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Delayed Luminescence of Luminol Initiated by a Membrane-Bound Peroxidase

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Abstract

The luminescense of the luminol–H_2O_2 system was initiated by either free or membrane-bound horseradish peroxideae (HRP). The instantaneous luminescene decayed rapidly and was followed by the delayed luminescence in the presence of excess luminol. The delayed luminescence was characterized by a chain reaction, in which luminescence intensity increased exponentially. Membrane-bound HRP demonstrated that the delayed luminescence took place even in the absence of HRP if the instantaneous luminescence was initiated by HRP. A mechanism for the nonenzymatic luminescence is proposed and discussed.

Index Entries: Delayed luminescence, of luminol; luminescence, delayed, of luminol; luminol, delayed luminescence of; membrane-bound peroxidase, and delayed luminescence; peroxidase, delayed luminescence of membrane-bound.

Introduction

Biochemical luminescence reactions have gained increasing attention in both the fundamental and applied biochemical sciences, particularly since the development of simple and sensitive photon-counting systems. The analytical application of bio-

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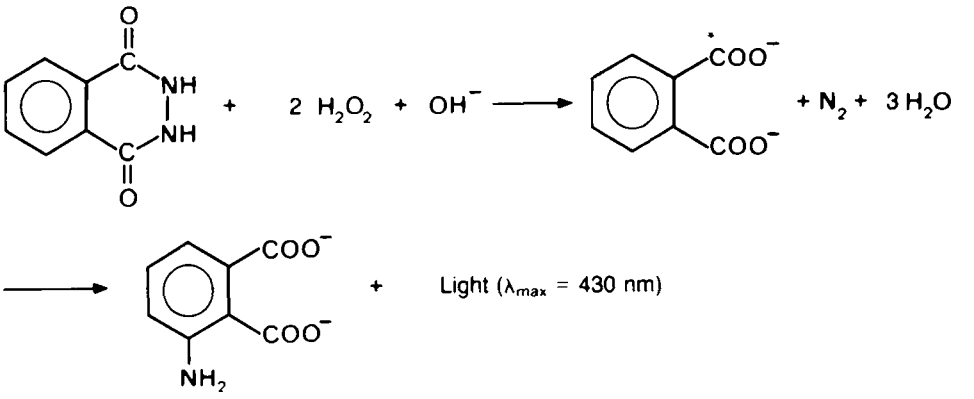
chemical luminescence has been proven feasible not only in enzyme assays, but also in immunoassays. Several novel devices have been proposed for the assay systems based on biochemical luminescence (*1–12*). In connection with the progress in luminescence analyses, extensive research has been carried out on biochemical luminescence reactions catalyzed by such enzymes as peroxidase (*13–16*), xanthine oxidase (*17–21*), lipoxygenase (*22*), and others (*23*, *24*).

Peroxidase is one of the enzymes commonly used to cause emission of visible light in the presence of luminol and H_2O_2, according to the following reaction sequence:



Although the reaction mechanism has not satisfactorily been established, luminol and H_2O_2 undergo luminescent reaction in two steps. The initial luminescence bursts and decays sharply, which is followed by the delayed luminescence. The delayed luminescence increases gradually, reaching a maximum. The luminescence process depends strongly on the molar ratio of luminol to H_2O_2. These facts suggest the inevitable characterization of the peroxidase-catalyzed luminol luminescence specifically in relation to analytical applications.

In our previous study (*12*), horseradish peroxidase (HRP) was immobilized in membrane matrix for the development of luminescent analytical devices. The membrane-bound HRP occasionally provided evidence for a new finding that the delayed luminescence occurs even in the absence of HRP, specifically when HRP is present at the initial stage of the luminescence reaction. This paper describes the characteristics of the delayed luminescence initiated by membrane-bound HRP.

Materials and Methods

Materials

Horseradish peroxidase (HRP, EC 1.11.1.7) (43.7 U/mg) was obtained from Amano Pharmaceutical Co., Ltd., (Nagoya). Collagen fibril was prepared by the method reported previously (*25*). Luminol (5-amino-2,3-dihydro-1,4-phthaladine dione) was purchased from Tokyo Kasei Co., Ltd. (Tokyo) and was used as a dimethylsulfoxide (DMSO) solution.

Preparation of HRP Membrane

A 2-mL solution of 25 mg/mL HRP was mixed with 50 g collagen fibril suspension (0.7% collagen by dry weight) and 50 mL H_2O and was cast on a Teflon board (6.5 × 25 cm^2) immediately after the evacuation of air bubbles from the suspension. The membrane was dried in a current of dry air at room temperature, and treated with 0.1*M* phosphate buffer (pH 7.0) containing 0.5% glutaraldehyde for 5 min. The preparation was washed carefully with water and 0.1*M* phosphate buffer, pH 7.0, which was then followed by drying at room temperature for 5 h. The membrane was noted as HRP membrane I.

In a similar fashion, 3 mL of 20 mg/mL HRP was mixed with 50 g of collagen fibril suspension (0.7% in dry weight) to prepare HRP membrane II. The membrane was treated with 1.0% glutaraldehyde (0.1*M* phosphate buffer, pH 7.0) for 30 s at room temperature.

Activity Measurement

Peroxidase activity was determined with HRP, H_2O_2, phenol (12.3 m*M*), and 4-aminoantipyrine (34 m*M*) at 37°C and expressed as μmol H_2O_2 converted per minute. Every reagent was prepared as a phosphate buffer solution (0.1*M*, pH 7.0). The rate of decomposition of H_2O_2 was measured by following the color development at 503 nm.

Each membrane was cut into small pieces (1 × 1 cm^2), and washed with 0.1*M* phosphate buffer of pH 7.0 at 37°C until no appreciable leakage of HRP was observed. Into a 100-mL flask were pipeted 1 mL of 57 m*M* 4-aminoantipyrine and 5 mL of 0.1*M* phosphate buffer (pH 7.0). The flask was shaken at 37°C. Three sheets of HRP membrane were immersed in the solution. Absorbance at 503 nm was monitored at intervals of 3 min. The apparent activities of HRP membranes I and II were 0.055 and 0.80 units/cm^2, respectively.

Measurement of Luminescence

Light emission was measured using a photon counter (Model 2000, SAI Technology Co., San Diego, CAL) equipped with an integrater.

Results

Enzyme Luminescence in the Free HRP–Luminol–H_2O_2 System

Figure 1 shows three typical luminescence patterns of the HRP–luminol–H_2O_2 reaction. A luminol solution was placed in the cuvette of a photon counter. Addition of H_2O_2 and native HRP initiated the emission of light, leading to an increase in output reading. The output reading followed the time course shown in Fig. 1, depending on a molar ratio of H_2O_2/luminol.

When H_2O_2 exceeded luminol in concentration, luminescence burst out and decayed gradually as curve (a) in Fig. 1. Curve (b) in Fig. 1 was obtained when the reaction between luminol and H_2O_2 was studied in a nearly 1:1 ratio. An initial

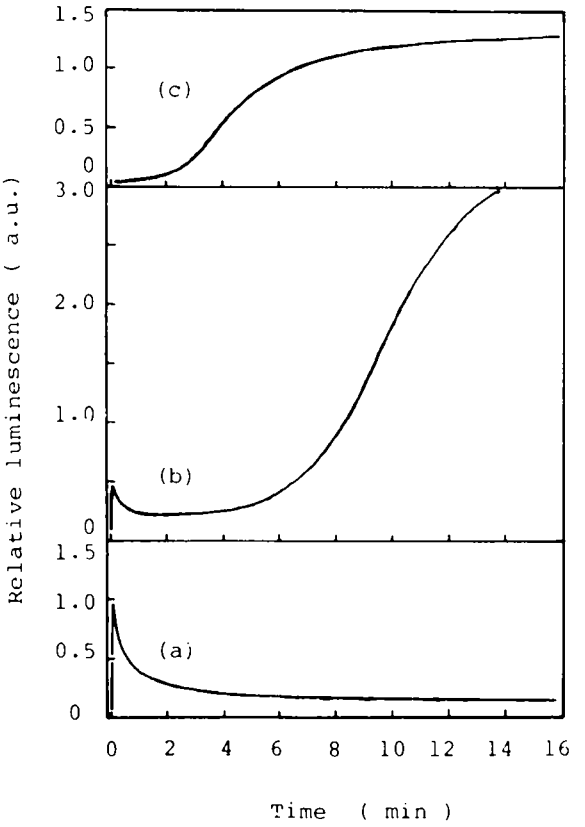


Fig. 1. Luminol luminescence at various molar ratios of H_2O_2/luminol. Luminol; 340 μM; HRP; 0.08 U/mL; H_2O_2: (a) 2.94 mM; (b) 540 μM; and (c) 120 μM.

sharp peak was followed by a gradually increasing curve. In contrast, luminescence changed as curve (c) in Fig. 1 in an excess of luminol. The initial sharp peak disappeared and a delayed luminescence intensively increased, reaching the maximum. These results clearly show that the HRP–luminol–H_2O_2 reaction involves two luminescence processes. Our efforts have been concentrated on the characterization of the delayed luminescence reaction.

The delayed luminescence reaction was distinctive in an excess of luminol. Figure 2 shows the dependence of the delayed luminescence on the concentration of H_2O_2. The initial luminescence appeared to increase exponentially with time; therefore, log [luminescense intensity] was plotted against time, which resulted in a straight line in the time range 3–6 min after a 3–4 min lag phase; this indicated that the delayed luminescence proceeded by a chain reaction, which might be derived from reactive intermediates generated in the course of the initial luminescence.

As shown in Fig. 2, the inclination of the curve depended in great measure on the concentration of H_2O_2. Its initial inclination corresponds to the initial change in the rate of reaction to time, since the intensity in luminescence corresponds to the rate of reaction; thus the inverse initial inclination of the curve was plotted against the inverse concentration of H_2O_2 in Fig. 3 in a manner similar to the Lineweaver-

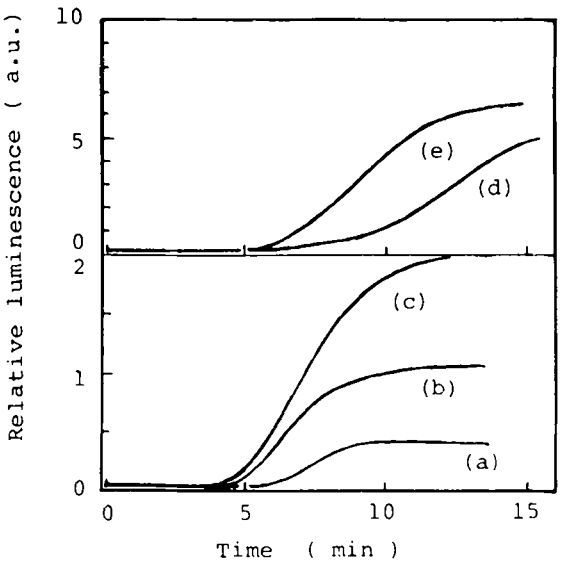


Fig. 2. Dependence of the delayed luminescence on the H_2O_2 concentration. Luminol; 1.28 m*M*; HRP, 0.06 U/mL, H_2O_2: (a) 3.5 μ*M*; (b) 7.0 μ*M*; (c) 18 μ*M*; (d) 35 μ*M*; and (e) 177 μ*M*.

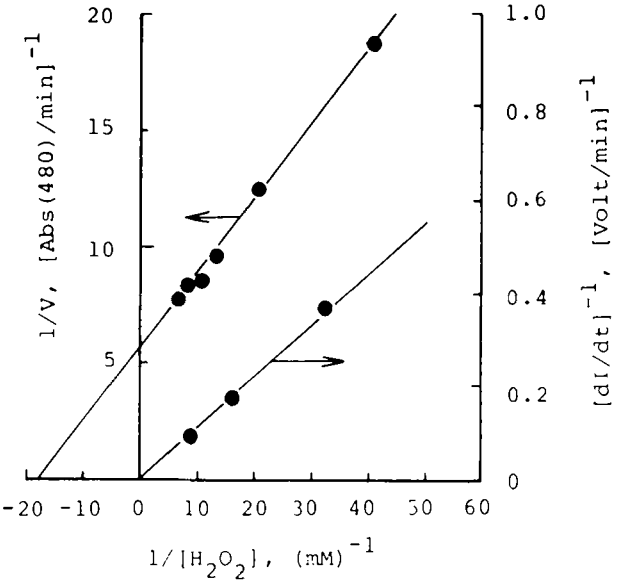


Fig. 3. Double reciprocal plots of HRP reactions coupled with either the luminol–H_2O_2 or the phenol–H_2O_2–4-aminoantipyrine system. Luminol–H_2O_2 system: 2.95 m*M* luminol and 0.04 U/mL HRP. Phenol–H_2O_2–4-aminoantipyrine system: 2.1 m*M* phenol, 1.5 m*M* 4-aminoantipyrine, and 0.015 U/mL HRP.

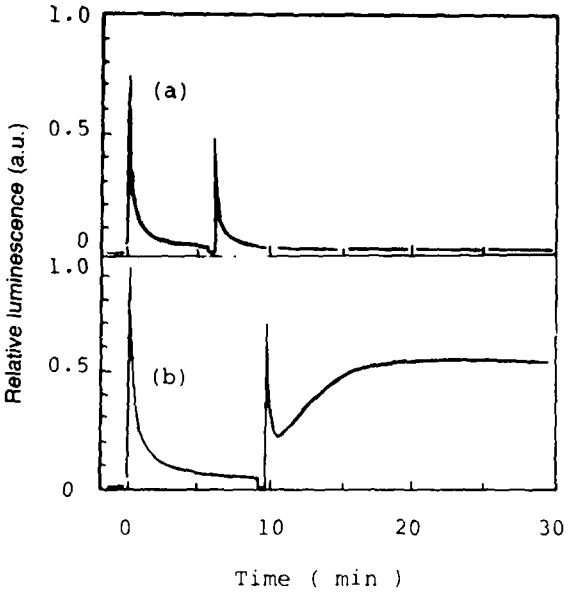


Fig. 4. The delayed luminiscence with the different catalytic activities of HRP membranes. Membrane I: 0.055 U/cm^2, 430 μM luminol, and 37 μM H_2O_2; membrane II: 0.80 U/cm$_2$, 360 μM, and 74 μM H_2O_2.

Burk plot. The result was a straight line that passed the origin. The significance of the double-reciprocal plot passing through the origin can be explained as follows. Fig. 4 shows that the inverse initial inclination of luminescence (dI/dt) is proportional to the inverse H_2O_2 concentration.

$$[dI/dt]^{-1} \propto [H_2O_2]^{-1} \tag{1}$$

whereas the luminescence intensity corresponds to the rate of reaction. We may write

$$I = [dS/dt] \tag{2}$$

By comparison with Eq. (1), we may obtain

$$dI/dt = d^2S/dt^2 \propto [H_2O_2] \tag{3}$$

Equation (3) suggests that the luminol–H_2O_2 system undergoes a chain reaction where the light intensity increases exponentially after the lag phase.

On the other hand, peroxidase activity was determined photometrically with phenol, H_2O_2, and 4-aminoantipyrine. No chain reaction should be induced by the generated phenoxy radical. The double reciprocal plot for the HRP–phenol–H_2O_2–4-aminoantipyrine system is also presented in Fig. 3. Lineweaver-Burk plot gave a K_m value for H_2O_2 as shown in Fig. 3. Cormier and Prichard reported that the effects of both luminol and H_2O_2 concentrations on the initial luminescence rate show normal Michaelis-Menten kinetics in their study of luminol luminescence, and that this does not necessarily lead to the conclusion that there is intermediate formation of an enzyme–substrate complex [26]. Therefore,

the participation of an enzyme–substrate complex was also not expected to be involved in the HRP-catalyzed reaction of phenol–H_2O_2–4-aminoantipyrine.

It is noted that there is a great difference between two straight lines involved in Fig. 3, which suggests that the delayed luminescence of the HRP–H_2O_2 reaction results from some other catalytic process than peroxidase catalysis.

Delayed Luminescence Initiated by Membrane-bound HRP

Both HRP membranes I and II caused luminol to emit light in the presence of H_2O_2 in the similar fashion to the free HRP–luminol system. However, little difference was found between membranes I and II. The time courses of luminescence are presented in Fig. 5 for membranes I and II. Membrane I induced the sharp initial peak of luminescence, but no appreciable delayed luminescence up to 30 min. When membrane I was shaken after 6 min, another sharp luminescence appeared. Membrane II also gave the initial sharp peak of luminescence, that was followed by the delayed luminescence. However, the delayed luminescence appeared after 20 min, and gradually increased. The shaking of the membrane prompted markedly delayed luminescence as shown in Fig. 5. Membrane I is different from membrane

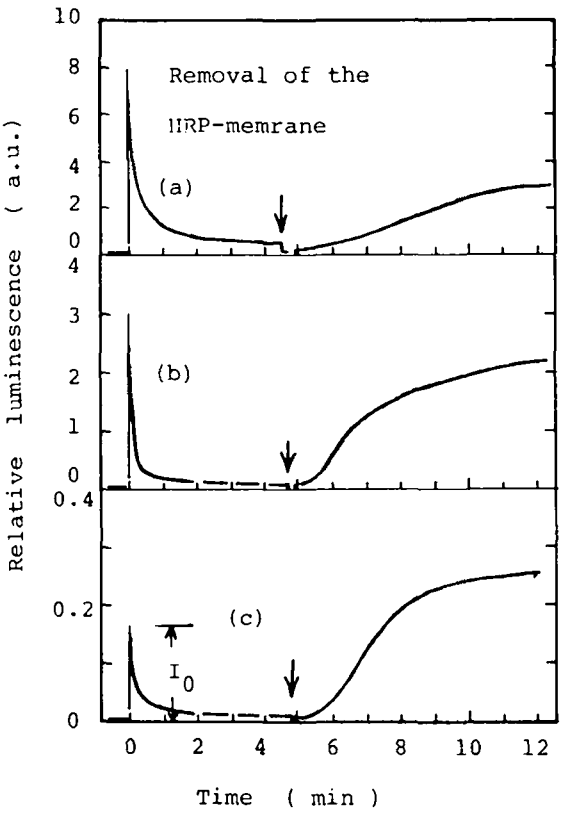


Fig. 5. Luminescence after the removal of the membrane-bound HRP. The membrane (Membrane II, 0.80 U/cm^2) was removed at the time indicated with an arrow. The intensity of the initial luminescence is termed I_0. H_2O_2: (a) 290 μM; (b) 120 μM; and (c) 24 μM.

II only in enzyme activity. The delayed luminescence resulted from only the membrane of high activity.

Since membrane-bound HRP can be easily taken out of the reaction system, the luminescence reaction of luminol takes place either in the presence or in the absence of HRP. The luminescence reaction of luminol was initiated in the presence of H_2O_2 by membrane II. The membrane was taken out after 5 min. Figure 5 shows the luminescence change owing to the above operation. An abrupt increase in luminescent intensity resulted from the contact of membrane-bound HRP to a reaction medium, which was followed by a gradual decay. When membrane II was taken out, the delayed luminescence occured. The intensity of luminescence both in the initial burst luminescence and in the delayed luminescence related remarkably to the concentration of H_2O_2. It should be noted that the delayed luminescence reaction occurred even in the absence of HRP.

Figure 5 shows that the intensity of the delayed luminescence increased exponentially; therefore, the delayed luminescence in the absence of HRP is also preceded by a chain reaction. The delayed luminescence after the removal of membrane II, however, had no induction phase, whereas the delayed luminescence in the presence of free peroxidase required a 3–4 min induction phase.

Characteristics of the Delayed Luminescence

The delayed luminescence chain reaction takes place either in the presence or in the absence of HRP. In both cases the initial luminescence gives a sharp intensity peak. The decay time of the peak depends markedly on the concentrations of H_2O_2 and luminol. The intensity of the delayed luminescence increases exponentially with a lag time, reaching the maximum. The peak intensity of the initial luminescence is termed I_0.

Figure 6 shows that the peak intensity of the initial luminescence (I_0), and the gradient of the delayed luminescence of time with free HRP (0.05 U/mL) at different concentrations of H_2O_2. The concentration of luminol was fixed at 1.29 m*M*. The initial luminescence intensity increased with increasing H_2O_2 concentration. The gradient of the delayed luminescence also increased, reaching the maximum at 20–50 μM H_2O_2. The delayed luminescence depended on a ratio of $[H_2O_2]$/[luminol]; hence it was distinctive in the range 0.01–0.1.

The characteristics of both initial and delayed luminescence derived from the HRP-membrane are shown in Fig. 7. It should be noted that the delayed luminescence was measured in the absence of the HRP-membrane, and that the HRP-membrane was present in the luminol–H_2O_2 system only during the initial luminescence. The HRP-membrane showed 0.8 U peroxidase activity/cm^2. The initial luminescence sharply increased with an increase in H_2O_2 concentration. When the initial luminescence decayed, the HRP-membrane was removed, which was followed by an increase in the delayed luminescence intensity. The gradient of the delayed luminescence changed, depending on a ratio of $[H_2O_2]$/[luminol] as presented in Fig. 7. The most distinctive delayed luminescence occurred around the molar ratio of 0.2.

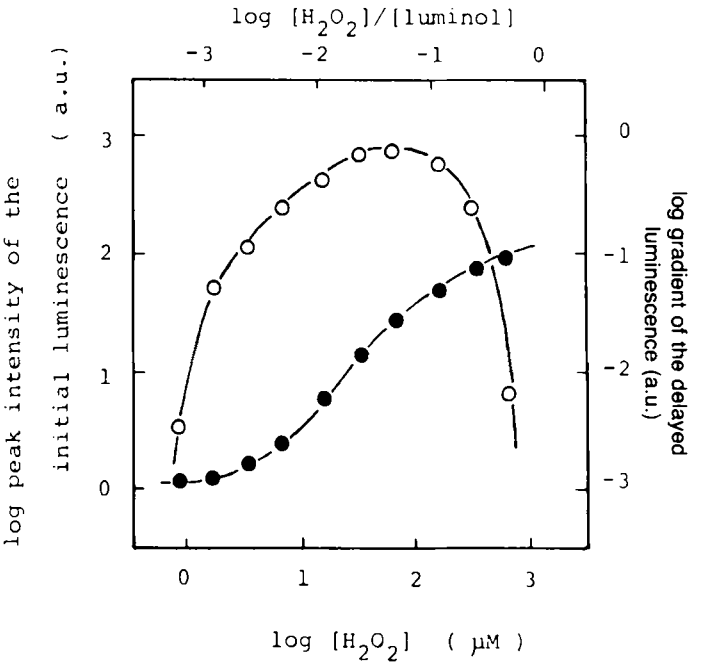


Fig. 6. The peak intensity of the initial luminescence I_0 (— ● —) and the gradient of the delayed luminescence (— ○ —) at various H_2O_2 concentrations. Luminol: 1.28 m*M*; free HRP: 0.05 U/mL.

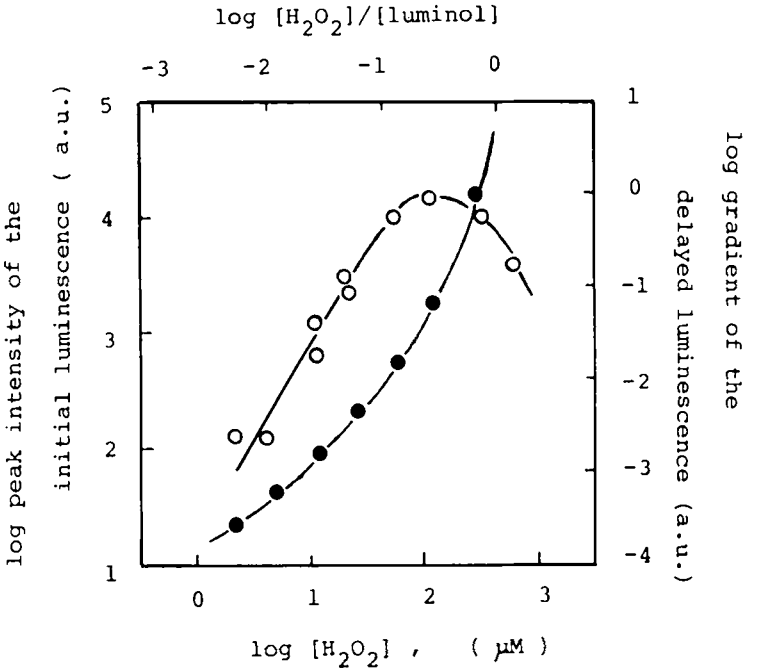
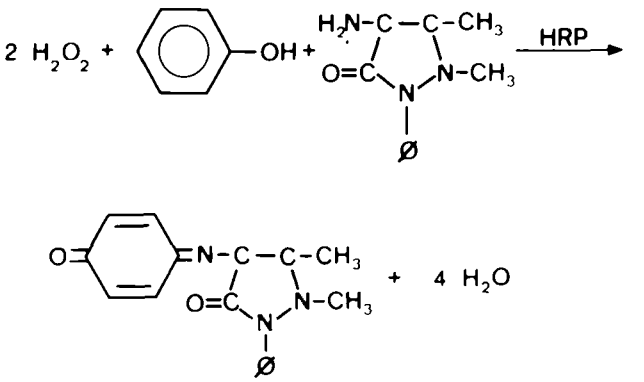


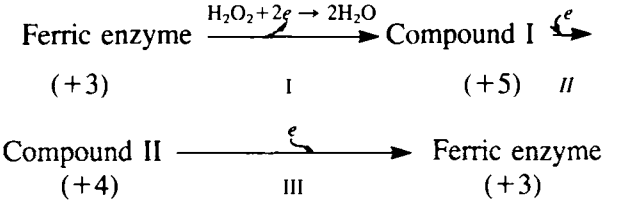
Fig. 7. The peak intensity of the HRP-membrane-catalyzed luminescence (— ● —) and the gradient of the delayed luminescence (— ○ —) after removing the membrane. Luminol: 340 μ*M*; membrane-bound HRP: 0.80 U/mL.

Discussion

The data presented in Figs. 1–3 indicate that the luminescent reaction of the HRP–luminol–H_2O_2 system undergoes a chain reaction, especially when the concentration of hydrogen peroxide is less than that of luminol. When compared with the experiment performed in HRP–phenol–H_2O_2–4-aminoantipyrine system (Fig. 3), where phenoxy radical is easily scavenged by 4-aminoantipyrine as shown in the following equation, a radical species of luminol is believed to play a key role in the HRP–luminol–H_2O_2 system.



The peroxidase-catalyzed reaction has been interpreted in relation to the peroxidase cycle (*27–29*):



where a number in a parenthesis denotes a relative oxidizing equivalent retained in the enzyme on the basis of the number of +3 to the native (ferric) enzyme. When luminol is used as an electron donor, Compounds I and II catalyze the oxidation of luminol [LH_2] to a radical species [LH·]. The luminol radical can then participate in either light emission or the generation of $O_2^{\cdot-}$ from dissolved oxygen in the manner (IV) shown in Fig. 8. The superoxide radical can oxidize luminol to a luminol radical with resulting formation of H_2O_2 (IV). The H_2O_2 generated oxidizes the enzyme by joining the so-called peroxidase cycle. According to the study of Cormier and Prichard, the luminol radical enters the light-emitting process as shown in the following equation (26):

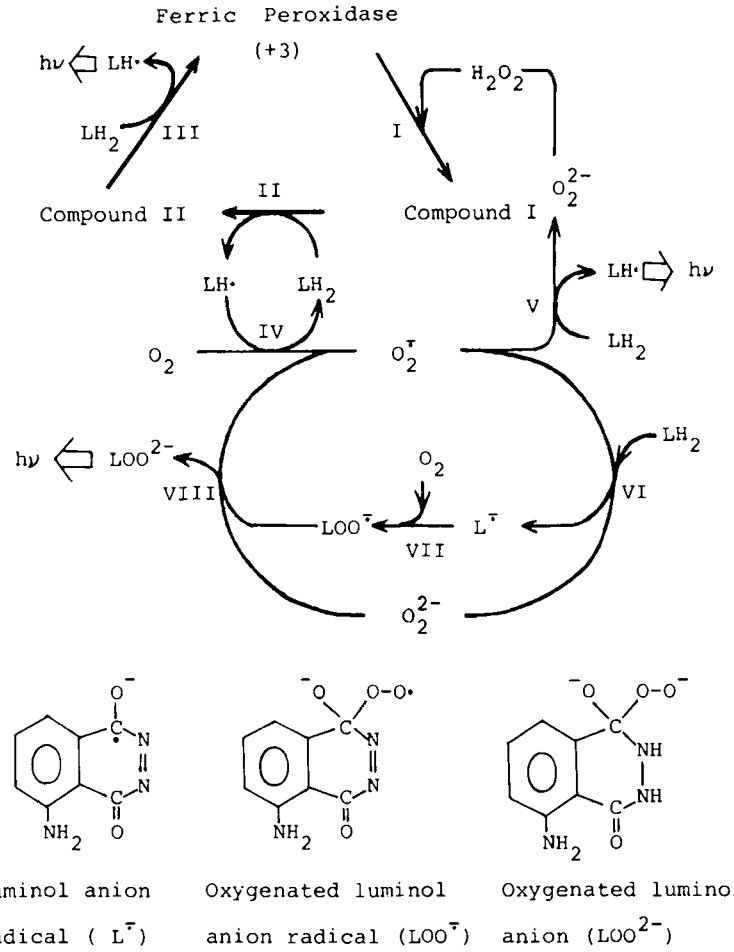


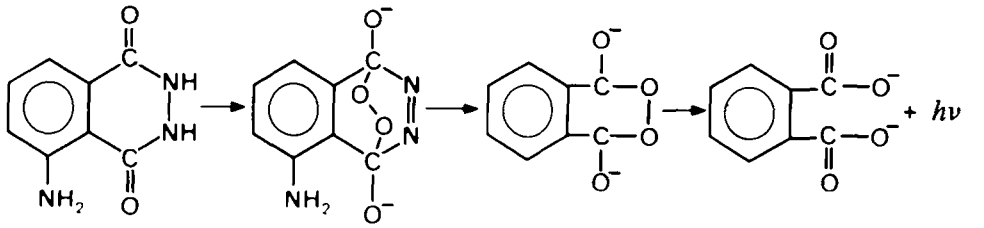
Fig. 8 Possible enzymatic and non-enzymatic luminescence processes. 5-Amino-1,4-phthalazine semidione and 5-amino-1,4-phthaladinedione-1-peroxyanion are termed as luminol anion radical ($L^{\cdot}$) and oxygenated luminol anion (LOO^{2-}), respectively.

$$2LH\cdot + H_2O_2 \rightarrow \text{Products} + h\nu$$

The peroxidase-catalyzed luminescence of luminol is well interpreted, as above. The primary process for the enzymatic luminescence is illustrated in Fig. 8 (I–V).

However, the data presented in Figs. 4 and 5 indicate that peroxidase-catalyzed luminescence or luminol accompanied not only the initial luminescence, but the delayed luminescence, and that the latter light-emitting reaction took place even in the absence of HRP provides that the reaction of luminol–H_2O_2 system was enzymatically initiated. Figure 7 shows that the delayed luminescence was most distinctive when hydrogen peroxide and luminol were used in the molar ratio of 0.2.

Although a luminol peroxide intermediate has never been isolated, up-to-date views of the luminescence of luminol (1) invoke formation of the compound (2) which degrades to the emitter (4) via the dioxethane intermediate (3) (*30*).



(1) (2) (3) (4)

In xanthine oxidase-induced chemiluminescence of luminol, light emission from luminol owing to the $O_2^{\cdot-}$ generated is observed (*20*). The reaction is inhibited by superoxide dismutase whereas the inhibitory effect of catalase is a relatively small one, if any (*31*). Whereas the luminol radical presumably reacts upon the oxygen molecule, since the latter is a characteristic biradical species, the reaction between an oxygenated luminol anion and hydrogen peroxide seems to proceed in a manner similar to that of a postulated Haber-Weiss reaction. Thus the enzymatically generated oxygen radical and luminol have sufficient potency to proceed along another path of luminescent chain reaction. As presented in Fig. 8, the oxygen radical can generate the luminol anion radical [$L^{\cdot-}$] (IV), although ionization of the luminol molecule either to the monoanion or the dianion is neglected in this report. The luminol anion radical is thus easily oxygenated, since both reactive substances concerned are radicals, which results in the generation of the oxygenated luminol anion with release of N_2.

Although key intermediates in the luminescent process are now being studied, the results of which will be presented in a subsequent paper, a postulated scheme consisting of $O_2^{\cdot-}$ – O_2^{2-}cycle is now expected to prove responsible for the nonenzymatic chain reaction.

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