**Enhancer Effect of Fluorescein on the Luminol–H$_2$O$_2$–Horseradish Peroxidase Chemiluminescence: Energy Transfer Process**

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The chemiluminescence of the luminol–H$_2$O$_2$–horseradish peroxidase system is increased by fluorescein. Fluorescein produces an enhancement of the luminol chemiluminescence similar to that of phenolphthalein, by an energy transfer process from luminol to fluorescein. The maximum intensity and the total chemiluminescence emission (between 380 and 580 nm) of luminol with fluorescein was more than three times greater than without fluorescein; however, the emission duration was shorter. The emission spectra in the presence of fluorescein had two maxima (425 and 535 nm) and the enhancement was dependent on pH and fluorescein concentration. A mechanism is proposed to explain these effects. © 1997 John Wiley & Sons, Ltd.

**INTRODUCTION**

Certain xanthene dyes, such as fluorescein, eosin Y, 4',5'-dibromofluorescein, phloxin B, 2',7'-dichlorofluorescein, erythrosin B and Rose Bengal produce chemiluminescent emission with hydrogen peroxide and horseradish peroxidase, in a buffer at pH 7.0 (1). Segawa et al. proposed a mechanism to explain these phenomena (2,3), in which fluorescein forms fluorescein radicals which react with oxygen to form singlet oxygen. The singlet oxygen transfers energy to fluorescein, which emits around 535 nm.

It is known that there is a chemiluminescent energy transfer from luminol to fluorescein. The chemiluminescence of this system shows two maxima of emission, one around 425 nm and another around 525 nm (4–6). Different mechanisms have been proposed to explain these observations: physical energy transfer (4–6) and chemical reactions between luminol and fluorescein involve the transfer of a peroxide from luminol to fluorescein (4) or an interaction between luminol radicals and fluorescein (5).

Numerous phenol derivatives enhance luminol–H$_2$O$_2$–horseradish peroxidase chemiluminescence (7–12). The spectra of the unenhanced and enhanced chemiluminescence are similar (7,8). This is because the chemiluminescent emission is from aminophthalate, which emits around 425 nm, and the enhancers simply accelerate the reaction. A mechanism has been proposed to explain these phenomena (13–15).

The maximal intensity of chemiluminescence observed for luminol in presence of fluorescein was about 100 times less than the maximal intensity of chemiluminescence of luminol with $p$-iodophenol emission.
(the most important enhancer known). Fluorescein is a weak enhancer, but produces two effects, an enhancement of the chemiluminescence of luminol with horseradish peroxidase, and an energy transfer from luminol to fluorescein.

This paper reports a study of the enhancer effect of fluorescein on the chemiluminescence of luminol with hydrogen peroxide and horseradish peroxidase (HRP) at pH 7.5–8.4. We propose a mechanism to explain these enhancer effects and energy transfer phenomena.

MATERIALS AND METHODS

Chemiluminescence experiments were performed in a Perkin-Elmer LS-50 (Beaconsfield, UK) luminescence spectrometer. The instrumental parameters were controlled by the Fluorescence Data Manager (FLDM) software (Perkin-Elmer). The light source was switched off and to detect light, the apparatus was set in the phosphorescence mode with a 0.00 ms delay-time and 60 ms gate-time or period of light observation. The photomultiplier voltage was set manually to 750 V. The slit-width of the emission monochromator was adjusted to maximum (20 nm) in order to detect the maximum radiant energy. The samples were placed in a quartz cuvette continuously stirred with a magnetic stirrer. The chemiluminescent reaction was triggered by injecting horseradish peroxidase with a syringe, through a septum.

The chemiluminescence spectra were recorded at wavelengths between 380 and 580 nm. The areas under these spectra were taken as the total chemiluminescence produced by the reaction. Chemiluminescence intensities against time curves were obtained by recording the chemiluminescence emitted during a time period at fixed wavelength. The emission of luminol without fluorescein was recorded at 425 nm, and the emissions of luminol with fluorescein at 425 nm and 535 nm respectively.

Fluorescence spectra were recorded in the Perkin-Elmer luminescence spectrometer or/and in SLM Aminco 48000S fluorometer (Urbana, IL).

Reagents

All solutions were prepared in distilled and demineralized water. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was prepared by dissolving 0.0913 g of luminol 97% from Sigma (St. Louis, MO, USA) in a small amount of NaOH and diluting to 50 mL with Tris-HCl buffer pH 8.4. Horseradish peroxidase 71.28 U/mL was prepared by diluting horseradish peroxidase Type VI-A (Sigma, 1100 U/mg) in Tris-HCl buffer pH 8.4. Hydrogen peroxide was prepared by diluting 2834 µL of hydrogen peroxide 6% w/v from Panreac (Montplet and Esteban S.A., Barcelona, Spain) and diluting to 50 mL with water. Fluorescein (3',6'-Dihydroxyspiro[isobenzofuran-1 (3H)-9H-xanthen]-3-one) 10 mmol/L was prepared by dissolving 0.094 g of fluorescein disodium (E. Merck AG, Darmstadt, Germany) in 25 mL of water.

Experimental procedure

Solutions of Tris-HCl buffer (1 mL, 0.1 mol at pH 7.5 or 8.4), luminol (20 µL, 0.01 mol), hydrogen peroxide (60 µL, 0.1 mol) and fluorescein disodium (different concentrations in cuvette), were pipetted into a quartz cuvette; this was filled to 2950 µL with distilled and demineralized water. The chemiluminescence reaction was triggered by injecting 50 µL of horseradish peroxidase (71.28 U/mL) with a syringe, through a septum. Immediately after this injection, the chemiluminescence intensity was scanned at wavelengths between 380 and 580 nm (Figs 1 and 2) and...
the areas under these chemiluminescence spectra were measured. Furthermore, the kinetics of light emission between 0 and 300 s were measured at both \( \lambda_{\text{em}} = 425 \) nm and \( \lambda_{\text{em}} = 535 \) nm, respectively.

The fluorescence spectra were obtained from a solution containing Tris-HCl buffer (1 mL, 0.1 mol/L at pH 8.4), luminol (20 \( \mu \)mol/L, 0.01 mol/L), hydrogen peroxide (60 \( \mu \)mol/L, 0.1 mol/L) and fluorescein di-sodium (different concentrations in cuvette); this cuvette was filled to 2950 \( \mu \)L with distilled and demineralized water. The chemiluminescence reaction was triggered by injecting 50 \( \mu \)L of horseradish peroxidase (71.28 U/mL). After reacting for 16 h the fluorescence spectra of this mixture were measured (\( \lambda_{\text{exc}} = 303 \) nm, wavelength of excitation of 3-aminophthalate).

**RESULTS AND DISCUSSION**

Fluorescein is a fluorescent molecule with a high quantum yield and a structure similar to phenolphthalain. Recently phenolphthalein has been found to be an enhancer of the luminol–H\(_2\)O\(_2\)–horseradish peroxidase chemiluminescence (10) by a mechanism similar to other phenol derivatives. Fluorescein is also an enhancer of this chemiluminescence, increasing the total chemiluminescence emission (between 380 and 580 nm) by threefold, and the maximum intensity also increases more than fivefold. Under the measurement conditions, no emission was observed from the luminol–H\(_2\)O\(_2\) and luminol–H\(_2\)O\(_2\)–fluorescein systems in the absence of peroxidase at either pH 7.5 or pH 8.4.

**Energy transfer**

Figs 1 and 2 show the chemiluminescent emission of the luminol–H\(_2\)O\(_2\)–horseradish peroxidase system at pH 7.5 and pH 8.4, respectively, against wavelength at different fluorescein concentrations. When the concentration of fluorescein increases, the total emission between 380 nm and 580 nm is enhanced and also the intensity at 535 nm increases while that at 420 nm decreases. This data suggests an energy transfer mechanism involving transfer of energy from luminol (donor) to fluorescein (acceptor). The emission at long wavelength, 535 nm, with the excitation lamp switched off, suggests that fluorescein absorbed part of the excited-state luminol energy and re-emitted it at longer wavelengths. In order to confirm this hypothesis, fluorescence energy transfer in the luminol–fluorescein system was studied. The emission spectra of luminol in the presence of increasing fluorescein concentrations at pH 8.4 (after 16 h of reaction) is shown in Table 1. Fluorescence energy transfer was revealed by the quenching of the luminol fluorescence and by the appearance of the sensitized fluorescence of the fluorescein. The similarity with the behaviour of this fluorescence energy transfer process (Table 1) confirms the hypothesis of chemiluminescence energy transfer in the luminol–fluorescein system.

The enhancement of energy transfer with fluorescein concentration, observed in Figs 1 and 2, can be ascribed to an increase of overlap between donor chemiluminescence and acceptor absorption, which modifies the chemiluminescence maximum from 425 to 410 nm. Emission from the acceptor occurs at wavelengths varying from 523 to 552 nm, depending on the fluorescein concentration (Table 1); this variation in wavelength maxima can be ascribed to a concentration effect. A similar effect has been described for 3-aminophthalimide, a molecule which displays inhomogeneous broadened spectra characterized by a shift in the luminescence spectra as the exciting light frequency was varied, consistent with selective excitation of various configurational states.
of solvated molecules (16–19). For such molecules at high concentration, the luminescence decay time depends on the recording wavelength. To confirm this behaviour in the luminol–fluorescein system, the decay time of concentrated fluorescein solutions was measured at two distinct pairs of wavelengths, and the emission spectra recorded at two excitation wavelengths. Using the phase-modulation approach (20), the decay time of fluorescein (3.33 mmol/L at $\lambda_{\text{exc}} = 495$ nm, $\lambda_{\text{em}} = 520$ nm and at $\lambda_{\text{exc}} = 400$ nm, $\lambda_{\text{em}} = 535$ nm) was calculated to be 4.3 and 6.2 ns, respectively. The emission maximum of fluorescein is 518 nm ($\lambda_{\text{exc}} = 495$ nm) and is concentration-independent. However, with excitation at 400 or 303 nm, emission maxima is a concentration-dependent parameter, shifting from 525 to 550 nm, or from 523 to 552 nm, respectively, and this explains the observed acceptor shift to longer wavelengths.

### Chemiluminescence enhancement

Fig. 3 shows the chemiluminescent emission vs. time, for a sample of luminol without fluorescein, measured at 425 nm (A), and for samples of luminol and fluorescein measured at 425 (B) and 535 nm (C), respectively. Chemiluminescence enhancement by fluorescein can be observed but the emission is for a shorter duration than without fluorescein. This fact suggests that fluorescein accelerates the conversion of luminol to aminophthalate, decreasing the concentration of luminol in the media, and then the chemiluminescence decays. To demonstrate this hypothesis, mixtures of luminol (20 μL, 0.01 mol/L), fluorescein (1 mL, 2 mmol/L), $\text{H}_2\text{O}_2$ (60 μL, 0.1 mol/L), peroxidase (50 μL, 71.3 U/mL) and Tris-HCl buffer (1 mL, 0.1 mol/L at pH 7.5) were prepared, adding water up to a final volume of 3 mL. Each mixture was reacted and, when their chemiluminescent emissions were negligible, different reagents (100 μL of luminol, 100 μL of peroxidase, 100 μL of $\text{H}_2\text{O}_2$ and 100 μL of fluorescein, respectively) were added to the four mixtures. Only the luminol injection produced a significant chemiluminescence enhancement: those of $\text{H}_2\text{O}_2$, peroxidase or fluorescein only produced a negligible chemiluminescence enhancement, demonstrating that the chemiluminescence decrease was caused by the low luminol concentration.

### Table 1. Wavelengths of the luminescence emission maxima of luminol-fluorescein system at pH 8.4

<table>
<thead>
<tr>
<th>Fluorescein (mmol/L)</th>
<th>Emission wavelengths (nm) using $\lambda_{\text{exc}} = 303$ nm</th>
<th>Emission wavelengths (nm) without excitation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda_1$</td>
<td>$\lambda_2$</td>
</tr>
<tr>
<td>0</td>
<td>422</td>
<td>–</td>
</tr>
<tr>
<td>0.067</td>
<td>415</td>
<td>523</td>
</tr>
<tr>
<td>0.667</td>
<td>406</td>
<td>533</td>
</tr>
<tr>
<td>3.333</td>
<td>–</td>
<td>546</td>
</tr>
<tr>
<td>6.233</td>
<td>–</td>
<td>553</td>
</tr>
</tbody>
</table>

$\lambda_1$: Luminol emission wavelength.
$\lambda_2$: Fluorescein emission wavelength.

These observations support the hypothesis that fluorescein increases the conversion of luminol to luminol radical, and so enhances the chemiluminescence of luminol. The enhanced chemiluminescence of luminol is transferred to fluorescein, with a new emission maximum appearing around 525 nm.

Fig. 4 shows the total luminol chemiluminescence emission spectra with H$_2$O$_2$ and horseradish peroxidase for different concentrations of fluorescein at pH 7.5 and 8.4. This data confirms that at pH 7.5 and 8.4, fluorescein increases the chemiluminescent emission (between 380 and 580 nm) of luminol by more than threefold. The blank was negligible; no emission was detected from luminol with H$_2$O$_2$ without peroxidase in presence or absence of fluorescein. The emission at low fluorescein concentration was greater at pH 7.5 than at pH 8.4 because the ratio between the concentrations of fluorescein anions ([FH$^-$]:[F$^-$]) decreases with pH. However, the enhancement observed at pH 8.4 was greater for higher concentrations of fluorescein than at pH 7.5.

**Mechanism of reaction**

The mechanism shown in Fig. 5 explains the observed enhancement as follows:

1. An enhancer effect on the chemiluminescence of luminol (LH$^-$) by fluorescein radical (F$^-$) that increases the formation of luminol radical (L$^-$) in stage 6 and, after the formation of luminol endoperoxide (LO$_2^-$) and excited 3-aminophthalate (AP$^+$) that then emits around 425 nm. The steps in the mechanism shown in Fig. 5 are based on those proposed for enhanced chemiluminescence by other classical enhancers (13–15).

2. An energy transfer from excited aminophthalate (AP$^+$) to fluorescein (F$^-$) to form excited fluorescein (F$^*$) that emits around 535 nm. These proposed steps are based on previous data (4–6).

3. An energy transfer from luminol endoperoxide (LO$_2^-$) to fluorescein (F$^-$) to form a fluorescein endoperoxide (FO$_2^-$) that decomposes, emitting a luminescence and liberating oxygen in the process.

\[
\begin{align*}
\text{HRP} + \text{H}_2\text{O}_2 & \rightarrow \text{HRP-H} + \text{H}_2\text{O} \\
\text{HRP-I} + \text{LH} & \rightarrow \text{HRP-II} + \text{L}^- + \text{H}_2\text{O} \\
\text{HRP-II} + \text{LH} & \rightarrow \text{HRP} + \text{L}^- \\
\text{HRP-I} + \text{FH} & \rightarrow \text{HRP-II} + \text{F}^- + \text{H}_2\text{O} \\
\text{HRP-II} + \text{FH} & \rightarrow \text{HRP} + \text{F}^- \\
\text{LH} + \text{F}^- & \rightarrow \text{L}^- + \text{F}^* \\
\text{L}^- + \text{FH} & \rightarrow \text{L}^- + \text{FH}^* \\
\text{L}^- + \text{H}_2\text{O}_2 & \rightarrow \text{LO}_2^- \\
\text{LO}_2^- & \rightarrow (\text{AP}^*) + \text{N}_2 \\
\text{AP}^* & \rightarrow \text{AP}^0 + \text{hv} (425 \text{ nm}) \\
\text{AP}^* & \rightarrow \text{AP}^0 + \text{hv} (535 \text{ nm}) \\
\text{LO}_2^- + \text{F}^- & \rightarrow \text{LO}^* + \text{FO}_2^-
\end{align*}
\]
This emission also occurred around 535 nm. These hypothetical steps proposed by us are based on previous data (4,5).

The enhancer effect of fluorescein is more important at pH 7.5 than at pH 8.4 for the same fluorescein concentration (Figs 1, 2 and 4). To explain these phenomena it is important to consider that horseradish peroxidase is a relatively specific hydrogen acceptor. Numerous phenol derivatives can act as hydrogen donors (7,8). However, only the acidic form of fluorescein (FH⁻) can act as a hydrogen donor, but not its basic form (F²⁻). The pKa of fluorescein is 6.7, and the ratios between the concentrations of protonated forms and the non-protonated forms of fluorescein ([FH⁻]:[F²⁻]) were 1: 6 at pH 7.5, and 1:50 at pH 8.4. Peroxidase compounds (HRP-I and HRP-II) only react with the protonated forms (FH⁻) to form fluorescein radicals (F⁻) in steps 4 and 5, but not with the non-protonated forms (F²⁻).

The emission of fluorescein without luminol was not detected in our measurement conditions at pH 7.5 or pH 8.4. For this reason, the contribution of the mechanism proposed by Segawa et al. (2,3), has not been considered to explain the chemiluminescence observed in fluorescein at pH 7.0 in the presence of H₂O₂ and horseradish peroxidase but without luminol, according to:

F⁻ + O₂ → O₂ singlet + decomposed fluorescein.
F²⁻ + O₂ singlet → (F²⁻) * + O₂.
(F²⁻) * → F⁻ + hν (535 nm).

CONCLUSIONS

The main conclusions of this work may be summarized as follows:

1. Fluorescein is an enhancer of the chemiluminescence of the luminol–H₂O₂–horseradish peroxidase system by a mechanism similar to phenolphthalein and other classical enhancers of this system.
2. The enhancement of fluorescein decreases when the pH increases because the concentration of protonated fluorescein (FH⁻) decreases.
3. The enhanced chemiluminescence of fluorescein is transferred partially to fluorescein by a physical mechanism and by a peroxide transference. A part of this energy absorbed by fluorescein is re-emitted by this molecule around 535 nm.
4. The chemiluminescence of fluorescein without luminol is negligible under these conditions.
5. Inhomogeneous broadening is the main way to explain the red-shift of fluorescein emission maxima when its concentration is increased.

Acknowledgement

This work was supported by the Comisión Interministerial de Ciencia y Tecnología (projects PB93-1006 and BIO94-0548).

REFERENCES