

Aniline derivatives as enhancers and inhibitors of the luminol–H₂O₂–horseradish peroxidase chemiluminescence: Effects of the Hammett constants of the substituents

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Abstract

The effects of monosubstituted anilines on the luminol–H₂O₂–horseradish peroxidase chemiluminescence were related with the type of substituent and position. Anilines substituted in *meta*- and *para*-positions by substituents with Hammett constants (σ) lesser than -0.27 produced an inhibition of the chemiluminescence. On the contrary, those anilines with substituents with Hammett constants between -0.27 and $+0.18$ produced an enhancer effect. Anilines with substituents with Hammett constants greater than $+0.18$ produced a slight enhancement or inhibition. An alike evolution was observed in *ortho*-substituted anilines. These effects were related with the reduction potentials of aniline radicals and the reaction rates of these anilines with the compounds of horseradish peroxidase. The data obtained permitted the prediction of the inhibitor or enhancer character of the aniline derivatives, and even the power of enhancement. © 1998 Elsevier Science S.A.

Keywords: Chemiluminescence; Luminol; Enhancement; Anilines

1. Introduction

Numerous phenol and aniline derivatives are hydrogen donors to horseradish peroxidase [1–4] and lactoperoxidase enzymes [5], however only some of them are enhancers of the luminol–H₂O₂–horseradish peroxidase chemiluminescence [6–11]. The enhanced chemiluminescence is very intense, prolonged and decays slowly. This enhancement has also been observed with 6-hydroxybenzothiazoles [12–14], naphthols [15], and arylboronic acids [16]. Numerous patents [8,17,18] and chapters of books [19–21] have been published in relation to this theme.

The use of enhancers facilitates a rapid and sensitive quantification of peroxidase conjugates. The wide applicability of this technique has been confirmed with a great range of ligand-binding assays. The enhanced chemiluminescence has been applied to immunoassay [6,12,13,22], assays of hydrolyase enzymes [23–25], detection and determination of enhancers [26,27], and determinations of low quantities of hydrogen peroxide [28].

However, little has been studied about the relation between the structures of phenols and anilines and their effects as enhancers and inhibitors of the luminol–H₂O₂–horseradish

peroxidase chemiluminescence. Qualitative relationships are found between the redox potentials of phenol derivatives and their effects as enhancers or inhibitors of this chemiluminescence [7,29]. The most reductor phenols are inhibitors of the chemiluminescence and the least reductor phenols are enhancers.

The present paper relates the enhancement and inhibition of the chemiluminescence produced by monosubstituted anilines with the Hammett constants (σ) of their substituents. The enhancer and inhibition effects are also related with the reduction potentials of the aniline radicals and with the reaction rates of anilines with the intermediates of horseradish peroxidase (HRP-I and HRP-II).

2. Experimental details

2.1. Instruments

The chemiluminescence experiments were carried out in a Perkin-Elmer LS-50 (Beaconsfield, UK) luminescence spectrometer with the light source switched-off. The apparatus was set in the phosphorescence mode with 0.00 ms of delay time and 10 ms of gate time. The slit width and wavelength of the emission monochromator were set at 20 nm and 425

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Table 1

Effects on luminol chemiluminescence of different *meta*- and *para*-substituted anilines against the Hammett constants of their substituents (σ), and logarithms of their rate constants of reaction with HRP-I ($\log k_4$) and HRP-II ($\log k_5$)

Aniline derivative	σ	$\log k_4$ (pH 7.0)	$\log k_5$ (pH 7.0)	Effect on the chemiluminescence (pH 9.5)
4- <i>N,N</i> -Dimethylaniline	-0.83			Inhibition
4-Morfolinoaniline				Inhibition
4-Aminoaniline	-0.66			Inhibition [8]
4-Aminophenylether				Inhibition [8]
4-Methoxyaniline	-0.27		6.824 [4]	Enhancement [8]
4-Benzoyloxyaniline				Enhancement [8]
4-Ethoxyaniline	-0.25			Inhibition [8]
4-Phenylloxyaniline				Enhancement [8]
4-Terbutylaniline	-0.20			Enhancement [8]
4-Methylaniline	-0.17	6.720 [1]	5.785 [4]	Enhancement [8]
Benzidine				Enhancement [8,11]
3-Aminoaniline	-0.16			Enhancement [8]
4,4'-Methyldianiline				Enhancement [8]
3-Methylaniline	-0.07		5.253 [4]	Enhancement [8]
2-Aminofluorene				Enhancement
4-Phenylaniline	-0.01			Enhancement [8]
Aniline	0.00	6.441 [1]	4.934 [4]	Enhancement [7,8]
4-Aminocinnamic acid	+0.03			Enhancement
3-Methoxyaniline	+0.12		4.797 [4]	Enhancement [8]
4-Phenylazoaniline				Enhancement
4-Iodoaniline	+0.18			Enhancement
4-Chloroaniline	+0.23		3.064 [4]	
4-Aminobenzoic acid	+0.35	3.672 [1]		Inhibition
3-Chloroaniline	+0.37		3.182 [4]	
4-Aminoacetophenone	+0.50			Weak enhancement
4-Aminobenzonitrile	+0.66			Inhibition
Asulam				Inhibition
3-Nitroaniline	+0.71			Inhibition [8]
4-Nitroaniline	+0.78			Weak enhancement [8]

nm, respectively. The photomultiplier voltage was set to 700 V. The samples were placed in a fluorescence cuvette continuously stirred with a magnetic stirrer.

2.2. Reagents

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) 97% and horseradish peroxidase, type VI-A (1100 U/mg) were supplied by Sigma (St. Louis, USA) and hydrogen peroxide 6% (p/v) by Montplet and Esteban, Barcelona, Spain.

The amines studied were 4-aminobenzoic acid (>99%), 4-iodoaniline (>97%), 4-amino-*N,N*-dimethylaniline (>97%), 4-aminobenzonitrile (>97%), 4-aminoacetophenone (>98%), 1-aminoanthracene (90%), 1-aminoanthracene (90%) and 4-aminocinnamic acid hydrochloride (>97%) (purchased from Fluka Chemie, Buchs, Switzerland); 2-fluoroaniline (>99%), 2-aminoacetophenone (98%), 1-aminopyrene (97%), 2-aminonaphthalene (95%), 2-aminobenzonitrile (98%), 2-aminofluorene (98%), 4-morfolinoaniline (>98%), 1-aminopyrene (97%), 2-aminonaphthalene (95%) and 4-phenylazoaniline (98%) (supplied by Aldrich-Chemical, Dorset, England); asulam 99.9% (methyl 4-aminophenylsulphonyl carbamate) (supplied by Dr. S. Ehrenstorfer, Augsburg, Germany).

The stock solutions were prepared in distilled and demineralized water or in dimethylsulfoxide for those low soluble compounds in water. Asulam was prepared in acetone.

2.3. Chemiluminescence reactions

A fluorescence cuvette was filled with 1 ml of 0.1 M buffer, 20 μ l of luminol 0.01 M, 60 μ l of hydrogen peroxide 0.1 M, a variable volume of aniline derivative and distilled and demineralized water up to 2950 μ l. The samples were continuously stirred with a magnetic stirrer and placed in the spectrometer. Ten seconds after the spectrometer began to record, the chemiluminescence reaction was triggered by injecting 50 μ l of horseradish peroxidase 75.9 U/ml with a syringe through a septum. The kinetics of the emission were recorded between 0 and 300 s. The emission areas between 0 and 300 s were measured.

3. Results and discussion

Tables 1 and 2 list a series of aniline derivatives with different substituents in *para*- and *meta*-positions (Table 1) and in *ortho*-positions (Table 2) to the group $-\text{NH}_2$. Fig. 1

Table 2

Effects on luminol chemiluminescence of different *ortho*-substituted anilines against the Hammett constants of their substituents (σ_p), and logarithms of their rate constants of reaction with HRP-I ($\log k_4$) and HRP-II ($\log k_5$)

Aniline derivative	σ_p	$\log k_4$ (pH 7.0)	$\log k_5$ (pH 7.0)	Effect on the chemiluminescence (pH 9.5)
2-Aminoaniline	-0.66			Enhancement [8]
2-Aminophenol	-0.36			Inhibition [8]
2-Methoxyaniline	-0.27		6.462 [4]	Enhancement [8]
2-Methylaniline	-0.17		5.140 [4]	Enhancement [8]
2-Phenylaniline	-0.01			Enhancement [8]
Aniline	0.00	6.441 [1]	4.934 [4]	Enhancement [7,8]
2-Fluoroaniline	+0.06			Weak enhancement
2-Iodoaniline	+0.18			Inhibition [8]
2-Chloroaniline	+0.23		3.004 [4]	Inhibition [8]
2-Bromoaniline	+0.23			Inhibition [8]
2-Aminoacetophenone	+0.50			Inhibition
2-Aminobenzonitrile	+0.66			Inhibition
2-Nitroaniline	+0.78			Inhibition [8]

Structure of some aromatic amines studied

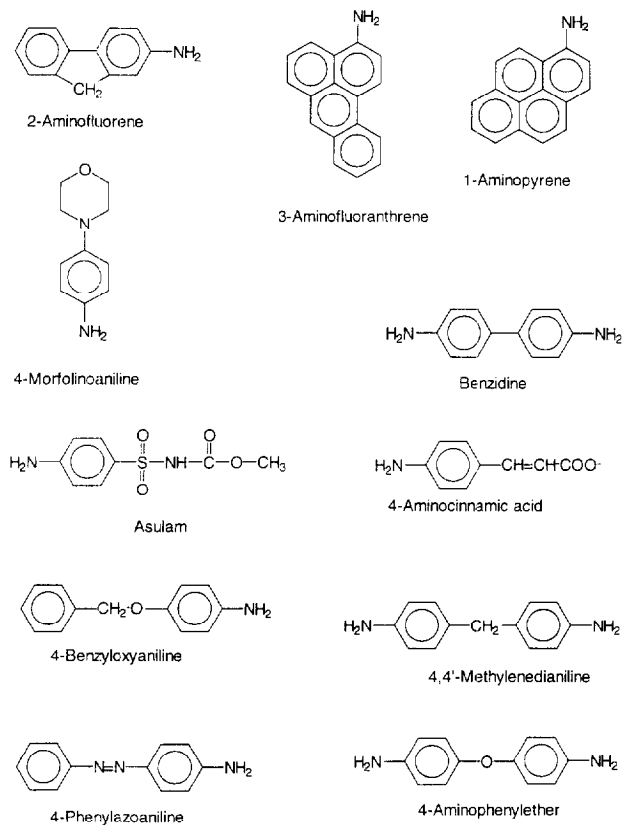


Fig. 1. Structure of some aromatic amines studied.

shows the structure of some anilines. Tables 1 and 2 arranged the aniline derivatives against the Hammett constants (σ) of their substituents and show the effects that these compounds produce on the luminol-H₂O₂-horseradish peroxidase chemiluminescence. The Hammett constants for *ortho*-position have been estimated similar to those for *para*-positions (σ_p) for the substituents of anilines. Table 3 shows a series of polycyclic aromatic amines against their effects as enhancers or inhibitors of the luminol chemiluminescence.

3.1. pH effects

The chemiluminescence of the luminol-H₂O₂-horseradish peroxidase system enhanced by phenols presents a pH optimum around 8.5 for the majority of them [21], however, greater differences in the pH optimum were observed when the enhancer added was an aromatic amine. Aniline produced

Table 3

Effects on luminol chemiluminescence of different polycyclic amines

Polycyclic aromatic amine	Effect on the chemiluminescence (pH 9.5)
1-Aminonaphthalene	Enhancement [8]
2-Aminonaphthalene	Enhancement
1-Aminoanthracene	Weak enhancement
2-Aminoanthracene	Enhancement [8]
1-Aminopyrene	Weak enhancement
3-Aminofluoranthrene	Enhancement [21]

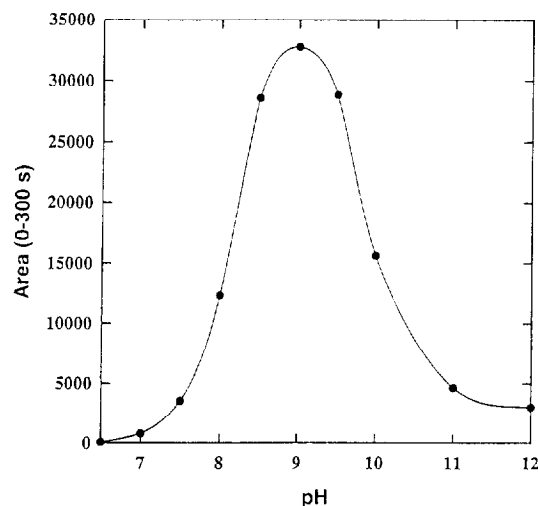


Fig. 2. Areas between 0 and 300 s of the chemiluminescent emission of luminol with 4-aminocinnamic acid against pH.

its maximal enhancement at pH 8.5 [7], 4-aminocinnamic acid at pH 9.0 (Fig. 2), 4-methoxyaniline at pH 9.5 [8], *o*-tolidine and benzidine at pH 10.5 [11].

3.2. Substituent effects

The inspection of Table 1 suggests the existence of three different zones. The first zone contains anilines with substituents with Hammett constants (σ) lower than -0.27 . These anilines inhibit the luminol chemiluminescence. The second zone contains anilines with Hammett constants between -0.27 and $+0.18$, these anilines enhance the chemiluminescence. The third zone presents anilines with substituents with Hammett constants greater than $+0.18$ that produced a slight enhancer effect or a slight inhibition effect.

Table 2 shows two different zones, the first zone has anilines with substituents with Hammett constants (σ_p) lower than 0.00 that produced an enhancement of the chemiluminescence. The second zone contains anilines with substituents with Hammett constants (σ_p) greater than 0.00 that produced a slight enhancement or a slight inhibition.

3.3. Mechanism of the enhanced chemiluminescence

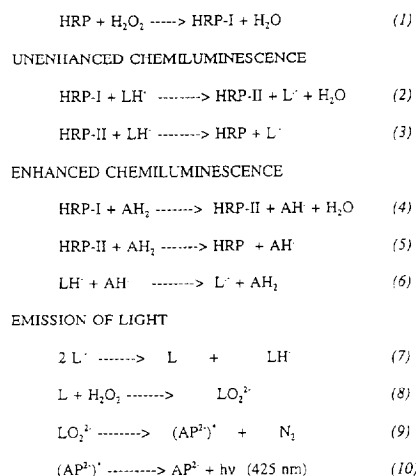
Fig. 3 shows the mechanism of the enhanced chemiluminescence of the luminol– H_2O_2 –horseradish peroxidase system [30–32]. The step six of this mechanism (Eq. (6)) is a reversible electron-transfer reaction between the enhancer radical and luminol [33]. The luminol chemiluminescence enhancers must display similar or greater reduction potentials than luminol, in this case equilibrium (Eq. (6)) proceeds to the right [34,33], as indicated by the Nernst equation:

$$E = E^\circ(AH/AH_2) - E^\circ(L^-/LH^-) - 0.059 \log \frac{[AH_2][L^-]}{[AH][LH^-]} \quad (1)$$

Aniline radicals which cannot oxidize luminol to luminol radical because their normal potentials of reduction are lower than that of luminol radical (~ 0.8 V) [35] behave in a different way, the equilibrium (Eq. (6)) of the mechanism does not occur towards the luminol radical formation. These anilines reduce luminol radicals to luminol and so inhibit the chemiluminescence [34,33].

3.4. Effects of the reduction potentials

Previous works have demonstrated that the reduction potentials of phenol radicals increase against the Hammett constants [35] so a similar increase for aniline radicals can be supposed. Unfortunately, only reduction potential data on very strong reductor character anilines are found in bibliography. Thus, the normal reduction potential of 4-aminoaniline and 4-aminophenol radicals are 0.73 V and 0.41 V, respectively at pH 7.0 [36,37]. Both anilines are inhibitors of the luminol chemiluminescence [8]. However for the



LH⁻: luminol, L⁻: luminol radical, AH₂: aniline, AH: aniline radical, L: diazaquinone, LO₂^{·-}: luminol endoperoxide, AP²⁻: 3-aminophthalate dianion, AP^{·-}: 3-aminophthalate excited

Fig. 3. Mechanism of the enhanced chemiluminescence of luminol with horseradish peroxidase.

other anilines, information on the relation between reduction potentials and Hammett constants can be deduced from the critical oxidation potentials of Fieser (E_c) and the half-wave potentials of polarography ($E_{1/2}$) of substituted anilines. The critical oxidation potentials of Fieser [38] increase with the Hammett constants (σ) of the substituents in *para*- and *meta*-positions according to the expression: $E_c = 0.45\sigma + 1.045$ (calculated from Refs. [38,39]). The sequence of the half-wave potentials of polarography ($E_{1/2}$) of substituted anilines is comparable to their normal redox potential so those are a measurement of the oxidizability of the anilines. The relation between half-wave potentials of polarography and the Hammett constants of the substituents for the *p*-substituted anilines is: $E_{1/2} = 0.50\sigma + 0.78$ [40] and for *p*- and *m*-substituted anilines: $E_{1/2} = 0.40\sigma + 0.72$ [41].

These data support the hypothesis that the reduction potentials of aniline radicals increase with the Hammett constants of their substituents. In conclusion, we can suppose that *meta*- and *para*-substituted anilines with substituents with Hammett constants greater than -0.27 and the studied anilines *ortho*-substituted, except 2-aminophenol, form radicals with reduction potentials greater than that of luminol. These aniline radicals can oxidize luminol to luminol radicals and enhance the chemiluminescence when the kinetics of the process permit it. However, *meta*- and *para*- aniline substituted by substituents with Hammett constants lower than -0.27 cannot oxidize luminol to luminol radical and inhibit the chemiluminescence.

On the other hand, anilines *meta*- and *para*-substituted with Hammett constants greater than $+0.18$ and aniline *ortho*-substituted with Hammett constants greater than 0.00 produce a slight enhancement or an inhibition of the luminol chemiluminescence. These anilines can oxidize luminol to luminol radical and enhance the chemiluminescence but the kinetic of the process does not permit this.

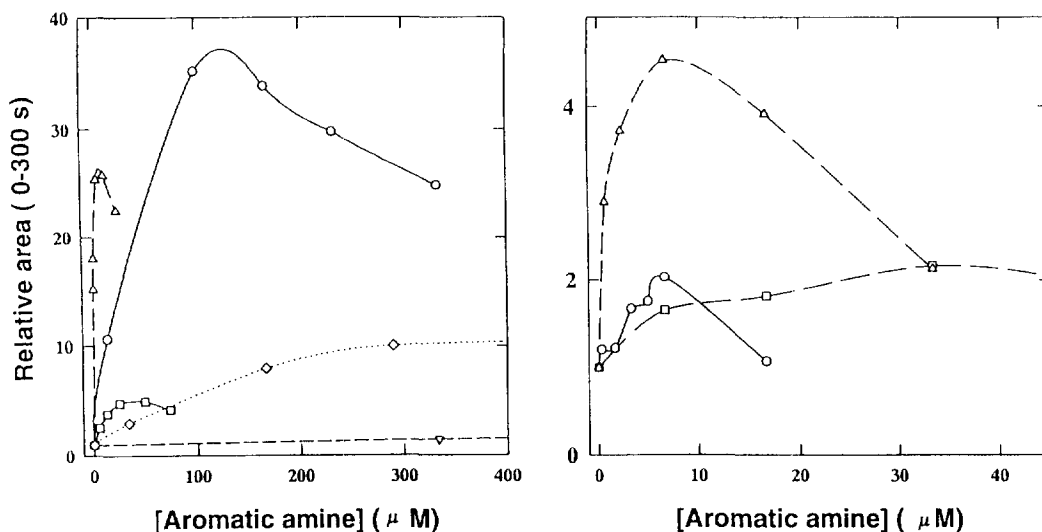


Fig. 4. Areas between 0 and 300 s of the chemiluminescent emissions of luminol with different enhancers against the concentration of each enhancer. Left: (\diamond) 4-iodoaniline in tetraborate buffer at pH 9.5; (∇) 4-aminoacetophenone in tetraborate buffer at pH 9.5; (Δ) 2-aminofluorene in tris-HCl buffer at pH 8.5; (\square) phenylazoaniline in tris-HCl buffer at pH 8.5; (\circ) 4-aminocinnamic acid in tris-HCl buffer at pH 9.0. Right: (\circ) 1-aminopyrene; (\square) 1-aminoanthracene; (Δ) 2-aminonaphthalene.

3.5. Kinetic effects

The reduction potentials of the enhancer radicals govern the equilibrium constant of the redox reaction (Eq. (6)). However, to achieve enhancement by an aniline derivative the luminol radicals must be produced through Eqs. (1)–(6) of the mechanism faster than by luminol without aniline through Eqs. (1)–(3).

The chemiluminescence increases with the concentration of luminol radicals [$L^{\cdot-}$] in the medium. This concentration increases with the formation rate of luminol radicals through Eqs. (1)–(3), which is given by the expression:

$$\frac{d[L^{\cdot-}]}{dt} = (k_2[HRP-I] + k_3[HRP-II])[LH^-] \quad (2)$$

where k_2 and k_3 are the second-order rate constants of reaction of luminol with HRP-I and HRP-II, respectively [42].

Similarly, the concentration of aniline radicals [A^{\cdot}] in the medium increase with the formation rate of these radicals through Eqs. (4) and (5), which is given by the expression:

$$\frac{d[AH^{\cdot}]}{dt} = (k_4[HRP-I] + k_5[HRP-II])[AH_2] \quad (3)$$

where k_4 and k_5 are the second-order rate constants of reaction of aniline with HRP-I and HRP-II, respectively [1,2]. Eq. (3) indicates that the aniline radical concentration increases with the reaction rate of aniline with peroxidase intermediates (HRP-I and HRP-II).

An increase in the aniline radicals concentration will shift the equilibrium (Eq. (6)) to the right, to the luminol radicals production, and the chemiluminescence will be enhanced. So a direct relation between chemiluminescence and the rate constants (k_4 and k_5) of the aniline with the peroxidase intermediates is deduced.

Previous works have demonstrated that the reaction rate of anilines with the peroxidase intermediates decrease against the Hammett constant of the substituents in the aniline [1–4]. Data in Tables 1 and 2 confirm these affirmations. The effects of anilines with different substituents (different Hammett constants, σ) on the chemiluminescence have been studied at different concentrations. Fig. 4 (left) shows the areas of the chemiluminescent emission against the concentration of 4-aminocinnamic acid, 4-aminoacetophenone, 2-aminofluorene, phenylazoaniline and 4-iodoaniline. The results show that at pH 9.5, 4-aminoacetophenone ($\sigma=0.5$) produces lower enhancement than 4-iodoaniline ($\sigma=0.18$) and the latter produces an enhancement lower than 4-aminocinnamic acid ($\sigma=0.03$) at pH 9.0. 2-Aminofluorene produces greater enhancement at pH 8.5 than 4-phenylazoaniline ($\sigma_{\text{aminopyrene}} < \sigma_{\text{4-phenylazoaniline}}$). Previous works have shown that, at low concentrations, aniline ($\sigma=0.00$) produces lesser enhancement than benzidine and *ortho*-tolidine ($\sigma < 0.00$) at pH 8.5 [7,11]. All these observations support that the enhancement produced by an aniline decreases with the Hammett constant of its substituent.

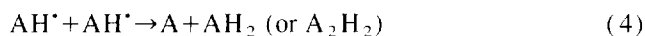
In consequence, the formation of luminol radicals, through Eqs. (4)–(6), decreases when the Hammett constant increases. Thus the enhancement is negligible for *meta*- and *para*-substituted anilines with Hammett constants greater than +0.18 and *ortho*-substituted anilines with Hammett constants (σ_p) greater than 0.00. This occurs like this because the rates of reaction of the anilines with the compounds of peroxidase decrease and in consequence the formation of luminol radicals decreases.

3.6. Synergism phenomena

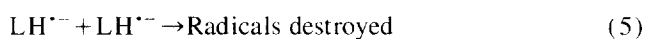
All these affirmations can be applied only for low concentrations of aniline, when aniline concentration increases syn-

ergism phenomena are observed. Thus, an aniline with lower Hammett constant than another can produce greater enhancement at low concentration than the other, however when the concentration of both anilines increase the second aniline (with higher Hammett constant) can produce greater enhancement than the first aniline (with lesser Hammett constant). As an example, benzidine produces greater enhancement than aniline at low concentration [7,11], however at greater concentrations the enhancement produced by aniline is greater than that produced by benzidine [7,11].

Fig. 4 (left and right) shows synergism cases, the chemiluminescence versus aniline concentration curves have no linear shape, even above certain concentrations the chemiluminescence decreases. Fig. 4 (left) shows that the optimal concentration (concentration of aniline that produces the maximal chemiluminescence) changes with the aniline used: the optimum concentration of aminoacetophenone ($\sigma=0.5$) is greater than that of 4-iodoaniline ($\sigma=0.18$) at pH 9.5; that of 4-iodoaniline ($\sigma=0.18$) at pH 9.5 is greater than that of 4-aminocinnamic acid at pH 9.0 ($\sigma=0.03$), and that of 2-aminofluorene at pH 8.5 lesser than that of 4-phenylazoaniline. The optimal concentration for each aniline increases with the Hammett constant (σ) but decreases with the reaction rate of the aniline with the compounds of peroxidase (HRP-I and HRP-II). In effect, high rate constant of aniline with HRP-I and HRP-II increases the concentration of aniline radicals in the medium. These aniline radicals can react among them and be destroyed producing synergism [34,33], indicates as the following equation [43,44]:



Other radical interactions which cause radical destruction are also possible:



At low concentration of aniline radicals, the equilibrium (Eq. (6)) will be shifted to the right to form luminol radicals and the destruction reactions are less important. When the concentration of aniline radicals (or luminol radicals) increase above a certain limit (different for each aniline) the effects of destruction of these radicals are more important than their formation. In consequence, the chemiluminescence decrease and the synergism effects are observed.

3.7. New enhancers

The previous observations permit predicting the enhancer or inhibitor behaviour of new compounds. Thus the inhibition by 4-*N,N*-dimethylaniline and 4-morfolinoaniline and the enhancement by 4-iodoaniline, 4-aminoacetophenone, 2-aminofluorene, phenylazoaniline and 4-aminocinnamic acid were found. In Tables 1 and 2 data about these compounds are indicated. Fig. 4 (left) shows the emission against concentration at the pH optimum for the new enhancers.

Table 3 and Fig. 4 (right) show the enhancer effects on the chemiluminescence of some polycyclic aromatic amines. All these amines are enhancers of the chemiluminescence at low concentration. New little enhancers such as: 1-aminopyrene, 1-aminoanthracene and 2-aminonaphthalene, have been found, however a clear relation between the polycyclic aromatic amines structure and enhancement cannot be established.

3.8. Conclusions

In consequence, the factors that determinate the enhancement of an aniline are three: (1) the potential of reduction of its aniline radicals; (2) the reaction rates of the aniline with HRP-I and HRP-II; (3) the maximal concentration of aniline radicals allowed in the medium.

Experiments have demonstrated that *meta*- and *para*-substituted anilines are enhancers when their Hammett constants are in the range between -0.27 and $+0.18$ and *ortho*-substituted anilines are enhancers when their Hammett constants are in the range -0.27 and 0.00 .

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