their 75% product recovery in *o*-nitrotoluene decomposition is significantly smaller than their product recovery in *p*-nitrotoluene decomposition, thus suggesting differences in mechanisms, it is possible to claim some degree of qualitative agreement. The validity of our experiments is now buttressed by the very satisfactory mass balance and the determination of rate expressions that are clearly explicable in terms of thermokinetic considerations. We have previously noted[1] our difficulty in understanding how all the *o*-methylphenyl radicals can be trapped in the laser experiments and our failure to do so using the same inhibitor as used by Gonzales and co-workers when the conditions for doing so are in fact more favorable. Aside from the possibility of experimental artifacts, the only remaining possibility is the definition of reaction conditions in the laser studies. That is, the stated temperatures are too low and the results will discriminate against the lower activation energy process. Note that aside from *o*-nitrotoluene decomposition the laser studies also led to some very novel and unexpected substituent effects. We have commented on these in our earlier paper.[1] This suggests the need for caution in the quantitative interpretation of laser pyrolysis data. In view of the recent development of the laser technique this caveat is hardly surprising. We note that in general the many advantages with regard to unambiguity in the determination of reaction mechanisms which are inherent to appropriate single pulse shock tube studies should also hold for the laser experiments. Furthermore, by the proper selection of internal standards and through variation of reaction conditions such as laser intensities, sensitizer concentrations, reaction pressures, etc., it may well be possible to factor out some of the quantitative discrepencies that we have noted.

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Reversible One-Electron Generation of 4a,5-Substituted Flavin Radical Cations: Models for a Postulated Key Intermediate in Bacterial Bioluminescence

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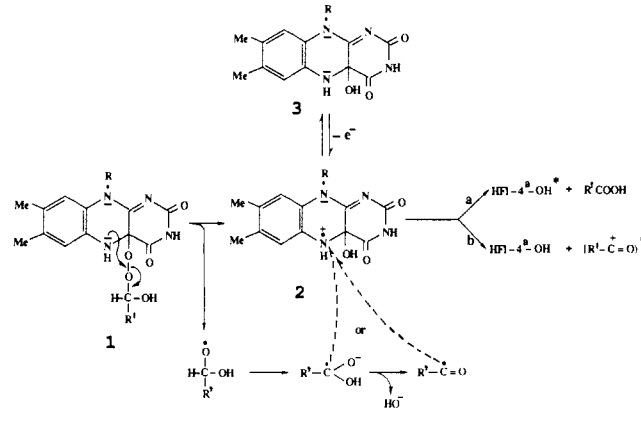
Abstract: A chemically initiated electron-exchange luminescence (CIEEL) mechanism is proposed for the bacterial bioluminescent reaction in which a 4a-hydroxyflavin radical cation is postulated to be a key intermediate. As model compounds, the electrochemical generation of 4a,5-substituted flavin radical cations from 5-ethyl-4a-hydroxy-3-methyl-4a,5-dihydrolumiflavin and 5-ethyl-4a-methoxy-3-methyl-4a,5-dihydrolumiflavin is demonstrated for the first time. These reversible one-electron oxidations were characterized in acetonitrile by cyclic voltammetry, controlled-potential coulometry, and spectroelectrochemistry. The electrogenerated radical cations can also undergo a one-electron oxidation and, after a subsequent chemical transformation, lead to the formation of a 5-ethyl-3-methyllumiflavinium cation. Both the intermediates and the final products of electrooxidation were spectrally and electrochemically identified.

The formation of a 4a-hydroperoxyflavin intermediate in the bioluminescent reaction catalyzed by bacterial luciferase has been well documented.[2-4] The flavin peroxide is proposed to react with an aliphatic aldehyde substrate to form a dihydroflavin 4a-peroxyhemiacetal **1**.[5] The decay of **1** leads to bioluminescent emission,[5,6] but the mechanism for the generation of the primary excited species is still not understood.

Schuster has elegantly demonstrated that a CIEEL (chemically initiated electron-exchange luminescence) mechanism can account for certain chemiluminescent, and possibly also for some bioluminescent, reactions.[7] Subsequently McCapra has shown that chemiluminescence derived from 10a-flavin adducts probably also follows a CIEEL mechanism.[8] Following these pioneering studies,

Scheme I



Mager and Addink have proposed a detailed CIEEL mechanism for the bacterial bioluminescence reaction (Scheme I).[9] Starting from dihydroflavin 4a-peroxyhemiacetal **1**, the first step is postulated to be an intramolecular one-electron rearrangement to give a 4a-hydroxyflavin radical cation **2** [(HFl-4a-OH)$^{\cdot+}$]. This latter

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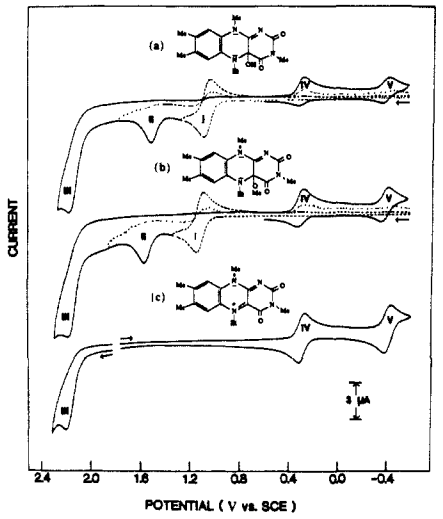


Figure 1. Cyclic voltammograms of (a) 1.05×10^{-3} M **4** (X = OH), (b) 1.0×10^{-3} M **4** (X = OMe), and (c) 8.4×10^{-4} M **7** in acetonitrile containing 0.1 M TBAP. Scan rate 0.1 $V \cdot s^{-1}$.

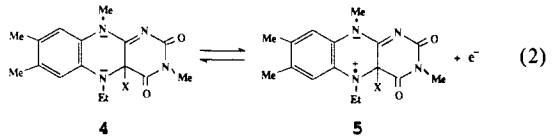
species is proposed to be a key intermediate in an electron back-transfer reaction leading either to an excited flavin pseudobase (pathway a) or to an excited acid or acylium cation (pathway b).[9]

Following the general CIEEL mechanism as proposed by Schuster,[7] the efficiency of a chemiluminescent reaction is primarily regulated by the step of one-electron transfer from an activator to an acceptor such as a peroxide. Furthermore, the rate constant of the limiting step is a function of the one-electron oxidation potential for the conversion of the activator to the corresponding activator radical cation. For Scheme I, the rate-limiting step would be the intramolecular one-electron transfer of compound **1** to form the flavin radical cation **2**. The redox potential of this reaction cannot be easily determined. The 4a-hydroxyflavin radical cation **2** should also be obtainable from the 4a-hydroxyflavin pseudobase **3** (HFl-4a-OH) by the loss of one electron (eq 1). As an approximation, the redox potential of the

$$\underset{\mathbf{3}}{\text{HFl-4a-OH}} \rightleftharpoons \underset{\mathbf{2}}{\text{(HFl-4a-OH)}^{\bullet+}} + e^- \qquad (1)$$

2/**3** couple should provide a thermodynamic indication as to how easily the flavin radical cation **2** can be generated. However, direct evidence for this one-electron redox reaction (eq 1) has never been demonstrated. This is primarily due to the instability of **3**,[4,5b] and most likely also that of **2**.

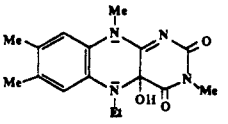
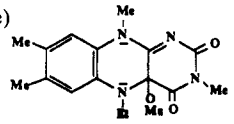
The N^1- or N^5-alkylated derivatives of **2** are expected to be less reactive than **2**, and model studies of N^1-alkylated flavins and 5-ethyl-4a-hydroxy(or methoxy)-3-methyl-4a,5-dihydrolumiflavin **4** have indeed provided evidence for the occurrence of a one-electron-transfer reaction yielding, respectively, 1,10a- and 4a,5-substituted flavin radical cations.[9-12] The interconversion of **4** to **5** is given by eq 2.



4 **5** (2)

In this report, the **4**/**5** redox couple was chosen as a model for **3**/**2**. This study provides unambiguous evidence for the reversible one-electron oxidation of **4** (X = OH) and **4** (X = OMe) (cf. **4** $\rightleftharpoons$ **5** + e^-). Thermodynamic half-wave potentials of these reactions and UV–visible spectra of the 4a,5-substituted flavin radical cations **5** are determined for the first time.

Table I. Initial Oxidation Potentials (V vs SCE) of the Flavin Derivatives **4** (X = OH, OMe) in Acetonitrile

compd	structure	$E_{1/2}$	E_p[a]
4 (X = OH)		1.05	1.50
4 (X = OMe)		1.11	1.59

[a] Scan rate 0.1 $V \cdot s^{-1}$.

Results and Discussion

Cyclic voltammograms of **4** (X = OH) and **4** (X = OMe) in acetonitrile containing 0.1 M TBAP are shown in Figure 1a,b. No oxidation/reduction processes of the compounds are observed on initial scans between −0.6 and +0.9 V vs SCE, but each voltammogram shows three oxidation processes at potentials positive of 0.9 V. These are labeled I–III. Moreover, after scanning to potentials positive of the second oxidations, two new redox couples (labeled as processes IV and V) appear on the reverse potential scan. These two processes are due to reactions involving products of the second electrooxidation (process II) and are not observed upon initial potential scans between −0.6 and +1.35 V (dashed line, Figure 1).

The first oxidation of each compound is reversible when the potential scan is reversed at 1.35 V (dashed line, Figure 1) and occurs at $E_{1/2}$ = 1.05 V for **4** (X = OH) and 1.11 V for **4** (X = OMe). These potentials are listed in Table I. The 60 ± 5 mV peak potential separations for $|E_{pc} - E_{pa}|$ and $|E_p - E_{p/2}|$ are in agreement with theoretical values for a reversible one-electron diffusion-controlled process.[13] Controlled-potential electrolysis at 1.30 V also gives a coulometric value of 1.0 ± 0.1 electrons abstracted in each oxidation and generates the 4a,5-substituted flavin radical cations **5** (X = OH) and **5** (X = OMe) as shown in eq 2.

Process II occurs at E_p = 1.50 V for **4** (X = OH) and at E_p = 1.59 V for **4** (X = OMe) and can be ascribed to the one-electron oxidation of **5** (X = OH, OMe) to give a flavinium dication **6** (X = OH, OMe). The voltammograms in Figure 1a,b are consistent with a fast chemical transformation following the generation of **6** in process II. This chemical reaction is proposed to be the formation of the flavinium cation **7** from **6**. Thus, process II is irreversible once the potential had been scanned positively from −0.6 to +1.8 V. The flavinium cation **7** can also be formed from a spontaneous decomposition of **5**,[11] but this pathway for the generation of **7** is relatively slow.

The cyclic voltammograms of **4** shows a third oxidation if the potential scan is swept to more positive potentials after the irreversible oxidation process II. This oxidation (process III in Figure 1a,b) occurs at E_p = 2.20 V for both **4** (X = OH) and **4** (X = OMe). We propose that this reaction involves the conversion of **7** (generated primarily from **6** formed in process II) to a flavinium dication radical **10**.

Two reversible one-electron reductions are observed at $E_{1/2}$ = 0.29 V (process IV) and $E_{1/2}$ = −0.41 V (process V) when the scan is swept in a negative direction after process II. These reactions are attributed to the reversible reduction of **7** to yield the flavosemiquinone **8** and the 1,5-dihydroflavin **9**. Under these conditions the overall oxidation/reduction of **4** (X = OH, OMe) is given by Scheme II where roman numerals I–V refer to the electrode reactions in Figure 1.

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Scheme II

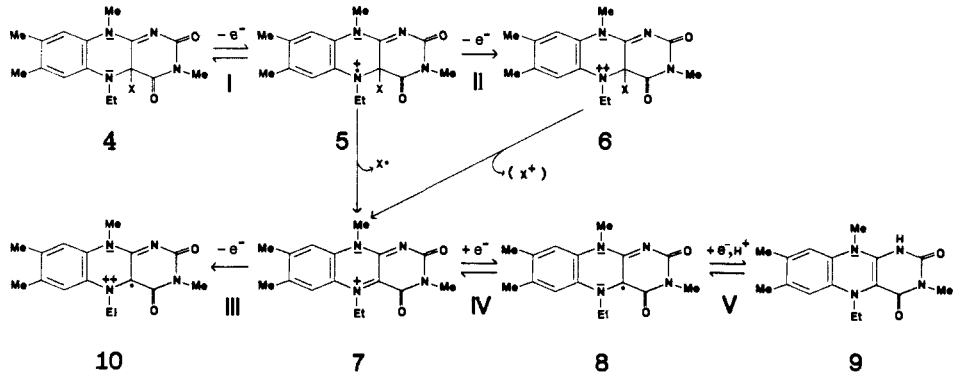
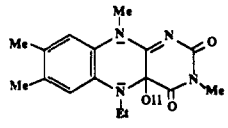
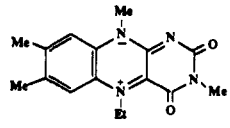
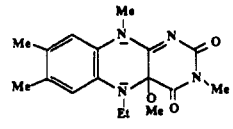
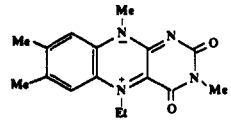
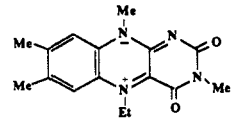
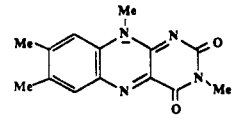
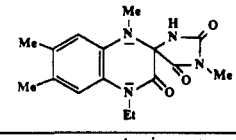


Table II. Oxidation and Reduction Potentials (V vs SCE) of Electrogenerated Flavin Products and Reference Compounds in Acetonitrile

init compd	electrode reactn	product	electrochemistry: oxidn E_p[a]	electrochemistry: redn $E_{1/2}$	
	2e oxidn[b]		2.20	0.29	−0.41
	2e oxidn[b]		2.20	0.29	−0.41
	none		2.20	0.29	−0.41
	none		1.83	−0.83	−1.86
	none		0.99[c] 1.55		

[a] Scan rate 0.1 V·s^{-1}. [b] Products generated in solution after electrooxidation at potentials greater than 1.3 V (see Figure 1). [c] $E_{1/2}$.

The electrochemical conversion of **7** → **8** → **9** was verified by cyclic voltammetric measurements carried out on an authentic sample of **7**. As shown in Figure 1c, **7** undergoes an irreversible oxidation (process III) at E_p = +2.20 V and two reversible one-electron reductions (processes IV and V) at $E_{1/2}$ = +0.29 and −0.41 V. All three potentials are identical with those for the compound generated after electrooxidation of **4** (X = OH, OMe) (Table II; Figure 1).

It should be noted that the cyclic voltammogram of **7** in dimethylformamide has been reported[14] to give a quasi-reversible oxidation at E_p = 1.08 V ascribed to the oxidation of **7** to **10**. We believe that this reaction corresponds to the oxidation of **4** (X = OH) to **5** (X = OH) as occurs in acetonitrile. We speculate that **7** can be rapidly converted to **4** (X = OH), possibly by reacting with traces of H_2O and/or basic impurities present in dimethylformamide. This is supported by the observation that the redox process I is not detected for **7** in acetonitrile (Figure 1c). Our hypothesis is further supported by the cyclic voltammograms of **7** and authentic **4** (X = OH) in dimethylformamide, both of which show a reversible oxidation at $E_{1/2}$ = 1.05 V. In this connection, the anodic wave reported to occur at $E_{1/2}$ = 1.07 V for 5-ethyl-4a-hydroperoxy-3-methyl-4a,5-dihydrolumiflavin in dimethylformamide[14] may well be associated with the oxidation

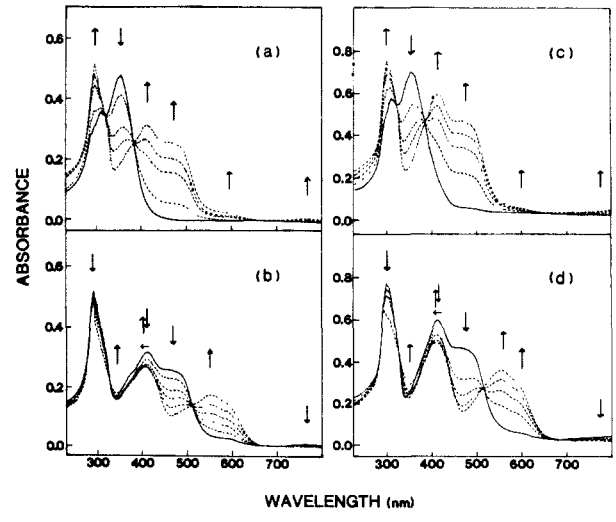


Figure 2. Electronic absorption spectra taken during controlled-potential oxidation of **4** (X = OH) and **4** (X = OMe) at 1.3 V in acetonitrile containing 0.2 M TBAP. Spectra were taken (a) during the first 14 s and (b) from 14 to 145 s for the controlled-potential oxidation of **4** (X = OH) and (c) during the first 9 s and (d) from 9 to 100 s for the controlled-potential oxidation of **4** (X = OMe). The initial spectra are shown as solid lines whereas the intermediate and final spectra are shown as dashed lines.

(14) Nanni, E. J.; Sawyer, D. T.; Ball, S. S.; Bruice, T. C. *J. Am. Chem. Soc.* **1981**, *103*, 2797.

of **4** (X = OOH) to **5** (X = OOH) rather than with the conversion of **7** to **10**.

Time-resolved electronic absorption spectra obtained during controlled-potential electrolysis of **4** (X = OH) at 1.3 V are shown in Figure 2a,b. These spectral results further confirm the chemical[11] and electrochemical conversions (this work) of **4** (X = OH) → **5** (→ **6**) → **7**. The original absorption band of **4** (X = OH) at 351 nm (ϵ = 9600 M^{-1} cm^{-1}, in acetonitrile, 0.2 M TBAP) decreases in intensity during the first 14 s of oxidation, while the shoulder at 307 nm increases and shifts to 298 nm. At the same time new bands grow in intensity at 412 and 470 nm (Figure 2a). The final spectrum shown in Figure 2a is ascribed to the radical cation **5** (X = OH). This product reaches a maximum concentration about 12 s after which the absorption bands at 298, 412, and 470 nm begin to decrease and a new band at 553 nm gradually appears (Figure 2b). Two isosbestic points at 323 and 509 nm are observed, and the final spectrum is identical with the known spectrum of **7**.[11]

Similar spectral changes are obtained during controlled-potential oxidation of **4** (X = OMe) at 1.3 V (Figure 2c,d). After electrolysis of **4** (X = OMe) for 9 s the bands of the radical cation **5** (X = OMe) at 298, 412, and 470 nm reach their maximum while the initial band at 351 nm (ϵ = 9200 M^{-1} cm^{-1}) disappears (Figure 2c). The final spectrum is obtained after 100 s and again shows the characteristic absorption band of **7** at 553 nm (Figure 2d).

The final absorption band at 553 nm in Figure 2b,d represents a yield of **7** (ϵ_{553} = 9000 M^{-1} cm^{-1})[11] at about 40–50%. An equilibrium between **7** and **4** (X = OH) is known to exist[12] (eq 3). Under our experimental conditions this reaction is shifted to the right for an initial acetonitrile solution of **4**.

$$7 + H_2O \rightleftharpoons 4\ (X = OH) + H^+ \qquad (3)$$

Exhaustive bulk controlled-potential electrolyses of **4** (X = OH, OMe) was carried out at 1.3 V in order to determine whether the yield of **7** is lowered by a reaction with H_2O in the acetonitrile. The final solution contained **7** at about 60% yield after the one-electron oxidation of 4. Acidification of the final reaction mixture did not show any additional formation of **7**, indicating that **4** was no longer present in the sample. Therefore, the reaction of **7** with H_2O for a reconversion to **4** cannot account for the less than stoichiometric yield of **7**.

Control experiments under identical conditions using solutions with known initial concentrations of **7** show that the 40% loss of **7** is not due to photochemical degradation. We speculate that other chemical conversions can occur and apparently yield one or more products that do not absorb in the visible region. Possible examples are ring-contracted derivatives like the C^{10a}-spirohydantoin; their formation from N^5-derivatized flavins has been previously demonstrated.[10a] The C^{10a}-spirohydantoin shows oxidation potentials close to those of **4** (X = OH, OMe) but does not exhibit any reductions between –0.6 and 0.9 V vs SCE (Table II).

Cyclic voltammetry of authentic 3-methyllumiflavin (Table II) also demonstrates that N^5-dealkylation does not take place during the electrochemical oxidation of **4** (X = OH, OMe).

In conclusion, we propose that **2** is a key intermediate for the formation of excited species in the bacterial bioluminescent reaction following a CIEEL mechanism (Scheme I). We believe that the **5**/**4** couple is a useful and relevant model of **2**/**3** for mechanistic investigations. In a different but closely related system, McCapra has used 10a-hydroxyflavin pseudobase as a model for bacterial bioluminescent reaction.[8] Bruice and colleagues have also successfully used 5-ethyl-4a-hydroperoxyflavins as a model to probe bacterial bioluminescence.[4] In their studies, the N^5-ethylated derivative of **1** would be formed from 5-ethyl-4a-hydroperoxyflavin and aldehyde. We propose that the decay of this N^5-ethylated derivative of **1** leads to the formation of **5**, and chemiluminescence occurs following a mechanism similar to the one shown in Scheme I. In fact, we have been able to initiate chemiluminescence in benzene from chemically generated **5** using dibenzoyl peroxide as an electron donor.[12]

The present work has been aimed to establish the existence of **5** as a new flavin radical species and to characterize it with respect to redox and spectral properties. The electrochemical results clearly show the first generation of **5** (X = OH, OMe) by a clean quantitative reversible one-electron transfer from **4**. Thus, these results provide the first unambiguous characterization of flavin redox systems relevant to bacterial luminescence following a radical mechanism. In this connection, a recent report concerning bacterial bioluminescence is also in favor of a CIEEL radical mechanism.[15] The reported supporting evidence is based on an apparent correlation of bacterial bioluminescence efficiencies with the redox potential of a series of flavin derivatives. However, the potentials do not refer to the redox systems as exemplified by eq 1 or 2, but rather to the "normal" flavin redox systems such as **7** ⇌ **8** ⇌ **9** (Scheme II).

Experimental Section

Instrumentation. Conventional and thin-layer cyclic voltammetric measurements were carried out with an IBM Model EC 225 voltammetric analyzer using a three-electrode system. Current–potential curves were recorded with a Houston Instruments Model 9002 A X-Y recorder. A Pt button served as a working electrode and a Pt wire as a counter electrode for obtaining conventional voltammograms. A saturated calomel electrode (SCE), which was separated from the bulk of the solution by a fritted-glass disk, was used as the reference electrode. A Princeton Applied Research Model 173 potentiostat was used for controlled-potential electrolysis and controlled-potential coulometry. Thin-layer cyclic voltammograms were carried out in the dark.

UV–visible spectra were recorded on an IBM 9430 spectrophotometer, and time-resolved spectroelectrochemical measurements were performed with a Tracor-Northern 6500 analyzer coupled with an IBM 225 voltammetric analyzer. An optically transparent platinum thin-layer electrode was used for the spectroelectrochemical experiments.[16] All electrochemical and spectroelectrochemical measurements were taken under an Ar atmosphere.

Chemicals and Reagents. 5-Ethyl-4a-hydroxy-3-methyl-4a,5-dihydrolumiflavin (**4**, X = OH), 5-ethyl-4a-methoxy-3-methyl-4a,5-dihydrolumiflavin (**4**, X = OMe), 5-ethyl-3-methyllumiflavinium (**7**) perchlorate, 3-methyllumiflavin, and the C^{10a}-spirohydantoin derived from **4** (X = OH) were prepared as described in the literature.[10b,11]

Acetonitrile was purchased from Mallinkrodt Inc. (water content 0.02%) and Aldrich Chemical Co. (water content <0.005%). Identical results were obtained when experiments shown in Figure 1 were carried out with use of acetonitrile from either of the two sources. Tetrabutylammonium perchlorate (TBAP) was purchased from Fluka Chemical Co, twice recrystallized from ethyl alcohol, and dried in a vacuum oven at 40 °C.

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