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ANALYTICAL USE OF A BIOCHEMICAL LUMINOUS MEMBRANE

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Luminous membranes were prepared by immobilizing peroxidase (POD) to collagen matrix. The POD luminous membrane generated luninescence in the presence of luminol and H_2O_2, and the peroxide was determined in the concentration range 10^{-6}–10^{-3} M by following luminescence emitted from the membrane. Glucose was determined using a luminous membrane in which POD and glucose oxidase (GOD) were coimmobilized. The luminous membranes appear to be feasible for the determination of enzyme substrates and enzyme activity.

In recent years, the firefly bioluminescent reaction has found potential application in many areas of analyses. The use of the firefly bioluminescent reaction is based on the fact that luciferase emits light quantitatively in the presence of luciferin, O_2, and ATP. ATP and some glycerides have been quantitated in samples such as urine, plasma, red blood cell extracts, and adrenal gland secretions by the luciferase chemiluminescent measurement (1–4). In the last several years, some other enzymes such as peroxidase have been found to emit light. These enzymatic chemiluminescent reactions provide a sensitive and specific method for the determination of biochemically important substances (5, 6).

The system chosen for this study was the luminol reaction catalyzed by peroxidase. Peroxidase was immobilized in a membrane matrix. The peroxidase-bound membrane emitted light in the presence of luminol and H_2O_2. This luminous membrane was applied to the determination of H_2O_2, because the luminescence intensity reflected the concentration of H_2O_2.

Many oxidases generate hydrogen peroxide with the resulting oxidation of a substrate. When an oxidase is coupled with the POD luminous membrane, the oxidase reaction can initiate the chemiluminescent reaction

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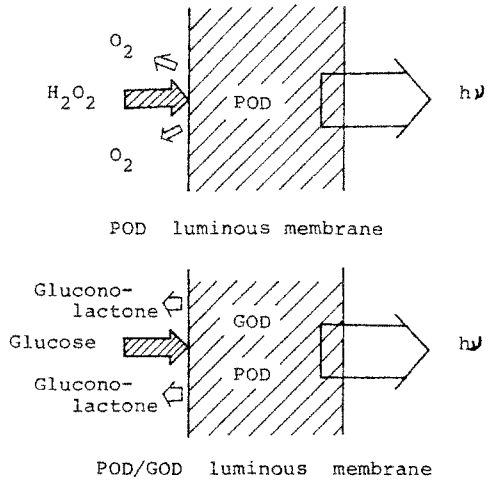


FIG. 1. Schematic representation of the POD and POD/GOD membranes. Chemiluminescence results from the catalytic action of the corresponding membrane-bound enzyme.

as follows:

$$\text{substrate} + O_2 \xrightarrow{\text{oxidase}} \text{product} + H_2O_2 \qquad (1)$$

$$H_2O_2 + \text{red} \xrightarrow[\text{(luminous membrane)}]{\text{POD}} \text{oxd} + H_2O + h\nu \qquad (2)$$

where red and oxd are the reductive and oxidative forms of luminol in this study. Either substrate or oxidase can be quantitated by measuring the chemiluminescence emitted from the POD luminous membrane. In the present investigation, glucose oxidase (GOD) was taken as a typical example. Both POD and GOD were immobilized in the same membrane matrix. The POD/GOD membrane became luminous in the presence of glucose, O_2, and luminol. The luminescence intensity reflected the concentration of glucose. This paper describes the applicability of the POD and POD/GOD luminous membranes to the chemiluminescent determination of H_2O_2 and glucose, of which schemes are represented in Fig. 1.

MATERIALS AND METHOD

Materials

Horse radish peroxidase (POD) (E.C. 1.11.1.7) and glucose oxidase (GOD) (E.C. 1.1.3.4) were supplied from Amano Pharmaceutical Co.,

Ltd., (Nagoya, Japan). Luminol was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution. A collagen fibril suspension was prepared as reported previously (7).

Preparation of Luminous Membranes

To 50 ml distilled water were added 50 mg POD a nd 50 g of 0.7% collagen fibril. The suspension was homogeneously mixed and cast on a Teflon plate (6.3 × 25 cm). After drying at room temperature for about 20 h, the membrane was peeled off and contacted with 0.05% glutaraldehyde for 5 min at pH 7.0. A POD luminous membrane was thus obtained. GOD (5 mg), POD (50 mg), and collagen fibril (50 g, 0.7% dry weight) were added to 50 ml of distilled water. A POD/GOD luminous membrane was prepared in the similar manner to the above.

Cellulose triacetate (250 mg) was dissolved in 5 ml dichloromethane. In the solution, 200 μl of 50% glutaraldehyde and 1 ml of 1,8-diamino 4-aminomethyl octane were added. The resulting solution was spread on a glass plate. After drying, the membrane was reacted with 1% glutaraldehyde and then with 1 mg/ml GOD at pH 7.6. The preparation was treated with 0.1 M $NaBH_4$ for 2 min, which was followed by thorough washing with water. This was termed a GOD membrane.

Activity Measurement of the Membrane-Bound Enzymes

Each luminous membrane was thoroughly washed and immersed in 0.1 M phosphate buffer of pH 7.0 for 24 h. Both the membrane and the washing solutions were assayed for enzyme activity.

The POD activity was determined with the usual colorimetric assay using H_2O_2, 4-amino antipyrine, and phenol. Into 3 ml of 0.46 mM H_2O_2, 1.45 mM 4-amino antipyrine, 1.96 mM phenol, and 0.1 M phosphate buffer of pH 7.0, 100 μl of the washing solution was added to determine the amount of POD leaked from the membrane. Absorbance at 480 nm was measured, and no appreciable increase was observed. This indicated that the leaking of membrane-bound POD was sufficiently prohibited. The activity of the POD membrane was determined in a similar way to that described above. The membrane-bound POD showed 55 U of peroxidase activity per cm^2 membrane.

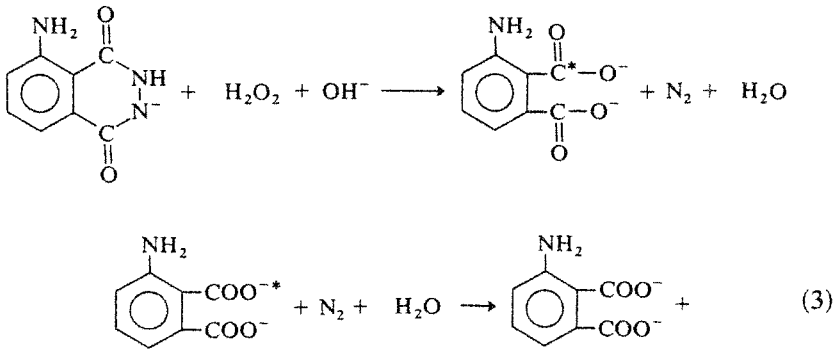
Luminescence Measurements

Chemiluminescence generated from a luminous membrane was measured using a photon counter (Model 2000) from SAI-Technology Co. (San Diego, Calif.) equipped with an integrator.

RESULTS

Response of a POD Luminous Membrane to H_2O_2

The luminol reaction catalyzed by POD is described as follows:



(3)

There are several factors influencing the chemiluminescence intensity. The optimum conditions of POD activity are different from those of the luminescence reaction. The luminescence intensity increases with an increase in pH. On the other hand, POD showed maximum activity at a slightly acidic pH. In consequence, the measurements were done at pH 7.0.

Into an aliquot of 0.1 M phosphate buffer (pH 7.0) contained in a vial for luminescence measurement, 50 μl of 8.54 mM luminol was added. A sheet of the POD luminous membrane was immersed. A requisite amount of H_2O_2 was then mixed with the solution to make the final volume 1 ml. After swirling for a few seconds, luminescence was recorded with a photon counter.

The typical response curves of the POD luminous membrane are presented in Fig. 2. The luminescence intensity sharply increased and then gradually decayed in a few minutes. The maximum intensity and integration of luminescence depended markedly on the concentration of H_2O_2, if a constant concentration of luminol was maintained.

The response of the POD luminous membrane to H_2O_2 depended on not only H_2O_2 but luminol. The molar ratio of H_2O_2 to luminol may be regarded as a determining factor. When the H_2O_2/luminol ratio was less than 0.5, the shape of each response curve agreed with the one presented in Fig. 2. However, the shape of the response curve was deformed as the H_2O_2/luminol ratio exceeded 0.5. It took a little longer time to reach the maximum intensity in luminescence along with a prolonged decay. A good linearity was not established between the concentration of H_2O_2 and the integrated luminescence under the above conditions. From these results, it

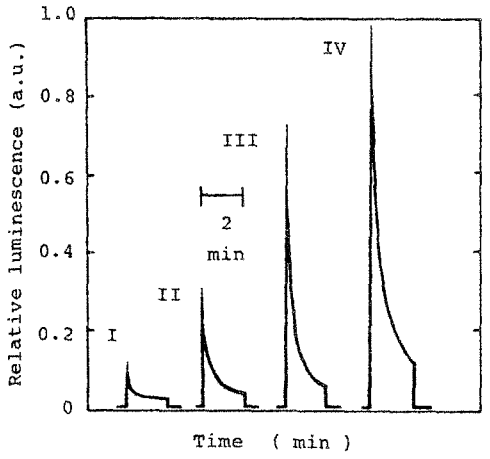


FIG. 2. Typical responses of the POD luminous membrane to H_2O_2. H_2O_2 concentrations: I, 4.7 μM; II, 11.8 μM; III, 23.5 μM; IV, 58.8 μM. Luminol concentration: 427 μM. Peroxidase activity: 55 U/cm^2. Chemiluminescence intensity is indicated in arbitrary units, where one unit corresponds to a 400 mV reading of the photon counter at a sensitivity of 8.0.

was concluded that H_2O_2 should be determined in the presence of excess luminol, using the POD luminous membrane.

Figure 3 shows the correlation between the concentration of H_2O_2 and the integrated luminescence. The concentration of luminol was 427 μM. Linearity was attained in the concentration range 10^{-6}–10^{-3} M H_2O_2. The POD luminous membrane was repeatedly used to obtain the calibration curve. The membrane was thoroughly washed with 0.1 M phosphate buffer of pH 7.0 after each measurement.

Various POD luminous membranes, which were different in specific activity, gave a good linearity between the concentration of H_2O_2 and the integrated chemiluminescence in the above concentration range. However, the slope depended slightly on the specific activity of the POD luminous membrane. The POD luminous membrane of which activity was 800 U/cm^2 showed a slope of 1.18 ± 0.06. The slope decreased to 1.08 ± 0.04 if the 55 U/cm^2 POD luminous membrane was used under the same conditions.

The standard deviation of the chemiluminescent measurement was determined with the POD luminous membrane at a specific activity of 55 U/cm^2. The POD luminous membrane was repeatedly reacted with 3.53 μM H_2O_2 at pH 7.2. The concentration of luminol was 569 μM. The

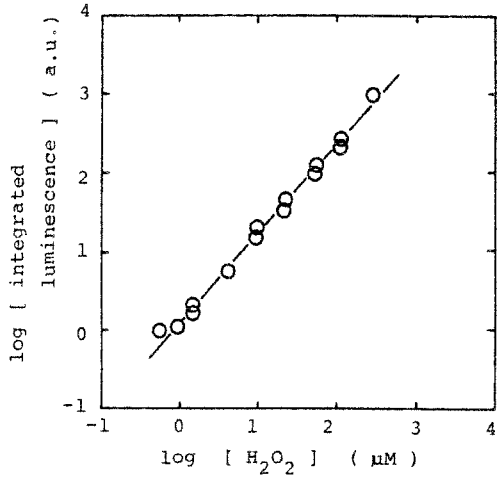


FIG. 3. Correlation between log(integrated luminescence) and H_2O_2 concentration. Luminol concentration: 427 μM. Integration: integrated for 2 min and expressed in arbitrary units.

integrated chemiluminescence was measured for 24 cases. The standard deviation yielded 16.8%. It is noted that the standard deviation markedly increased with an increase in repeated running. The first five runs gave a standard deviation of 5.7%. Moreover, the standard deviation was depressed in the lower concentration range of H_2O_2.

Determination of Glucose Using the POD/GOD Luminous Membrane

The POD/GOD luminous membrane catalyzes the oxidation of β-D-glucose and the luminol reaction:

$$\begin{aligned} \beta\text{-D-glucose} + H_2O + O_2 &\longrightarrow \text{gluconolactone} + H_2O_2 \\ H_2O_2 + \text{luminol} &\longrightarrow \text{product} + H_2O + h\nu \end{aligned} \tag{4}$$

Glucose may be quantitated by following the luminescence generated from the POD/GOD luminous membrane.

The POD/GOD luminous membrane showed 52 U/cm^2 of POD activity and 10 U/cm^2 of GOD activity at 30°C. A sheet of POD/GOD luminous membrane (1×1 cm^2) was placed in a vial for chemiluminescence measurement, in which 569 μM luminol and 0.1 M phosphate buffer of pH 7.0 were contained. A glucose solution was injected in the vial. After immediate mixing, chemiluminescence was recorded. The time course of chemiluminescence is shown for typical cases in Figs. 4 and 5. The shape of

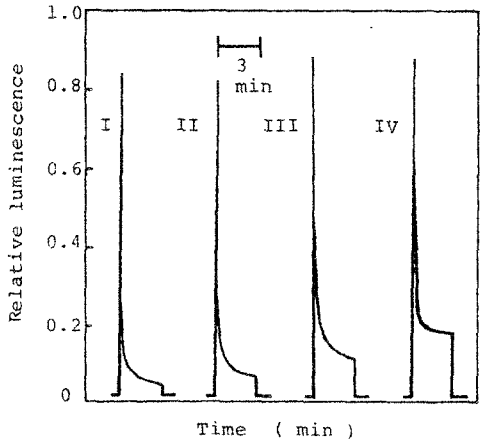


FIG. 4. Responses of the POD/GOD luminous membrane to glucose. Glucose concentration: I, 4.0 μM; II, 8.0 μM; III, 20.0 μM; IV, 40.0 μM. Luminol concentration: 569 μM. Luminescence intensity: expressed in arbitrary units, where one unit corresponds to a 50 mV reading of the photon counter at a sensitivity of 8.0.

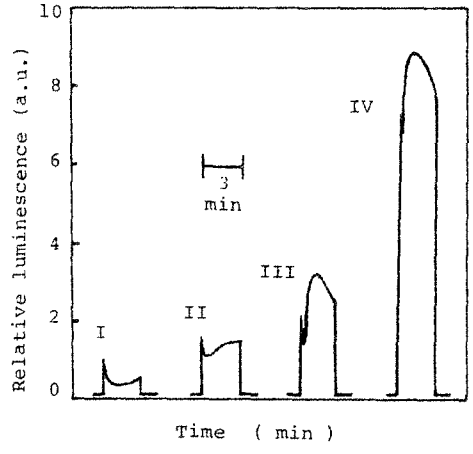


FIG. 5. Responses of the POD/GOD membrane to glucose. Glucose concentration: I, 100 μM; II, 200 μM; III, 400 μM; IV, 1000 μM. Luminol concentration: 569 μM. Luminescence intensity: expressed in arbitrary units, where one unit corresponds to 50 mV reading of the photon counter at a sensitivity of 8.0.

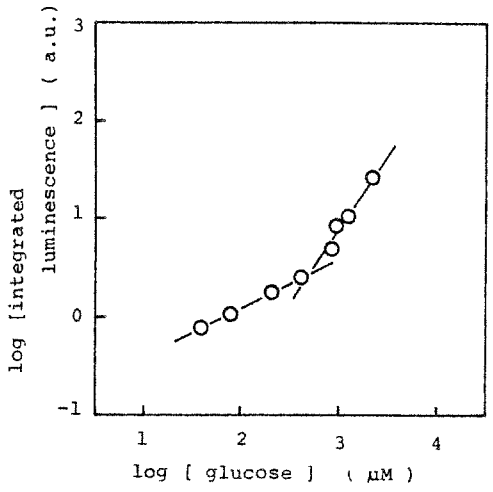


FIG. 6. Log(integrated luminescence) versus log[glucose] curve with the POD/GOD membrane. Luminol concentration: 569 μM. Integration: integrated for 3 min and expressed in arbitrary units.

the response curve depended considerably on the concentration of glucose. In the concentration range above 100 μM, the response curve was markedly deformed as compared to those in the lower concentration range.

The observed chemiluminescence was integrated for 2 min. The integrated chemiluminescence was plotted against the concentration of glucose. Two straight lines were obtained, as shown in Fig. 6. This result indicates that the integrated chemiluminescence reflects the concentration of glucose in two ways. In the lower concentration range 5–50 μM, the slope of the straight line was one-third of the one in the range 50–500 μM. As shown in Figs. 4 and 5, the response curves were considerably different in shape in these two concentration ranges. However, glucose was determined in the concentration range 5–500 μM.

Determination of Glucose Using the POD Luminous Membrane with the GOD Membrane

The GOD membrane, in which GOD was covalently immobilized to a cellulose triacetate membrane, was coupled with the POD luminous membrane for the determination of glucose. Glucose is oxidized by the GOD membrane. The POD luminous membrane catalyzes the chemiluminescence reaction of luminol and H_2O_2 generated by the GOD reaction. The measurements were made in a similar manner to the above.

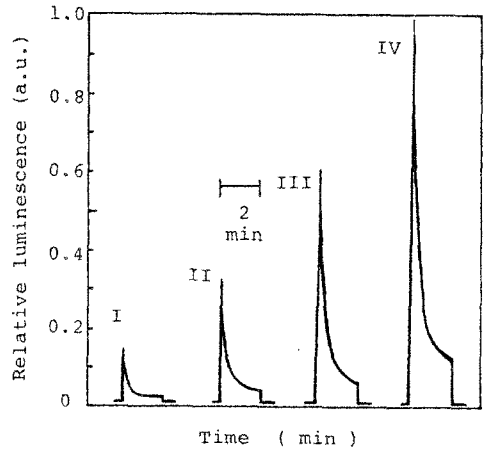


FIG. 7. Chemiluminescence of the POD membrane coupled with the GOD membrane in the presence of glucose. Glucose concentration: I, 50 μM; II, 125 μM; III, 250 μM; IV, 500 μM. Luminol concentration: 428 μM. Luminescence intensity: expressed in arbitrary units, where one unit corresponds to a 150 mV reading of the photon counter at a sensitivity 8.0.

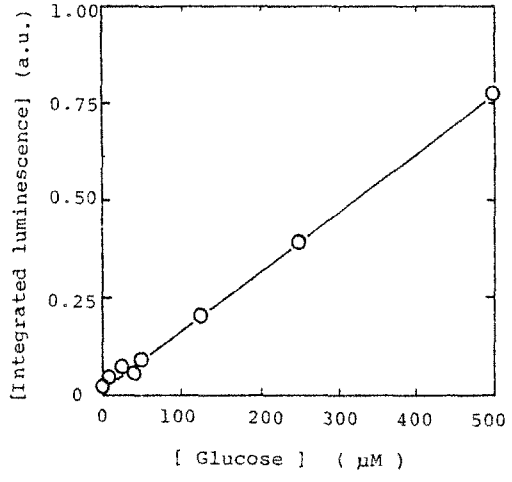


FIG. 8. Calibration curve for the determination of glucose by the POD membrane coupled with the GOD membrane. Luminol concentration: 428 μM. Integration: integrated for 2 min and expressed in arbitrary units.

Figure 7 shows the typical response curves when various concentrations of glucose were determined. The concentration of luminol was maintained at 482 μM. The shape of each curve was identical in the concentration range 10–500 μM glucose.

A calibration curve is shown in Fig. 8. A good linearity was obtained between the integrated luminescence and the concentration of glucose over the range 10–500 μM. It shows the feasibility of the luminous membrane in the chemiluminescent determination of glucose.

DISCUSSION

The POD luminous membrane emitted light only in the presence of H_2O_2 and such a reductant as luminol. Of several reductants surveyed, luminol was most effective on chemiluminescence. Under appropriate conditions, the chemiluminescence intensity of the POD luminous membrane reflected sensitively the concentration of H_2O_2. This leads to the chemiluminescent determination of H_2O_2 using the POD luminous membrane. As H_2O_2 is generated by many oxidase reactions, the POD luminous membrane should be applicable to the chemiluminescence determination of a specific substrate that couples with these oxidases. The determination of glucose is described as an example in this paper. Furthermore, the membrane can be used for the quantitation of these oxidases.

The POD luminous membrane provides several advantages over soluble POD. For example, since the immobilized enzyme is localized on the membrane, the membrane-bound POD is reusable. This might be advantageous specifically for practical purposes.

Brown-colored precipitate appears after the luminol reaction. The precipitate adsorbs partly on the POD luminous membrane, which causes a gradual inactivation of the membrane-bound POD. Thus the membrane was thoroughly washed after each measurement.

The chemiluminescent determination of glucose was performed with the POD/GOD membrane and with the POD and GOD membranes. The POD and GOD membranes were superior to the POD/GOD membrane in responding to glucose, because GOD in the latter membrane was not efficiently coimmobilized with POD. If the GOD activity of the POD/GOD membrane is increased, the membrane responds more sensitively to glucose.

The luminous membrane can be incorporated in a continuous analytical system, which offers the selective and sensitive chemiluminescent determination of biologically important substances.

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