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Fluorometric Determination of Hydrogen Peroxide with 4-Amino-1*H*-1,5-benzodiazepine-3-carbonitrile Hydrochloride

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The ring transformation reaction of nonfluorescent 4-amino-1*H*-1,5-benzodiazepine-3-carbonitrile hydrochloride (1) with hydrogen peroxide to fluorescent benzimidazole was applied for the determination of hydrogen peroxide by fluorometry. Peroxidase was employed as a catalyst in the reaction. The measurable range was from 5.880×10^{-7} mol/l to 1.176×10^{-5} mol/l of hydrogen peroxide. Although the detection limit is not as good as that with diacetyldichlorofluorescin (detection limit of about 10^{-8} mol/l), **1** has the advantages of better solubility in water and stability against oxidation by air.

Keywords——fluorometry; enzymatic reaction; peroxidase; benzimidazole; hydrogen peroxide; ring transformation

Fluorometry has been used for the determination of hydrogen peroxide, utilizing chemiluminescence-generating reactions. Many reagents have been developed for this purpose. For example, homovanilic acid (nonfluorescent) is well known to react with hydrogen peroxide in the presence of peroxidase (POD) to give a fluorescent dimer,[2] which might be formed by phenolic oxidation of the acid with hydrogen peroxide.

In a previous study,[3] we found that 4-amino-1*H*-1,5-benzodiazepine-3-carbonitrile hydrochloride (1) readily reacted with hydroxylamine to give a ring-transformed compound, 5-(*o*-aminoanilino)-4-cyanoisoxazole. In place of this nucleophile, hydrogen peroxide was found to be able to react with **1**. This paper deals with **1** as a reagent for the fluorometric determination of hydrogen peroxide.

Materials and Methods

Chemicals and Reagents——All chemicals used were of analytical reagent grade, and distilled and deionized water was used. POD (from horseradish, 200 U/mg) was purchased from Boehringer Mannheim Co., Ltd., and was employed without purification. Reagent **1** was synthesized as described previously.[4]

Preparation of Solutions——A solution of 1/30 M KH_2PO_4 and 1/30 M Na_2HPO_4 was used to prepare buffer solutions. POD solutions were prepared in phosphate buffer at 20 U/ml. A solution containing 0.5 mg of **1** in 100 ml of water was prepared freshly before use. Hydrogen peroxide solution (30% in water) was diluted with water and titrated with 0.1 N $KMnO_4$.

Apparatus——A Shimadzu Rf 510 spectrofluorophotometer was used for measurement of fluorescence intensity. Infrared (IR) and ultraviolet (UV) spectra were obtained on a Jasco RA-1 (Japan Electroscopic Co., Ltd.) and a Hitachi 200-20 spectrophotometers, respectively. Mass (MS) spectra were recorded on a JMS-O1S spectrometer (Japan Electron Laboratory, Ltd.)

Procedure——One ml of a solution of H_2O_2 was added to a mixture of 2 ml of buffer, 1 ml of the reagent, and 1 ml of POD solution. The mixture was warmed at 37°. After allowing it to cool to room temperature, the fluorescence intensity was measured at 468 nm (excitation wavelength, 395 nm).

Isolation of Benzimidazole——POD (50 mg) was added to a solution containing 0.5 g of **1** and 2 ml of H_2O_2 in 1 liter of water. After 1 week at room temperature, the solution was extracted with chloroform.

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2) G.G. Guilbault, D.N. Kramer, and E. Hackley, *Anal. Chem.*, **39**, 271 (1967).
3) Y. Okamoto, K. Takagi, and T. Ueda, *Chem. Pharm. Bull.*, **28**, 567 (1980).
4) Y. Okamoto and T. Ueda, *Chem. Pharm. Bull.*, **23**, 1391 (1975).

The chloroform layer was separated, dried over Na_2SO_4, and concentrated under reduced pressure at 40° to give benzimidazole as a powder in 5.9% yield (15.75 mg).

Results and Discussion

The nonfluorescent compound **1** was found to be converted to benzimidazole (which is fluorescent) in an alkaline medium or in the presence of ferrous sulfate. An attempt to use these reactions for the determination of hydrogen peroxide by fluorometry failed because of strong background fluorescence caused by by-products. However, the product which was obtained from the reaction of **1** with hydrogen peroxide in the presence of POD also showed IR and MS spectral patterns identical with those of authentic benzimidazole (Chart 1). Figure 1 shows the fluorescence spectrum of this reaction system. Since the background at 468 nm is very weak, optimum conditions for the determination of hydrogen peroxide were surveyed.

pH

The first parameter to be investigated was the effect of the pH of the reaction medium on the fluorescence intensity. Figure 2 shows a plot of fluorescence intensity as a function of pH. The optimum response is observed at pH 6.61.

POD Concentration

Optimum concentration of POD was examined when the other parameters were fixed (see Fig. 3). A final concentration of 4 U/ml of POD was selected (hereafter, all concentrations represent final concentrations in the reaction mixture).

Reagent Concentration

Figure 4 indicates that the optimum concentration of **1** for 5.880×10^{-6} mol/l of H_2O_2 was 1.134×10^{-5} mol/l. However, this concentration was not applicable for higher concentrations of H_2O_2 because the fluorescence intensity increased only slowly with increasing concentration

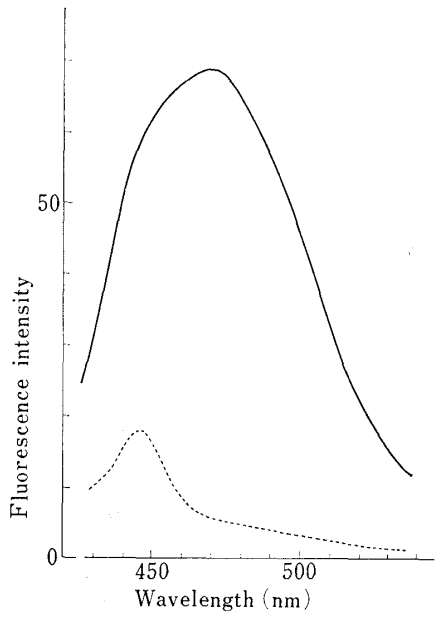

Fig. 1. Fluorescence Spectra (Excitation Wavelength, 395 nm)

(——): A solution containing **1** was reacted with H_2O_2 in the presence of POD. Conditions: reagent 2.268×10^{-4} mol/l, H_2O_2 8.820×10^{-6} mol/l, POD 4 U/ml, pH 6.58, reaction time 25 min.
(------): A solution of **1** (2.268×10^{-4} mol/l).

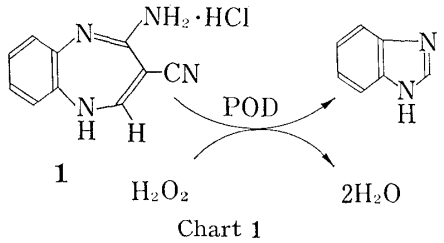

Chart 1

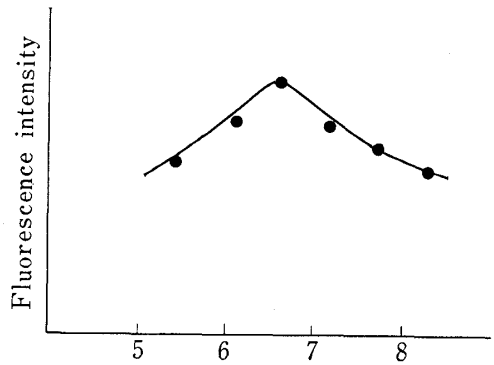

Fig. 2. Effect of pH on the Fluorescence Intensity

Conditions: H_2O_2 2.940×10^{-6} mol/l, reagent 2.268×10^{-5} mol/l, POD 4 U/ml, reaction time 25 min.

of H_2O_2. This problem was overcome by the use of a higher concentration of **1**. When 2.268×10^{-5} mol/l of **1** was used, the measurable range was expanded to 1.176×10^{-5} mol/l of H_2O_2.

Reaction Time

Fluorescence intensity was measured as a function of reaction time. The optimum reaction time is more than 10 min, as shown in Fig. 5.

Response to H_2O_2

Figure 6 shows that a plot of fluorescence intensity *vs.* concentration of H_2O_2 was linear from 5.880×10^{-7} to 1.176×10^{-5} mol/l (20 to 400 ng/ml). The limit of detection, for a signal-

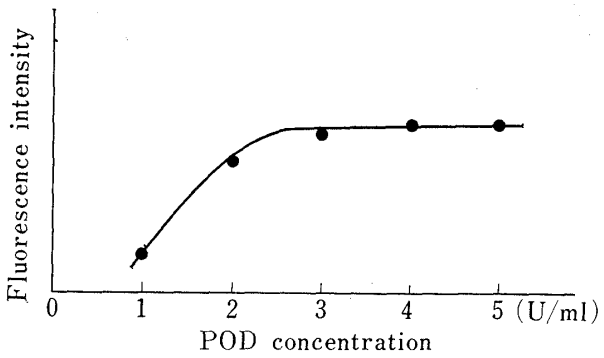

Fig. 3. Effect of POD Concentration on the Fluorescence Intensity

Conditions: pH 6.61, H_2O_2 5.880×10^{-6} mol/l, reagent 2.268×10^{-5} mol/l, reaction time 25 min.

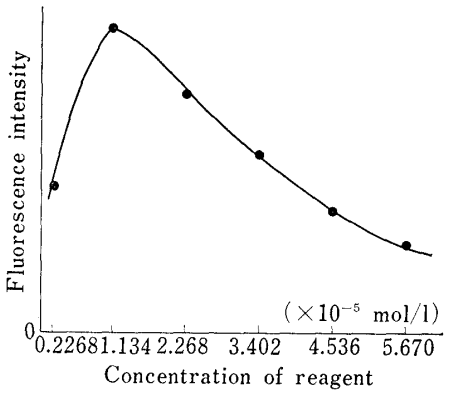

Fig. 4. Effect of Reagent Concentration on the Fluorescence Intensity

Conditions: pH 6.61, H_2O_2 5.880×10^{-6} mol/l, POD 4 U/ml, reaction time 25 min.

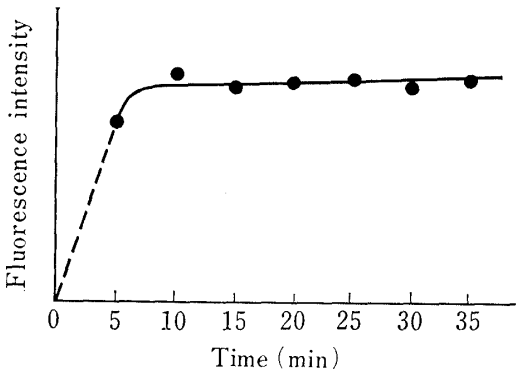

Fig. 5. Effect of Reaction Time on the Fluorescence Intensity

Conditions: pH 6.61, H_2O_2 5.880×10^{-6} mol/l, reagent 2.268×10^{-5} mol/l, POD 4 U/ml.

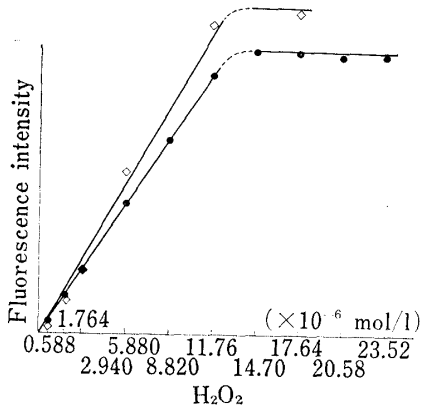

Fig. 6. Standard Curves for H_2O_2

Conditions: pH 6.61, reagent 2.268×10^{-5} mol/l, POD 4 U/ml, reaction time 25 min.
●: Total volume of sample, 10 ml.
◇: Total volume of sample, 5 ml.
The signal-to-noise ratio was 2.3 at 5.880×10^{-7} mol/l of H_2O_2.

TABLE I. UV Absorption Data for Reagent 1

Conditions	UV $\lambda_{max}^{H_2O}$ nm (log ε)	
Fresh, or after 1 week at 4°	200 (4.09)	268.5 (4.25)
After 1 month at room temp.	200 (3.82)	268.5 (3.81)

to-noise ratio of 2, was found to be 5.880×10^{-7} mol/l (20 ng/ml) of H_2O_2. The coefficient of variation was 3.20% at 1.176×10^{-5} mol/l of H_2O_2 in this system ($n=10$). In a 10 ml system,[5] it was 1.67% at 1.176×10^{-5} mol/l and 4.10% at 5.880×10^{-6} mol/l of H_2O_2 ($n=10$). The limit of detection of this method is not as good as that of the method using diacetyldichlorofluorescin (2),[6] which is in the range of 10^{-8} mol/l. Since **2** is not soluble in water, and is oxidized easily by air, an organic solvent and a stabilizer are required for the system. However, **1** has the advantages of high solubility in water, and stability against oxidation by air. The stability of **1** in water was checked by UV spectroscopy (see Table I). No spectral change was observed for at least a week at 4°. However, **1** was slowly decomposed at room temperature, and after 1 month, the absorbance at each absorption maximum decreased.

Compound **1** might be useful as a reagent for the fluorometric determination of hydrogen peroxide in the range of 10^{-7} mol/l. However, the use of this method with glucose oxidase (GOD)[7] was not suitable for the determination of glucose because the standard curve was linear only in the range of 5.551×10^{-6} mol/l to 1.665×10^{-5} mol/l.

5) Total volume, 10 ml; the volume of each solution (reagent, POD, and H_2O_2) is 1 ml, and that of buffer is 7 ml.
6) A.S. Keston and R. Brandt, *Anal. Biochem.*, **11**, 1 (1965).
7) GOD (20 U/mg) was purchased from Boehringer Mannheim Chemical Co., and was used at a concentration of 4 U/ml.