bound glycoprotein,[5] all have some structural similarities and catalyze the overall above reaction.

Bioluminescence Reactions

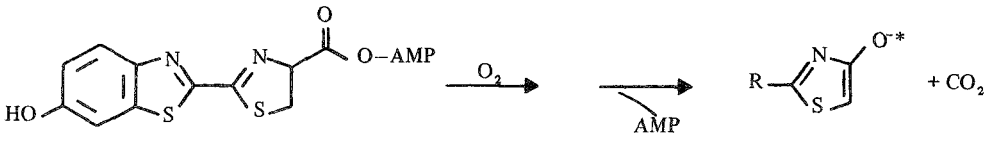
There are also many other bioluminescent organisms, but their light-emitting reactions are diverse and quite different from that of the bacterial system.[6] Luciferases include all such enzymes which catalyze reactions in which one of the products formed is in an electronically excited state. They are also similar by virtue of the fact that all (of those characterized) are oxygenases, requiring molecular oxygen as a substrate and incorporating it into the product. To accomodate these facts, the time-honored generalized scheme for bioluminescent reactions[7] may be written in a slightly altered way in the form of an internal monooxygenase:[8]

$$LH_2 + O_2 \xrightarrow{\text{luciferase}} H_2O + [L{-}O{-}]^* \rightarrow h\nu\ (\text{light}) \quad (2)$$

where LH_2 is used to designate a reduced substrate (luciferin), whatever it may be, while [L–O–]* designates the enzyme-bound, excited-state intermediate.

Actually, in those systems where the chemistry is known, including the bacterial reaction described above, none follow this exact formulation and differ from one another in several

ways. For example, there are different luciferins, cofactors, and metal requirements. In the firefly (*Photinus*), *Cypridina* and *Renilla* systems, the net reaction involves an oxidative decarboxylation and the incorporation of oxygen into products.[8,9] Among these, the firefly reaction is unique in that the luciferin reacts first with ATP (catalyzed by firefly luciferase) to form luciferyl-AMP, the substrate which then reacts with oxygen.[10] (see Structure 1).



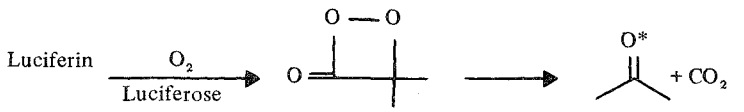
Firefly luciferyl adenydate

Product (emitter)

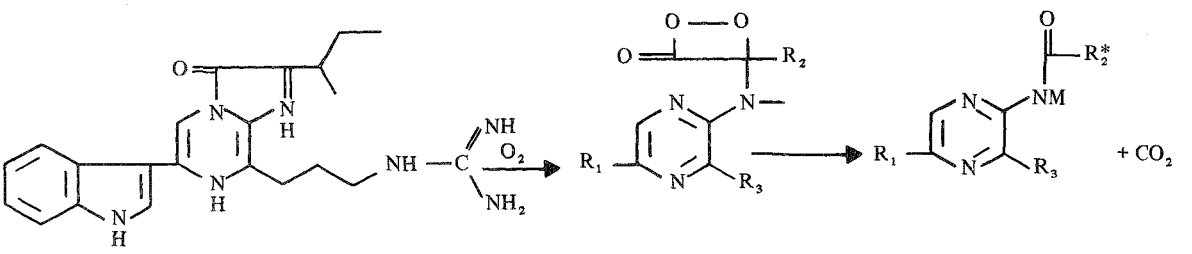
Structure 1

Based on this first reaction step with ATP, firefly luciferase (molecular weight ~100,000) is widely used as an assay for ATP.[11]

The structures of *Cypridina* and *Renilla* luciferins are similar to one another but quite different from the firefly.[8] Nevertheless, the net reactions are the same and the mechanisms may also be very similar. Based on theoretical[12] and experimental considerations,[13,14] a reaction mechanism was proposed involving a hypothetical dioxetanone intermediate, the cleavage of which would generate an electronically excited carbonyl (the emitter) and CO_2, which is indeed produced in all these cases. Thus, the general case (Structure 2) is illustrated for *Cypridina* (Structure 3).



Structure 2



Cypridina luciferin

Proposed dioxetanone intermediate

Product (emitter)

Structure 3

It is clear from model experiments with synthetic dioxetanes and dioxetanones that electronically excited fragments are produced upon breakdown of ring peroxides.[6,15] But it is still uncertain whether or not such a mechanism pertains with these luciferins. Studies concerned with the fate of labeled oxygen atoms have given different results in different enzyme systems and laboratories.[16-18] A report favoring a linear-peroxide mechanism[19] is balanced by one which supports the occurrence of the dioxetanone intermediate[20]

The Organisms: Luminous Marine Bacteria

Bacteria which are capable of emitting light occur ubiquitously in the oceans from the poles to the equator and from the surface to the depths. They can be isolated either by plating

sea water directly on a suitable medium or by enrichment on the surface of fish or squid. In both cases, observation in the dark allows one to pick the luminous organisms. Such bacteria can also be isolated directly from special photogenic organs of fish and squid (Figure 1) where they are maintained in pure culture as symbionts in a chemostat-like fashion.[20a] In these cases, the function of the light emission clearly relates to the use of the photogenic organ by the host.[20b] In luminous bacteria which occur free in sea water, the functional importance of light emission is less evident. Since the luminescent system is both inducible and subject to repression, luminous bacteria may actually be nonluminous under certain circumstances and have other specific functions, including those of enteric symbionts.[2]

CONTROL OF LUCIFERASE SYNTHESIS

Autoinduction

An unusual mechanism controls the synthesis of the luminescent system, apparently in all species of luminous bacteria (Figure 2). Luciferase and other components of the luminescent system are not synthesized at low cell densities during growth in a shake flask; synthesis at a rate faster than growth occurs at higher cell densities. This is explained by postulating that the cells produce a substance, termed autoinducer,[21,22] that accumulates in the culture medium and induces the synthesis of the components of the luminescent system. Although the identity of the substance is not known for any species, several natural isolates of *Photobacterium fischeri* have been found which differ in the amount of autoinducer produced.[23] A bright strain (MJ-1) produces ten times the normal levels of autoinducer, while a dim strain (B-61) produces very low levels. Using a bioassay (Figure 3), it has been shown that added autoinducer stimulates luciferase synthesis. Autoinducer is produced continuously during growth and accumulates in the medium but not preferentially within the cells. Although autoinduction occurs in all luminous species, luciferase

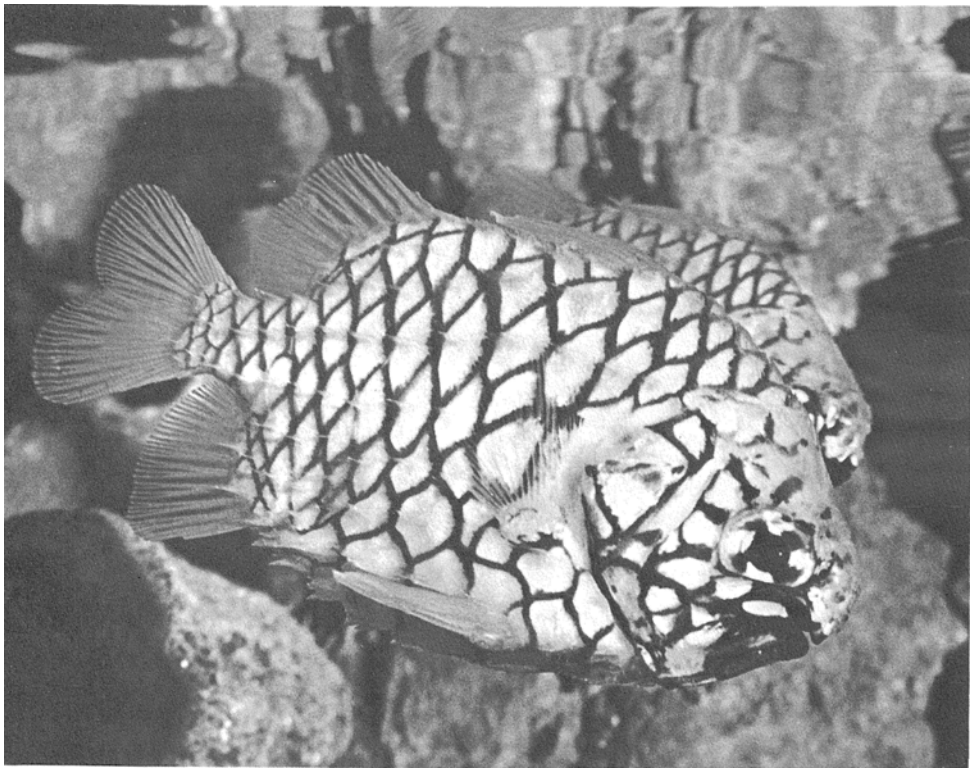


FIGURE 1. Light organs located just beneath the eyes and behind the mouth contain luminous bacteria (*Photobacterium fischeri*). (Courtesy of Steinhart Aquarium, San Francisco, Cal.)

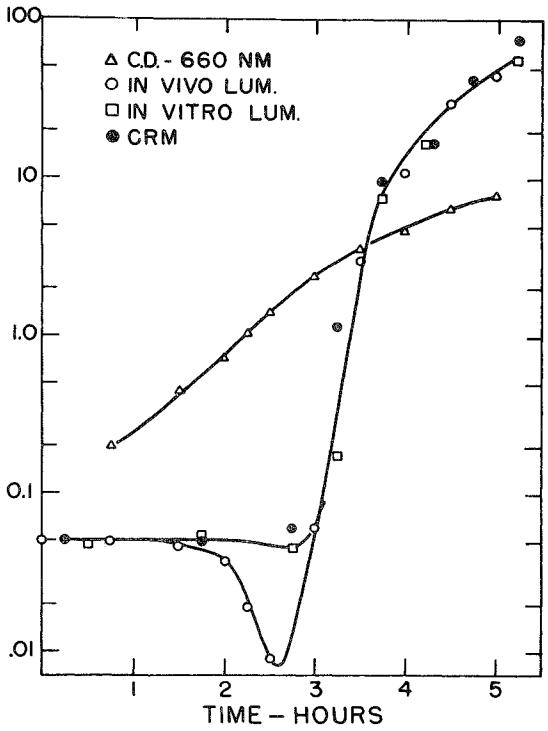


FIGURE 2. Autoinduction of the bioluminescent system. The experiment shows the time course of growth (O.D. 660 nm) and luminescence (1 unit = 2×10^{10} quanta/sec/ml) of *Beneckea harveyi*, along with measurements of the activity of extractable luciferase and of antigenically cross-reacting material (CRM) to antibody against luciferase. (From Nealson, K. H., Platt, T., and Hastings, J. W., *J. Bacteriol.*, 104, 313, 1970. With permission.)

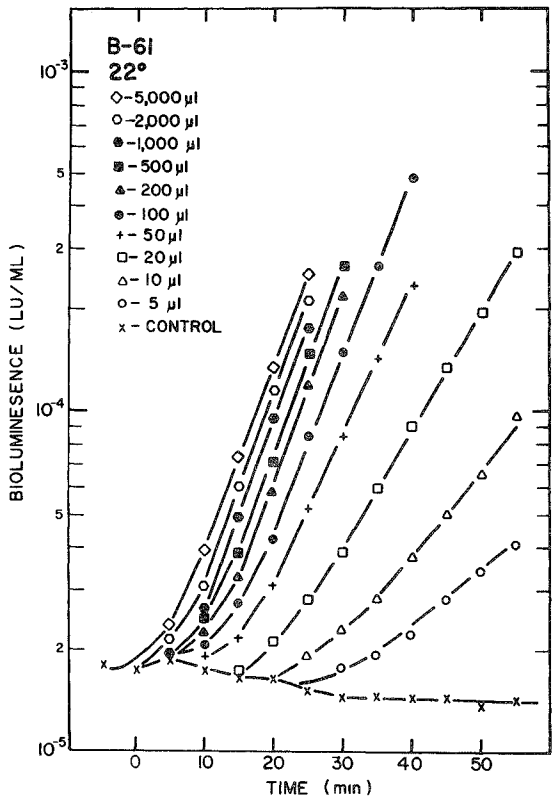


FIGURE 3. This figure shows the stimulation of bioluminescence by exogenous autoinducer, added to a culture similar to one in Figure 2 just after inoculation, well before autoinduction. The autoinducer, isolated as the supernatant from fully induced cultures, was added in different amounts as indicated. (Reproduced, with permission from Hastings, J. W. and Nealson, H. H., *Annu. Rev. Microbiol.*, 31, 549, 1977. Copyright © 1977 by Annual Reviews Inc.)

synthesis appears to be at least partially constitutive in some strains.[24,25]

Arginine and Salt Effects

Although autoinduction occurs in a minimal medium, bioluminescence is severely repressed, with only very low levels of luciferase per unit cell mass being produced.[26] Additional autoinducer does not stimulate luminescence, but arginine does,[26-28] but only if autoinduction has occurred (Figure 4). Certain compounds related to arginine are also active. Citrulline and arginosuccinate, the immediate biosynthetic precursors of arginine, stimulate, as does proline.[27,28]

Mutants which have "escaped" the arginine requirements have been isolated by selecting those which are brighter than wild type on minimal medium.[28] These are called minimal-bright (MB) mutants and possess correspondingly greater amounts of luciferase. Such mutants are also luciferase overproducers when grown on a complete medium. The reason for the escape from the arginine requirement is not known. MB mutants apparently do not overproduce arginine, are not altered in cAMP levels, and produce normal levels of autoinducer. Thus, neither the mode of action of arginine nor the character of the MB mutants is known.

The luminescence of *Beneckea harveyi* grown in a minimal medium is greater with 1% than with 3% NaCl, due to the synthesis of more luciferase and other components of the luminescent system.[29,30] This may be related to the arginine effect. In minimal medium, cells grown with 1% NaCl exhibit less stimulation by arginine, and the luminescence of MB mutants is

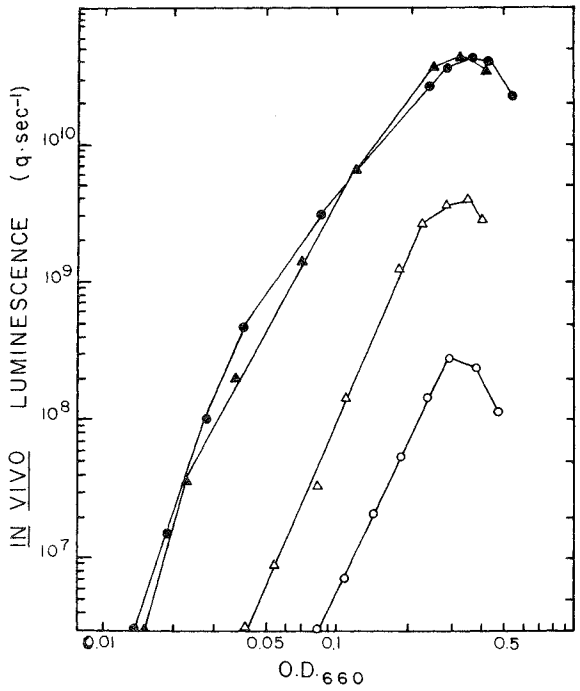


FIGURE 4. Effect of arginine on the development of in vivo luminescence during growth in minimal medium of both wild-type and the MB mutant MB-4. Cells from late-exponential-phase cultures were diluted 200-fold into fresh minimal medium. Arginine (final concentration, 3×10^{-3} *M*) was added at this time. Cell density (abscissa) and in vivo luminescence (ordinate) of the culture were measured with a side-arm flask. Symbols: solid symbols, MB-4; open symbols, wild type; circles, no addition; and triangles, arginine.

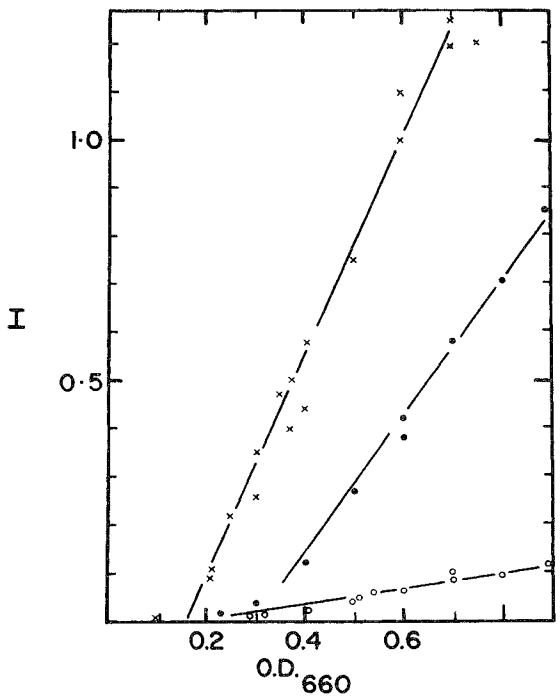


FIGURE 5. Effect of glucose and cAMP on the luciferase content of luminous bacteria growing in a complete medium plus glycerol. With an exponentially growing culture of strain MAV as an inoculum, cells were diluted into fresh medium to an O.D. of about 0.005, and 4-ml aliquots of this inoculum was dispensed into 16 × 125 mm screw cap culture tubes containing the indicated additions and shaken vigorously. Tubes were removed and the cells were harvested and assayed for luciferase content. The data from several experiments are plotted on a single graph. Luciferase content, expressed in light units (1 unit = 2.2×10^{10} quanta/sec), is plotted (ordinate) as a function of the cell density (abscissa). Symbols: × — ×, control; no glucose or cAMP; O — O, glucose; • — •, glucose plus 0.5 mg/ml cAMP. (From Nealson, K., Eberhard, A., and Hastings, J. W., *Proc. Natl. Acad. Sci. U.S.A.*, 69, 1073, 1972. With permission.)

not dependent on NaCl as is the wild type. Neither the salt nor the arginine effect occurs in a complete medium.

Catabolite Repression

The synthesis of the luminescent system of *B. harveyi* is subject to catabolite repression; glucose represses luciferase synthesis and cAMP reverses this repression (Figure 5).[31] As in other catabolite-sensitive systems, the control overrides inducer. Thus, luciferase can be viewed as a "nonessential" enzyme having an important function under certain conditions while being repressed under others. Mutants resistant to catabolite repression have been isolated as "bright on glucose" (BG) phenotypes[32] and partially characterized.[33]

Ulitzur and Yashphe[34] isolated a mutant which is nonluminous unless supplied with exogenous cAMP. The mutant is pleiotropic, being deficient for a variety of other functions which are also deficient in the absence of cAMP. An unusual feature is that, in the absence of added cAMP, the development of luminescence in the mutant is stimulated by low concentrations of cGMP.[35] At higher cGMP concentrations, inhibition of the development of luminescence occurs; in the presence of cAMP, cGMP has an inhibitory effect only.

Oxygen

Oxygen is important not only as a substrate for the luminescence reaction, but in the control of the synthesis of the luminous system.

Oxygen can limit the aerobic growth of luminous bacteria at concentrations where it is still fully active as a substrate for the luminous reaction.[36] This is because oxygen has an extremely high affinity for luciferase.[37] At these low oxygen concentrations where growth is limited, the synthesis of luciferase is similarly limited in some species of luminous bacteria (*B. harveyi* and *P. leiognathi*) but not in others (*P. phosphoreum* and *P. fischeri*).

LUCIFERASE STRUCTURE AND CHEMISTRY

Purification and Properties

All bacterial luciferases possess a heterodimeric ($\alpha\beta$) structure with molecular weights in the range of 76,000 ± 4000. All exhibit specificity for $FMNH_2$, reaction with aldehyde, and slow turnover. The exact turnover rate is dependent on the chain lengths of the aldehydes used and may differ significantly and characteristically with different luciferases. In the past, all bacterial luciferases have been considered to be structurally simple, possessing no metals, prosthetic groups, or nonamino acid residues.[38,39] However, luciferase from a bright strain of *P. leiognathi* has recently been reported to be a membrane-bound glycoprotein whose activity is labile to lysozyme.[5] The relationship of this new luciferase to those which have been well characterized (from *B. harveyi*, *P. fischeri*, and *P. phosphoreum*) is not yet known, but it is interesting to note that it has recently been shown in *B. harveyi* that a number of membrane polypeptides are coinduced with luciferase and that these same polypeptides are also lacking in certain dark mutants.[40]

Soluble bacterial luciferase is released into the medium from cells osmotically lysed in distilled water. In fully induced cells lysed at the peak of luminescence, luciferase constitutes about 5% of the soluble protein, a value typical for induced enzymes. The enzyme is purified in several steps, the first of which involves batch adsorption to Whatman DEAE-cellulose.[41,42] Luciferase is eluted preferentially and then fractionated by precipitation with ammonium sulfate. After redissolving and dialysis, the enzyme is subjected to column chromatography on Sephadex® A-50, yielding a product approximately 80% pure. For more highly purified preparations, the protein may be reprecipitated and chromatographed on a second column.

Subunit Structure

B. harveyi luciferase is a heterodimer of molecular weight 79,000 whose two nonidentical subunits, α (molecular weight = 42,000) and β (molecular weight = 37,000), can be isolated in quantity of DEAE-Sephadex chromatography in 5 *M* urea[3,41] (Figure 6). Individual subunits are inactive but, when recombined under appropriate conditions, they recover nearly full activity.[43] For both *P. fischeri* and *B. harveyi* luciferases, there is evidence that the two subunits have some degree of homology.[44,45] for *B. harveyi*, their synthesis is induced and concerted.[46,47] For any given luciferase, the two subunits are functionally distinct. Substrate ($FMNH_2$ and aldehyde) binding and catalytic properties appear to reside on the α subunit; the β subunit, which is absolutely required for luminescence activity, is considered regulatory and may be involved in the association of the enzyme with the membrane. Its specific function remains undefined.

Chemical Modification of Luciferase

Succinylation of luciferase results in a nearly complete inactivation and the remaining activity has an altered rate of turnover.[48,49] Hybrid luciferases formed with native α and succinylated β exhibit moderate ($\sim$ 50%) activity and no change in the turnover rate, while luciferases having a native β and succinylated α have a very low activity and show the same altered turnover rate as the succinylated dimer ($\alpha_s\beta_s$) (Figure 7). These results indicate that the two subunits are functionally distinct and suggest that α is the catalytic subunit. This interpretation has been confirmed by mutant enzyme analysis.

Luciferase contains a particularly reactive sulfhydryl group in or near the active center of the enzyme. Its modification results in a loss of activity; aldehyde, $FMNH_2$, and FMN protect the reactive sulfhydryl from modification.[50,51] Studies with (^{14}C)-N-ethylmaleimide have shown it to be located on the α subunit in a tryptic peptide with the sequence phe-gly-ile-cys-arg.[52] However, a direct involvement of this reactive sulfhydryl in catalytic function was not shown; its pK_a was found to be 9.4, which did

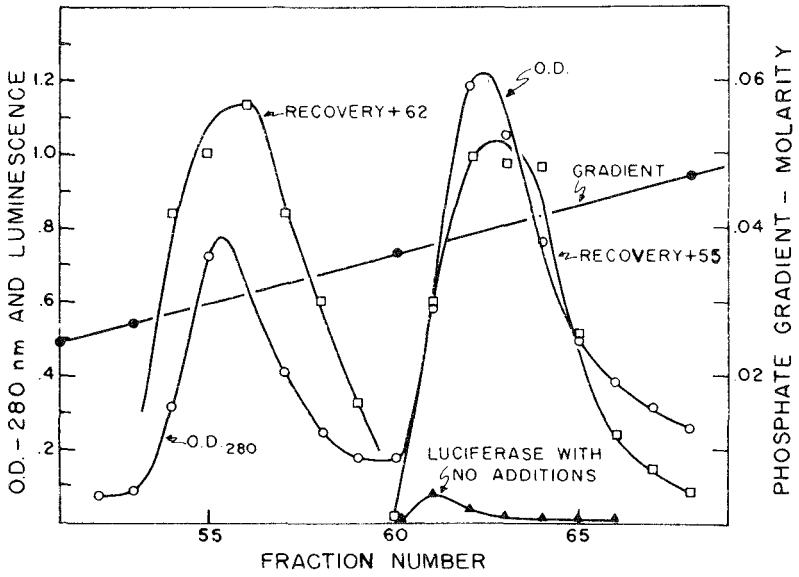


FIGURE 6. Separation of *B. harveyi* luciferase subunits by DEAE-cellulose chromatography in the presence of 8 *M* urea eluted with a linear phosphate gradient at pH 7.0. Recovery of luciferase activity was measured 14 hr after dilution into buffer to lower the urea concentration. The recovery of luciferase activity occurs in the region of the α subunit (tubes 60 to 68) if some β (tube 55) is added, and some α (tube 62) is added in the region of the β. With no additions, some recovery occurs in the region of overlap (▲), where some of both subunits are present in the tubes. (From Hastings, J. W., Weber, K., Friedland, J., Eberhard, A., Mitchell, G. W., and Gunsalus, A., *Biochemistry*, 8, 4681, 1969. With permission.)

not correlate with the pH dependence of $FMNH_2$ binding, the stability of the intermediate, or quantum yield of the reaction.

A histidyl residue on the α subunit has also been implicated as playing an essential role in the mechanism of the luciferase reaction.[53] Luciferase is inactivated by ethoxyformic anhydride with a concomitant increase in ethoxyformylhistidyl derivatives. Substrates protect against chemical inactivation; in hybrid luciferases, modification of two hystidyl residues on the β subunit is without effect on activity while modification of a single histidyl on the α causes inactivation.

Sequential modification of these "essential" residues, cysteinyl and histidyl, showed that modification of one did not affect the reactivity of the remaining one, suggesting that chemical modification had not caused a major change in protein conformation.[54] However, after modification of the hystidyl residue, $FMNH_2$ and aldehyde no longer protected the cysteinyl residue against modification, whereas after modification of the cysteinyl residue, substrates still protected the histidyl residue against modification. The results were taken as evidence that the histidyl and not the cysteinyl residue of luciferase is essential for the binding of substrates.

Binding Properties

Although luciferase activity is highly specific for $FMNH_2$, other flavins and flavin analogues exhibit a low but authentic activity,[55,56] and binding of oxidized flavin to luciferase can be measured by absorption changes (Figure 8) and fluorescence quenching (Figure 9).[42,57] Studies of the structural requirements for interacting with luciferase indicate a broader specificity for the oxidized than for the reduced forms of the flavins and differences in the nature of the interactions of the two forms.[58]

When the $\alpha\beta$ heterodimeric structure of luciferase was elucidated,[43] the participation of two reduced flavins per dimer seemed an interesting possibility. However, binding-site determinations by a kinetic method showed that luciferase possesses only a single $FMNH_2$ binding site per dimer.[58a] Furthermore, the chemical modi-

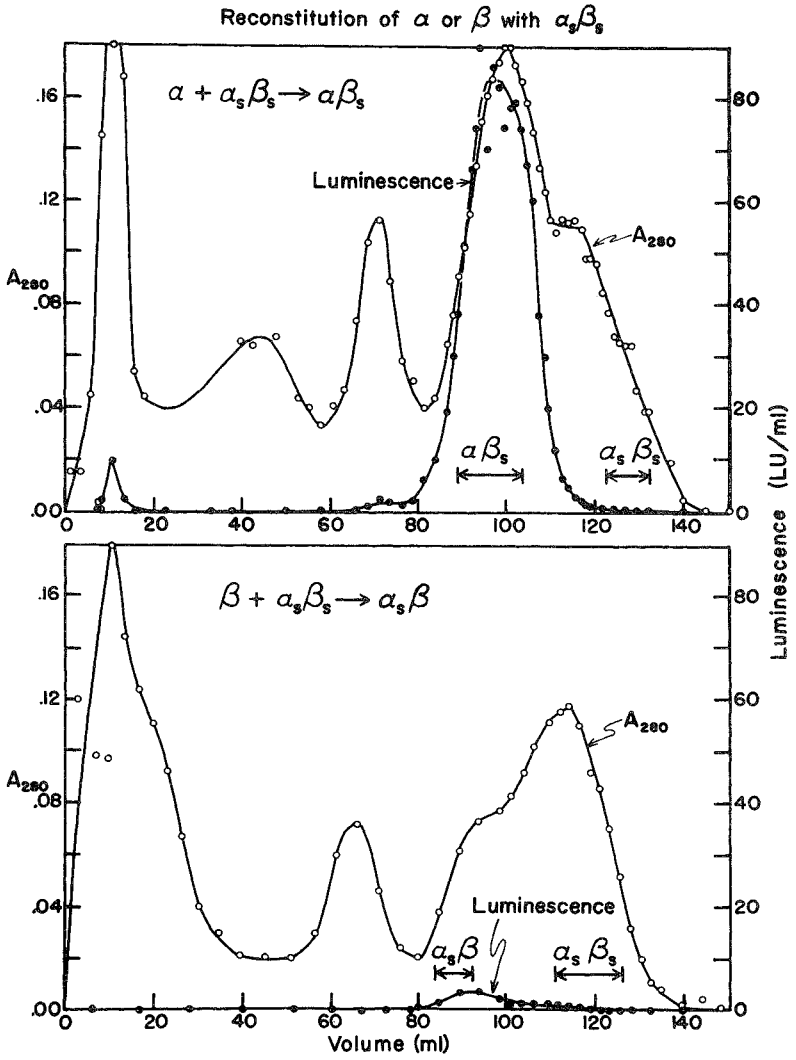


FIGURE 7. Purification of $\alpha\beta_s$ and $\alpha_s\beta$. Succinylated luciferase was denatured in 5 *M* urea in the presence of either α or β subunit and renatured by dilution in buffer at pH 7.0. After dialysis and concentration, each was subjected to chromatography on DEAE-Sephadex. The fractions were collected and analyzed for absorbance at 280 nm (A_{280},O) and luminescence activity (LU/ml•). (From Meighen, E. A., Nicoli, M. Z., and Hastings, J. W., *Biochemistry*, 10, 4062, 1971. With permission.)

fication studies and mutant analyses referred to above indicated that only the α subunit participates in the catalytic step. The unconditional demonstration that the luciferase molecule has only one binding site for $FMNH_2$ with an affinity relevant to activity is provided by CD studies of bound $FMNH_2$. Figure 10 gives molar ellipticity spectra for free $FMNH_2$, free luciferase in the presence of dithionite, and an equimolar mixture of $FMNH_2$ and luciferase. The relatively large signal at 370 nm for the mixture is indicative of a complex between reduced flavin and luciferase. The stoichiometry of this complex was examined by mixing equimolar solutions of $FMNH_2$ and luciferase in different proportions; the CD signal at 370 nm varies as a function of the relative mole fraction of the reagents (Figure 11). Equilibrium considerations do not rule out the possibility that sequential binding of two or more $FMNH_2$ molecules to luciferase occurs during a catalytic cycle. This was examined by experiments in which the bio-

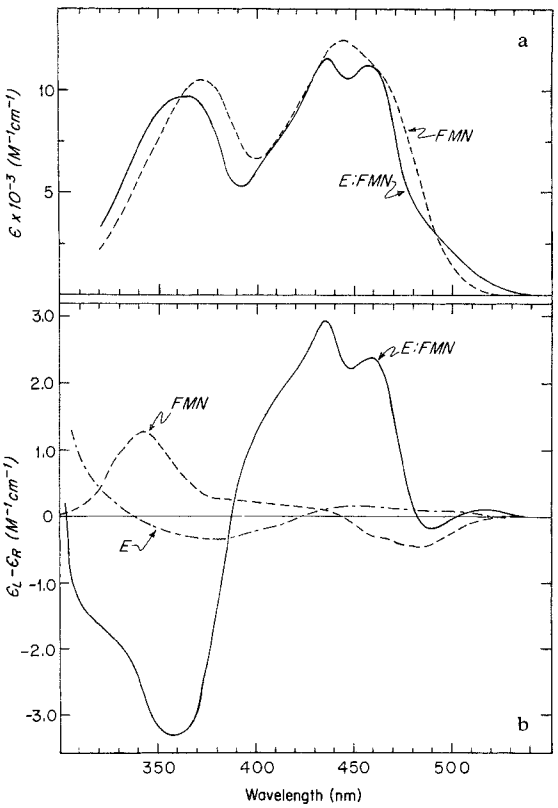


FIGURE 8. (a) Circular dichroism spectra of free (– – –) and luciferase-bound (—) FMN at 6 ± 1°C. (b) Optical absorption spectra of free (– – –) and luciferase-bound (—) FMN at 2 ± 2°C. The luciferase-bound FMN spectrum was obtained by correcting for contributions of protein (– · –). (From Nicoli, M. Z., Baldwin, T. O., Becvar, J. E., and Hastings, J. W., *Flavins and Flavoproteins*, Singer, T. P., Ed., Associated Scientific Publishing, Amsterdam, 1976, 87. With permission.)

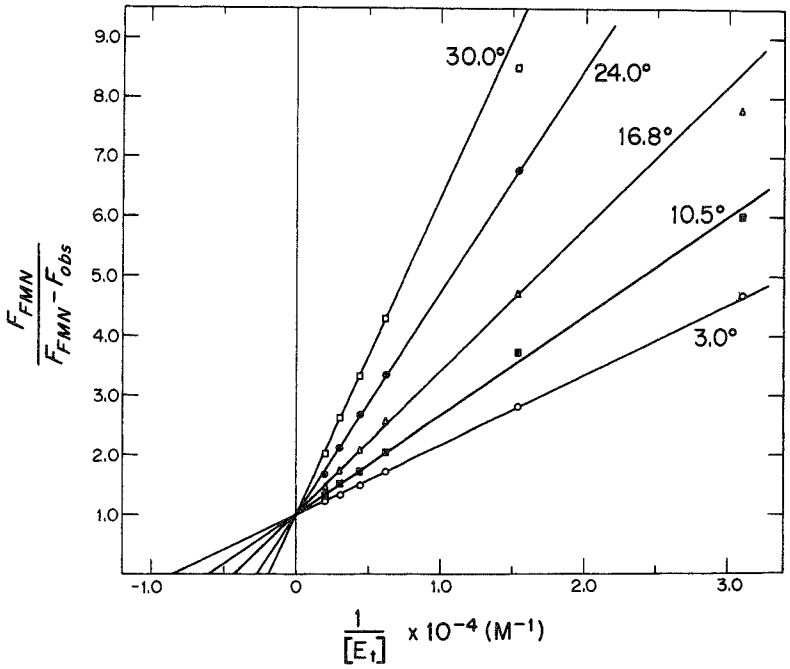
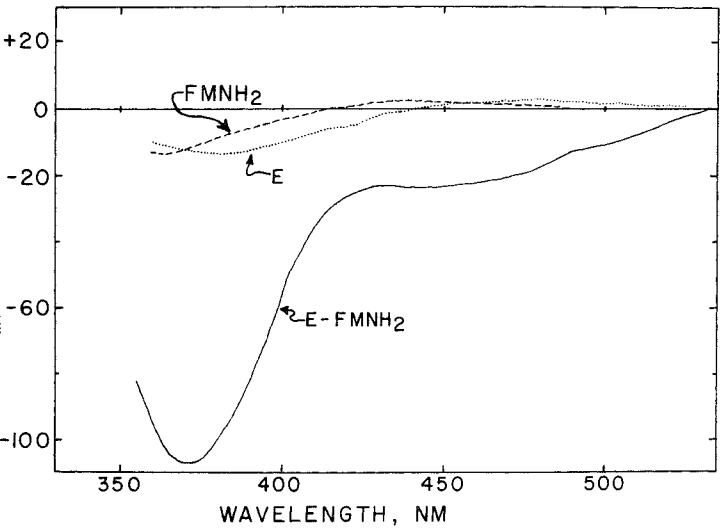


FIGURE 9. Quenching of the fluorescence of 8.9×10^{-6} *M* FMN as a function of luciferase concentration. Samples in 0.05 *M* bis-tris, pH 7.0, were excited at 450 nm and fluorescence (F_{obs}) was monitored at 525 nm. (From Baldwin, T. O., Nicoli, M. Z., Becvar, J. E., and Hastings, J. W., *J. Biol. Chem.*, 250, 2763, 1975. With permission.)



URE 10. Circular dichroism spectra of $FMNH_2$ (– – –), wild-type luciferase ···), and the luciferase-$FMNH_2$ complex (—) at 6 ± 1°C, pH 7.0, in the presence xcess sodium dithionite, 0.1 *M* bis-tris, 0.1 *M* NaCl, and 0.2 m*M* dithiothreitol, 7.0. (From Becvar, J. E., Baldwin, T. O., Nicoli, M. Z., and Hastings, J. W., *ins and Flavoproteins,* Singer, T. P., Ed., Associated Scientific Publishing, sterdam, 1976, 94. With permission.)

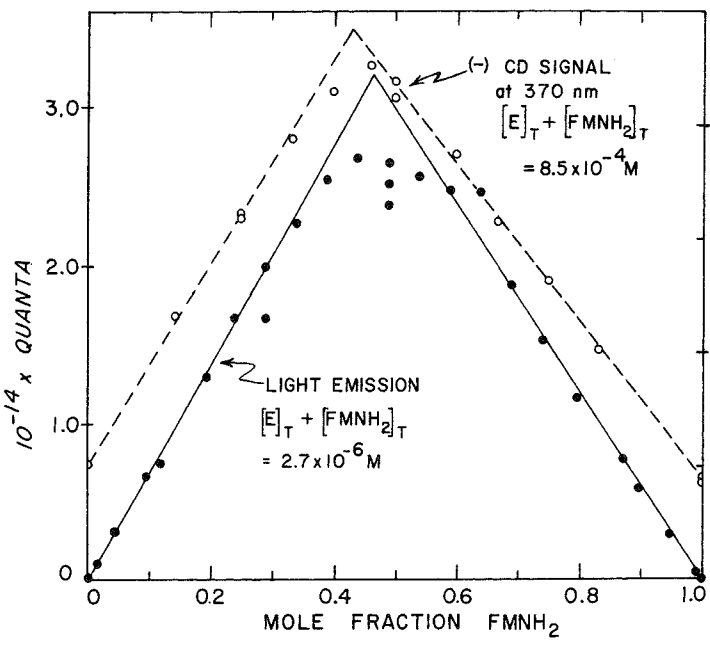


FIGURE 11. Analysis of the $FMNH_2$:luciferase stoichiometry by a Job p CD and light-emission measurements. Wild-type luciferase and FMN solu each at 0.85 m*M*, were mixed in various proportions in the presence of exc thionite. The negative 370-nm CD signal (○, – – –, right ordinate) which from the mixtures at 6 ± 1°C is plotted as a function of the mole fraction of Fl in the mixture. The light-emission data (•, —, left ordinate) was obtained by r luciferase and purified FMN solutions in various proportions in 50 m*M* phos buffer, pH 7.0, 0.2% bovine serum albumin, and a slight excess of dithioni volume of 1.2 ml. The luminescent reaction was then initiated by injecting d (0.5 m*M*), giving a final total concentration of flavin plus luciferase of 2. Total quanta were calculated as I_o/k, where I_o is the peak intensity and k decay constant of light emission. (From Becvar, J. E., Baldwin, T. O., Nicc Z., and Hastings, J. W., *Flavins and Flavoproteins,* Singer, T. P., Ed., Asso Scientific Publishing, Amsterdam, 1976, 94. With permission.)

luminescence from enzyme-$FMNH_2$ mixtures upon addition of excess O_2 and decanal was measured as a function of mole fraction of $FMNH_2$ (Figure 11). The data extrapolate to a maximum in light emission at mole fraction near 0.5, where $FMNH_2$ concentration equals enzyme concentration.

Luciferase also has a single binding site for the fluorescent probe, 8-anilino-1-naphthalenesulfonate (ANS). ANS acts as a luciferase inhibitor competitive with $FMNH_2$ but does not significantly displace FMN from binding to luciferase.[59] This provides a distinction between the FMN and $FMNH_2$ binding by luciferase. The distance between the "essential" sulfhydryl group and the ANS site was calculated to be about 20 Å[60] from fluorescence energy transfer studies.

Bacterial luciferase appears to have a specific binding site for phosphate associated in some fashion with the flavin binding site. The turnover rate of the enzyme is significantly affected by phosphate concentration.[61] Meighen and MacKenzie[55] have shown that a negative charge at least four methylene groups removed from the isoalloxazine ring is required for strong binding of the flavin to luciferase. This negative charge is provided by the phosphate of $FMNH_2$, but a carboxylate will also suffice. Furthermore, Meighen and MacKenzie found that the quantum yield of the bioluminescence reaction with reduced riboflavin as substrate is dramatically enhanced by the presence of high inorganic phosphate concentrations in the buffer, although the binding affinity for reduced riboflavin is not altered. This phosphate effect has been confirmed with isolated luciferase-bound peroxyriboflavin intermediate.[103]

Phosphate also has a dramatic effect upon the structure and stability of the luciferase. The enzyme is rapidly inactivated by all proteases which have been tested;[62] the rate of inactivation is paralleled by loss of the α subunit, which is converted from 42,000 to about 28,000 daltons plus several smaller fragments. The β subunit is not attacked by proteases under nondenaturing conditions.[63] The rate of proteolytic and thermal inactivation is decreased at least 20-fold in the presence of FMN, phosphate, sulfate, or certain other anions.[64] This suggests that these bindings are accompanied by protein structure changes which result in an enhanced quantum yield and protein stability.

Photoexcitable Luciferase

Luminous bacteria possess a special type of luciferase, photoexcitable luciferase. It is an inactive luciferase possessing a bound flavin. In *B. harveyi* and *P. fischeri,* this protein has been shown to possess bioluminescence activity which can only be initiated by photoexcitation of the bound flavin (no added flavin is needed). This protein appears to be a product of luciferase turnover in vivo, since dark "aldehyde" mutants[2] do not have photoexcitable luciferase even though they possess luciferase. Such mutants, if supplied with exogenous aldehyde so that they emit light during growth, do possess photoexcitable luciferase.[65]

Mutant Luciferases

Identification of dark mutants which have altered luciferases is made easy by the fact that the activity of the enzyme in vivo can be followed directly by light emission. Furthermore, alterations which affect the function of the active center can be detected by the fact that the rate constant for the decay of luminescence in the $FMNH_2$-initiated in vitro assay is a direct measure of the reaction turnover time, the catalytic cycle, independent of enzyme concentration.

Three different phenotypic features, altered kinetics, altered binding, and thermal instability, have been used in the identification of luciferase mutants.[66-68] All involve an initial selection for mutants which are dim in vivo. Those with altered kinetics are then detected by the different rate constant when the luciferase is assayed in vitro. All of those so selected have been found to possess lesions in the α subunit, not the β, as determined by properties of hybrid luciferases (Table 1). These mutants may also exhibit other differences, such as binding affinities for either $FMNH_2$, aldehyde, or both (or neither). This confirms the identity of α as the catalytic subunit.

The detection of luciferase mutants can also be made by determining the binding affinity for $FMNH_2$ of the luciferase of dim mutants; the few so selected do not also exhibit kinetic differences.

Luciferases altered so that they are more

thermally unstable are detected by first selecting mutants which are dim at an elevated temperature (40°C) but bright at a lower temperature (25°C). Such mutants include at least three major classes (Figure 12): those which fail to synthesize aldehyde (TSAS), those which fail to synthesize the luminescence system (TSLS), and those with a temperature sensitive luciferase (TSL). This last class can be screened for by selecting those in which the luminescence in vivo is lost rapidly when cultures grown at 25° are transferred to 40°, as shown in Figure 13. Temperature-sensitive mutants of other classes retain light-emitting capacity for a much longer time after transfer.[66]

LUCIFERASE INTERMEDIATES AND REACTION MECHANISM

The Long-lived Intermediate and the Single Turnover Assay

The bacterial luciferase reaction is unusual

TABLE 1

Characteristics of the Mutant Luciferases

Mutant	Relative peak luminescence[a] *in vivo* 20°C	36°C	Thermal stability,[b] $T_{sm,50}$% (°C)	Apparent first-order rate constant for the decay of luminescence in vitro[d] (sec^{-1}) with *n*-decylaldehyde	Substrate dissociation constants: Reduced flavin mononucleotide ($M \times 10^4$)	*n*-Decylaldehyde[f] ($M \times 10^4$)
Wild type	100	70	45	0.22	0.0069	0.068
TSL-14(α)	35	0.47	22	0.22	—	—
TSL-11(α)	130	0.23	26	0.21	—	—
TSL-5H(α)	43	6.3	28	0.23	—	—
TSL-18(α)	42	0.11	28	0.17	—	—
TSL-4H(β)	100	1.6	24	0.21	—	—
TSL-20(β)	125	0.070	25	0.21	—	—
TSL-2(β)	105	0.10	26	0.21	—	—
TSL-1(β)	72	0.0083	29	0.22	—	—
AK-6(α)	1.3	—	44	0.0086	3.2	0.069
AK-20(α)	0.13[c]	—	45	0.033	0.0013	0.51
AK-16(α)	20	—	44	0.14	0.0086	0.066
AK-15(α)	43	—	44	0.092	0.46	0.040
AK-7(α)	0.62	—	33	0.084	0.083	2.2
AK-24A(α)	20	—	42	0.33	0.12	0.086
AK-9(α)	1.2	—	36	0.012[e]	0.0063	0.65
AK-17(α)	1.4	—	41	0.16	0.0072	0.16

[a] For a lawn of the bacteria in a 100 × 15 mm petri dish, prepared by spreading 2.5 ml of 0.5% agar culture medium containing 25 μl of an overnight liquid culture. All strains ending in "H" were derived from a mutant of the wild type designated HTB-1. Peak luminescence of HTB-1 at 20°C is the same as the wild type, but at 36°C, it is six times brighter than the wild type.

[b] Temperature for 50% inactivation by preincubation in assay buffer for 5 min.

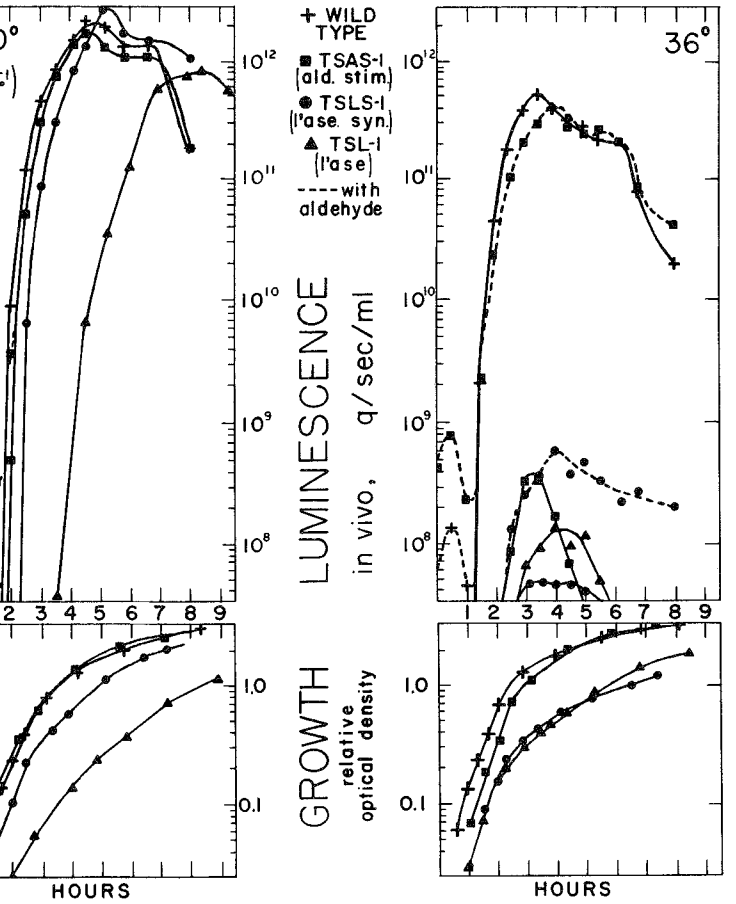
[c] The luminescence of these mutants in liquid cultures is stimulable more than 150% by added decanal.

[d] As aldehyde concentration is increased, the observed first-order rate constant changes from the endogenous (no aldehyde) value to a value characteristic of the chain length of the aldehyde present. This table lists the maximum or minimum value reached as aldehyde concentration was increased. Usually, this value was reached at high concentrations of aldehyde, levels which caused a considerable inhibition of I_{max}.

[e] In these cases, the decay rate constant continues to diverge from the value without aldehyde even as the maximum (soluble in assay buffer) is reached. Therefore, these reported constants may not represent the true k_b.

[f] This value should be interpreted only as a relative indication of aldehyde affinity, since it is calculated assuming the stock aldehyde is pure and that all the aldehyde is in aqueous solution.

(From Cline, T. W. and Hastings, J. W., *Biochemistry*, 11, 3359, 1972. With permission.)



URE 12. The three classes of temperature-sensitive mutants of bioluminescent eria. The upper panels show luminescence and the lower show growth for cells oth the permissive (26°C for TSL-1, 30°C for all others) and restrictive temper-es for conditional luminescence mutants. Luminescence is measured in quanta second per milliliter of culture at the time after inoculation indicated on the issa. Aldehyde stimulability was tested in all experiments; in those cases where sitive response was obtained, the level of luminescence reached with added *n*-nal is indicated by a dotted line. (From Cline, T. W. and Hastings, J. W., *Proc. Acad. Sci. U.S.A.*, 68, 500, 1971. With permission.)

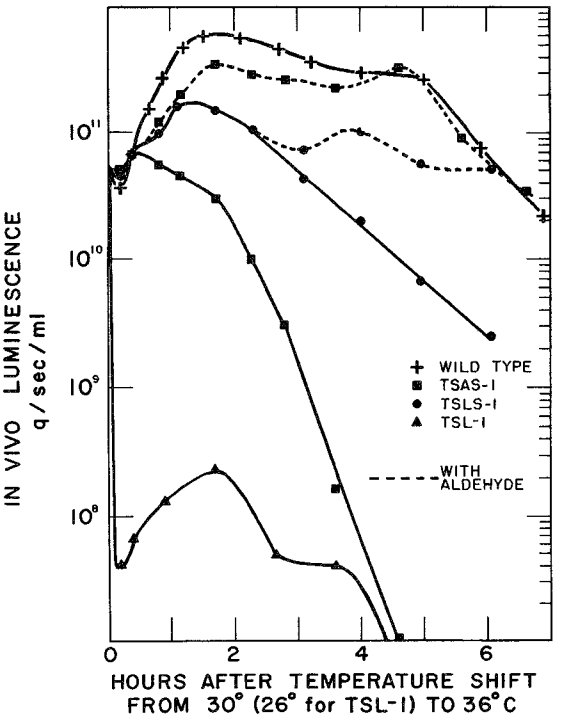


FIGURE 13. Effects of a shift from the permissive to the restrictive temperature on the luminescence of growing cells. The shift occurred early in the luciferase synthesis phase, when less than 5% of maximum luminescence had been reached. When *n*-decanal stimulated luminescence, the level reached is indicated by a dotted line. (From Cline, T. W. and Hastings, J. W., *Proc. Natl. Acad. Sci. U.S.A.*, 68, 500, 1971. With permission.)

because the enzyme turnover rate is extremely slow, involving an enzyme intermediate which has a half-life of about 20 sec at 20°C.[69] At subzero temperatures, where its lifetime is measured in weeks, it has been isolated, purified, and characterized as a luciferase-bound peroxydihydro-FMN.[75]

The in vitro assay at +20°C (Figure 14) can be carried out by rapidly mixing $FMNH_2$ with luciferase in the presence of oxygen and long-chain aldehyde; the instantaneous rate of the reaction is measured directly as the light intensity in photons per second. The fact that the free substrate ($FMNH_2$) is rapidly autoxidized (< 1 sec)[70] means that the substrate is offered as a pulse whose duration is short compared to the lifetime of the intermediate(s). Therefore, the assay in vitro involves only a single turnover of enzyme, the specific rate of which depends on the chain length of the fatty aldehyde (Figure 14, inset). Furthermore, an enzymatic reaction which results in little or no light emission, but also involves the long-lived intermediate, can occur with $FMNH_2$ and O_2 alone, i.e., without aldehyde. There is now a substantial body of evidence for the detailed scheme for the proposed reaction pathway (Figure 15).

Intermediate I: Luciferase-$FMNH_2$

The reaction of reduced flavin with luciferase results in the formation of luciferase-bound $FMNH_2$, designated as intermediate I. As mentioned above, this has been detected and studied by circular dichroism spectroscopy in the presence of excess reducing agent and shown to involve a 1:1 reduced flavin-luciferase complex for *P. fischeri* and *B. harveyi* luciferases[71,72] (Figure 8). A similar stoichiometry was found for the *P. phosphoreum* enzyme.[73] In early studies,[39,69] evidence was reported leading to the postulate that this step involved the reduction of an enzyme disulfide to a dithiol with concomitant flavin oxidation. Subsequent attempts to repeat these experiments failed, and recent experiments have reconfirmed that it is not valid: intermediate I prepared without excess reducing agent is in fact a reduced flavin-luciferase complex involving no flavin oxidation, and the native enzyme contains no disulfide bonds.[74]

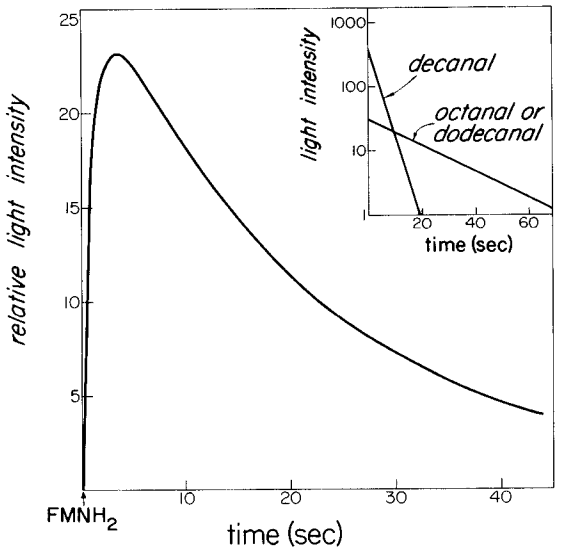


FIGURE 14. Time course of the bioluminescenct reaction initiated with $FMNH_2$ with long-chain aldehyde. The effect of aldehydes of different chain length upon the decay of the reaction is illustrated in the inset.

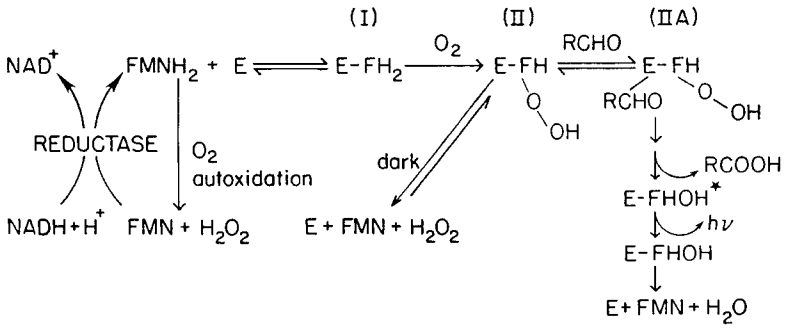


FIGURE 15. Reaction sequence and intermediate hypothesized for the reaction of bacterial luciferase with $FMNH_2$.

Intermediate II: Luciferase-peroxy $FMNH_2$

Reaction of I with one molecule of oxygen[73] is extremely rapid, forming a second intermediate (II), which because of its relatively long lifetime can be accumulated, purified, and studied using low-temperature techniques.[75] After isolation and purification by column chromatography at low temperatures (−20 to −30°C), it can be made to give rise to bioluminescence, with a quantum yield equal to that of the overall reaction, simply by injection into buffer-containing aldehyde at +20°C. Oxygen is not required for this reaction.[76] This oxygen-containing intermediate differs spectrally from both oxidized and reduced flavin; it has absorption in the visible range, peaking at 372 nm (Figure 16), and fluorescence emission centered at about 490 nm (Figure 17). In its color, the fluorescence corresponds closely to the bioluminescence emission (Figure 18),[77] and although the intermediate cannot itself be the emitter (the aldehyde has not yet reacted), its structure may provide a clue to that of the emitter. Similar properties and absorption spectra have been reported both for the intermediate from *P. fischeri* (in aqueous solution at 2°)[78,79] and that from *P. phosphoreum*,[80] the latter retracting an earlier report.[81]

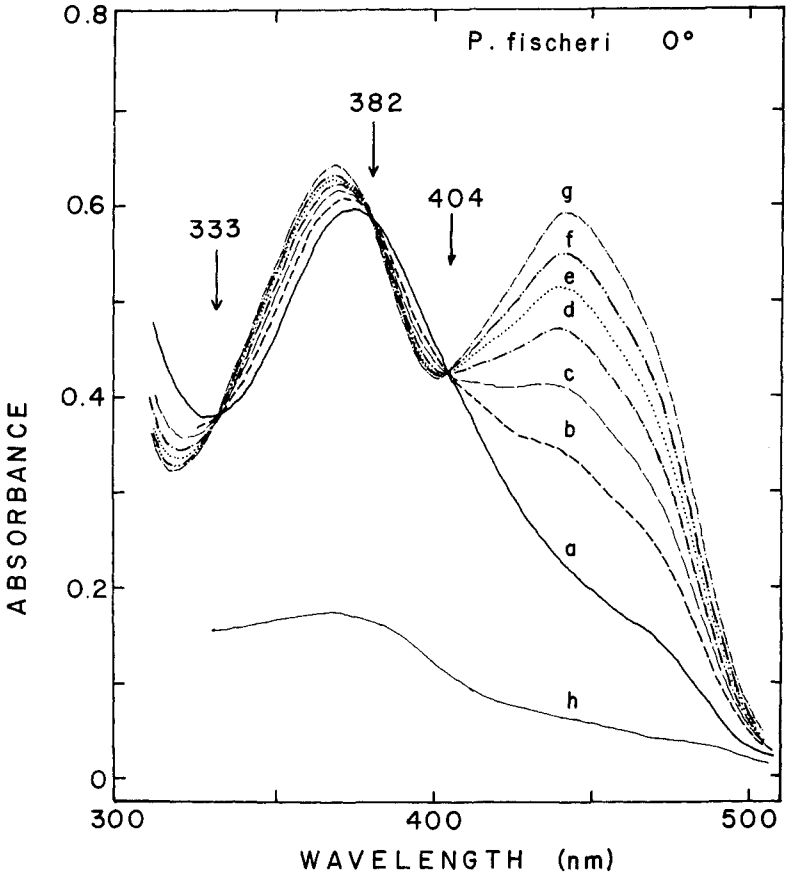
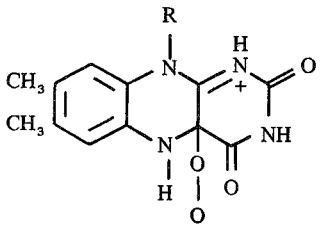


FIGURE 16. Time-dependent spectral changes of isolated *Photobacterium fischeri* intermediate II. An isolated fraction was placed into a cuvette maintained at 0°C and spectra (a) to (g) were recorded successively with time. The minutes which had elapsed since application of the mixture onto the column are (a) 13.1, (b) 22.1, (c) 31.2, (d) 44, (e) 58, (f) 81, and (g) 169. These times represent the moment when 440 nm was recorded for each spectrum. A spectrum of the *P. fischeri* luciferase at the same concentration as in the intermediate sample (A_{280} = 10.5/cm) is also shown (h). (From Becvar, J. E., Tu, S.-C., and Hastings, J. W., *Biochemistry*, in press. With permission.)



Structure 4

The intermediate is postulated to be the 4a-peroxy adduct of $FMNH_2$.[75] Spectral properties (absorption and fluorescence) of 4a-substituted flavins resemble those of the luciferase intermediate. Furthermore, a 4a-substituted flavin hydroperoxide recently synthesized by Bruice's laboratory does, in fact, emit light (low quantum yield) when reacted with aldehyde.[82] More recent experiments[83] suggest that the reaction mechanism involves the reaction of aldehyde with the peroxyreduced flavin, as earlier postulated.[84]

H_2O_2-Initiated Luminescence

The mechanism postulated above suggests that bioluminescence should be obtained from H_2O_2 and FMN in lieu of $FMNH_2$. This has been found to be so.[85,86]

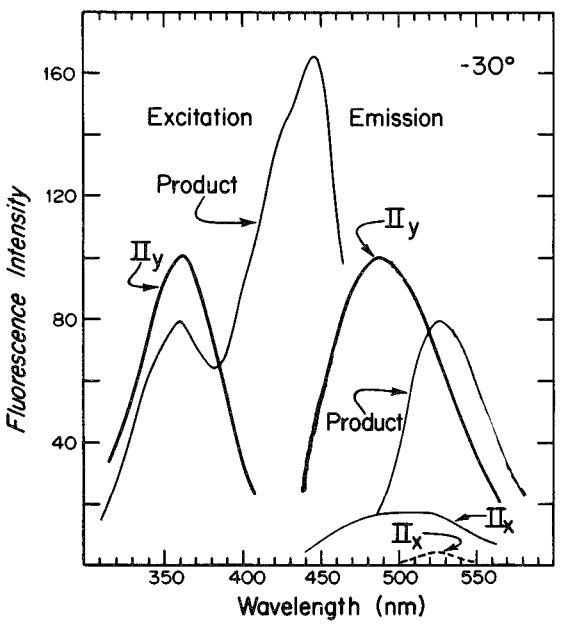


FIGURE 17. Fluorescence excitation and emission spectra for the intermediate II and the product of the reaction (FMN). (From Balny, C. and Hastings, J. W., *Biochemistry*, 14, 4719, 1975. With permission.)

The "Dark" and "Light" Pathways: Intermediate IIA

In the absence of aldehyde, intermediate II breaks down to yield H_2O_2 and FMN (Figure 15); the pathway may be viewed as an enzyme-mediated flavin oxidation which is much slower than the same reaction in the absence of enzyme (autooxidation). Using purified intermediate II, the identity and stoichiometry of the products via this pathway were determined to be 1 mol each of H_2O_2 and FMN per mole of enzyme intermediate.[1,77] A very small amount of bioluminescence, between 10^{-2} and 10^{-4} of that with aldehyde, is also produced.[52,87] In the presence of aldehyde, an intermediate (IIA) is formed whose continued reaction via several additional intermediates results in a high quantum yield

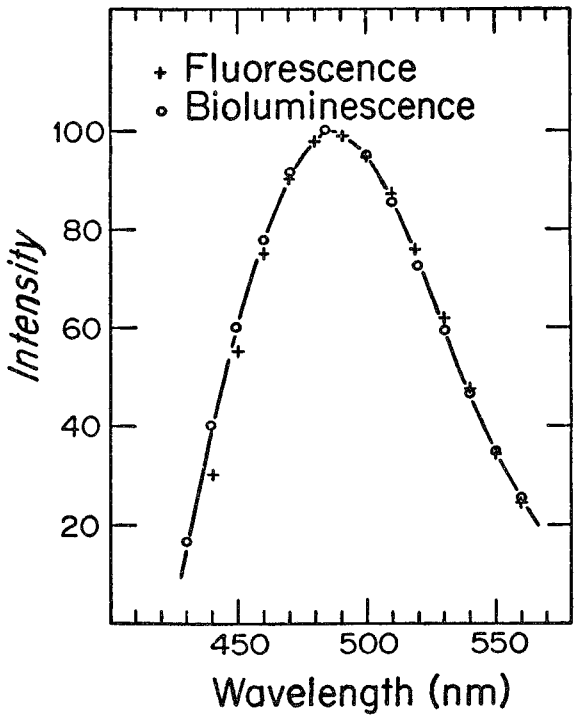


FIGURE 18. Emission spectrum of bioluminescence (O), measured directly from the cuvet during warming of the intermediate in the presence of aldehyde, plotted together with the fluorescence emission spectrum of the intermediate (+). Ordinate, intensity of bioluminescence and fluorescence normalized at the peak. (From Balny, C. and Hastings, J. W., *Biochemistry*, 14, 4719, 1975. With permission.)

emission.[69] The formation of the enzyme-aldehyde intermediate complex has been detected by optical absorption spectroscopy at −30°C.[1] In the in vitro reaction, the conversion of aldehyde to acid concomitant with light emission has been shown by several workers.[88-91] The other products of the reaction, starting with intermediate II and aldehyde at −30° and warming to obtain light emission, have proved to be FMN but not H_2O_2 (Figure 16).[1]

Reaction Mechanisms

Several specific mechanisms have been proposed for the bacterial luciferase reaction.[84,89,92] Lowe et al.[92] suggested that aldehyde reacts with the dihydroperoxy flavin at the N-5, that a four-membered flavin-oxazetidine ring is an intermediate, and that the product FMN is the emitter. In the McCapra-Hysert[89] mechanism, aldehyde is postulated to react with $FMNH_2$ prior to the entry of oxygen. This does not conform to the observation that $FMNH_2$ reacts with oxygen first.[69] Eberhard and Hastings[84] postulated that intermediate II is the luciferase-bound 4a-hydroperoxide of $FMNH_2$ and that the light-emitting steps involve the reaction of II from a mixed flavin-aldehyde peroxide followed by a Baeyer-Villiger type rearrangement.

In the reaction, at least one additional intermediate must be involved between breaking of the aldehyde C-H bond and production of the emitter, as deduced from the following observations.[104] Upon reacting decanal with intermediate II at 2°C, a rapid absorbance increase (complete within 0.1 sec after mixing) is observed at 380 nm; this is followed by two phases of absorbance decrease (the first complete within 1 sec and the second continuing with a half time for decay of about 10 sec). When this reaction is examined using ^{2}H-decanal, only the slower second phase of absorbance decrease is altered in rate; a kinetic isotope effect (k_H/k_D) of about 4 is apparent for this absorbance change, in contrast to the isotope effect of 1.7 for the decay of light intensity. The magnitude of the former isotope effect makes it reasonable to associate the C-H bond breaking with this particular absorbance change. Since the half time for the decay of light intensity at this temperature is about 60 sec, the rate-limiting step for the formation of the emitter occurs subsequent to breaking of the C-H bond of the aldehyde.

Emitting Chromophore

The emitter is, of course, an intermediate of special interest. It has always seemed that excited FMN could be the emitter in a reaction where oxidized FMN, a highly fluorescent molecule, is the product. However, there are substantial arguments against FMN itself as the emitter.[56,93] Its fluorescence emission is centered at 530 nm while the bioluminescence peaks around 490 nm, a difference difficult to attribute solely to environmental effects. A more recent and compelling observation is the fact that luciferase-bound FMN is actually nonfluorescent.[42]

Certain flavin analogues with different fluorescence emissions exhibit low but authentic activity with bacterial luciferase, and the color of the resulting bioluminescence is different with different analogues.[56] The fluorescence-emission spectrum of flavin can be altered by substitution or protonation.[94,95] Most important, the isolated luciferase intermediate II exhibits a fluorescence emission attributable to its flavin moiety which exactly matches the bioluminescence emission.[77] Thus, the emitting species in the mechanism proposed is postulated to be the 4a-hydroxy substituted flavin, designated as (E-FHOH)*. Upon light emission, H_2O and FMN are formed as the chemical products. The latter, because of its low affinity for luciferase, dissociates.

MEMBRANES AND BACTERIAL BIOLUMINESCENCE

Bacterial luciferase is a very soluble protein, and until recently, there has been no evidence that its activity might be associated with membranes. However, the biochemical link of luminescence with electron transport, its aldehyde requirement, and its sensitivity in vivo to anaesthetics[96] suggested involvement of membranes. Balakrishnan and Langerman[5] now report that treatment with deoxycholate is needed to achieve the solubilization of luciferase activity in extracts of S-1 strain of *P. leiognathi* and that the purified material is a glycoprotein whose activity is lost upon treatment with lyso-

zyme. In *B. harveyi,* it has been reported that there are membrane polypeptides implicated in the bioluminescence system.[40] Cells lacking the system were obtained by two independent means, i.e. by harvesting the wild type prior to induction, by using a mutant lacking the luminous system. In both cases, at least four membrane polypeptides were absent, and also in certain temperature sensitive dark mutants at the nonpermissive temperature. Differences between such luminous and nonluminous cells were also observed by electron microscopy of replicas of freeze-cleaved membranes.

ENERGY REQUIREMENTS

The energies involved in bioluminescent reactions are considerably higher than those encountered in other biological reactions; photons at 500 nm are equivalent to about 50 kcal (210 kJ) per einstein.[97] Thus, such light-emitting reactions are highly exergonic,[98] the equivalent of perhaps 6 ATP per photon. But if the quantum yield is only about 0.1 and a luciferase cycle which fails to emit a photon is equally costly energetically, the cell foregoes the production of about 60 ATP molecules per photon. Fully induced bioluminescent bacteria (with maximum luciferase) emit light at an appreciable rate, somewhere between 10^3 and 10^5 photons per second per cell, depending on the strain.[7,99]

The question as to what this apparently significant energy expenditure (both photic and biosynthetic) represents to the cellular economy has often been raised, especially in connection with the possible selective advantage of dark strains. Eymers and Van Schouwenburg[100] were able to deduce from KCN inhibition studies that up to 20% of the oxygen taken up was utilized in the luminescent system. Watanabe et al.[25] reported that at the peak, 0.045 photons were emitted per O_2 consumed in *P. phosphoreum.* Based on a bioluminescence quantum yield of 0.2, they then estimated that 23% of the total cellular O_2 uptake was due to the luciferase system. Similar values have been obtained in our own laboratory. In all these experiments, the rate of oxygen consumption per unit of cell mass varies by a factor of two or less over the course of growth in a shake flask (before and after induction) while the luminescence varies by a factor of 1000. Thus, finely tuned control mechanisms must operate if, as would be deduced, the amount of oxygen utilized by the light-emitting system varies relative to that consumed in the respiratory pathway over the course of the growth cycle, while total consumption remains constant.

In view of this apparently major energy commitment to luminescence, Ulitzur and Hastings[101] measured ATP levels per cell and found a significant (about tenfold) drop prior to and during the period of induction of the luminescence system. The dip did not occur in those mutants which failed to synthesize the luminescence system, including the cAMP mutant. The fact that the activity per se of the luminescent system is not wholly responsible was indicated by the fact that mutants with a temperature-sensitive luciferase still showed a dip in ATP at the restrictive temperature. Aldehyde-deficient mutants fell into two classes: in one class (such as mutant M-17), there was no dip, showing that it is also not the synthesis of the luciferase which is responsible; in others including the mutant TSAS-1, the dip was even more pronounced than in the wild type. The results suggested that a metabolite belonging to the pathway of aldehyde synthesis is involved in the dip in the ATP pool.

FUNCTIONS OF BIOLUMINESCENCE IN BACTERIA

If one considers all of the factors involved in the synthesis of luciferase and the luminous system — inhibitors, autoinducer, catabolite repression, arginine, and oxygen — then the regulation of the synthesis of the luminous system can involve a multiplicity of controls in a given species. It is easy to envisage different conditions under which some of the factors might come into play and that different factors might be significant in some species and not in others. For example, autoinducer could not accumulate with bacteria free in seawater, whereas with bacteria living as symbionts within a light organ, it would. Thus, autoinduction could be viewed as an "environmental sensing" mechanism which functions to turn off the synthesis of the luminescent system when

the cell density is low, when bioluminescence may have no functional importance. A different example would be under certain nutrient-rich conditions, possibly in the gut of fish, where luminescence as such may also not be functional; catabolite repression might operate and repress luciferase synthesis. There are species in which low oxygen inhibits growth but not luciferase synthesis. These might inhabit a niche, perhaps some type of luminous organ, where oxygen is poised at this level permitting luciferase synthesis and luminescence but little or no cell growth.

The elucidation of mechanisms controlling transcription and their applicability for the different species will be of utmost importance in building models to explain the ecology of the luminous bacteria and to understand their physiology under various "life styles." Differences in species may offer clues regarding both their versatility and their role(s) in the marine environment.

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