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Water Quality Monitoring Using an Enhanced Chemiluminescent Assay Based on Peroxidase-Catalyzed Peroxidation of Luminol

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Abstract

Enhanced chemiluminescence (ECL) describes the phenomenon of the light output increase in the reaction of oxidation of luminol catalyzed by horseradish peroxidase (HRP) in the presence of certain phenolic compounds. This work summarizes the effects of preincubation of certain substances with HRP on the chemiluminescent reaction intensity. Preincubation of herbicide, detergent, surfactants (Brij-96 and Tween-20), phenol, metal ions (mercury, cobalt, and nickel), and bactericide with HRP had an inhibitory effect on the enzyme activity. HRP-preincubation with metal ions (cadmium, magnesium, and zinc), as well as with some insecticides, stimulated the chemiluminescent intensity. Calibration graphs were obtained to demonstrate the possibility to determine the pollutant concentration. Light emission from the peroxidase catalyzed enhanced chemiluminescence is affected by a wide number of chemicals and, therefore, the method can be used for on-site monitoring of water quality. A rapid and simple assay to detect water contamination has been developed.

Index Entries: Horseradish peroxidase; luminol; water pollutants.

Introduction

The generation of municipal and industrial wastewater is a growing problem that requires strict control. Metals are widely used in industry owing to their multiple properties that make them of great use. The presence

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of metal ions in different aqueous media and especially in natural water presents an environmental threat. At the same time, according to the data presented by Arévila et al. *(1)*, the agricultural returns in Mexico constitute 46% of the total of the unloading and equals 282 m^3/s and, consequently, to almost 77% of the extracted water. The actions undertaken to control the nonpoint sources of water pollution (water of agricultural returns, industrial surpluses, and so on) were insufficient mainly owing to the difficulties in controlling and monitoring of such types of pollution *(2)*. This situation requires the development of new methods for controlling and monitoring water pollution.

The enhanced chemiluminescent reaction of luminol peroxidation catalyzed by horseradish peroxidase (EC 1.11.1.7) presents evident advantages in the development of the techniques that permit the presence of environment pollutants to be detected. The high selectivity, the simplicity, and the extreme sensitivity of the chemiluminescent methods explain the success of its recent utilization *(3)*. Chemiluminescence is generated when a chemical reaction gives an electronically excited product, which emits radiation to convert back to the resting state. In the case of luminol oxidation catalyzed by peroxidase, the decay of aminophthalate dianion emits light (λ = 425 nm) *(4–6)*.

The discovery of an enhanced substrate for HRP-catalyzed chemiluminescence has led to the development of commercial kits for sensitive immunoassays to substitute for ^{125}I labels and radioactivity in conventionally used radioimmunoassay systems. However, the exact mechanism of enhancement is still unresolved *(3,6–9)*. The HRP-catalyzed enhanced chemiluminescence provides a sound basis for an assay of enzymatically generated H_2O_2, an enzyme immunoassay and DNA dot-hydrolyzation assay *(3)*. Much of interest in this reaction has focused on its analytical application because it is possible to control the reaction conditions in such a way that the light intensity is linearly dependent on the concentration of a reactant or the catalyst.

In this work we present some advances in the development of chemiluminescent assay for detecting water pollution. Some potential pollutants, e.g., divalent metal ions, agricultural pollutants, phenol, cyanide, surfactants, and detergent that can cause environmental problems and have short- and long-term effects on human health, affect the reaction catalyzed by HRP. The enhanced chemiluminescent reaction of luminol peroxidation has been demonstrated to be useful for monitoring of water pollution.

Material and Methods

HRP (type IV A, 1100 U/mg), Trizma, hydrochloric acid, 5-amino-2,3-dihydrophthalazine-1,4-dione (luminol), *para*-iodophenol, EDTA, hydrogen peroxide, NaOH were purchased from Sigma Chemical Co. (St. Louis, MO). The following model substances were used in the experiments as

potential pollutants: detergent Ariel (Procter & Gamble, Mexico), surfactants—Tween-20 and Brij-96 (Sigma), phenol (Sigma). Agrochemical products were provided by Mexican Biochemical Group (Saltillo, Coahuila, Mexico): as a bactericide Agrimicin contained streptomycin and oxytetracycline as active compounds, as a herbicide paraquat and Rugby, Pounce, Dominex insecticides that contain cadusafos, permethrin, and alfamethrin, respectively. The salts of metals (mercury, nickel, cobalt, magnesium, cadmium chlorides, and zinc sulfate) were of analytical grade and were purchased from DEQ (Mexico). Chemiluminescence intensity was measured using an EMILITE EL 1003 portable luminometer (Russia).

Preliminary Study of Luminol Peroxidation System

The stock solutions of luminol (1.2 m*M*) and *para*-iodophenol (0.8 m*M*) were prepared in 1 *M* NaOH solution (50 μL) and 0.1 *M* Tris-HCl buffer, pH 8.5 using bidistilled water. The *para*-iodophenol was used as an enhancer. The stock solutions were kept in amber flasks at 4°C. Storage of the stock solutions of *para*-iodophenol and luminol under such conditions permits their use for 45 d.

The measurements of chemiluminescent intensity were performed in 0.1 *M* Tris-HCl buffer containing 2 m*M* of EDTA (or without EDTA), pH 8.5. Aliquots of luminol, *para*-iodophenol, and H_2O_2 were added to a cuvet to a final volume of 1.01 mL. The background reaction was recorded. The reaction was initiated by addition of 10 μL of enzyme solution. To select optimal conditions for light emission concentrations of luminol, *para*-iodophenol and H_2O_2 were varied over the range of 0.01–0.1 m*M*, 0.01–0.04 m*M*, and 0.001–0.075%, respectively.

Study of the Effect of Different Potential Pollutants on Enhanced Chemiluminescence (ECL) Intensity in the Reaction of Luminol Peroxidation

The selected concentrations of reagents were used to record the effects of different potential pollutants on the ECL reaction. To carry out this study, 20 μL of the enzyme solution was preincubated with 20 μL of the pollutant solution prior to assay during 1 min at 25°C. Thereafter, 20 μL of this mixture was added to initiate the reaction of luminol peroxidation as described previously. The same procedures were performed using bidistilled water (as control) instead of the pollutant solution. The ECL intensity profiles were recorded and the maximum intensity was used to plot the calibration graphs. Each measurement was in triplicate, as well as every one of the assays.

Results and Discussion

The mechanism of the horseradish peroxidase (HRP)-catalyzed enhanced chemiluminescence has been widely studied *(7–10)*, and several mechanisms for the peroxidase-enhancer-luminol-hydrogen peroxide system have been proposed, however, they are not as yet fully characterized

(10). The generally accepted scheme of Thorpe and Kricka *(7,8)* proposes a three-step mechanism: step I, HRP interacts with the peroxide, yielding HRP compound-I and water; step II, the HRP compound-I reacts with the enhanced substrate, like *para*-iodophenol, to form the HRP compound-II and generate enhancer free radicals; and step III, the enhancer free radicals react with luminol, which is believed to generate luminol free radicals. The luminol radicals in turn generate the luminol endo-peroxide which decays into the excited 3-aminophthalate dianion responsible for light emission.

Preliminary Study of Luminol Peroxidation System

According to the results of different authors *(10–12)*, the kinetics of enhanced HRP reaction is affected by reagent concentrations and purity, the type of buffer used in the system, etc. Thus, the preliminary study of the ECL system was performed to define the optimal reaction conditions.

Using 0.1 *M* Tris-HCl buffer, pH 8.5, without EDTA, light emission was observed in the absence of hydrogen peroxide or the enzyme (background signal). It has been demonstrated earlier *(13)* that the emission of light is induced by the luminol oxidation catalyzed by ions of transition metals including iron, but the catalytic efficiency of the haem iron is much greater than that of a free salt. This is probably owing to its ability to promote the formation of the perhydroxyradical intermediate, which is a primary oxidant for luminol oxidation *(13)*. Therefore, in a nonperoxidative metal porphyrin chemiluminescence system, metal porphyrins generate light from high pH luminol solutions. This reaction is called nonperoxidative because peroxide is not added to the reaction solution *(14)*. To simplify the procedure of ECL detection and to avoid the subtraction of the background intensity, it was proposed to use 0.1 *M* Tris-HCl buffer, pH 8.5, containing 2 m*M* EDTA. It was demonstrated that EDTA allows the background light emission to be eliminated and does not inhibit the ECL reaction catalyzed by HRP.

The limitation of the ECL sensitivity is associated with a lag time period time *(14)*. This phenomenon is associated with a delay in light emission after mixing of all reactants (hydrogen peroxide, luminol, enhancer, and HRP). Because the lag time phenomenon affects the HRP-induced chemiluminescence only at low HRP concentrations, the inhibition of detection sensitivity makes the dependence of light output on HRP concentration nonlinear *(12)*. In this study, the maximum ECL intensity was studied as a function of the enzyme concentration (Fig. 1). The obtained curve is nonlinear. Although it is not shown in the figure, a remarkable lag period was observed for the systems with the enzyme concentration less than 22.5 ng/mL. According to these results it was proposed to use an enzyme concentration of 45 ng/mL. There was virtually no lag period in light emission from the system that contained this enzyme concentration. At the same time, a gradual change in the intensity was observed, e.g., the maximum value of intensity was recorded after several seconds, whereas at the higher enzyme concentrations a drastic change of the ECL response made the maximum intensity estimation difficult.

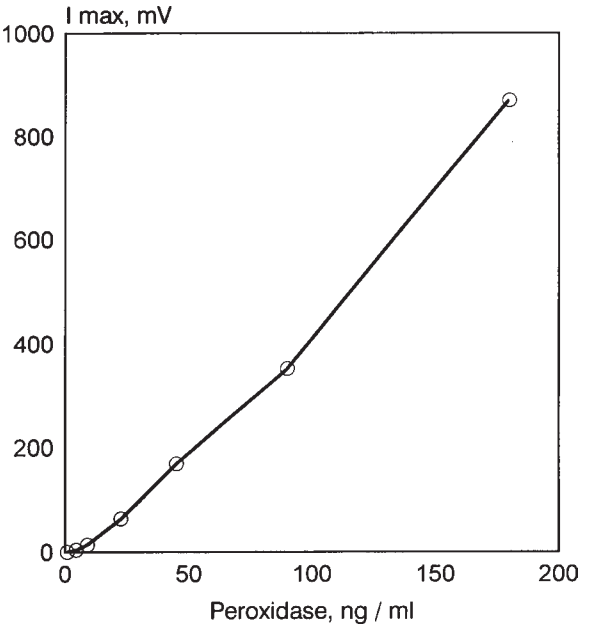

Fig. 1. Maximum ECL intensity from luminol peroxidation reaction as a function of the HRP concentration.

Figures 2 and 3 demonstrate that the maximum ECL intensity is the function of concentrations of luminol and *para*-iodophenol in accordance with the Michaelis-Menten equation. A series of parallel lines (Figs. 2 [bottom] and 3 [bottom]) were obtained in Lineweaver-Burk (L-B) coordinates. The tangent values of these lines give the constant value of $I_{max\ app}/K_{m\ app}$ ratio due to a simultaneous change in $I_{max\ app}$ and $K_{m\ app}$ (Figs. 2 and 3). In the further experiments, 0.03 and 0.01 m*M* concentrations of *para*-iodophenol and luminol, respectively, were used, providing high and reliable values of maximum ECL intensity.

The maximum ECL intensity is presented in Fig. 4, as a function of hydrogen peroxide concentration. The maximum ECL intensity was reduced significantly when the concentration of this cosubstrate was more than 0.015%. Meanwhile, HRP compound-III can be formed by the oxidation of HRP compound-II by an excess of H_2O_2 or by the reaction of native HRP with superoxide anion. The HRP compound-III is less reactive than compounds I and II *(15)* and it could be a reason for the decrease in maximum ECL intensity. The optimal concentration of hydrogen peroxide (0.015%) was chosen and it was used in the further experiments.

Study of the Effect of Different Potential Pollutants on Enhanced Chemiluminescence (ECL) Intensity in the Reaction of Luminol Peroxidation

Reactions that proceed via a free radical intermediate are inhibited by substances that react with and quench free radicals. HRP enhancement

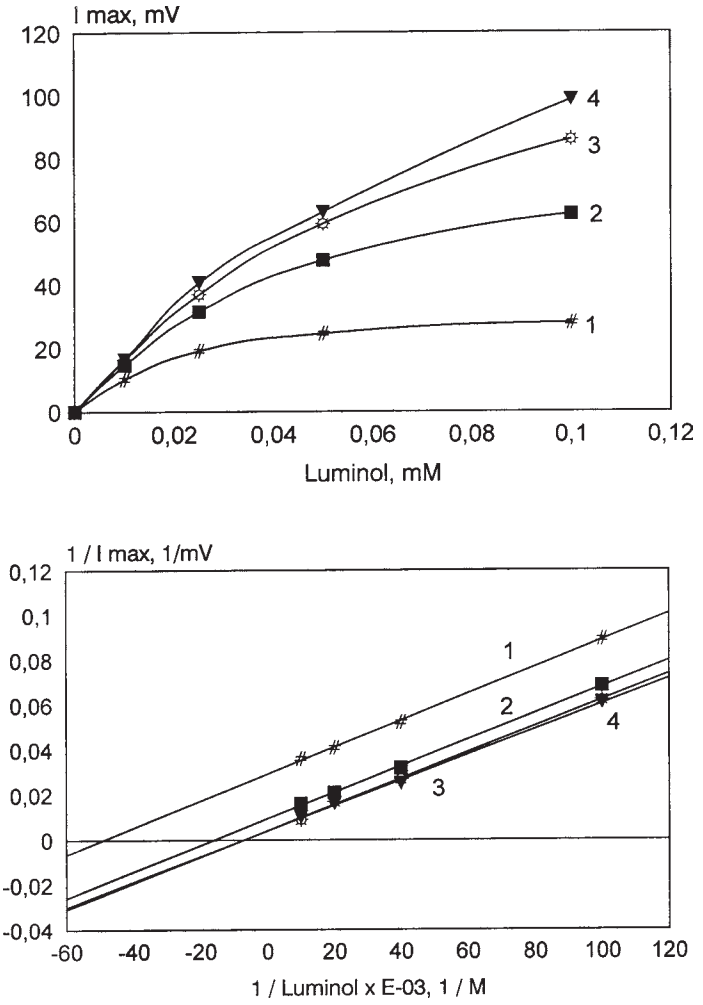

Fig. 2. **(Top)** Maximum ECL intensity from luminol peroxidation reaction as a function of luminol concentration in the presence of different concentrations of *p*-iodophenol: (1) 0.01 m*M*, (2) 0.02 m*M*, (3) 0.03 m*M*, (4) 0.04 m*M*. **(Bottom)** Presentation of obtained curves in L-B coordinates. Note: $I_{max\ app}/K_{m\ app} = 1.67$ mV/μ*M*.

occurs via formation of free radicals of enhancer that presumably diffuses away from HRP and subsequently interacts with luminol. Various substances are known to quench the chemiluminescence of luminol. ECL of the HRP system was found to be sensitive to certain biologically important compounds; addition of minute amounts elicited either quenching or enhancement of the intensity of chemiluminescence. It was reported early that some substances (cisteamine, glutatione, tyrosine, sulfite ion, ascorbate, and so on) are able to inhibit HRP-catalyzed ECL *(15–19)*. Antioxidants act effectively as quenching agents. A decrease as well as a delay of light emission was observed in the peroxidation of luminol by HRP in the presence of such reductants as reduced pyridine nucleotides *(16)*. The

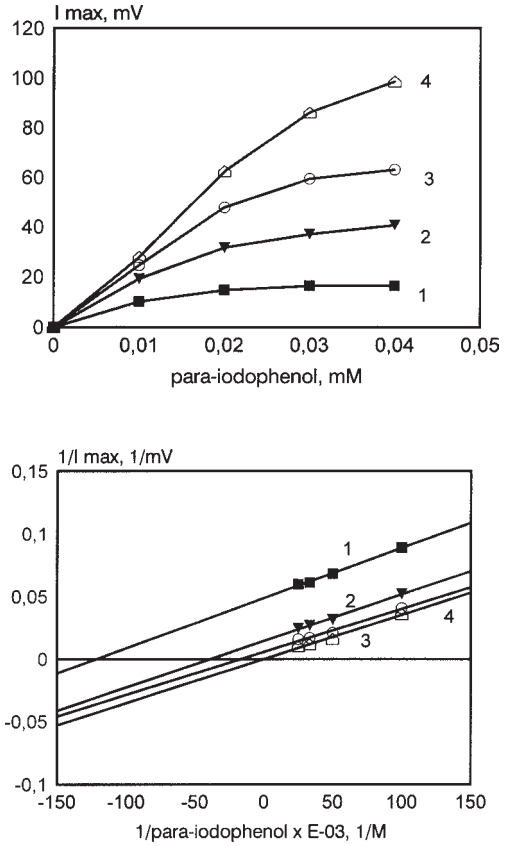

Fig. 3. **(Top)** Maximum ECL intensity from luminol peroxidation reaction as a function of the *para*-iodophenol concentration in the presence of different concentrations of luminol: (1) 0.01 m*M*, (2) 0.025 m*M*, (3) 0.05 m*M*, (4) 0.1 m*M*. **(Bottom)** Presentation of obtained curves in L-B coordinates. Note: $I_{max\ app}/K_{m\ app} = 2.5$ mV/μ*M*.

addition of such antioxidants as ascorbate in the course of development of ECL signal temporally causes a delay in light output *(17)*. Similarly, other substances could react with *para*-iodophenol and luminol radicals, thereby preventing formation of luminol radicals or oxidation to aminophthalate and subsequent light emission.

Thus, the hypothesis guiding these experiments was that the presence of pollutants resulting in free radical quenching or enhancement of their production should change significantly light emission. These effects can be used for pollutant detection.

There are many potential toxic chemicals that are likely to have a wide environmental impact. For example, agriculture uses some substances such as bactericides, herbicides, insecticides, and so on, generally of synthetic origin, there are discharges of detergents and surfactants, liberation of some metal ions. Altogether these pollutants can damage the health and the environment due to their accumulation and slow elimination *(1,2)*.

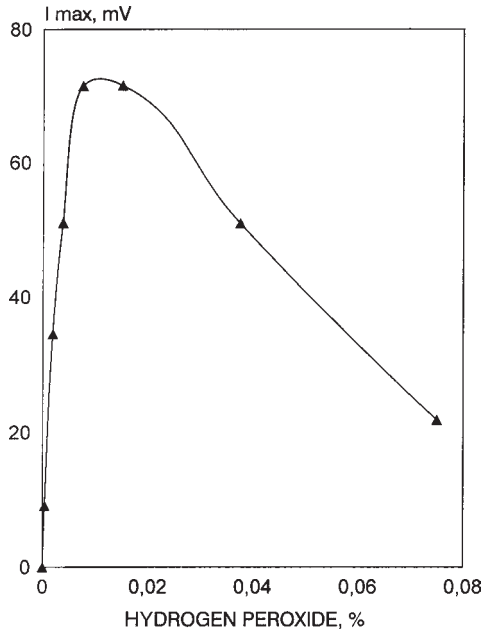

Fig. 4. Maximum ECL intensity from the luminol peroxidation reaction as a function of hydrogen peroxide concentration.

In this study, the effect of several substances on the light emission from luminol peroxidation was studied. Under the applied conditions (pollutant concentration at the level of milligrams per milliliters and incubation period), the chemicals used as potential pollutants can be classified in two principal groups:

1. provoking a decrease in the ECL intensity;
2. provoking an increase in the ECL intensity.

Such chemicals as phenol, herbicide, detergent Ariel, laboratory surfactants, bactericide, cyanide, and some metal ions (mercury, cobalt, and nickel) are found within the first group (Figs. 5–7). These substances provoke different changes in the ECL intensity profile (Fig. 8). For example, phenol, detergent, and bactericide reduce the maximum intensity but not the tangent of the initial part of the intensity-time profile, whereas in the case of herbicide and metal ions, both the decrease in the maximum ECL intensity and a change in the tangent are observed (Fig. 8). The relationships that characterize the dependence of the maximum intensity on the pollutant concentration (Figs. 5–7) were obtained for every one of the mentioned substances. Preincubation of enzyme with surfactants Brij-96 and Tween-20 provoke the decrease in the ECL intensity using higher concentrations than in the case of detergent Ariel (Fig. 6). Within the applied concentration range, linear dependencies were obtained for herbicide, detergent, and bactericide (Fig. 5), whereas the quantitative detection of

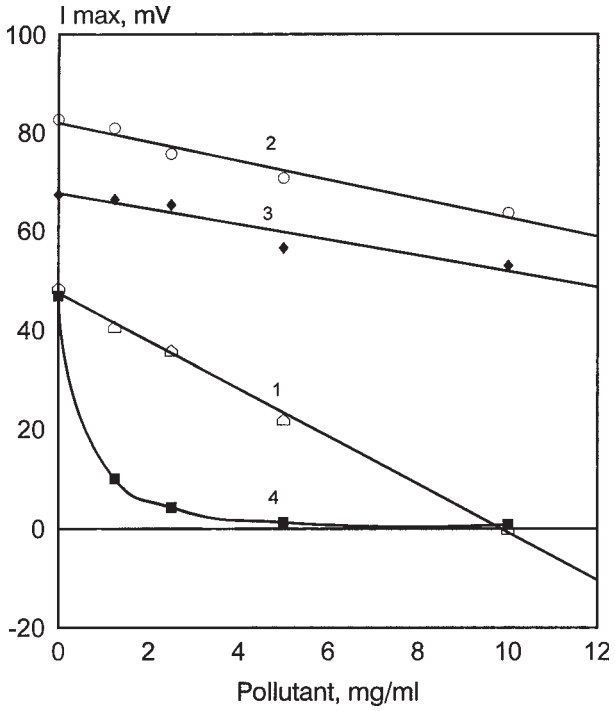

Fig. 5. Maximum ECL intensity as a function of concentration of (1) herbicide, (2) detergent Ariel, (3) bactericide, and (4) phenol.

phenol was possible till the concentration of 5 mg/mL and the obtained curve was nonlinear (Fig. 5). The effect of phenol and cyanide on the ECL reaction was studied at the concentration level of micrograms per milliliter (Figs. 9 and 10). At phenol and cyanide concentrations less than 2 and 1.25 μg/mL, respectively, the increase in maximum intensity was detected. The initial part of calibration graphs may be presented as a linear function (Figs. 9B and 10B).

The tested potential pollutants can be listed by their inhibiting capacity in the following order (Figs. 5 and 6): phenol cyanide>herbicide> bactericide>detergent Ariel>surfactants.

It is known that metal ions affect the chemiluminescent intensity when luminol is mixed with hydrogen peroxide in alkaline solutions (in the absence of peroxidase). This catalytic effect was observed with Cu (II), Ni (II), Mn (II), Fe (III), and Co (II) ions, and it was applied to develop an assay for their determination at the level of nanograms per milliliter *(13)*. Contrary to nonenzymatic luminol peroxidation, preincubation of HRP with Hg (II), Co (II), and Ni (II) ions inhibits the luminol peroxidation reaction (Fig. 7). It is possibly owing to the partial enzyme inactivation. The effect of the different metal ions on the ECL intensity was described with the different calibration graphs (Fig. 7). For mercury ions the function was linear within a range of concentrations from 0.04 mg/mL to 0.1 mg/mL, for

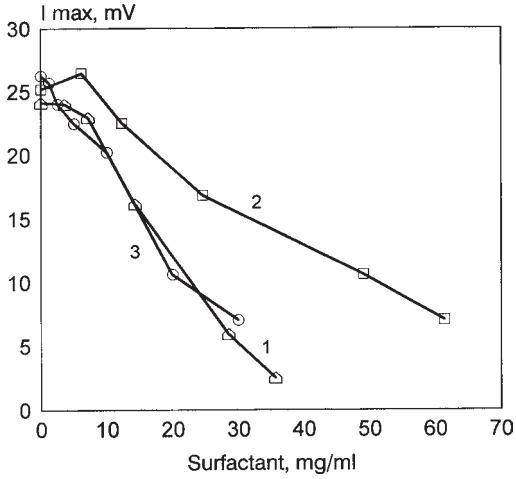

Fig. 6. Maximum ECL intensity as a function of concentration of (1) Tween-20, (2) Brij-96, and (3) detergent Ariel.

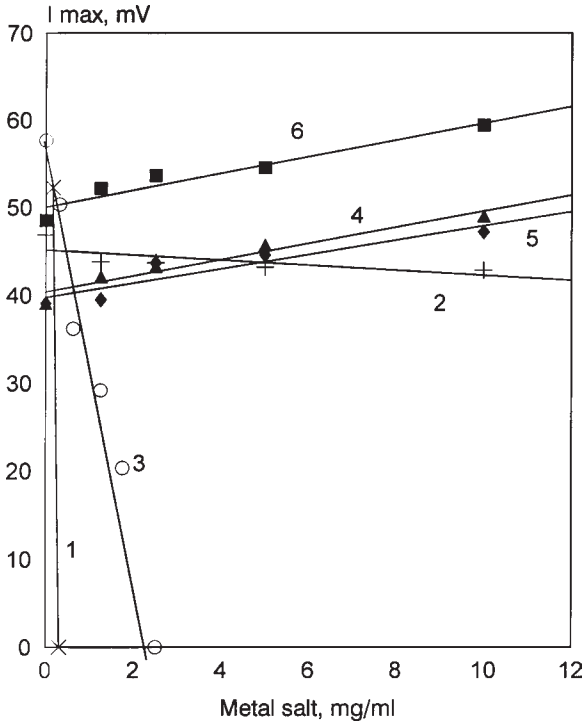

Fig. 7. Maximum ECL intensity as a function of concentration of (1) mercury chloride, (2) nickel chloride, (3) cobalt chloride, (4) magnesium chloride, (5) cadmium chloride, and (6) zinc sulfate.

cobalt ions—from 0.01 mg/mL to 2.5 mg/mL, and in the case of nickel ions—from 1.25 mg/mL to 10 mg/mL. In addition, a change in the tangent of the initial part of the ECL intensity profile was measured (Fig. 8). Accord-

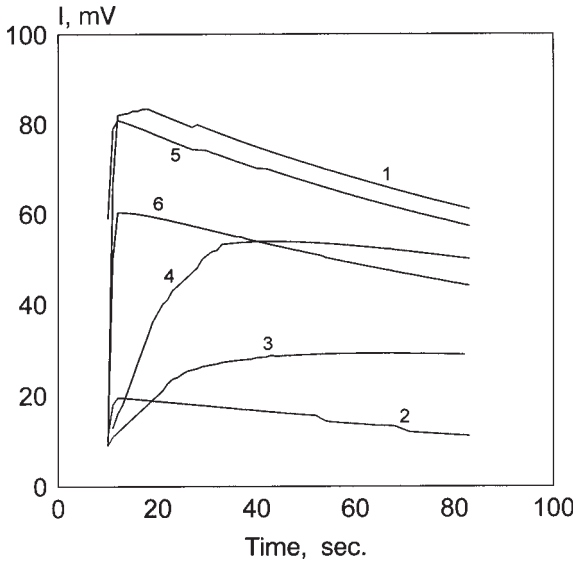

Fig. 8. ECL intensity-time profile in the absence of pollutant substances in water (1, control) and in the presence of: (2) phenol, (3) Co (II) ions, (4) herbicide, (5) detergent Ariel, and (6) bactericide.

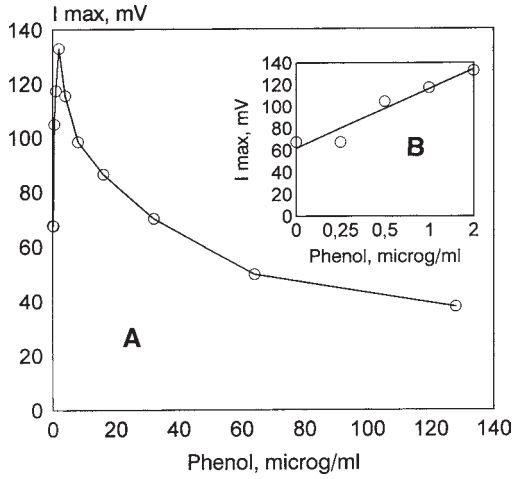

Fig. 9. Maximum ECL intensity as a function of concentration at μg/mL of phenol: **(A)** the used concentration range, **(B)** the initial part of curve.

ing to the inhibitory effect, the ions inhibitors can be listed in the following order: mercury >cobalt > nickel.

Figures 7 and 11 demonstrate that the ECL intensity was increased after preincubation of enzyme with the emulsions of some insecticides (Rugby, Pounce, Dominex) and with solutions containing zinc, magnesium and cadmium ions. The increase in the intensity is not very significant within the concentration range used and the duration of incubation. Fur-

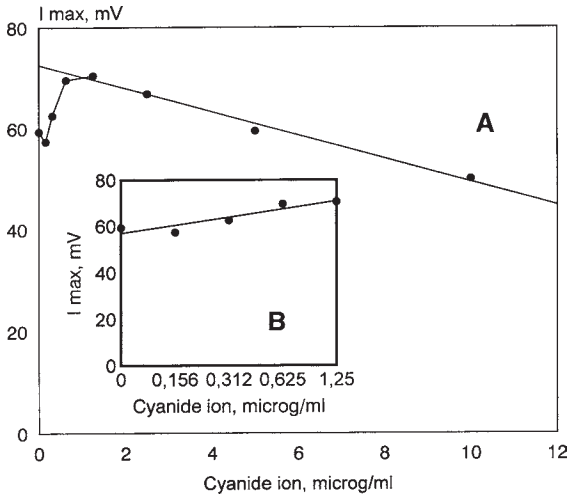

Fig. 10. Maximum ECL intensity as a function of concentration at μg/mL of cyanide: **(A)** the applied concentration range, **(B)** the initial part of curve.

thermore, the maximum intensity was a linear function of the concentration of these ions, whereas the calibration graphs obtained for insecticides were nonlinear (Fig. 11).

Thus, we demonstrate that the ECL system of luminol peroxidation catalyzed by HRP can be applied as an analytical tool for detection of a great variety of chemicals in water solutions. The ECL intensity varies in the presence of enzyme inhibitors and quenchers or enhancers of radicals *(17–22)*. Therefore, this system is suitable to monitor environmental contamination. Analyzing the results obtained in this work, we conclude that the ECL reaction in several cases can be used for qualitative or quantitative detection of water pollutants. The detection technique is faster than other known methods *(23)*. It does not require a special treatment of a sample such as extraction or concentration of the pollutant. Using the ECL system, it is possible to propose a method employing small amounts of the enzyme, which, furthermore, is quite stable in aqueous solution. We expect that the method developed in this work will be rapid, direct, portable, and compact as soon as the results can be obtained on-site or in line, avoiding transportation and sample conservation problems. The reagents and the equipment used in the chemiluminescent method are not expensive and the method does not require special training of the technicians. The development of this method presents a new perspective in the field of bioanalytical science.

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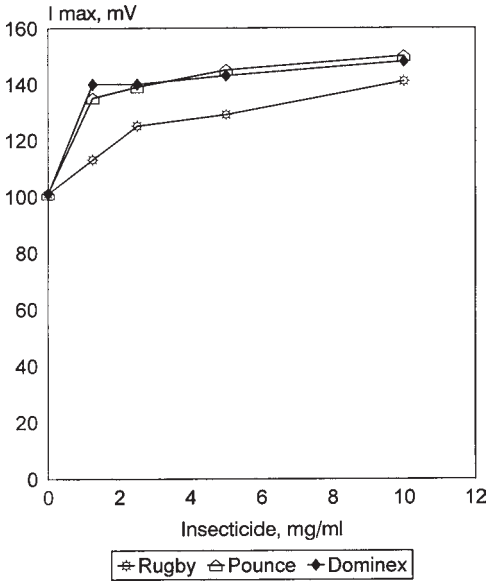

Fig. 11. Maximum ECL intensity as a function of concentration of some insecticides.

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