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Electrobioluminescence – a novel method for understanding bioluminescent mechanisms[§]

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Abstract. The chemistry of the formation of excited states in living systems is probed by using the electrochemical techniques. A new method of using a living animal as an electrode provides mechanistic details on the various steps involved and the overall energetics of the process of excited state formation. This is discussed in detail with reference to the newly developed earthworm electrode which glows upon potential sweep or step programme in an electrochemical cell.

Keywords. Electrobioluminescence; earthworm bioluminescence; firefly bioluminescence; electron injection; cyclic-voltammetry of living animals.

1. Introduction

The phenomenon of some living animals generating bioluminescence has been known and investigated (Adams and Cilento 1982; Hastings 1983a) for understanding the chemistry of the excited states. The approach has been to isolate the chromophore and the enzyme for simulating the observed emission features. The energetics of such reactions have been considered in detail (Kosower 1981; Ward and Cormier 1978; Hastings 1983b) in selected systems. An interesting outcome of these studies has been the identification of new pathways for excited state formations and a realization that some of the living systems produce excited states at efficiencies > 90%. So far it has seldom been possible to study bioluminescent mechanisms in the living state. A method which has recently been developed called electrobioluminescence (EBL) offers some unusual advantages for investigating the mechanistic schemes, sensitivity to external conditions in generating the excited states and the effect of electrolytes on the production efficiencies of excited states. This has been based on the generation of bioluminescence by living animals under the influence of an externally impressed voltage (Ismail and Santhanam 1984; Limaye and Santhanam 1986a and b). It may be considered as stimulated bioluminescence where a chemical reaction is initiated by the application of an appropriate voltage resulting in the overall production of the excited state. A photon is emitted upon the excited state returning to the ground state. A primary requisite for the study is that the animal chosen is amenable for its development as an electrode.

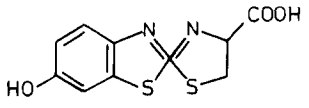
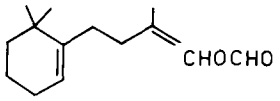
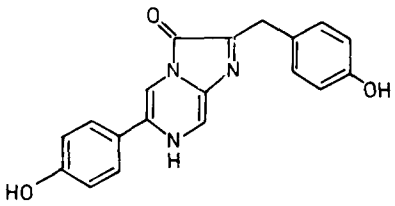
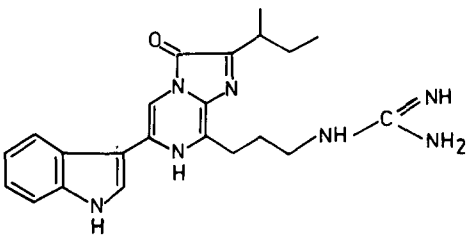
The formation of the excited state in living systems is caused by the generation reaction

$$\mathrm{L} + \mathrm{Lase} \xrightarrow{\mathrm{R}} \text{products} + h\nu, \qquad (1)$$

where L represents luciferin, Lase represents the enzyme luciferase and R represents the activator. The chromophore of luciferin is significantly different

[§] Dedicated to Prof. K S G Doss on his eightieth birthday.

Table 1. Nature of luciferins present in organisms.

Photogen	*Organism*	λ_{max}(nm)
	Firefly	552–590
	Bacteria	470–500
	Latia	535
	Coelenterazine (Renilla, Aequorea)	470
	Cypridina	462
Earthworm luciferin (?)	Lampito mauritii	495

from one species to another. The EBL reaction may also be represented by (1). A few examples of luciferins are shown in table 1.

2. Chemical mechanisms: from chemical bonds to photons

One of the complex questions that arise in considering reaction (1) is about the nature of the chemical process which would release enough energy in one step to form the excited state. Here the nature of L and R would control the reaction. Another important question is the efficiency of bioluminescence and how it changes from one system to another. The answers to these questions can be generated by a study of EBL; it provides a comparison of *in vivo* to *in vitro* bioluminescence.

Three types of chemical mechanisms that are generally considered suitable for bioluminescence are reviewed here and discussed in detail below:— (a) Electron transfer reactions of radical ion annihilation, (b) peroxide type reactions and (c) reactions involving specific ions.

(a) *Electron transfer reactions of radical ion annihilation*: The chemiluminescence that is often produced by the reaction of oppositely charged aromatic ion radicals in aprotic solvents has been considered as a one-step excitation to give the product in the excited state. It is considered as an electron transfer reaction with no intermediate formation of a bond. A typical reaction is shown in figure 1. Here the free energy released in the electron transfer reaction is taken as one parameter and the changes in solvation as the other (Periasamy and Santhanam 1977; Kulkarni *et al* 1978a, 1978b). Marcus (1965, 1970) pioneered this model based on dielectric polarization, where

$$G_{\text{pol}} = m^2 e^2 \left(\frac{1}{2r_+} + \frac{1}{2r_-} - \frac{1}{R} \right) \left(\frac{1}{D_O} - \frac{1}{D_S} \right) \tag{2}$$

r_+ and r_- are the radii of the cation and anion, R the interionic distance, D_O and D_S the optical and dielectric constants and m the reaction coordinate for solvent dipole reorientation. The above expression can be simplified to $G_{\text{pol}} = m^2\ \lambda$ where

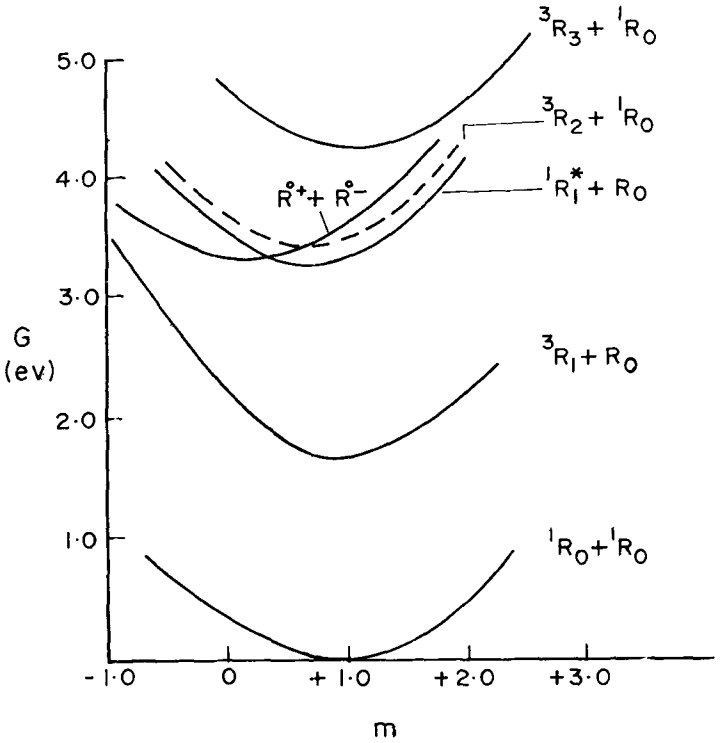


Figure 1. Free energy curves for electron transfer bioluminescence. $^1R_0$ = ground singlet state, $^3R_1$ = first triplet state, $^3R_2$ = second triplet state, $^1R^*$ = first excited single state, $\dot{R}^+$ = free radical cation, and $\dot{R}^-$ = free radical anion; m represents the parameter defined in the text.

λ constitutes the product of the parameters in the brackets multiplied by the charge. The free energy of the product molecules is considered with

$$\Delta G_{\text{pol}} = E_{S_1} \text{ (or } E_{T_1}) + (m-1)^2,$$

where E_{S_1} and E_{T_1} are the energies of first singlet and first triplet states respectively. The region of intersection of the separated ions and products is defined by m^* which has direct relationship to E_{S_1} or E_{T_1} and the ΔG^0 of the reaction. The accessibility of the product states is given by the intersection region and the rate of formation of the product state is defined by

$$k_s = gpz \exp\left\{-\frac{\lambda}{4RT}\left[1+\frac{E_{S_1}+\Delta G^0}{\lambda}\right]^2\right\}, \tag{3}$$

where g is the spin statistical factor, p the probability factor and z the collision frequency. For the triplet state E_{S_1} will have to be replaced by E_{T_1} and the appropriate ΔG^0 (Periasamy and Santhanam 1977).

The free energy of the reaction is calculated from the electrochemical potentials. For the reactions

$$R + e \rightleftarrows R^{\cdot -}, \; E^0 (R/\dot{R}^-), \tag{4}$$

$$R - e \rightleftarrows R^{\cdot +}, \; E^0 (\dot{R}^+/R), \tag{5}$$

$\Delta G^0 = E^0_{(R/R^-)} - E^0_{(R^+/R)}$. The ΔG^0 is generally large for several electron transfer reactions of the above type and in the range of 1–4 eV; in several cases the emitting state is the excited singlet state. Thus, with the available energy, chemi-excitation is in general possible. Table 2 lists selected examples of the electron transfer reactions. However, in some situations ΔG^0 is less than the excited singlet state and in such situations, the energy is enough to populate the accessible triplet state ($\Delta G^\circ \geq E_T$). The resulting triplets annihilate to produce the excited singlet state of the molecule (Periasamy *et al* 1973). The chemi-excitation process is generally efficient in electron transfer reactions. However, this type of electron transfer reactions has not been considered in bioluminescence until recently, mainly

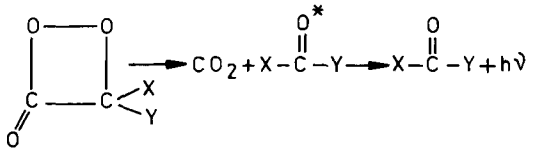
Table 2. Electron transfer reactions[a].

Type of Reaction	$-\Delta G^0$ (eV)	E_{S_1} (eV)	E_{T_1} (eV)	λ (nm)
$DPA^- + DPA^+$	3·08	3·00	1·84	440
$Rub^- + Rub^+$	2·29	2·30	1·10	600
$AN^- + AN^+$	3·36	3·20	1·80	420
$DPA^- + TMPD^+$	1·97	3·00 (DPA)	1·80 (DPA)	440
$Rub^- + TMPD^+$	1·56	2·30 (Rub)	1·10 (Rub)	600

[a] Reactions carried out in aprotic solvents containing 0·1 M $(C_4H_9)_4NClO_4$. DPA = 9,10-diphenylanthracene, Rub = rubrene, AN = anthracene, TMPD = N,N,N^1,N^1 tetramethyl-*p*-phenylene diamine.

because bioluminescence requires oxygen and free radicals (anion or cation) are unstable in the presence of oxygen. Hence the pathways for bioluminescence have to be modified in the light of this factor; in these situations also, electron transfer reactions must be involved before the excited state is formed, and hence, a hybrid reaction mechanism should be considered.

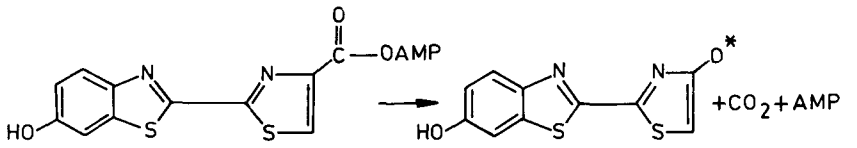
(b) *Reactions of the peroxide type*: The reaction mechanisms considered here use oxygen as the component of the reaction and the peroxide $-O-O-$ bond is formed prior to the formation of the excited state. The discovery by Kopecky and Mumford (1969) that isolated ring peroxides produce high yields of electronically excited products led to several postulates of peroxides in bioluminescence. The breakdown of the peroxide ring can generate about 4 eV which would be enough to form a product in an excited state. McCapra and Richardson (1964) proposed the $-O-O-$ bond formation in bioluminescent systems such as firefly and cypridina. The following sequence has been visualized



(6)

Chart 1.

X and Y represent the chromophores. Kosower (1981) considered the energetics of the above reaction scheme in detail and suggested that the thermal decomposition of the $-O-O-$ bridged compound may not be a one-step process but may be caused by an electron transfer step (see subsequent section). Interestingly it has been shown (Stone 1968) that for every O_2 (the earlier step to the formation of $-O-O-$ bridge) that is consumed in the reaction, a CO_2 molecule and a carbonyl moiety are produced. A quantum yield close to unity has been demonstrated (Seliger and McElroy 1960). Later Plant *et al* (1968) used labelled ^{14}C-carboxy luciferin and demonstrated the quantitative liberation of CO_2 in the reaction. The overall reaction can be represented as

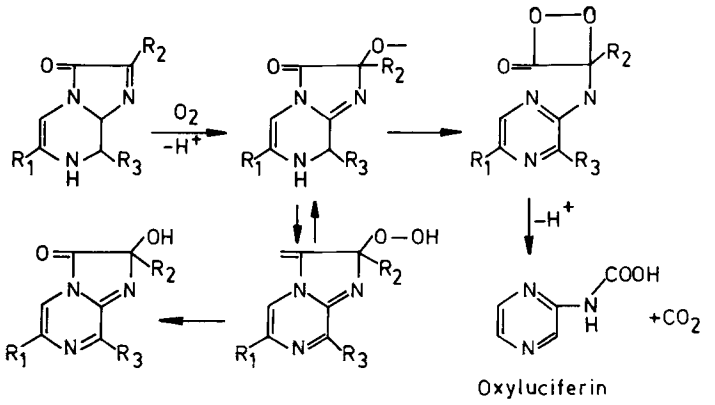


(7)

Chart 2.

The formation of ring peroxides in bioluminescent reactions opens up new pathways for combining electron transfer reactions and the excited state formation.

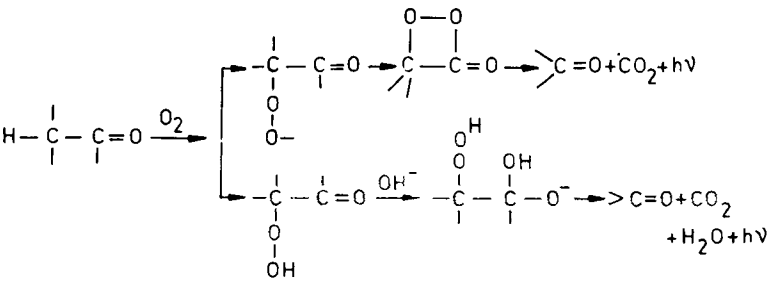
With cypridina and coelenterazine such peroxides have been demonstrated (Adams and Cilento 1982). The reaction mechanism for cypridina luciferin is shown below.



(8)

Chart 3.

The proposed oxidative mechanisms were discussed by Shimomura *et al* (1977) and the proposed mechanism is shown below.



(9)

Chart 4.

As can be seen in the above reaction scheme, the upper path involves a dioxetane bridge formation and the latter involves the participation of the solvent. The experimental results support the upper pathway. The two mechanisms were differentiated by using labelled $H_2^{18}O$ and $^{16}O_2$ and $H_2^{16}O$ and $^{18}O_2$(DeLuca and Dempsey 1973; Shimomura *et al* 1977).

Peroxide bridge formation is also recognized in aequoera (jellyfish). However the photoprotein will emit light upon addition of calcium ion (Shimomura and Johnson 1975). Here an enzyme substrate peroxide intermediate is produced and stored in the cell. The intermediate is able to react with calcium and emit flashes (Hastings and Morin 1969).

Although cyclic peroxide formation is overwhelmingly established, laboratory experiments with such cyclic peroxides generate triplet states rather than excited singlet states; this step requires thermal decomposition. If the triplet state is produced, then the excited singlet state formation would require an energy

doubling scheme in the overall reaction (such as triplet-triplet annihilation). A process such as this would result in the reduction of overall efficiency. As the bioluminescent reactions produce the excited singlet state of the emitter in high yield, the reaction scheme requires a higher energy conservation step. Hence an electron transfer scheme [such as that discussed in (a)] and a peroxide mechanism would be required. The electron transfer scheme is by far the most efficient chemi-excitation process. One such proposal is that the disintegration of peroxide caused by an electron transfer reaction (Adams and Cilento 1982; Koo and Schuster 1977) charge annihilation and chemi-excitation follows the above reaction. With a view to test such a process the following reaction has been performed between several radical cations (R^+) and diphenyl peroxide (DPP). The following reaction sequence is proposed.

$$R + DPP \rightarrow (R^+ + DPP^-)_{cage}. \tag{10}$$

With the low stability of the radical anion, it undergoes decarboxylation.

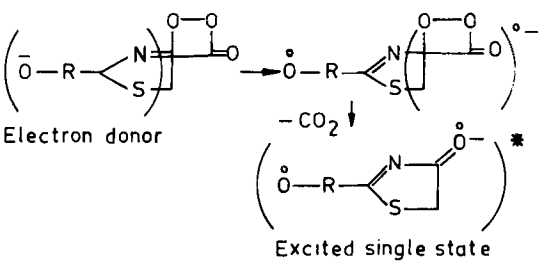
$$(R^+ + DPP^-)_{cage} \rightarrow (R^+ + BC^- + CO_2)_{cage}. \tag{11}$$

BC is the product of the decarboxylation of DPP (benzocoumarin). As BC^- is a powerful reducing agent (E^0 of the redox couple is very negative), the electron transfer reaction in the cage will occur to produce the excited state.

$$(R^+ + BC^- + CO_2)_{cage} \rightarrow ({}^1R^* + BC + CO_2)_{cage}. \tag{12}$$

Since the cage reaction should be sensitive to the dielectric constant of the medium, the above reaction occurs spontaneously in solvents like DME, THF, etc.

For bioluminescence the following charge transfer resonance structure of the product state is expected on the basis of the above scheme.



(13)

Chart 5.

This pathway can produce high quantum yields.

(c) *Reactions involving specific ions*: A specific ion requirement has been noticed in the bioluminescence of marine animals. *Renilla* bioluminescence requires Ca^{2+} for binding to the protein (Cormier *et al* 1970, Cormier 1978). The nerve impulse is linked through Ca^{2+} and the protein; the latter is a single peptide chain of molecular weight 18,500 and contains two high affinity Ca^{2+} binding sites. The native protein carries luciferin (one mole) and is referred to as blue fluorescent protein (BP–LH_2) [the luciferin (LH_2) is bound to the protein]. Here the linkage

has been considered to be noncovalent. The addition of BP–LH_2 to luciferase does not produce emission of light because bound LH_2 is sterically hindered for the reaction. When Ca^{2+} is added to it, the ion displaces the LH_2 due to a conformational change in BP–LH_2 and light emission occurs.

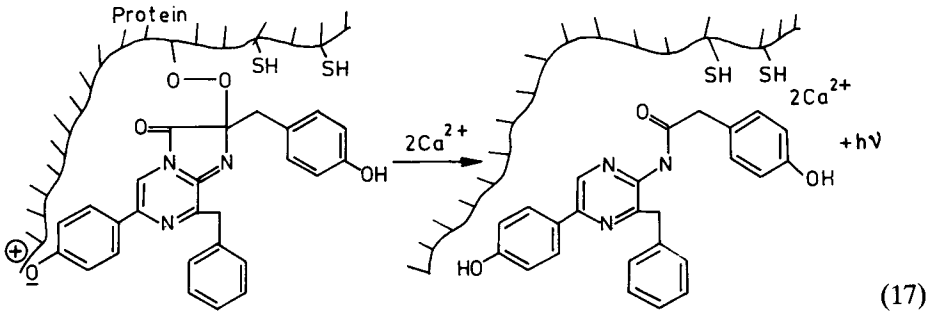
The following sequence of reactions is required for the ion formation

$$BP-LH_2+Ca^{2+} \rightleftarrows BP-Ca+LH_2, \tag{14}$$

$$BP-Ca+LH_2+E \rightleftarrows E-LH_2+Ca-BP, \tag{15}$$

$$E-LH_2+Ca-BP \rightarrow \text{Oxyluciferin}^*-E+CO_2. \tag{16}$$

In contrast to aequorea, the emission of radiation occurs only upon the addition of Ca^{2+} (Shimomura and Johnson 1975); the substrate (LH_2) is bound to the enzyme and its release occurs upon addition of Ca^{2+}. The reaction sequence may be visualized as



(17)

Chart 6.

2.1 *Mechanisms investigated by EBL*

The *in vivo* understanding of bioluminescence is most challenging due to the interlinking of reactions. Self-triggering mechanisms generally operate to produce bioluminescent chemicals. However, some animals generate bioluminescence under chemical or electrochemical excitation–which may be termed external control. Of these two types, chemical excitation results in the excitor being a component of the reaction scheme and often this results in the destruction of the animal. The electrical excitation offers an advantage over this except that in this case electrochemical reactions may be occurring on the surface of the animal. The development of an earthworm (*Lampito mauritii*) electrode (Limaye and Santhanam 1985) has the potentiality for investigating bioluminescent reaction mechanisms. This electrode is used in an electrochemical cell carrying an electrolytic solution; the role of specific ion requirement can be investigated by this method. Here the excited state is produced on the surface of a "live electrode".

A typical set up for EBL is shown in figure 2. The earthworm electrode (EWE) is prepared by carefully washing the earthworm in distilled water and following the procedure described earlier (Limaye and Santhanam 1986a). Using the EWE as the working electrode and a large mesh platinum grid [1·0 cm × 1·0 cm] as the auxiliary electrode, the potential of the EWE is swept linearly with time and the resulting current is measured. A saturated calomel electrode (SCE) is used as a reference

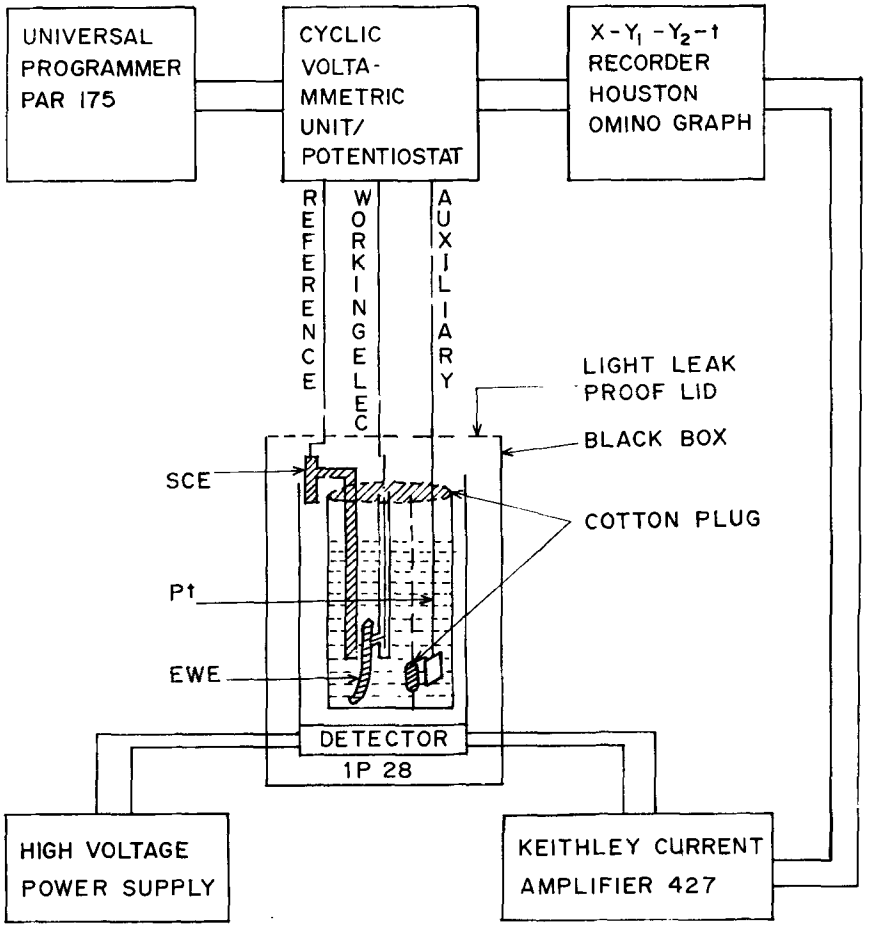


Figure 2. Experimental set-up for EBL. Reference, working and auxiliary electrodes are as shown.

electrode in this scheme. The recording of the current-voltage curve provides the onset potential for luminescence and the resulting current as a measure of the reactions at the electrode. The onset potential is characteristic of the reaction initiating the electron transfer step. With EWE, the onset potential is −0·56 V vs SCE and suggests a 2*e* reduction of O_2 as the initiator of the luminescence. Experiments conducted by saturating the electrolyte solutions with O_2 and removal of O_2 by degassing produce drastic changes in the bioluminescence output. Further the absence of emission in the $FeSO_4$ medium due to Fenton's reaction supports the reduction scheme above (see subsequent discussions).

It is important to note that the potential sweep towards positive potentials does not generate luminescence at this electrode even though during this sweep a few μA current flow occurs. Thus it is the electron input to this electrode that is generating the bioluminescence on a living animal.

2.2 *The ionic role in bioluminescence*

The influence of the electrolyte in the medium produces marked changes in the output of the EWE. Table 3 shows the EBL output of the EWE in different electrolytes. Ca^{2+} in the medium produces the highest EBL output, while Fe^{2+} or Fe^{3+} or Cu^{2+} in the medium produces the lowest (Limaye and Santhanam 1986a). The influence of Ca^{2+} on the bioluminescence output has been investigated by isolating the bioluminescence chemicals. Experiments *in vitro* do not produce higher luminescence output than has been obtained with other electrolytes such as K_2SO_4 or $MgSO_4$. The chemiluminescent kinetics in the above experiments were very similar. These results in comparison with EBL suggests Ca^{2+} is not an ingredient of the bioluminescent scheme as in *aequorea*; it does not release the substrate-bound protein. The highest EBL output in Ca^{2+} medium arises from the process of coelomic cell displacement on the electrode. A relative comparison of the EBL emission durations in Ca^{2+} to any other ion such as K^+ or Mn^{2+} is much longer i.e. 320 s to 100 s; the rate of decay in Ca^{2+} solution is slower than in K^+ or Mn^{2+} ion solution. The EBL output shows a correlation with action potential; the amplitude of the action potential is related to Ca^{2+} concentration. It changes from -30 mV to $+30$ mV from negligible Ca^{2+} concentration 0·1 mM to 100 mM. Ito *et al* (1970) obtained a linear relationship between Ca^{2+} concentration and the intracellularly evoked spikes. These plots had a slope of (26)/10 mV$\times$(Ca^{2+}). The membrane potential of the longitudinal muscle does not show a change from its -35 mV upon addition of Ca^{2+}.

The EWE does not generate any luminescence in solutions of Fe^{2+}. Two possible explanations may be offered; (a) it is known to decompose H_2O_2 through (Halliwell and Gutteride 1984),

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}, \tag{18}$$

Table 3. Relative photonic output of EWE in different electrolytes[a].

Medium	Relative photonic output
0·1 M K_2SO_4	1·00
0·1 M $FeSO_4$	0·00
0·1 M $Fe_2(SO_4)_3$	0·00
0·1 M $CuSO_4$	0·00
0·1 M $CaCl_2$	1·98
0·1 M $MnCl_2$	0·63

[a] The potential step programming is done from 0 to $-1·0$ V. The data collected is an average of 25 worms. The EWE is stable after the experiment. The earthworm is not visually damaged. The relative photonic output is calculated with reference to the output in 0·1 M K_2SO_4.

(b) the reduction potential of Fe^{3+} is +0·46 V vs SCE and the reduction of this ion will precede O_2 reduction at the EWE. The effect of Fe^{3+} on EWE output is more complex; the presence of Fe^{3+} in bioluminescent *in vitro* reaction does not affect the emission intensity. Hence the enzyme in activation of the reaction (1) by specific binding of Fe^{3+} or Fe^{2+} can be conclusively ruled out. With iron salts, Haber–Weiss reaction

$$H_2O_2 + O_2^- \rightarrow OH^{\cdot} + OH^- + O_2 \tag{19}$$

is also likely to occur; in this situation the EBL reaction will have to be considered as occurring as follows

$$O_2 + e \rightleftarrows O_2^-, \tag{20}$$

$$2O_2^- + 2H^+ \rightarrow 2HO_2 \rightarrow H_2O_2 + O_2 \tag{21}$$

or

$$O_2 + 2e \rightarrow O_2^{--} + 2H^+ \rightarrow H_2O_2\,. \tag{22}$$

The rate of formation of H_2O_2 in mitochondria (Boveris and Turrens 1980) reflects the rate of univalent reduction of oxygen and it has been demonstrated to reach 15% of the total O_2 consumption (Boveris and Turrens 1980). Hence the presence of Fe^{3+} in the medium appears to inactivate the displacement of coelomic cells on the EWE. For similar reasons Cu^{2+} also inhibits the EBL output.

2.3 *Correlation of the Faradaic current and EBL*

The Faradaic current during EBL has been shown to have a quantitative correlation to the photons liberated (Limaye and Santhanam 1986a); this produced a correlation of electron input to the photon output. Figure 2 shows the results obtained with EWE in a K_2SO_4 electrolyte. The linearity observed in this correlation suggests the absence of other biological stimulii on this electrode. It is noteworthy that in none of the experiments with EWE has there been an output of one photon per electron input. Considering the first step in EBL as oxygen reduction (initiator of bioluminescence) consumption of 2*e* would be expected. The observed correlation here suggests there are other side reactions operating along with the above reaction. Nevertheless in the above reaction a maximum efficiency of 50% is achieved for the best EWE prepared in our experiments.

The general current decay pattern of a species in solution undergoing an electrode reaction by a process of diffusion control, is defined by (Bard and Faulkner 1980)

$$i_f = nFAD^{1/2}C^*/(\pi^{1/2}\, t^{1/2}), \tag{23}$$

and the current decaying pattern at an EWE markedly deviates from the above definition because of significant convection at the electrode due to the orderly movement of the earthworm. This aspect is reflected in the current-time curve shown in figure 3. The extent to which these oscillations occur in a slow sweep electrochemical experiment (such as cyclic voltammetry) is too small for its

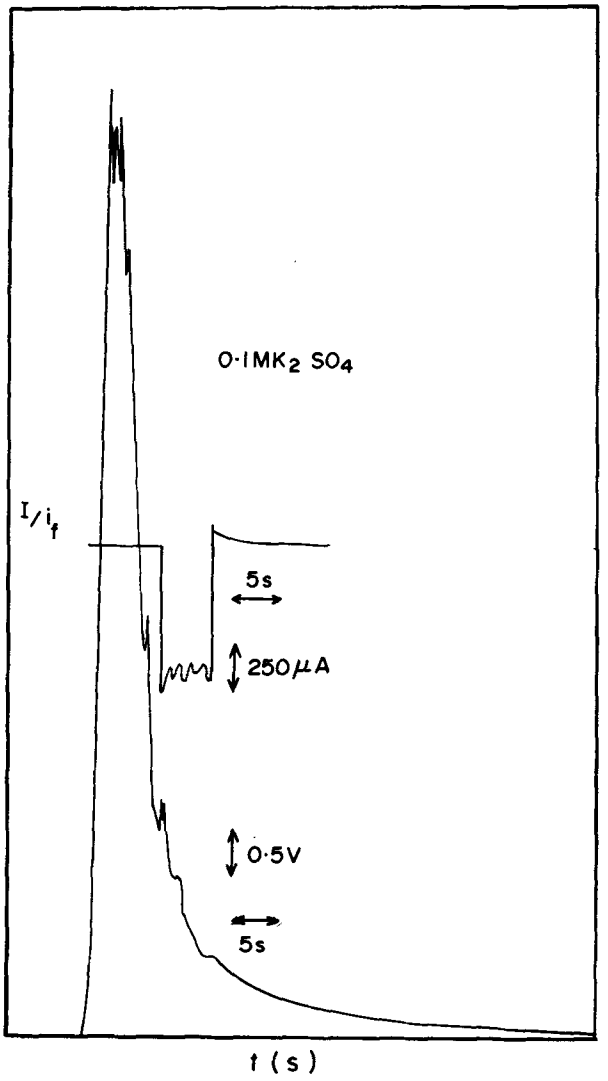


Figure 3. Current-time curve (at the centre) for potential step electrolysis of the *Lampito mauritii* electrode in 0·1 M K_2SO_4 media. Step potential 0 to −1·0 V vs SCE. i_f represents the current. Intensity-time curve during the above potential step. I = intensity.

observations by the slow perturbation techniques. Nevertheless the electron input to photon output correlations discussed earlier would be significant.

2.4 *Experiments with dissected EWE*

The data on the distribution of coelomocytes in the EWE have been obtained by using EBL. Here the major question that has arisen is whether the coelomocytes are displaced to the surface of the EW uniformly or there are marked regional differences. Four types of experiments have been performed (Limaye and Santhanam 1986a), using a) dissected *pre-clitellar* EWE, b) dissected *post-clitellar*

EWE, c) dissected *pre-clitellar* EWE, and d) undissected *post-clitellar* EWE. Figure 4 shows the differences in the EBL outputs of these electrodes. A significant feature of these experiments has been that a *post-clitellar* electrode always produces an order of magnitude higher photonic output. This difference stems from the excretary acts in the two regions; the excretion is accomplished by a funnel and nephrostone which opens into the coelom.

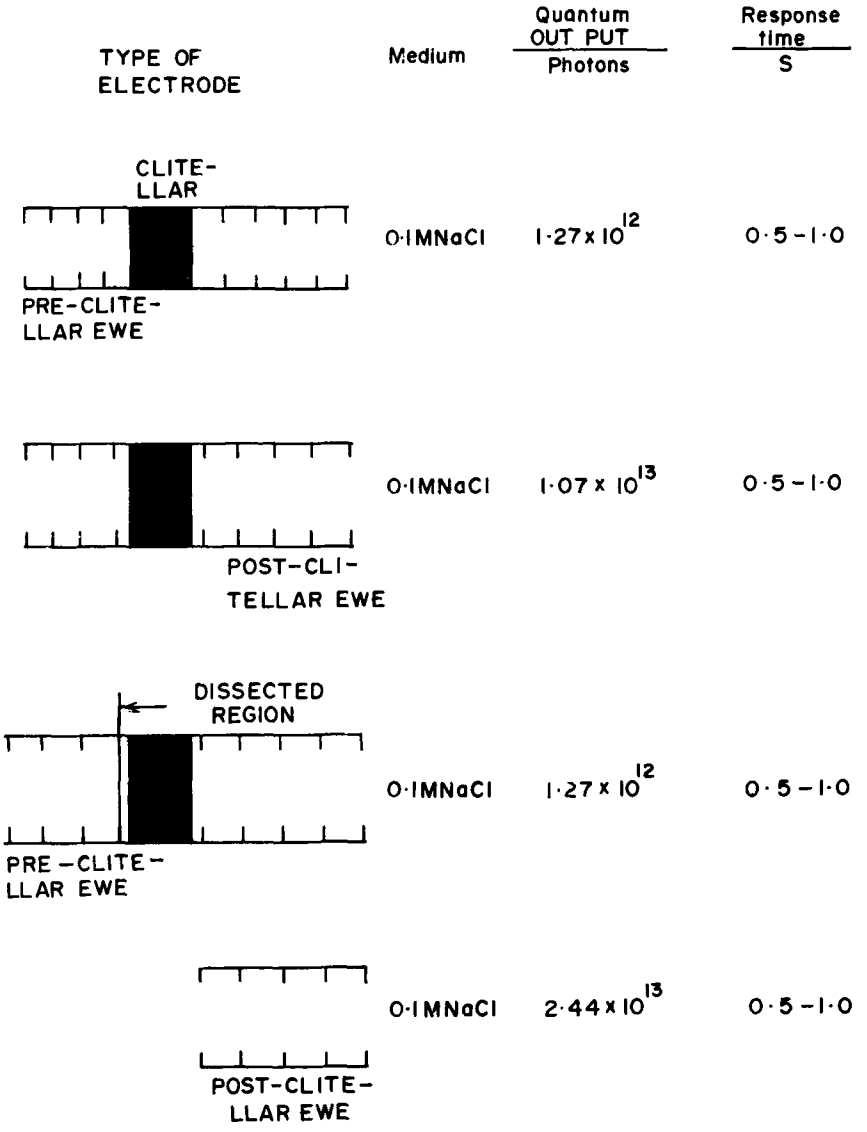
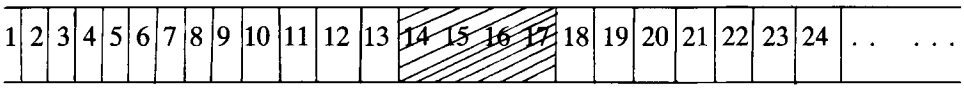


Figure 4. Representation of the output photons and response times at an EWE. The electrode type is shown diagrammatically.

The EBL of *Lampito mauritii* may be considered as segmentally displaced coelomic cells; the segmental displacement may be considered as shown below.



$$\text{The total EBL output} = \sum_{i=1}^{13} (h\nu)_i + \sum_{i=18}^{n} (h\nu)_i + \sum_{i=14}^{17} (h\nu)_i, \tag{24}$$

i refers to the segmental number. The last term contributes very little to the total output and hence is neglected and therefore,

$$I = \int_0^\infty I_1 \, dt + \int_0^\infty I_2 \, dt + \int_0^\infty I_3 \, dt + \ldots., \tag{25}$$

where I_1, I_2, $I_3 \ldots$ are considered the intensities of each of the segments and are time dependent. Since the photon is released in the biochemical reaction,

$$\frac{dI}{dt} = \left[\frac{dS}{dt}\right] \frac{(I_{max}-I)^2}{K_S I_{max}}, \tag{26}$$

and when I reaches zero the above expression simplifies to

$$\frac{dI}{dt} = \left[\frac{dS}{dt}\right] \frac{I_{max}}{K_S}, \tag{27}$$

where S refers to substrate concentration and K_S is Michaelis constant. The above discussion shows the segmental displacement when it occurs in EBL, the microscopic analysis shows the variance in the emission intensity distribution. However, from the above results it is clear that

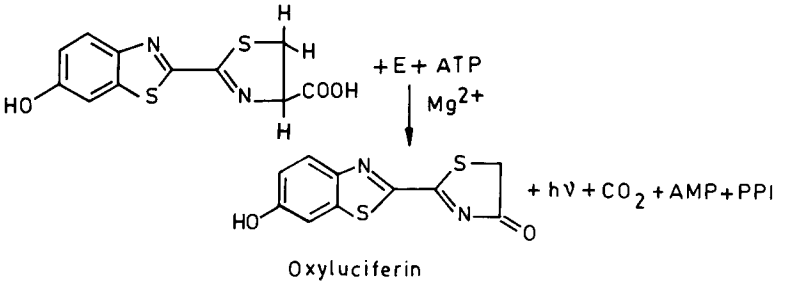
$$\sum_{i=1}^{13} (h\nu)_i < \sum_{i=18}^{n} (h\nu)_i, \tag{28}$$

and furthermore $\sum_{i=18}^{n} (h\nu)_i$ in the dissected EWE is nearly the same (factor of two difference) as the live *post-clitellar* EWE. The electrolyte consumption by the EWE (through the mouth) does not result in the sustained production of coelomic cells; if it is so then the production rate is small. Consequently, it follows that the EBL of the EWE arises from the *post-clitellar* region. A closer examination of the current-time response at an EWE shows negligible Faradaic current flowing upon reversal of the potential setp (i.e. 0 V) when a *post-clitellar* EWE is used; with the *pre-clitellar* electrode it is significant.

3. Development of a firefly electrode

The larvae of the firefly *Pteroptyx malaccae* generate pulsed light emission with $\lambda = 530$ nm at 500 msec intervals. The emission features are represented by a short

intensity spike followed by a large intensity spike in the ratio of 1:2. The development of a firefly electrode has generated data on the factors affecting the emission intensity. Unlike in the case of an EWE where the application of a voltage causes the electrode to glow for a fixed period of time, the emission intensity is reduced in the firefly electrode. The recovery of the original emission occurs after about 8·0 sec. The interesting aspect of this electrode is its sensitivity to adaptability for an electrolytic medium. With $CaCl_2$ as the electrolyte, the emission intensity decreases slowly and after about 138 sec, the intensity reaches very low levels; at the end of the experiment the firefly is not destroyed (cf. the effect of $CaCl_2$ with the earthworm). The Ca^{2+} appears to inhibit the generalized firefly reaction



(29)

Chart 7.

probably by binding specifically to E. The first step in the above reaction is the enzyme-bound luciferyl adenylate and pyrophosphate (PPi). Preliminary investigations have shown that in EBL, oxygen is reduced causing a lower photonic output. Currently investigations are in progress to correlate the photonic output with Faradaic current flow and for a fuller understanding of the reaction mechanism. It appears from the results that oxygen is a component of the reaction (29) and that peroxide intermediate formation is less likely for the generation of the excited state.

4. Conclusions

The development of EBL has generated results for a mechanistic understanding of bioluminescent reaction schemes. A significant feature of this method is the *in situ* probing of the excited state formation. With the earthworm electrode, it has been conclusively shown that ions like Fe^{2+} or Fe^{3+} inhibit the bioluminescent reaction; this does not occur when the bioluminescent reaction is carried out with the isolated chemiluminescent material. Furthermore, the enhancement of the emission of radiation with EWE in the presence of Ca^{2+} follows a mechanism different from the conventional reaction mechanism operating with marine bioluminescence. It is also shown that the EBL of the earthworm electrode predominantly arises from the *post-clitellar* segments; the emission intensity is a

composite of the reactions occurring in the individual segments and is caused by the overlapping decay curves.

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