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Relation between the structure of some heterocyclic derivatives and other compounds, and their effects as enhancers or inhibitors of the luminol–H_2O_2–horseradish peroxidase chemiluminescence

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Abstract

The enhancement or inhibition produced by 4-hydroxyazobenzene, 4′-hydroxyazobenzene-2-carboxylic acid (HABA), 7-hydroxycoumarin, 7-hydroxy-4-methylcoumarin, 7-hydroxycoumarin-4-acetic acid, 4-hydroxypyridine, 6-hydroxyquinoline, vanillin, *p*-rosolic acid, phenylacetate, 4-phenylphenolacetate, 5-aminoquinoline and 5-aminoisoquinoline on the luminol–H_2O_2–horseradish peroxidase chemiluminescence were studied. These phenomena were compared with the effects produced by some enhancers known with similar structures to the previous compounds, such as, phenol, 4-phenylphenol, 4-hydroxycinnamic acid, 2-naphthol, 4-hydroxybenzaldehyde, phenolphthalein and 1-aminonaphthalene. The enhancer activity was related to particular structures of each compound, reaction rate constants with horseradish peroxidase, pH and enhancer concentration. © 1997 Elsevier Science S.A.

Keywords: Chemiluminescence; Luminol; Enhancers; Inhibitors

1. Introduction

The enhanced chemiluminescence of the luminol–H_2O_2–horseradish peroxidase system is very intense, prolonged and decays slowly. This has determined its great application to immunoassay [1–3] and other analytical applications [4,5], mainly bound to biochemistry [6,7].

There are numerous aromatic compounds with hydroxy or amino groups that produce an important enhancement of the luminol–H_2O_2–horseradish peroxidase chemiluminescence. Some of these compounds are 4-hydroxycinnamic acid, 4-phenylphenol, 2-naphthol [8], 1-aminonaphthalene [9], phenol, 4-hydroxybenzaldehyde [10], phenolphthalein and phenol red [11].

It is known that few structural differences convert an enhancer of this chemiluminescence to an inhibitor [12,13]. An example, 4-hydroxycinnamic acid, is an important enhancer of the chemiluminescence of luminol with hydrogen peroxide and peroxidase, however, 4-hydroxy-3-methoxycinnamic acid is an inhibitor of this chemiluminescence [12].

This paper studies the increase of the emission of light of a luminol–hydrogen peroxide–horseradish peroxidase reaction, when some heterocyclic compounds with hydroxy or amino groups or phenol derivatives are added. Our attention has been centred on the increase of the light emission from this reaction produced by the enhancer. The paper shows a series of aromatic compounds with hydroxy or amino groups that have structures similar to other known enhancers. The compounds studied were 4-hydroxyazobenzene, 4′-hydroxyazobenzene-2-carboxylic acid (HABA), 7-hydroxycoumarin, 7-hydroxy-4-methylcoumarin, 7-hydroxycoumarin-4-acetic acid, 4-hydroxypyridine, 6-hydroxyquinoline, vanillin, *p*-rosolic acid, phenylacetate, phenylphenolacetate, 5-aminoquinoline and 5-aminoisoquinoline. Some of the compounds studied are new enhancers for the luminol–H_2O_2–horseradish peroxidase chemiluminescence, and others are inhibitors. One of the compounds studied, 4-hydroxyazobenzene [8], produces an enhancement similar to 4-iodophenol, the most used enhancer.

2. Experimental

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

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tris–HCl buffer (pH 8.5). Horseradish peroxidase was prepared from horseradish peroxidase type VI-A (1100 U mg^{-1}, Sigma) by diluting in 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, Barcelona) and diluting to 25 ml with distilled and demineralized water.

The compounds tested were: 4-hydroxyazobenzene, 4'-hydroxyazobenzene-2-carboxylic acid, 5-aminoquinoline, 4-hydroxypyridine, 6-hydroxyquinoline and *p*-rosolic acid (4-[bis(4-hydroxyphenylmethylene]-2,5-cyclohexadien-1-one) from Fluka; 7-hydroxycoumarin, 7-hydroxy-4-methylcoumarin, 7-hydroxycoumarin-4-acetic acid, 4-phenylphenol acetate, phenyl acetate and 5-aminoisoquinoline from Aldrich; vanillin (4-hydroxy-3-methoxybenzaldehyde from Sigma.

The stock solutions were prepared in distilled and demineralized water or in dimethylsulfoxide for those compounds little soluble in water.

2.2. Instrumentation

The measures were carried out in a Perkin-Elmer LS-50 (Beaconsfield, UK), luminescence spectrometer with the light source switched off. The spectrometer was set in the phosphorescence mode with a delay time of 0.00 ms and a gate time of 10 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 reactants were introduced in a quartz cuvette and stirred with a magnetic stirrer. The chemiluminescent reaction was triggered by injecting horseradish peroxidase with a syringe through a septum.

2.3. Chemiluminescent reactions

The chemiluminescent reactions were carried out in a quartz cuvette. The concentration of the reactants in cuvette were [luminol] = 6.7 μM, [hydrogen peroxide] = 2 mM, [horseradish peroxidase] = 1.27 U ml^{-1}, [buffer] = 0.033 M. The buffers used to study the pH influence were potassium dihydrogen phosphate with NaOH (pH 6.5), tris–HCl (pHs 7.0, 7.5, 8.0, 8.5, 9.0), sodium tetraborate with NaOH (pH 9.5), sodium bicarbonate with NaOH (pHs 10, 11) and potassium chloride with NaOH (pH 12). The mixture was continuously stirred with a magnetic stirrer.

3. Results and discussion

Fig. 1 shows the structures of some compounds studied in this paper. Some of the heterocyclic derivatives or phenol derivatives produced an enhancement of the luminol–H_2O_2–horseradish peroxidase chemiluminescence, these were studied against pH (Fig. 2) and against the concentration of each enhancer (Fig. 3).

3.1. Heterocyclic derivatives

7-Hydroxycoumarin, 7-hydroxy-4-methylcoumarin and 7-hydroxycoumarin-4-acetic acid have similar structures to *p*-coumaric acid, other classic enhancer [14]. 7-hydroxycoumarin-4-acetic acid produced an inhibition of this chemiluminescence. In contrast, 7-hydroxycoumarin and 7-hydroxy-4-methylcoumarin produced enhancement of the luminol chemiluminescence. Fig. 2 shows that the optimum

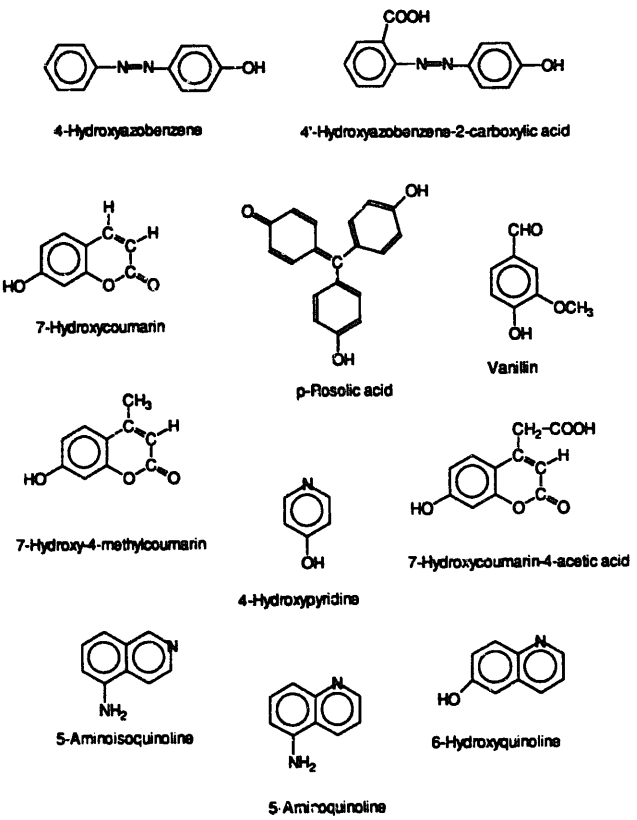

Fig. 1. Structure of some compounds studied.

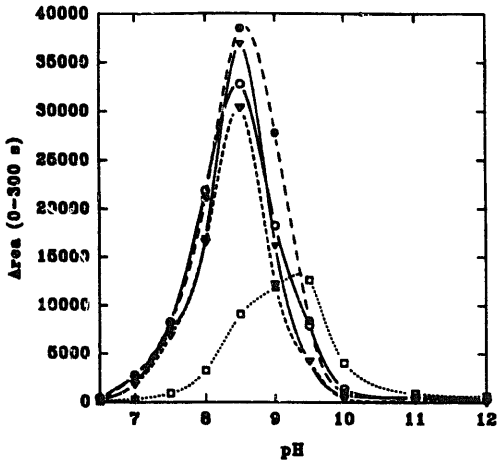

Fig. 2. Areas of the chemiluminescent emission between 0 and 300 s against the pH for each enhancer. (●) 4'-hydroxyazobenzene-2-carboxylic acid (16.67 μM); (▼) 7-hydroxy-4-methylcoumarin (500 μM); (○) 4-hydroxyazobenzene (2 μM); (▽) 7-hydroxycoumarin (333.33 μM); (□) 5-aminoisoquinoline (33.33 μM).

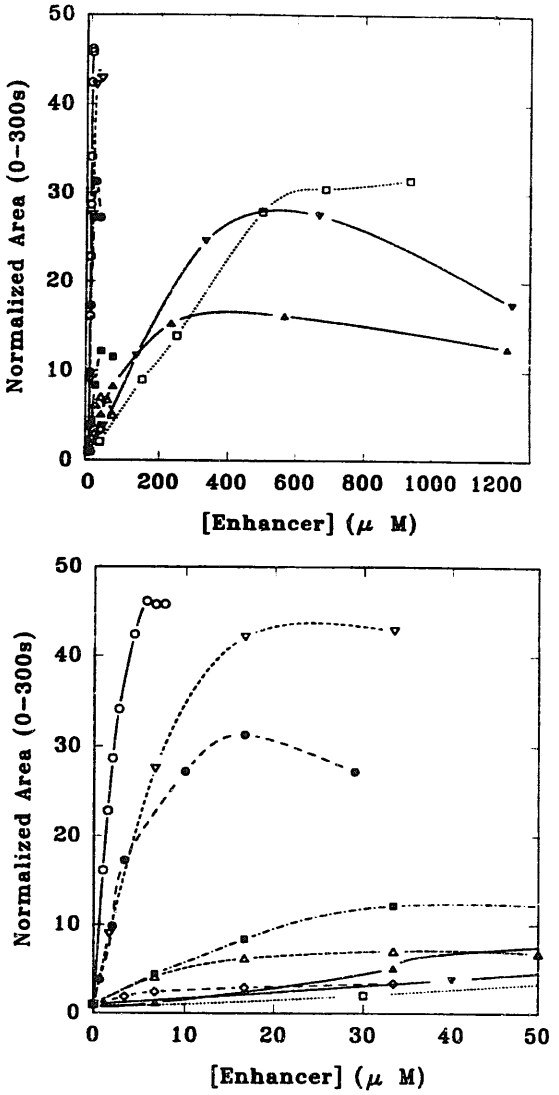

Fig. 3. Normalized areas (area without enhancer = 1) of the chemiluminescent emission between 0 and 300 s against the concentration of enhancer. (●) 4′-hydroxyazobenzene-2-carboxylic acid at pH 8.5; (▿) *p*-iodophenol at pH 8.5; (□) 7-hydroxy-4-methylcoumarin at pH 8.5; (○) 4-hydroxyazobenzene at pH 8.5; (▼) 7-hydroxycoumarin at pH 8.5; (■) 5-aminoisoquinoline at pH 9.5; (△) 5-aminoquinoline at pH 9.5; (◇) *p*-rosolic acid; (▲) vanillin.

pH (pH with the greatest enhanced chemiluminescence) was 8.5 for both compounds. Fig. 3 shows that the chemiluminescence enhanced by 7-hydroxycoumarin had an optimum concentration (concentration with the greatest emission of chemiluminescence) at 500 μM and enhanced by 7-hydroxy-4-methylcoumarin had an optimum concentration at 800 μM.

2-Naphthol is another enhancer of the chemiluminescence of luminol with peroxidase [14], however, 6-hydroxyquinoline having a resembling structure but with a nitrogen in 1-position, produced an inhibition of the chemiluminescence.

5-Aminoisoquinoline and 5-aminoquinoline are two enhancers with a structure similar to 1-aminonaphthalene (another enhancer known [9]), but with a nitrogen atom in the positions 1 and 2 respectively. Both compounds produced an important enhancement of the chemiluminescence. The chemiluminescence enhanced by 5-aminoisoquinoline had an optimum pH at 9.5 (Fig. 2). The optimum concentration for 5-aminoquinoline and 5-aminoisoquinoline were 40 μM, in both cases (Fig. 3).

7-Hydroxypyridine is an inhibitor of the luminol chemiluminescence, but its structure is similar to phenol, another enhancer known [10], but with a nitrogen atom in position 1.

3.2. Phenol derivatives

Vanillin (4-hydroxy-3-methoxybenzaldehyde) showed an enhancer effect, the structure of this compound is similar to the structure of 4-hydroxybenzaldehyde but with a methoxy group. This methoxy group in *ortho*-position to the hydroxy group increases the rate of reaction of vanillin with the second compound of horseradish peroxidase [15] (HRP-II, $\log k_2 = 6.114$), against the rate of reaction of 4-hydroxybenzaldehyde [16] ($\log k_2 = 3.722$). Fig. 3 shows its enhanced chemiluminescence against concentration. This increase in the rate of reaction determined that vanillin produced more enhancement at lower concentration (0.1 mM) than 4-hydroxybenzaldehyde [10]. Another consequence is that vanillin had an optimum concentration (0.5 mM) less than 4-hydroxybenzaldehyde (5 mM) [10].

The enhancer effect of 4-hydroxyazobenzene and 4′-hydroxyazobenzene-2-carboxylic acid were discovered by Kricka et al. [8]. However, the chemiluminescence of the luminol–H_2O_2–horseradish peroxidase system enhanced by these compounds has not been as studied as the chemiluminescence enhanced by *p*-iodophenol, *p*-coumaric acid or 4-phenylphenol [14]. The structures of both compounds are similar to the structures of 4-phenylphenol, but with a substituent in the *para*-position (group phenylazo) more electron withdrawing than the phenyl group. Fig. 2 shows the enhanced chemiluminescence produced by 4-hydroxyazobenzene and 4′-hydroxyazobenzene-2-carboxylic acid against pH, both compounds had 8.5 as optimum pH. Fig. 3 shows the enhanced chemiluminescence against concentration of both compounds, the optimum concentration for 4-hydroxyazobenzene was 5 μM, and for 4′-hydroxyazobenzene-2-carboxylic acid was 15 μM. These phenol derivatives are two important enhancers of this chemiluminescence. 4-Hydroxyazobenzene produced an enhancement similar to *p*-iodophenol (Fig. 3), the most important enhancer discovered, yet.

p-Rosolic acid is another enhancer having a similar structure to phenolphthalein and phenol red, two other enhancers known and studied [11]. The chemiluminescence enhanced by this compound against its concentration is shown in Fig. 3.

3.3. Esters of phenol derivatives with acetic acid

4-Phenylacetate and 4-phenylphenolacetate are two esters of acetic acid with phenol and 4-phenylphenol respectively. Both compounds produced an enhancement of the chemilu-

minescence of luminol with peroxidase and hydrogen peroxide at pH 8.5. We think that the enhancement occurs because phenyl acetate and 4-phenylacetate suffer fast hydrolysis that render phenol and 4-phenylphenol, respectively, two known enhancers [8,10]. A similar effect was observed in 2-naphthylacetate at pH 8.5, this compound suffers a fast hydrolysis to form 2-naphthol [17]. This phenomenon has been applied to the determination of serum cholinesterase by a chemiluminescent method [7] and by a fluorescent method [17].

4. Conclusions

7-Hydroxycoumarin, 7-hydroxy-4-methylcoumarin, vanillin, *p*-rosolic acid, phenylacetate, 4-phenylphenolacetate, 4-hydroxyazobenzene, 4′-hydroxyazobenzene-2-carboxylic acid, 5-aminoquinoline, and 5-aminoisoquinoline are found to be enhancers of the luminol–H_2O_2–horseradish peroxidase chemiluminescence. On the other hand, 4-hydroxypyridine, 7-hydroxycoumarin-4-acetic acid and 6-hydroxyquinoline are found to be inhibitors. We observed that few structural differences turn an enhancer of this chemiluminescence (phenol, 2-naphthol, 4-hydroxycinnamic acid) into an inhibitor (4-hydroxypyridine, 6-hydroxyquinoline, 7-hydroxycoumarin-4-acetic acid, respectively), or an enhancer (4-hydroxybenzaldehyde) into a greater enhancer (vanillin). The structural differences affect reaction rates of the compounds studied with horseradish peroxidase (this occurs with phenol and aniline derivatives [15,16], as vanillin against 4-hydroxybenzaldehyde), and also, these structural differences affect redox potentials of the radicals formed by these compounds (this occurs with phenol derivatives [12,18]), which react with luminol to form luminol radicals [14]. These changes in the rates of reaction or in the redox potentials could affect the enhancement or inhibition of the chemiluminescence.

4-Phenylphenol acetate and phenyl acetate are two pro-enhancers of this chemiluminescence. These compounds produce enhancement of the chemiluminescence when they suffer hydrolysis to render phenol or 4-phenylphenol.

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