ELSEVIER

Journal of Photochemistry and Photobiology
A: Chemistry 87 (1995) 99–103

Journal of PHOTOCHEMISTRY AND PHOTOBIOLOGY A:CHEMISTRY

Chemical indicators as enhancers of the chemiluminescent luminol–H_2O_2–horseradish peroxidase reaction

A. Navas Díaz, F. García Sánchez *, J.A. González García

Departamento de Química Analítica, Facultad de Ciencias, Universidad de Málaga, 29071 Málaga, Spain

Received 1 August 1994; accepted 27 September 1994

Abstract

The chemiluminescent emission from the luminol–H_2O_2–horseradish peroxidase system was enhanced between 2 and 80 times, during the first 5 min, by chemical indicators, such as phenolphthalein, cresolphthalein, phenol red, cresol red, benzidine and *o*-tolidine. Phenol derivatives showed emission maxima at pH 8.5, while aniline derivatives showed emission maxima at pH 10.5. The enhancers with a methyl group at the ortho or para position to the OH and NH_2 groups in the benzene ring showed an inhibitory effect when their concentrations were increased.

Keywords: Chemiluminescence; Chemical indicators; Luminol-H_2O_2-horseradish peroxidase

1. Introduction

Certain benzothiazole [1,2], phenol [3], naphthol [4] and aromatic amine [5,6] derivatives enhance the light emission from the horseradish peroxidase–luminol–H_2O_2 system. The intense, prolonged light emission is easily measured. Peroxidase and horseradish peroxidase (HRP) conjugate concentrations can be assayed sensitively in seconds. The applicability of these enhanced chemiluminescent reactions to immunoassays and enzymatic assays has been demonstrated in several cases [7–10].

Nevertheless, a large number of phenol, aniline and benzothiazole derivatives do not produce an enhancement of light emission when incorporated into the diacylhydrazide-peroxidase oxidation reaction. Substituent effects have been studied and compared in several benzothiazoles and para-substituted halophenols [4].

In this paper, we have studied the enhancing or inhibitory effects of some indicators. The majority of chemical indicators are derivatives of phenol, aniline or naphthol. The chemical indicators studied in this work were violet crystal, methylene blue, methyl yellow, phenolphthalein, phenol red, *o*-cresolphthalein, cresol red, calcon, fluorescein, *o*-tolidine and benzidine. Their effects on the chemiluminescent horseradish peroxidase–luminol–H_2O_2 system were studied. The enhanced chemiluminescence was measured at different pH values and concentrations. The effect of a CH_3 group at the ortho or para position to the OH and NH_2 groups in the benzene ring was studied and compared for benzidine and *o*-tolidine, phenolphthalein and *o*-cresolphthalein and phenol red and cresol red.

2. Experimental details

2.1. Materials

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was prepared by dissolving 0.0913 g of luminol (97%, Sigma, St. Louis, MO, USA) in a little NaOH and diluting to 50 ml with Tris-HCl buffer (pH 8.5). Horseradish peroxidase was prepared by diluting 0.0010 g of horseradish peroxidase (Sigma, 1100 U mg^{-1}) in 10 ml of Tris-HCl buffer (pH 8.5). Hydrogen peroxide was prepared by diluting 1.42 ml of hydrogen peroxide (6% w/v, Panreac, Montplet and Esteban S.A., Barcelona, Spain) and diluting to 25 ml with bidistilled water.

Fig. 1 shows the structures of the tested indicators: phenol red (4,4'-(3H-2,1-benzoxathiol-3-ylidene)bisphenol-S,S-dioxide), phenolphthalein (3,3-bis(4-hydroxyphenyl)-1-(3H)-isobenzofuranone) and violet crystal from Panreac; *o*-cresolphthalein (3,3-bis-

* Corresponding author.

SSDI 1010-6030(94)03970-4

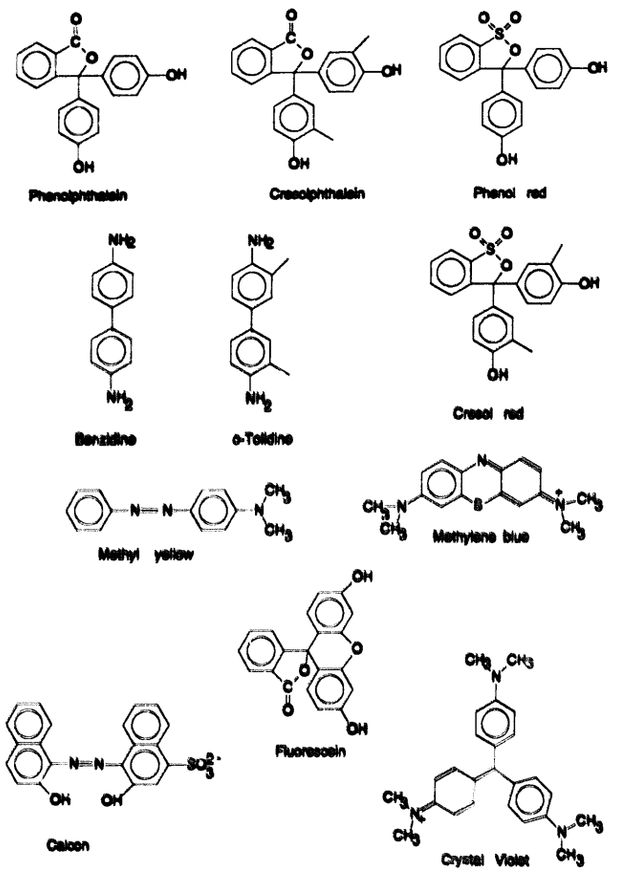

Fig. 1. Structures of the indicators studied.

(4-hydroxy-3-methylphenyl)-1-(3H)-isobenzofuranone) and cresol red ((4,4'-(3H-2,1-benzoxathiol-3-ylidene)-bis-2-methylphenol)S, S-dioxide) from UCB, Brussels, Belgium; calcon (1-(2-hydroxy-1-naphthylazo)-2-naphthol-4-sulphonic acid), fluorescein (3',6'-dihydroxyspiro[isobenzofuran-1-(3H)-9'-[9H]-xanthen]-3-one) and methylene blue (3,7-bis(dimethylamino)-phenothiazin-5-ium chloride) from E. Merck AG, Darmstadt, Germany; benzidine ([1,1'-biphenyl]-4,4'-diamine) and *o*-tolidine (3,3'-dimethyl-[1,1'-biphenyl]-4,4'-diamine) from Sigma. These solutions were prepared in bidistilled water, in ethanol (99%) or in ethanol–water mixtures (50:50 v/v) with concentrations ranging from 10^{-5} to 10^{-2} M.

2.2. *Instruments*

The chemiluminescence experiments were carried out using a Perkin–Elmer LS-50 (Beaconsfield, UK) luminescence spectrometer with the light source switched off. The apparatus was set in the phosphorescence mode with a delay time of 0.00 ms and a gate time of 60 ms. The slit width of the emission monochromator was set at 20 nm with $\lambda_{em} = 425$ nm and the photomultiplier voltage was set manually to 700 V. 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.

2.3. *Chemiluminescent reactions*

Stock solutions were prepared in bidistilled water or in water–ethanol mixtures. Solutions of Tris-HCl buffer (1 ml, 0.1 M at pH 8.3), luminol (20 μl, 0.01 M), hydrogen peroxide (60 μl, 0.1 M) and indicator (20 μl, 1 mM, in water or an ethanol–water mixture) were pipetted into a quartz cuvette; this cuvette was filled to 2950 μl with bidistilled water. The chemiluminescent reaction was triggered, 10 s after the spectrometer began to record, by injecting 50 μl of horseradish peroxidase (73 U ml^{-1}) with a syringe through a septum. The kinetics of light emission between 0 and 300 s were recorded and the areas under the emission curve were measured. The percentage of ethanol in the cuvette was below 0.5%.

2.4. *pH studies*

The pH effects were studied for the following concentrations of the enhancers: phenolphthalein (20 μl, 1 mM) in ethanol (99%); *o*-cresolphthalein (20 μl, 1 mM) in ethanol (99%); *o*-cresol red (60 μl, 0.33 mM) in water; phenol red (250 μl, 8×10^{-5} M), benzidine (20 μl, 1 mM) and *o*-tolidine (20 μl, 1 mM) in a mixture of ethanol and water (50:50 v/v). The percentage of ethanol in the cuvette was always smaller than 0.7%. The assays were carried out with 1 ml of buffer solutions in the following ranges for each enhancer: potassium dihydrogen phosphate buffer (pH 6–7.5); tris(hydroxymethyl)aminomethane buffer (pH 7.5–8.5); borax buffer (pH 8.5–10); sodium bicarbonate buffer (pH 10–11); disodium hydrogen phosphate buffer (pH 11–11.5); KCl/NaOH buffer (pH 11.5–12.5).

2.5. *Concentration studies*

We studied the effect of enhancer concentration on the chemiluminescence. The ranges of concentration for these compounds were as follows: phenolphthalein (3.33–250 μM), phenol red (2.7–50 μM), cresol red (0.22–11.1 μM), cresolphthalein (1.7–66.7 μM), benzidine (0.67–33.33 μM) and *o*-tolidine (0.67–6.67 μM).

3. Results and discussion

Fig. 1 and Table 1 show the structures and responses respectively of the tested indicators. Only the compounds with OH or NH_2 groups joined to the benzene

Table 1
Relative chemiluminescence emission (blank = 1) from the luminol–horseradish peroxidase–H_2O_2 system at pH 8.5 following the addition of different chemical indicators. Experimental conditions: [Tris-HCl] = 0.1 M (pH8.5); [Luminol] = 66.7 μM; [H_2O_2] = 2 mM; [Horseradish peroxidase] = 1.2 U ml^{-1}; [Chemical indicator] = 6.67 μM

Compound	I_{max} (relative) [a] (0–300 s)	Relative area [b] (0–300 s)
Blank	1.000 ± 0.048	1.000 ± 0.042
Crystal violet	1.083 ± 0.168	1.118 ± 0.096
Methylene blue	0.797 ± 0.042	0.648 ± 0.118
Methyl yellow	0.730 ± 0.041	0.498 ± 0.100
Phenolphthalein	1.596 ± 0.204	1.315 ± 0.042
Phenol red	4.371 ± 0.424	2.612 ± 0.049
o-Cresolphthalein	2.448 ± 0.193	1.562 ± 0.129
Cresol red	1.765 ± 0.060	1.280 ± 0.043
Calcon	2.053 ± 0.387	0.568 ± 0.090
Fluorescein	0.824 ± 0.132	0.781 ± 0.047
o-Tolidine	20.240 ± 0.423	3.783 ± 0.085
Benzidine	28.930 ± 0.004	3.407 ± 0.032

[a] Maximum intensity of chemiluminescence and standard deviation.
[b] Area beneath the chemiluminescence emission curve between 0 and 300 s and standard deviation.

ring enhance the chemiluminescence. Compounds with $N(CH_3)_2$ groups show no enhancement.

Six enhancers of the chemiluminescence from the luminol–H_2O_2–horseradish peroxidase system were studied: phenolphthalein, phenol red, cresolphthalein, cresol red, benzidine and *o*-tolidine. All of these compounds are phenol or aniline derivatives.

3.1. pH effects

Fig. 2 shows the effect of pH on the chemiluminescence for the six enhancers. All of these compounds have chemiluminescence intensity maxima at pH 8.5; nevertheless, the relative standard deviations (RSDs) of the intensity maxima without enhancer are larger than 100% at pH ⩾ 9.5. Therefore we used only the area under the emission curve during the first 5 min for the pH studies. Phenolphthalein, phenol red, cresolphthalein and cresol red have area maxima between 0 and 300 s at pH 8.5, whereas benzidine and *o*-tolidine have area maxima at pH 10.5. Fig. 3 shows these phenomena for benzidine. This behaviour can be related to the concentrations of protonated (benzidine-H_2^{2+}) and non-protonated (benzidine) forms of benzidine and *o*-tolidine at pH 8.5 and pH 10.5.

3.2. Effects of enhancer concentration

The structural difference between phenolphthalein, phenol red and benzidine vs. cresolphthalein, cresol

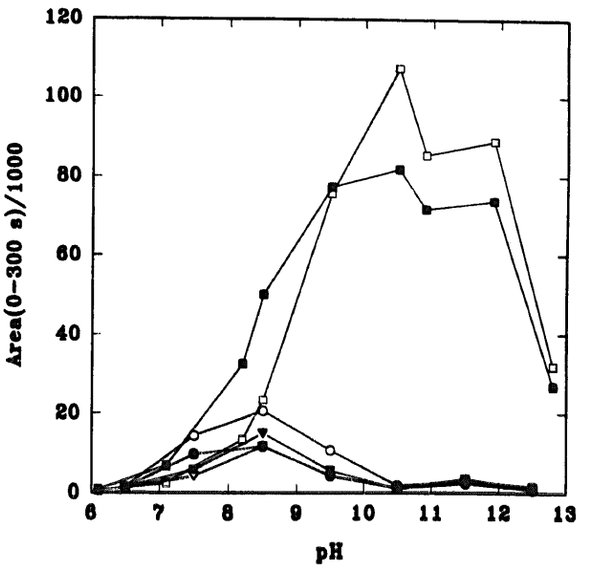

Fig. 2. Area (divided by 1000) beneath the chemiluminescence emission curve between 0 and 300 s vs. the pH, in the presence of different enhancers. Experimental conditions: [Luminol] = 66.7×10^{-6} M; [H_2O_2] = 2×10^{-3} M; [Horseradish peroxidase] = 1.2 U ml^{-1}; [Indicator] = 6.7×10^{-6} M. □, Benzidine; ■, *o*-tolidine; ●, phenolphthalein; ○, phenol red; ▼, cresolphthalein; ▽, cresol red.

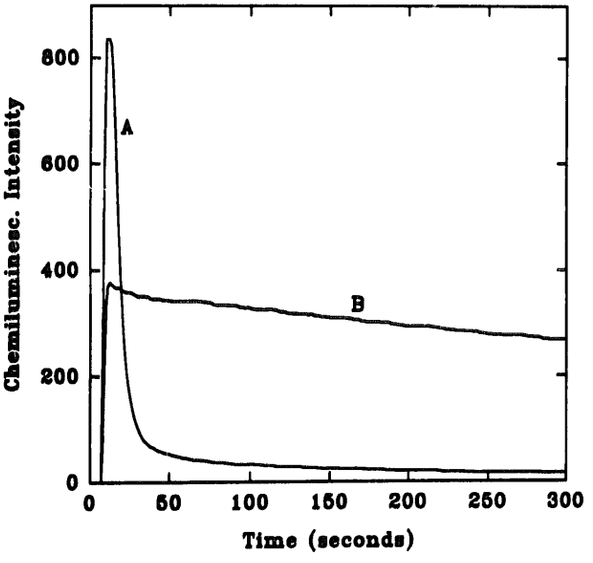

Fig. 3. Chemiluminescence intensity vs. time for the luminol–H_2O_2–horseradish peroxidase system with benzidine at different pH values. (A) Benzidine at pH 8.7. (B) Benzidine at pH 10.9.

red and *o*-tolidine is the presence of a CH_3 group in the last three compounds. This group is in the ortho position to the OH and NH_2 groups in the benzene ring. Fig. 4 shows the area beneath the emission curve vs. the enhancer concentration at pH 8.5, and Fig. 5 shows the same for benzidine and *o*-tolidine at pH 10.5. As the enhancer concentrations are increased,

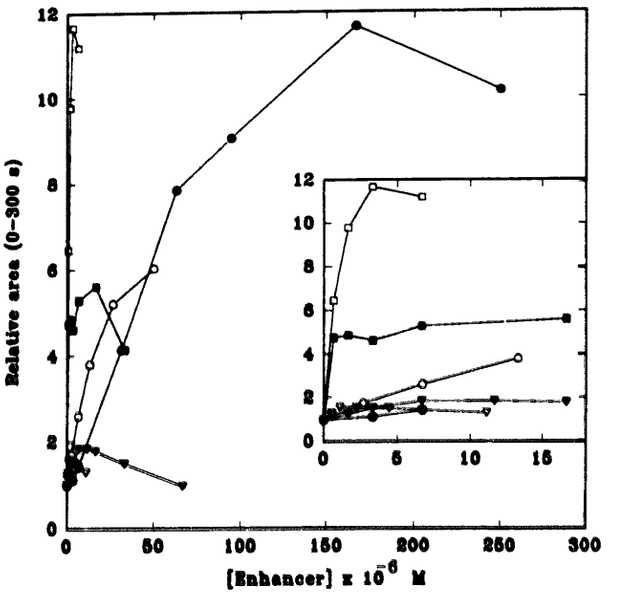

Fig. 4. Relative area (blank = 1) beneath the chemiluminescence intensity curves between 0 and 300 s vs. enhancer concentration. Experimental conditions: [Tris-HCl] = 0.033 M at pH 8.5; [Luminol] = 66.7×10^{-6} M; [H_2O_2] = 2×10^{-3} M; [Horseradish peroxidase] = 1.2 U ml^{-1}. ■, Benzidine; □, *o*-tolidine; ●, phenolphthalein; ○, phenol red; ▼, cresolphthalein; ▽, cresol red.

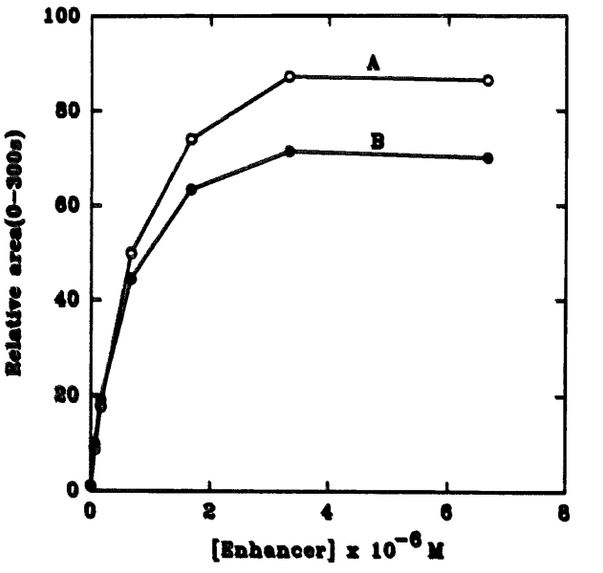

Fig. 5. Relative area (blank = 1) beneath the chemiluminescence curve between 0 and 300 s vs. concentrations of benzidine (A) and *o*-tolidine (B) at pH 10.5. Reactant concentrations: [Bicarbonate buffer] = 0.033 M at pH 10.5; [Luminol] = 66.7×10^{-6} M; [H_2O_2] = 2×10^{-3} M; [Horseradish peroxidase] = 1.22 U ml^{-1}.

cresolphthalein, cresol red and *o*-tolidine inhibit the enhanced chemiluminescence more strongly than phenolphthalein, phenol red and benzidine respectively (Fig. 4). A similar effect was observed for *p*-cresol and phenol [11].

The CH_3 groups and, probably, the aliphatic chains at the ortho or para position to the OH and NH_2 groups in the benzene ring lead to a decrease in the chemiluminescence from the luminol–H_2O_2–horseradish peroxidase system, when the enhancer concentration is increased. In addition, it is known that benzyl radicals (Ph-$CH_2^{\cdot}$) are very stable, and that horseradish peroxidase catalyses the hydroxylation of benzyl methyl groups [4]. We suggest that there may be interactions between the enhancer radicals (Ar-$O^{\cdot}$) or hydroxyl radicals ($OH^{\cdot}$) formed, and the CH_3 groups of the enhancers; these interactions could destroy the phenoxy radicals (CH_3-Ph-$O^{\cdot}$) formed.

Fig. 5 shows that benzidine enhances, by about 80 times, and *o*-tolidine, by about 70 times, the non-enhanced blank chemiluminescence at pH 10.5. We must emphasize that the blank chemiluminescence at pH 10.5 is less than that at pH 8.5, whereas the enhanced chemiluminescence with benzidine or *o*-tolidine exhibits greater emission at pH 10.5 than at pH 8.5 (Fig. 2).

We can see in Fig. 1 that phenolphthalein and fluorescein have similar structures, the only difference being the ether function in the meta position to the OH group which joins two phenolic rings in fluorescein. Studies using β-resorcilic acid (2,4-dihydroxybenzoic acid) and 4-hydroxybenzoic acid as enhancers of the luminol–H_2O_2–horseradish peroxidase system have shown that benzoic acid is a greater enhancer than β-resorcilic acid at pH 8.5 [12]; the only difference between the two is the OH group in a meta position to the other OH group in β-resorcilic acid. However, phenolphthalein enhances the chemiluminescence at 425 nm, whereas fluorescein decreases this emission. We believe that this is due to energy transfer from 3-aminophthalate to fluorescein. Emission occurs at 425 and 530 nm [13,14]. In addition, some xanthene dyes (fluorescein, 4',5'-dibromofluorescein, phloxin B, 2',7'-dichlorofluorescein, eosin Y, erythrosin B and rose bengal) produce chemiluminescence in the presence of H_2O_2 and horseradish peroxidase (without luminol) at about pH 7.0 with $\lambda_{em} \approx 530$ nm [15,16]. Therefore, in our measurement conditions, luminol and fluorescein will compete to react with H_2O_2 and horseradish peroxidase.

3.3. Chemiluminescence emission spectra

The chemiluminescence spectra vs. the emission wavelength for non-enhanced and enhanced luminol–H_2O_2–peroxidase reactions are similar, but with different intensities. The emission spectrum is independent of the enhancer. We suggest a mechanism similar to that proposed in Refs. [17–19] to explain these effects.

$$\text{Peroxidase} + H_2O_2 \longrightarrow \text{Compound I} + H_2O \quad (1)$$

Step (1a), non-enhanced

$$\underset{\text{(luminol)}}{\text{Compound I}} + LH^- \longrightarrow \underset{\text{(luminol radical)}}{\text{Compound II}} + L^{\cdot -} + H_2O \quad (2)$$

$$\text{Compound II} + LH^- \longrightarrow \text{Peroxidase} + L^{\cdot -} \quad (3)$$

Step (1b), enhanced

$$\underset{\text{(enhancer)}}{\text{Compound I}} + EH \longrightarrow \underset{\text{(enhancer radical)}}{\text{Compound II}} + E^{\cdot} + H_2O \quad (4)$$

$$\text{Compound II} + EH \longrightarrow \text{Peroxidase} + E^{\cdot} \quad (5)$$

$$E^{\cdot} + LH^- \longrightarrow EH + L^{\cdot -} \quad (6)$$

Step (2)

$$L^{\cdot -} + O_2 \longrightarrow L + O_2^{\cdot -} \quad (7)$$

(ion radical superoxide)

$$L^{\cdot -} + O_2^{\cdot -} \longrightarrow LO_2^{2-} \quad (8)$$

(luminol endoperoxide)

$$LO_2^{2-} \longrightarrow AP^{2-*} + N_2 \quad (9)$$

(excited 3-aminophthalate dianion)

$$AP^{2-*} \longrightarrow AP^{2-} + h\nu \quad (10)$$

(3-aminophthalate dianion)

Acknowledgment

We thank the Comisión Interministerial de Ciencia y Tecnología (Project PB93-1006).

References

[1] T.P. Whitehead, G.H.G. Thorpe, T.J.N. Carter, C. Groucutt and L.J. Kricka, *Nature, 305* (1983) 158.
[2] G.H.G. Thorpe, S.B. Moseley, L.J. Kricka, R.A. Stott and T.P. Whitehead, *Anal. Chim. Acta, 170* (1985) 101.
[3] G.H.G. Thorpe, L.J. Kricka, S.B. Moseley and T.P. Whitehead, *Clin. Chem., 31* (1985) 1335.
[4] L.J. Kricka, R.A.W. Stott and G.H.G. Thorpe, in W.R.G. Baeyens, D. De Keukeleire and K. Korkidis (eds.), *Luminescence Techniques in Chemical and Biochemical Analysis*, Marcel Dekker, New York, 1991, p. 599.
[5] L.J. Kricka, A.M. O'Toole, G.H.G. Thorpe and T.P. Whitehead, U.S. Patent 4,729,950, 1988.
[6] D.S. Milbrath, Eur. Patent Application, 219352, 1987.
[7] H.J. Stiles, H.J. Bicknell and J.A. Matthews, *J. Biolumin. Chemilumin., 1* (1986) 53.
[8] D. Samuel, T.G. Harrison and A.G. Taylor, *J. Biolumin. Chemilumin., 2* (1988) 251.
[9] S. Steuber, J.Y. Caille and F. Horchner, *Acta Trop., 44* (1987) 459.
[10] L.J. Kricka and D. Schmerfeld-Pruss, *J. Biolumin. Chemilumin., 6* (1991) 231.
[11] F. García Sánchez, A. Navas Díaz and J.A. González García, *J. Luminesc.*, in press.
[12] A. Navas Díaz, F. García Sánchez and J.A. González García, *J. Biolumin. Chemilumin.*, in press.
[13] Y. Haas and E. Würzberg, *J. Phys. Chem., 83* (21) (1979) 2692.
[14] J. Hadjianestis and J. Nikokavouras, *J. Photochem. Photobiol. A: Chem., 69* (1993) 337.
[15] T. Segawa, T. Kamidate and H. Watanabe, *Anal. Sci., 6* (1990) 763.
[16] T. Segawa, T. Kamidate and H. Watanabe, *Bull. Chem. Soc. Jpn., 66* (1993) 2237.
[17] M.J. Cormier and P.M. Prichard, *J. Biol. Chem., 243* (1968) 4706.
[18] A. Lundin and L.O.B. Hallander, in J. Scholmerich, R. Andreesen, A. Kapp, M. Ernst and W.G. Wood (eds.), *Bioluminescence and Chemiluminescence, New Perspectives*, Wiley, Chichester, 1987, p. 555.
[19] G.H.G. Thorpe and L.J. Kricka, in J. Scholmerich, R. Andreesen, A. Kapp, M. Ernst and W.G. Wood (eds.), *Bioluminescence and Chemiluminescence, New Perspectives*, Wiley, Chichester, 1987, p. 199.