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Chemical Mechanisms in Bioluminescence

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There is surely no more dramatic natural phenomenon than bioluminescence.[1] If chemists were readily persuaded to investigate the chemistry of natural coloration, how can they resist examining the flashing multicolored lighting display of bioluminescent creatures?

This "cold" light is used for almost every conceivable purpose.[1-3] Fireflies flash with a pattern which serves to distinguish species and sex, while in the sea the fish Argyropelecus[3] matches the ambient light from above, disguising its silhouette; the deep sea fish Pachystomias has a red "headlight" to seek out prey, which have the disadvantage (common to many deep sea animals—except Pachystomias!) of being almost blind to red light. In general[1,2] the light is used in courtship displays, shoaling and communication, differentiation of the sexes, finding and attracting prey, distracting predators, and camouflage.

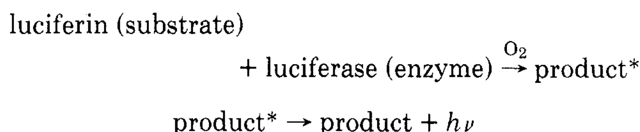
Light in the depths of the ocean has a maximum intensity in the blue-green region (475 nm) and the eyes of most of the inhabitants have probably developed optimum efficiency around that wavelength. It is thus not surprising to find that most marine bioluminescence is also in the blue-green. We are naturally interested in the molecular evolution which has provided this useful color range.

Although terrestrial luminescent organisms such as the firefly, glowworm, and certain click-beetles are best known, most of the other examples are in the sea, ranging in complexity from microscopic bacteria and plankton to fish of many species. Two-thirds of the organisms in the upper 2000 m of the oceanic water column are bioluminescent,[2] with the maximum incidence of luminescence occurring at 800 m. So widespread a phenomenon must be a strongly selected trait, and it should be possible to follow, in outline at least, the structural and mechanistic evolution of bioluminescent systems. Efficient *chemi*luminescence is rare, and we might expect that several totally unrelated phyla would develop bioluminescent processes based on the same or very similar chemiluminescent reactions. On present evidence, this appears to be the case.

Frank McCapra was born in Glasgow in 1934. He received the B.Sc. degree from the University of Glasgow, and studied at Imperial College, London, for the Ph.D. with Sir Derek H. R. Barton. Following 1 year as postdoctoral fellow at Johns Hopkins University with W. D. McElroy, and then 2 years as I.C.I. Fellow at the University of Glasgow, he joined the faculty of the University of British Columbia in 1962. He moved to his present post as Reader in Chemistry at the University of Sussex in 1966. Dr. McCapra's research is mainly concentrated in the study of the mechanisms of chemiluminescence and bioluminescence, enzyme models, and biosynthesis.

Biochemistry

There has been a steady growth in our knowledge of the finer details of the biochemical systems which generate light.[4-6] The discovery of the classical reaction of a separable enzyme and substrate (a luciferase and a luciferin) provided a readily applied procedure for investigation of the inevitably small quantities of specimens obtainable. This reaction is represented in its simplest form by the Cypridina system:



$$\text{luciferin (substrate)} + \text{luciferase (enzyme)} \xrightarrow{O_2} \text{product}^*$$

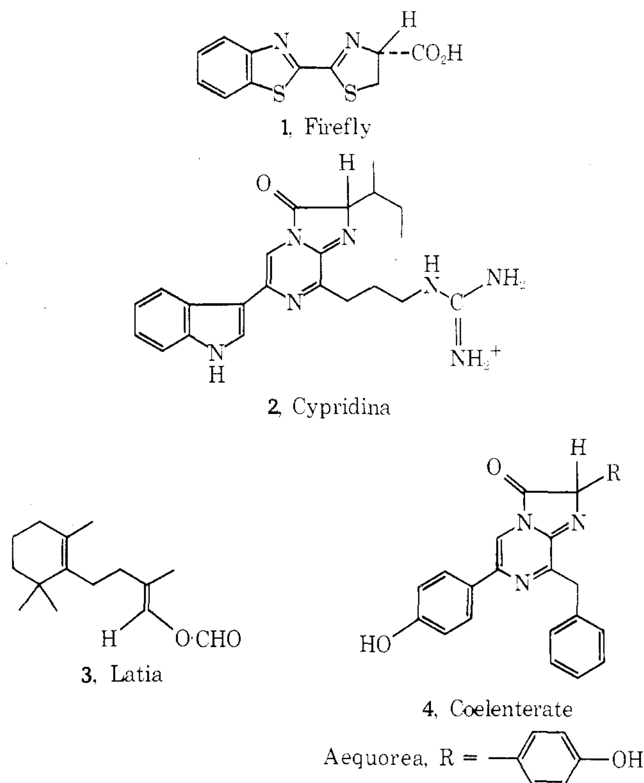
$$\text{product}^* \rightarrow \text{product} + h\nu$$

One would expect the fluorescence spectrum of the product to match that of its chemiluminescence. Although this is often the case, there are exceptions. The luciferin also normally undergoes a nonenzymatic chemiluminescent reaction, albeit with lower quantum yield.

Later a more complex system was isolated with no separable substrate.[7] A single protein—called a photoprotein—merely required the addition of calcium ion to trigger the luminescent reaction. Oxygen is unnecessary, in contrast to the classical system. These observations were first made on the jellyfish Aequorea[7] and subsequently on the comb-jelly Mnemiopsis.[8] Further work has shown that Coelenterates of various sorts[9,10] possess a common luciferin, and that the pho-

(1) E. N. Harvey, "Bioluminescence", Academic Press, New York, N.Y., 1952.
(2) J. R. Badcock and N. R. Merrett, *Nat. Environ. Res. Counc. News J.*, **5**, 4 (1972).
(3) E. Denton, *Sci. Am.*, **224**, 64 (1971).
(4) F. H. Johnson, *Compr. Biochem.*, **27**, 79 (1967); J. W. Hastings, *Annu. Rev. Biochem.*, **37**, 597 (1968).
(5) F. H. Johnson and Y. Haneda, Ed., "Bioluminescence in Progress", Princeton University Press, Princeton, N.J., 1966.
(6) M. J. Cormier, D. M. Hercules, and J. Lee, Ed., "Chemiluminescence and Bioluminescence", Plenum Press, New York, N.Y., 1973.
(7) O. Shimomura, F. H. Johnson, and Y. Saiga, *J. Cell. Comp. Physiol.*, **59**, 223 (1962).
(8) W. W. Ward and H. H. Seliger, *Biochemistry*, **13**, 1500 (1974); S. J. Girsch and J. W. Hastings, *Am. Soc. Photobiol. Abstr.*, 183 (1973).
(9) M. J. Cormier, J. E. Wampler, and K. Hori, *Fortschr. Chem. Org. Naturst.*, **30**, 1 (1973), and references cited.
(10) K. Hori and M. J. Cormier, ref. 6, p 361; J. G. Morin and J. W. Hastings, *J. Cell Physiol.*, **77**, 305 (1971); O. Shimomura and F. H. Johnson, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 1546 (1975); W. W. Ward and M. J. Cormier, *ibid.*, **72**, 2530 (1975); O. Shimomura, S. Inoue, and T. Goto, *Chem. Lett.*, 247 (1975).

Scheme I
Luciferins



toprotein is in essence the luciferase to which is bound the luciferin and oxygen. The nature of the oxygen binding is not yet known.

Although chemical studies of the chemiluminescent luciferins and model compounds have concentrated on the last energy-yielding reaction, much of the effort of biochemists has been directed toward learning about the preliminary steps in the sequence. These are often directly relevant to the later excitation step, examples being the formation of the firefly luciferin adenylate[11] and activation of Renilla luciferin by enzymatic hydrolysis of the protecting sulfate.[9] There are also several organisms whose biochemistry is fairly well understood, but whose chemistry cannot yet be fruitfully discussed.[1,4,5]

Luminescent Reactions of the Luciferins and Their Models

Three distinctly different luciferin structural types (**1–4**) have been discovered so far.[9,10,12–14] One specific luciferin structure, **4**, is common to all the luminescent Coelenterates[10] hitherto examined.

Bioluminescence is remarkably efficient. Quantum yields for the bacteria are 0.12–0.17,[15–17] for Cypridina 0.28,[18] Renilla 0.04,[9] and the firefly a prodigious 0.88.[19] (A useful subdivision of the quantum yield is made by stating ϕ, the overall quantum yield based on substrate consumed, in terms of these factors: ϕ_c, the ordinary chemical yield of excited product, ϕ_f, the fluorescence quantum yield of this product, and ϕ_e, the proportion of molecules entering the excited state, so that $\phi = \phi_c\phi_f\phi_e$. The greatest theoretical interest is in the last, derived by measuring the others.) When chemists first became interested in bioluminescence it seemed inconceivable that any simple chemical reaction could produce almost no ground-state product, especially since chemiluminescence quantum yields as then known were typically a thousand-fold less!

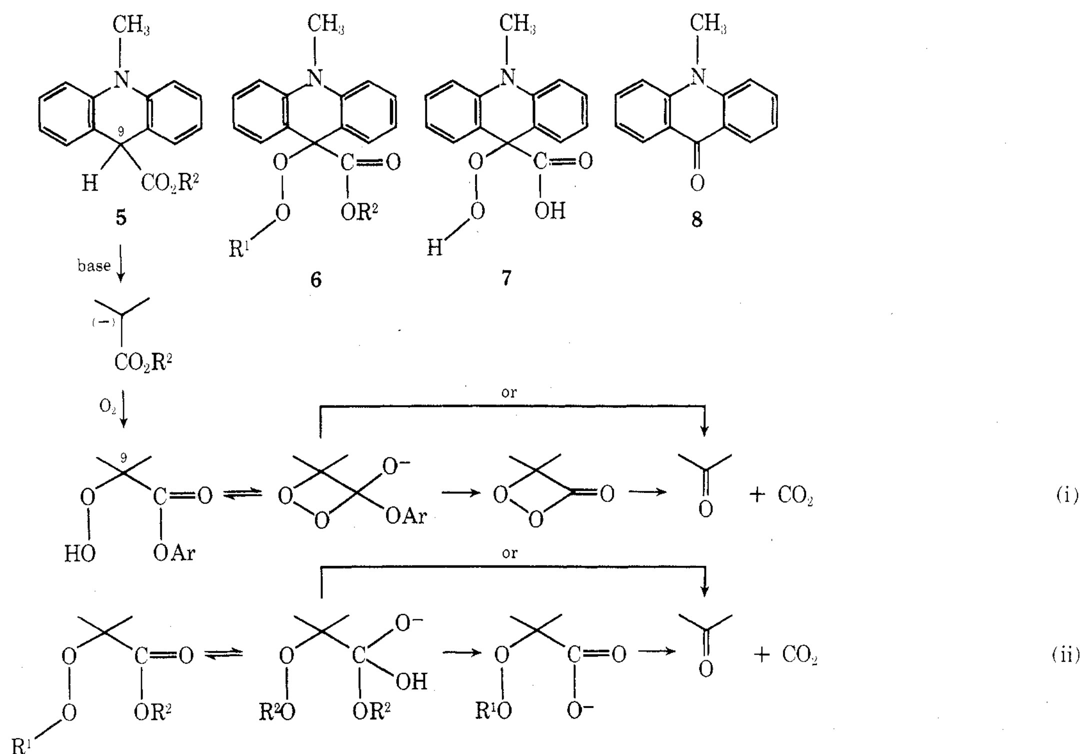
Chemiluminescent organic compounds[20–23] fall into three categories relevant to bioluminescence. Firstly there are many weakly chemiluminescent reactions (quantum yields in the range 10^{-15} to 10^{-3}). These are difficult to investigate since often the excited *products* are formed in these same impossibly low yields. Nevertheless it is conceivable that efficient excited-state formation followed by rapid quenching is occurring, particularly in a nonenzymatic reaction, so that these reactions may not be excluded permanently from consideration.

In the second group are a number of respectably luminescent compounds (quantum yields from 0.01 to 0.50) whose structures or mechanism do not appear to be analogous to the known characteristics of the bioluminescent system. Compounds such as luminol and the hydrazides[22] are probably of this sort. The third category is derived from a consideration of luciferin structures, their inherent reactivities, the nature of the enzymes, and study of model compounds. These reactions are all peroxide decompositions[23–25] without detectable free-radical intermediates.

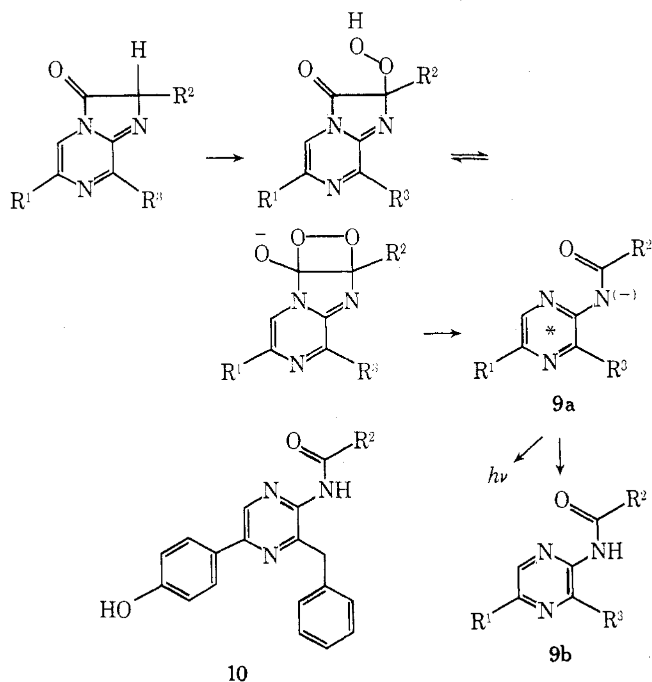
The key feature of the luciferins has always seemed to us to be the juxtaposition of autoxidizable CH grouping and an active ester or amide. It should be possible to synthesize model compounds with these features which would be chemiluminescent. Thus active acridancarboxylic esters[25] (**5**) exemplify in almost all respects the properties of the luciferins, and have played a large part in establishing the current mechanism which is sketched in Scheme II. Support for this mechanism also derives from its success in predicting the structure of the firefly luciferin product[26,27] long before its identification[28] in vivo and in making a cor-

(11) W. C. Rhodes and W. D. McElroy, *J. Biol. Chem.*, **233**, 1528 (1958).
(12) E. H. White, F. McCapra, G. F. Field, and W. D. McElroy, *J. Am. Chem. Soc.*, **83**, 2402 (1961).
(13) Y. Kishi, T. Goto, Y. Hirata, O. Shimomura, and F. H. Johnson *Tetrahedron Lett.*, 3427 (1966); T. Goto, S. Inoue, S. Sugiura, K. Nishikawa, M. Isobe, and Y. Abe, *ibid.*, 4035 (1968).
(14) O. Shimomura and F. H. Johnson, *Biochemistry*, **7**, 1734 (1968).
(15) O. Shimomura, F. H. Johnson, and Y. Kohama *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 2086 (1972).
(16) F. McCapra and D. W. Hysert, *Biochem. Biophys. Res. Commun.*, **52**, 298 (1973).
(17) D. K. Dunn, G. A. Michaliszyn, I. G. Bogacki, and E. A. Meighen, *Biochemistry*, **12**, 4911 (1973).
(18) F. H. Johnson, O. Shimomura, Y. Saiga, L. C. Gershman, G. R. Reynolds, and J. R. Waters, *J. Cell. Comp. Physiol.*, **60**, 85 (1962).
(19) H. H. Seliger and W. D. McElroy, *Biochem. Biophys. Res. Commun.*, **1**, 21 (1959); *Arch Biochem. Biophys.*, **88**, 136 (1960).
(20) F. McCapra, *Prog. Org. Chem.*, **8**, 231 (1971); K. D. Gundermann, "Chemilumineszenz Organischer Verbindungen", Springer-Verlag New York, New York, N.Y., 1968.
(21) E. H. White and R. B. Brundrett, ref 6, p 231, and references cited.
(22) E. H. White and D. F. Roswell, *Acc. Chem. Res.*, **3**, 54 (1970); E. H. White and M. J. C. Harding, *J. Am. Chem. Soc.*, **86**, 5686 (1964).
(23) M. M. Rauhut, *Acc. Chem. Res.*, **2**, 80 (1969).
(24) F. McCapra and D. G. Richardson, *Tetrahedron Lett.*, 3167 (1964).
(25) F. McCapra, *Pure Appl. Chem.*, **24**, 611 (1970); F. McCapra, M. Roth, D. Hysert, and K. A. Zaklika, ref 6, p 313.
(26) F. McCapra, Y. C. Chang, and V. P. Francois, *Chem. Commun.*, 22 (1968).
(27) T. A. Hopkins, H. H. Seliger, E. H. White, and M. W. Cass *J. Am. Chem. Soc.*, **89**, 7148 (1967); E. H. White, E. Rapaport, T. A. Hopkins, and H. H. Seliger, *J. Am. Chem. Soc.*, **91**, 2178 (1969).
(28) E. H. White, E. Rapaport, H. H. Seliger, and T. Hopkins, *Bioorg. Chem.*, **1**, 92 (1971); N. Suzuki and T. Goto, *Tetrahedron*, **28**, 4075 (1972).

Scheme II



Scheme III



rection[29] of an erroneous structure in the Cypridina reaction scheme.[13]

The principal advantages of the acridan esters are that the intermediate peroxides, unlike those of the luciferins and most of our other models, are available in pure form and that the products, as in the luciferins, are highly fluorescent. We have recently completed a reexamination of the reaction, with the results summarized below. Fundamentally the problem is to distinguish between two mechanisms (eq i and ii, Scheme II). Our present view is that eq i represents a satisfactory light-yielding path (singlet excited state formed in 10% yield), whereas eq ii does not. An obvious difference between the two mechanisms lies in the source of the second oxygen atom of the CO_2 produced. Unfortunately the reaction only takes place effectively at pH greater than 7, where exchange of the CO_2 oxygen with water can be too rapid for unambiguous interpretation. As we point out later, we feel that this is a problem with the luciferins also.

The more cogent arguments[25,26,30] can be summarized as follows.

(a) The quantum yield (ϕ_e) of singlet excited state for compound **6a** (R^1 = H; R^2 = Ph) is 0.1, whereas for **6b** ($R^1 = CH_3$, R^2 = Ph) is about 1.5×10^{-5}, both yields being based on the amount of **8** formed. A hydrolytic route must operate for **6b;** clearly the decomposition of the tetrahedral intermediate, however it occurs, cannot be substantially chemiluminescent.

(b) **7** forms **8** with no detectable light emission ($\phi < 10^{-10}$). The yield of **8** is quantitative.

(c) Alkyl esters of **6** (R^1 = H; R^2 = alkyl) give quantum yields of around 1×10^{-5}. Hydroperoxide anions will not readily expel alkoxy groups. Thus the quantum yield should be related to the alcohol (or phenol) pK_a (see Figure 1) since the competing hydrolysis is a dark reaction. Active esters seem to have their counterpart in the structures of the luciferins.

(d) As expected, there is no significant difference in quantum yield for a phenyl ester (e.g., **6,** R^1 = H; R^2 = Ph) in dry ethanol as solvent, as compared to that in aqueous solution. Since alkyl esters are both slower reacting and *very* much less efficient, external attack by ethanol (and hence water) cannot be a major reaction.

Cypridina Luciferin and the Coelenterate Systems. The presence of an indole group in Cypridina luciferin (**2**) was apparent before the whole structure became known. Various indole derivatives are weakly

(29) F. McCapra and Y. C. Chang, *Chem. Commun.*, 1011 (1967).

(30) F. McCapra, R. A. Hann, and K. A. Zaklika, unpublished observations.

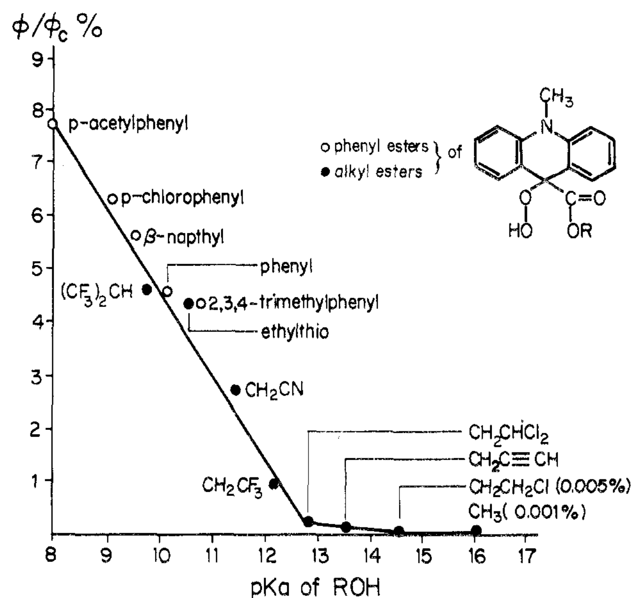
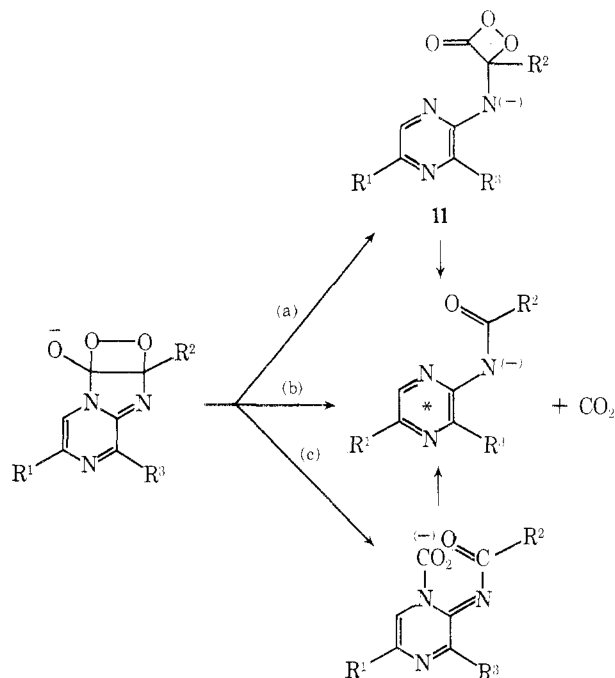


Figure 1. Reaction of acridinium salts with hydrogen peroxide at pH 11. All quantum yields are based on yield (ϕ_c) of 8 formed. The group R is as indicated, with the obvious exception of the ethyl thioester.

Scheme IV



chemiluminescent,[31] and it once seemed possible that this portion of the luciferin molecule was responsible for the excitation. However, the structure has in addition the characteristics of the model compounds, being autoxidizable and having an active acyl grouping. Thus, upon protonation on the nonpyrazine nitrogen, the carbonyl assumes properties resembling those of an acylpyridinium salt, being reactive toward nucleophiles. It is thus possible to predict[29] the product and excited state of the natural system.

The amide **9b** (see **2** for R groups) and CO_2 were subsequently proved to be products of the enzymic reaction,[32,33] as required. An interesting feature is that the amide is only weakly fluorescent in aqueous solution, but is strongly so in aprotic solvents[34] and when enzyme bound.[35] Modification of the properties of the excited state by the enzyme is always a possibility. The distinction between the cyclic (eq i) and hydrolytic (eq ii) routes using $^{18}O_2$ and $H_2{}^{18}O$ has been unequivocally made in favor of the former.[36] Since there is no likelihood of exchange of $^{18}O_2$ with water or CO_2, the incorporation of 80 atom % of $^{18}O_2$ into the CO_2 produced demands the mechanism of eq i or a close relative. The fluorescence spectrum of the anion **9a** and the chemiluminescence emission match exactly.

The structure of Renilla luciferin, in its activated form (**4**), seems identical in essence, and one would expect the same mechanism to operate. It is therefore surprising that use of $^{18}O_2$ and $H_2{}^{18}O$ supports the hydrolytic mechanism.[37] Unlike the evidence in favor of $^{18}O_2$ participation, this interpretation is never free from doubts about rapid exchange in the CO_2, even though control reactions were carried out. Our misgivings are reinforced by the observation that in the Cypridina system exchange can be shown to rise to an extent sufficient to explain the anomaly unless the concentration of luciferin is kept high.[36] In both the Renilla experiments and in the case of the firefly system to be discussed, the luciferin concentrations were unfortunately low enough as to cast serious doubt on the experimental results. The chemiluminescence quantum yield is still too low (in spite of efforts in our own and other laboratories) to allow unambiguous labeling studies at present.

Coelenterate luciferin (**4**) must surely produce the excited product in the same way as does that of Cypridina.

The Emitting Species in the Coelenterates. In Cypridina, as already mentioned, the light emitted (λ_{max} 460 nm) corresponds to the fluorescence of the anion **9a.** Although it is virtually certain that the coelenterates Obelia, Campanularia, Clytia, Lovanella, Pelagia, Mnemiopsis, and others all possess the same or a closely related luciferin, the color of the light emitted varies considerably.[9] There are two reasons for this perhaps useful specificity. The first is inherent in the organization of the whole system in the organism. The bioluminescent reaction in Renilla in particular takes place in an organelle, called a lumisome by Cormier,[38] in which there is a fluorescent green protein (λ_{max} emission 509 nm). Energy transfer from the initial excited state of **9a** (see **4** for R groups) generates the green emission.

Not all coelenterates use this energy-transfer system, and emission in these cases occurs directly from the amide anion. Synthesis of compounds with the essential features of the coelenterate luciferin allowed the study of the chemistry of the light emission.[39,40] By treating the luciferin with base too weak to ionize the phenolic

(31) F. McCapra, D. G. Richardson, and Y. C. Chang, *Photochem. Photobiol.*, **4**, 1111 (1965); F. McCapra and Y. C. Chang, *Chem. Commun.*, 522 (1966); G. E. Philbrook, J. B. Ayers, J. F. Garst, and J. R. Totter, *Photochem. Photobiol.*, **4**, 869 (1965); N. Sugiyama, M. Akutagawa, T. Gasha, Y. Saiga, and H. Yamamoto, *Bull. Chem. Soc. Jpn.*, **40**, 347 (1967).

(32) T. Goto, *Pure Appl. Chem.*, **17**, 421 (1968).

(33) H. Stone, *Biochem. Biophys. Res. Commun.*, **31**, 386 (1968).

(34) T. Goto, S. Inoue, S. Sugiura, K. Nishikuwa, M. Isobe, and Y. Abe, *Tetrahedron Lett.*, 4035 (1968).

(35) O. Shimomura, F. H. Johnson, and T. Masugi, *Science*, **164**, 1299 (1969).

(36) O. Shimomura and F. H. Johnson, *Biochem. Biophys. Res. Commun.*, **44**, 340 (1971); **51**, 558 (1973).

(37) M. DeLuca, M. Dempsey, K. Hori, J. E. Wampler, and M. J. Cormier, *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 1658 (1971).

(38) J. M. Anderson and M. J. Cormier, ref 6, p 387; *J. Biol. Chem.*, **248**, 2937 (1973).

(39) F. McCapra and M. J. Manning, *J. Chem. Soc., Chem. Commun.*, 467 (1973).

(40) K. Hori, J. E. Wampler, and M. J. Cormier, *J. Chem. Soc., Chem. Commun.*, 492 (1973).

hydroxyl group (see **4**), one can still generate the product in the excited state *as the amide anion.* Attempts to obtain a matching fluorescence spectrum (λ_{max} 473 nm) by adding base to the isolated product (**10**) fail since the phenol ionizes first. The amide thus cannot be more acidic in the excited state[41] than the phenol, and the anion **9a** must arise directly from the oxidation, with a very short lifetime. The intermediate (**11**) of path a (Scheme IV) should have a lifetime more than sufficient for protonation, and paths b or c are perhaps more likely. Deprotonation of an excited state without loss of excitation is well known, but as yet there is no example of loss of CO_2 to give a similar result as is required by path c. An obvious third possibility is a concerted reaction (path b).

The wavelength produced in the reaction ranges from 525 nm (dianion excited state) through 473 nm (**9a,** $R^{1\bullet}$ = HOC_4H_4, un-ionized) to 414 nm (both groups unionized) depending on the base strength of the catalyst. The emission of the ether ($R^1 = PhCH_2OC_6H_4$) at 473 nm confirms the assignments, but it is not yet known whether the 414-nm emission reflects an increased rate of protonation of **9a** or acid catalysis of the peroxide addition reaction.

The Firefly. Firefly luciferin (1) was the first to have its structure elucidated,[12] and the major problems have shifted to details of the enzyme luciferase.[43] As the acyl adenylate, its reactions should be simulated by active esters of the acridan type. Nevertheless, labeling studies[44] using $^{18}O_2$ and $H_2{}^{18}O$ give apparently unambiguous support for the hydrolytic mechanism in the luciferin–luciferase reaction. However, the recent demonstration[45] that $^{18}O_2$ is extensively incorporated into the CO_2 in the chemiluminescent reaction in dry dimethyl sulfoxide, together with the possibility of exchange of the CO_2 oxygen with water in the enzyme-catalyzed case, cast doubt on this result. Although the peroxy acid (**14,** R = H) has so far proved impossible to prepare, we believe we have managed to make (in situ) acid **12** (R = CH_3). This decomposes extremely rapidly *without light emission* to **13** (R = CH_3). This is an important observation since, although the exact energy of the red fluorescent excited state is not known with certainty, it may be lower than 54 kcal mol^{-1}. Most blue chemiluminescent reactions probably cannot sustain the loss of 30-kcal strain energy by foregoing the cyclic pathway, but it was always possible that the lower requirements of the firefly excited state could be met by the hydrolytic route.

The cyclic mechanism (Scheme VI) was suggested independently by two laboratories.[26,27] White and his coworkers have also shown that the yellow light (λ_{max} 562 nm) of the firefly is actually the result of deprotonation of the initially formed excited state.[14] It is possible to produce emission from this initial state also, even in living fireflies which normally show yellow. Other species emit red (ca. 575 nm) light, and we can guess that the initial state emits in these cases. These

(41) We have measured the pK_a's of several fluorescent amides in both ground and excited states; the changes are small (0 to −1 pK_a unit) for the amides so far studied.[42]

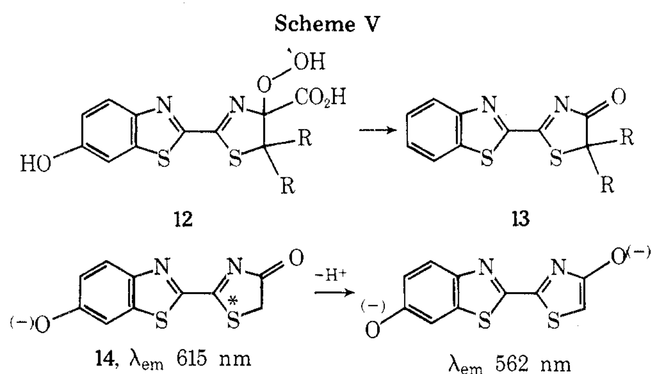
(42) F. McCapra and P. V. Long, to be published.

(43) W. D. McElroy and M. DeLuca, ref 6, p 285.

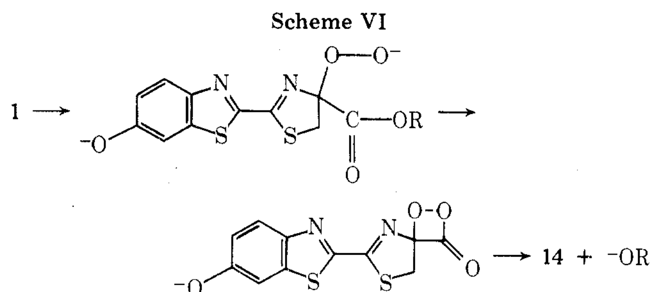
(44) M. Deluca and M. E. Dempsey, *Biochem. Biophys. Res. Commun.*, **40**, 117 (1970); W. D. McElroy in ref 6.

(45) E. H. White, J. D. Miano, and M. Umbreit, *J. Am. Chem. Soc.*, **97**, 198 (1975).

Scheme V



Scheme VI



R = adenosine monophosphate, phenyl, or ethoxyvinyl

observations make an interesting contrast with the behavior of Cypridina and coelenterate luciferins.

Role of the Enzyme. The luciferases are classified as oxygenases by the reactions they catalyze. These reactions resemble, in outline at least, those of other oxygenases such as those of catechol and tryptophan. The major similarities are the C–C bond cleavage and incorporation of the atoms of O_2 into the products. A striking difference, however, is that other oxygenases almost invariably have an oxidative cofactor based on heme or Fe^{III}. There is certainly no redox prosthetic group in the Cypridina, Coelenterate, and firefly luciferases, so that the reaction resembles an autoxidation. Indeed it is difficult to see a role for the enzyme in the oxidation step *other than removal of the active proton* on the luciferins (see Scheme II), with oxidation by atmospheric oxygen occurring by cage recombination of superoxide ion, in the fashion characteristic of certain carbanion oxidations.[46] However, there is very little relevant information, and this is certainly an area that will repay detailed investigation. Other functions of the enzyme concern catalysis of the attack of peroxide (or, less likely, water) on an active ester. Certain of our model compounds react (at pH 8) at a rate comparable to that of the bioluminescent reaction. The details of this aspect of the enzymic catalysis are probably related in principle to the action of hydrolytic enzymes.

It is often remarked that the efficiency of bioluminescence cannot be matched by that of chemiluminescence. This is only partly true, since chemiluminescent reactions can have quantum yields (ϕ_e) of 0.1 to 0.50[21,23,25] and some useful model compounds are in this category.[45] However, the enzyme does enhance the efficiency to a significant extent. It has been argued that there is a distinctive pathway unavailable to the nonenzymatic reaction.[44] We believe it unlikely that the enzyme will choose to transform a known nonchemiluminescent pathway, rather than to enhance an already

(46) G. A. Russell, A. G. Bemis, E. J. Geels, E. G. Janzen, and A. J. Moye, *Adv. Chem. Ser.*, **No. 75,** 174 (1967).

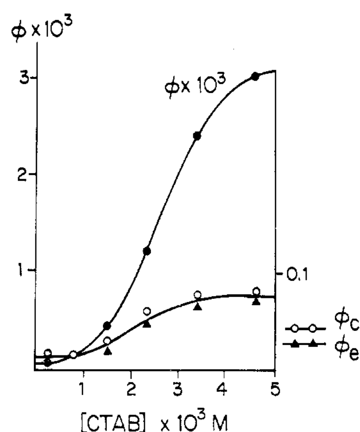


Figure 2. Reaction of acridan ester (5, R = C_6H_5) in aqueous alkaline solution with varying cetyltrimethylammonium bromide (CTAB) concentration. Overall quantum yield ϕ increases 130-fold, and the rise in ϕ_e shows evidence of discrimination against competing dark reactions. ϕ_f of 8 is unaffected by CTAB.

partially successful route. This enhancement obviously includes avoidance of side reactions.

The emitter from Cypridina is not appreciably fluorescent in aqueous solution, but nonpolar and aprotic solvents markedly increase ϕ_f.[34] Binding to the enzyme not only produces the maximum fluorescence yield but also shifts the spectrum to the observed bioluminescence position.[35] This behavior is, of course, known for other, even nonbiological, substrates. Supposing that the chemical and fluorescence yields are close to unity (as is probably the case for the enzymatic firefly reaction), can the enzyme influence the number of molecules entering the excited state? Changes which are known to enhance fluorescence, such as in rigidity, dielectric constant, or specific binding, could all result in enhanced ϕ_e.

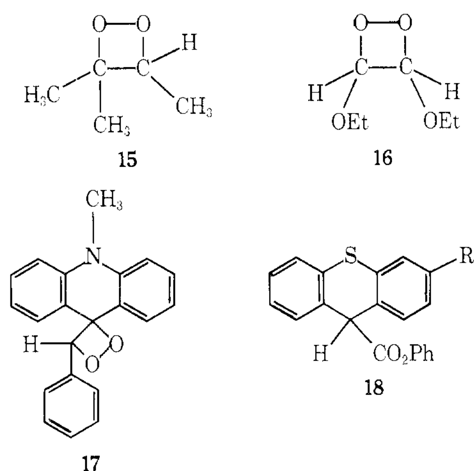
Unfortunately there is as yet no evidence for this, the most intriguing, of the three possible enzymic activities. Since the luciferase contains no auxiliary oxidative cofactors (for the oxidation and later steps) and simple conditions (base and aprotic polar solvent) applied to the luciferin afford rapid, moderately efficient light emission, it is possible that micelles would mimic the properties of the enzyme[47] and allow some insight into its behavior.

We used the acridan phenyl ester (5, R = Ph) as substrate since we could study both the "luciferin" and the derived peroxide. Furthermore, ϕ_f for the product, *N*-methylacridone, is invariant with micellation. There is the rate increase expected by analogy with other reactions in micelles but, more significantly, ϕ_e also increases (up to 200-fold, Figure 2). In other words a greater proportion of the product is appearing in the excited state.

It would be dramatic indeed if the partition between excited and ground states were influenced, but the explanation is probably more prosaic. The peroxide **6** (R^1 = H; R^2 = Ph) shows *no* increase in ϕ_e, indicating that the micelle does not assist the excitation reaction, but must operate before formation of the peroxide. The most probable explanation is that the rate of *oxidation* is enhanced, thereby decreasing the formation of *N*-methylacridone via the competing hydrolysis to carboxylic acid, which of course oxidizes without light

(47) T. Goto and H. Fukatsu, *Tetrahedron Lett.*, 4299 (1969).

Scheme VII



emission. We are presently trying to find out whether the effect operates on the ionization to the carbanion or on its oxygenation. Whatever the cause, the reaction at least superficially resembles the oxidation carried out by a luciferase.

The Excitation Step. The structures and probable mechanism of reaction of the luciferins and model compounds pointed to the intermediacy of a four-membered peroxide ring—a dioxetane or dioxetanone. These compounds had been implicated in a variety of contexts but had never been isolated. After our suggestion[48] that they were likely to be prime intermediates in bioluminescence, and that the antiaromatic nature of the transition state may lead to excitation, Kopecky and Mumford[49] reported the luminescence of the first isolable dioxetane (**15**).[50] Wilson and Schaap[51] made another fundamental contribution by obtaining the *cis*-diethoxydioxetane **16** from reaction of diethoxyethylene with singlet oxygen. Their investigation, which set a pattern for future studies, showed very high yields of (triplet) excited products. We had previously shown that luminescence from fluorescent singlet states was possible by this route,[52,53] but had been unable to isolate our presumed dioxetane (**17**) (see Scheme VII).

Several important questions must yet be answered if these results are to be applied to bioluminescence. The most important of these is that isolable dioxetanes appear to produce triplet states[54] much more effectively than they do singlets.[55] Given the low probability of triplet to singlet transfer, the reactants in bioluminescence must produce singlet states directly and almost exclusively. Further, theoretical justification for efficient excited-state production has concentrated on dioxetanes, whereas the luciferins so far discussed ap-

(48) F. McCapra, *Chem. Commun.*, 155 (1968).

(49) K. R. Kopecky and C. Mumford, *Can. J. Chem.*, **47**, 709 (1969).

(50) For reviews of dioxetane chemistry as it concerns luminescence, see D. C.-S. Lee and T. Wilson, ref 6, p 265, and N. J. Turro, P. Lechtken, N. E. Schore, G. Schuster, H.-C. Steinmetzer, and A. Yekta, *Acc. Chem. Res.*, **7**, 97 (1974). For an excellent review with a wider scope, see E. H. White, J. D. Miano, C. J. Watkins, and E. J. Breaux, *Angew. Chem., Int. Ed. Engl.*, **13**, 229 (1974).

(51) T. Wilson and A. P. Schaap, *J. Am. Chem. Soc.*, **93**, 4126 (1971).

(52) F. McCapra and R. A. Hann, *Chem. Commun.*, 442 (1969).

(53) Dioxetanes of this sort are difficult to isolate owing to their much faster decomposition, but singlet yields appear to be very high, perhaps as great as 20%: K. A. Zaklika, unpublished observations.

(54) N. J. Turro and P. Lechtken, *J. Am. Chem. Soc.*, **94**, 2886 (1972).

(55) However, some alkyl-substituted dioxetanes give relatively high yields of excited singlet carbonyl products. No pattern in the relative singlet–triplet yields with changes in substitution has emerged as yet. See T. R. Darling and C. S. Foote, *J. Am. Chem. Soc.*, **96**, 1625 (1974).

pear to utilize dioxetanones. It is not clear whether such considerations apply to the latter, although simple dioxetanones also decompose to excited triplet products in high yield.[56] Another difficulty is that the lifetimes of simple dioxetanes are all considerably greater than those of the intermediates in bioluminescence. However, dioxetanones have much shorter lifetimes,[56] and the effect of substitution must be considered.

One view of the decomposition of dioxetanes, based on kinetic evidence, is that it is a nonconcerted reaction requiring the formation of a diradical by complete cleavage of the peroxide bond.[57] While this may apply to simple dioxetanes, those related to the luciferins may decompose in an almost concerted fashion, giving high yields of singlet excited states. In this connection it is noteworthy that various *tert*-butyl peresters show a lowering in activation energy for homolytic decomposition ascribed to the onset of concertedness.[58]

The distinction between concerted and nonconcerted decomposition may not be easy to make, since the breakage of the C–C bond cannot be far behind that of the O–O bond. This is implied in our correlation diagram[20,59] which, together with that of Kearns,[60] would seem to provide an adequate basis for discussion in spite of subsequent calculations[61] and restatements of the problem.[62] In the many model compounds we have studied we recognize a feature, shared with the luciferins, which leads to a greater rate of decomposition and enhanced (singlet) quantum yields. Conjugation of a strongly electron-releasing group with the incipient carbonyl seems to be of special importance. For example, ϕ_e (singlet) for the oxidation of the thioxanthene derivative **18**, R = H, is at least ten times less than for **18**, R = HO. Such substituents probably operate through a variety of interconnected influences such as a reduction in energy of the excited state, increasing charge transfer character in the π,π^* transition, and a weakening of the C–C bond.

We have assumed and tried to show that a dioxetane-like intermediate is involved in certain bioluminescent reactions. Any alternative mechanisms to be considered should respond to the features mentioned above. Moreover, our experience with model compounds suggests that the new bonds formed in the decomposition should be part of the chromophoric system of the excited molecule (i.e., they should not be σ in character). At least one molecular fragment so formed must have a thermodynamically accessible excited state. Since the energy of decomposition will be distributed among the fragments, probably in proportion to their degrees of vibrational freedom and the strengths of the bonds made, there must not be a relatively loose, highly dissociated transition state. Electron-transfer reactions often satisfy these criteria, but many alternative fragmentation reactions do not.

(56) W. Adam and J. C. Liu, *J. Am. Chem. Soc.*, **94**, 2894 (1972); W. Adam, G. A. Simpson, and F. Yang, *J. Phys. Chem.*, **78**, 2559 (1974).

(57) H. E. O'Neal and W. H. Richardson, *J. Am. Chem. Soc.*, **92**, 6553 (1970).

(58) P. D. Bartlett and R. R. Hiatt, *J. Am. Chem. Soc.*, **80**, 1398 (1958); P. D. Bartlett and C. Richardson, *ibid.*, **80**, 1756 (1958).

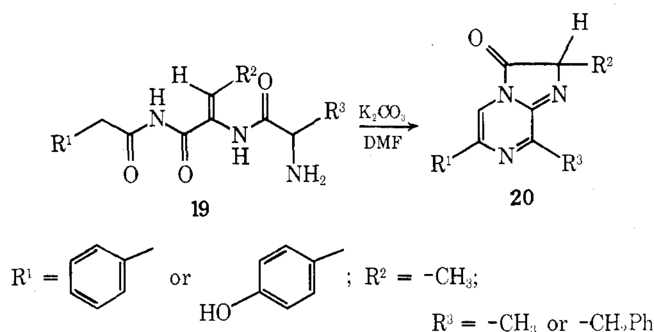
(59) To our knowledge, the first attempt to use a correlation diagram in this way was described by us at the Symposium on Orbital Symmetry Correlations in Organic Reactions, Cambridge, England, Jan 7–9, 1969. Helpful correspondence with Professor Lionel Salem (Orsay) at that time is gratefully acknowledged.

(60) D. R. Kearns, *Chem. Rev.*, **71**, 395 (1971).

(61) E. M. Evleth and G. Feler, *Chem. Phys. Lett.*, **22**, 499 (1973); D. R. Roberts, *J. Chem. Soc., Chem. Commun.*, 683 (1974); M. J. S. Dewar and S. Kirschner, *J. Am. Chem. Soc.*, **96**, 7578 (1974); M. J. S. Dewar, S. Kirschner, and H. W. Kellmar, *ibid.*, **96**, 7579 (1974).

(62) N. J. Turro and A. Devquet, *J. Am. Chem. Soc.*, **97**, 3859 (1975).

Scheme VIII



Biosynthesis

The luciferins constitute a novel group of natural products, and the question arises as to how they are formed. Capturing or cultivating sufficient living specimens is often a formidable task. The value of biosynthetically patterned synthesis in stimulating reasonable hypotheses is therefore considerable. One would also eventually like to be able to trace the chemical evolution of the luminescent system. This possibility is especially attractive in view of the almost "accidental" nature of the chemiluminescence—one need only add base in the presence of air for a bright and instantaneous flash of light.

Cypridina and Coelenterate Luciferins. The structure of Cypridina luciferin suggests its biosynthesis from three amino acids (trytophan, isoleucine, and arginine). We felt that these should be initially assembled by the obvious peptide linkages, giving the intermediate **19**, and that this compound would perhaps follow the biosynthesis.

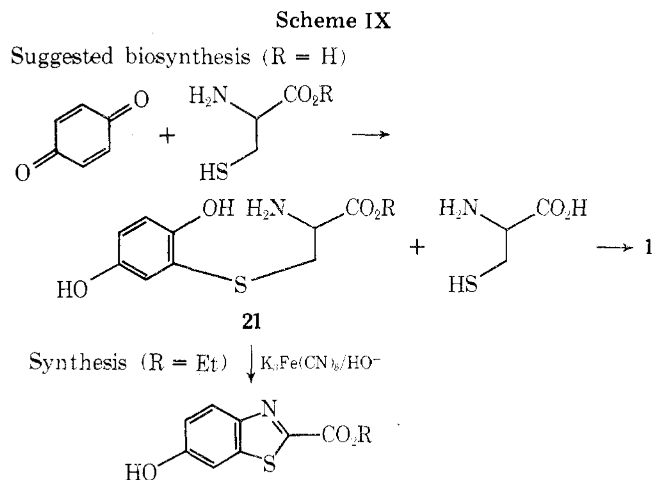
The modifications (decarboxylation and oxidation) are all frequently found in natural amino acid derivatives. Base-catalyzed cyclization of **19** proceeds to the luciferin model **20** in quantitative yield,[63] lending support to the proposal (see Scheme VIII). Although the idea should not be taken too far, we felt that with the possible incorporation of other amino acids, related luciferins may be rather common. Indeed, at that time the working structure[9] for Renilla luciferin did not contain the tripeptide backbone, and we started a synthesis[39] of a more likely analogue. Using dehydrovaline for convenience, we synthesized the structure **19**. It can be seen that Coelenterate luciferin is derived in principle from tyrosyltyrosylphenylalanine, as is the luciferin of the totally unrelated squid, Watasenia.[64] It will be interesting to see how this theme develops as other of the thousands of luminescent marine organisms are investigated.

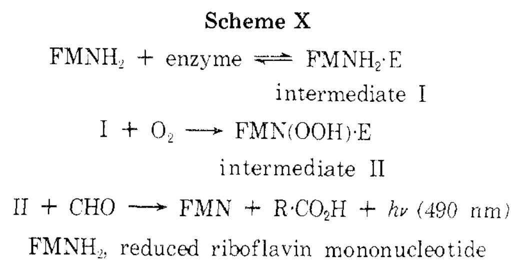
Firefly Luciferin. Terrestrial bioluminescent creatures are far less common, with the firefly dominating the scene. Information is accordingly less available, and it is too early to say whether similar relationships are to be expected. However, a luciferin (very different from that of the marine organisms) is common to all firefly species so far examined.[65] The major

(63) F. McCapra and M. Roth, *J. Chem. Soc., Chem. Commun.*, 894 (1972).

(64) S. Inoue, S. Suguiura, H. Kakoi, K. Hasizume, T. Goto, and H. Iio, *Chem. Lett.*, 141 (1975); T. Goto, H. Iio, S. Inoue, and H. Kakoi, *Tetrahedron Lett.*, 2321 (1974).

(65) H. H. Seliger and W. D. McElroy, *Proc. Natl. Acad. Sci. U.S.A.*, **52**, 75 (1964).





question concerns the derivation of the benzothiazole ring, a rare structure in nature.[66] The occurrence of benzoquinone (from tyrosine) in the Coleoptera[67] seems to indicate a probable biosynthesis (Scheme IX).

Oxidation of the adduct of cysteine ethyl ester and benzoquinone (**21**) by $K_3Fe(CN)_6$ gives the benzothiozole directly in good yield. An intermediate benzothiazine is involved. Our preliminary examination[68] of the in vivo biosynthesis in Pyrophorus pellucens shows that cysteine is incorporated, and that C-2 of the benzothiazine *may* be the linking carbon between the benzothiazole and thiazoline portions of the molecule. The incorporation confirms that luciferin is synthesized during the lifetime of the organisms, refuting a suggestion that adult fireflies emerge with luciferin sufficient for their short lifetime.[69]

Problems in Progress—Bacterial Luminescence

Chemiluminescence is not confined to any one reaction mechanism, in spite of the rarity of *efficient* light emission. Nevertheless, at the present time there are no clearly discerned alternatives to peroxide decomposition and few models, other than dioxetanes, which can be applied to bioluminescence. The marine bacteria (e.g., *Photobacterium phosphoreum*) do not have a classical luciferin, and may provide an example of a new chemiluminescent reaction. As yet there is no known chemical model for the system, and details of the crucial excitation step are missing. The basic scheme which must be interpreted chemically has been provided particularly in recent years by the extensive and ingenious biochemical investigations of Hastings and his collaborators.[70]

Reduced flavin mononucleotide ($FMNH_2$) reversibly binds to the enzyme. Free $FMNH_2$ autoxidizes extremely rapidly, so that intermediate II is left to decay in first-order fashion. Reaction of this intermediate with a long-chain aldehyde (C_6–C_{18}) then produces light. The enzyme has been purified to a most satisfactory level in Hastings' laboratory, and much useful and detailed information concerning it obtained. However, we are chiefly interested in the excitation step. The presence of the obvious product of aldehyde oxidation—the corresponding fatty acid—as a contaminant delayed recognition that this is indeed formed stoichiometrically with a quantum yield of 0.12.[15–17] At one time we suspected that the α-methylene group, by analogy with the chemistry of the luciferins and carbonyl compounds generally, was involved in the oxidation, but further investigation failed to support this idea.

Hastings' superb study of the luciferase at low temperature[71] has shown that intermediate II is a peroxide which reacts with aldehyde to give light. We are thus dealing with an aldehyde oxidase, as suggested by McElroy some time ago.[72] This could be taken as an indication that something akin to a Baeyer–Villiger or Dakin reaction is occurring. So far we have been unable to generate light by model reactions of this sort, and we have shown that previous evidence[73] in favor of such a mechanism is not valid.[74]

The quantum yield[4,75] with respect to FMN is much greater than 1.0, requiring that it be recycled, yet it is clear from the wavelength of light emitted that FMN itself is not the excited product. To accommodate these facts it was suggested[76] that the monocation $FMNH^+$ is the emitter. The spectrum measured is actually that of the doubly protonated molecule, and we feel that alternatives must be sought. Although our original proposal[16] must be modified in view of Hastings' results, the scheme has some useful features, notably those involving the reversible ring opening of the flavin.

Thus for this system we seem to be faced at present with two distinct prescriptions for future hypotheses: either a hitherto unexplored chemiluminescent reaction is operating, or the enzyme is participating in such a way as to exclude the organic chemist and his crude models. These are only the main ingredients of present and future mechanisms, and essential details which would afford better answers may have been ignored. However, "the best conclusion for a piece of research is not an exclamation point but a question mark".[77]

(66) For a review of similar reactions see R. H. Thomson, *Angew Chem., Int. Ed. Engl.*, **13**, 305 (1974), and L. Minale, E. Tattorusso, S. De Stefano, and R. A. Nicolaus *Gazz. Chim. Ital.*, **100**, 461 (1970), for a related in vitro experiment.

(67) J. Weatherstone, *Q. Rev., Chem. Soc.*, **21**, 287 (1967).

(68) F. McCapra and Z. Razavi, *J. Chem. Soc., Chem. Commun.*, 153 (1976).

(69) A recent paper presents evidence suggesting that the luciferin oxidation product is recycled, but this does not of course cast light on the biosynthesis de nova; see K. Okada, H. Iio, I. Kubota, and T. Goto. *Tetrahedron Lett.*, 2771 (1974). Seliger (ref 6, p 335) claims that the amount of luciferin present in a firefly lantern is sufficient for the number of photons produced in its lifetime, making recycling seem unnecessary.

(70) J. W. Hastings, Q. H. Gibson, J. Friedland, and J. Spudich, ref 4, p 151; J. W. Hastings, *Curr. Top. Bioenerg.*, **1**, 113 (1966); J. W. Hastings and C. J. Balny, *J. Biol. Chem.*, **250**, 7288 (1975). See also ref 9.

(71) J. W. Hastings, C. Balny, C. Le Peuch, and P. Douzou, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 3468 (1973).

(72) W. D. McElroy in "Light and Life", W. D. McElroy and B. Glass, Ed., Johns Hopkins University Press, Baltimore, Md., 1961, p 309.

(73) D. Bentley, A. Eberhard, and R. Solsky, *Biochem. Biophys. Res. Commun.*, **56**, 865 (1974); A. Eberhard and J. W. Hastings, *ibid.*, **47**, 348 (1972).

(74) F. McCapra and R. Hart, unpublished observations; Dr. Eberhard has kindly informed us of his difficulty in repeating these results also.

(75) M. J. Cormier and J. R. Totter, *Biochim. Biophys. Acta*, **25**, 229 (1957); J. Lee and H. H. Seliger, *Photochem. Photobiol.*, **4**, 1046 (1965).

(76) M. Eley, J. Lee, J. M. Lhoste, C. Y. Lee, M. J. Cormier, and P. Hemmerich, *Biochemistry*, **9**, 2902 (1970).

(77) "From my Life", Memoirs, Richard Willstätter, W. A. Benjamin, New York, N.Y., 1965, p 194.