NOTES

FLUOROMETRIC DETERMINATION OF HYDROGEN PEROXIDE WITH
CATALASE-HANTZSCH REACTION SYSTEM USING METHYL ACETOACETATE

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A new method for the fluorometric determination of hydrogen peroxide is described. The method is based on the combination of catalase reaction and the Hantzsch Reaction. The fluorometric characters of the Hantzsch Reaction were investigated using three reagents, acetylacetone, ethyl acetoacetate and methyl acetoacetate. Then methyl acetoacetate was found to be the best of the three reagents.

INTRODUCTION

The enzymatic oxidation of amine in monoamine oxidase reaction is accompanied by production of ammonia, hydrogen peroxide and the corresponding aldehyde from the amine. Therefore, it could be expected that the activity of monoamine oxidase was estimated by the quantitative measurement of hydrogen peroxide in the amine oxidase reaction. Many methods, for example enzymatic colorimetry¹⁾ or fluorometry²⁾, are available for measurement of hydrogen peroxide. These methods are combined with peroxidase and various indicator substrates, which are oxidized by hydrogen peroxide in the presence of peroxidase as a catalyst to yield a chromogen or a fluorophore. However, the effect of peroxidase, which is an oxidation catalyzer of hydrogen peroxide, is not so specific for the substrate, and the oxidation of an indicator substrate by hydrogen peroxide catalysed with peroxidase is easily influenced by the presence of a reducing substance such as ascorbic acid or a reducing substrate such as catecholamine. That is a reason why catecholamines have not been used as substrates for determination of monoamine oxidase activity in the peroxidase method.

On the other hand, it has been known that catalase converts methanol to formaldehyde through the action of hydrogen peroxide³⁾. Formaldehyde reacts with ammonia and acetylacetone(AA) or ethyl acetoacetate(EA) under mild conditions, and the corresponding lutidine derivatives(the Hantzsch Reaction) were formed as follows,

This principle was applied for the colorimetric⁴⁾ and fluorometric⁵⁾⁶⁾ determination of traces of formaldehyde.

A combination of catalase reaction and the Hantzsch Reaction was successfully carried out for a colorimetric determination of uric acid in serum and urine using uricase-catalase system⁷⁾, but the detection limit of uric acid in this method was not so low. It would be desirable to develop a more sensitive method for the deter-

mination of hydrogen peroxide in the study of biological systems.

We planned to combine catalase reaction and fluorometric detection of lutidine derivative formed by the Hantzsch Reaction. In the Hantzsch Reaction, three reagents, AA, EA and methyl acetoacetate(MA) which had not been used before were tested, and MA was found to be the best reagent of the three reagents with respect to fluorescence intensity. The study was performed in order to develop a new enzymatic technique for the sensitive determination of oxidative enzyme activities such as monamine oxidase.

MATERIALS AND METHODS

Reagents

AA, EA, MA and ammonium acetate were obtained from Wako Junyaku(Osaka). A 0.3% solution of hydrogen peroxide was prepared by diluting a 30% stock solution(Merck, Rahway, NJ, U.S.A.) with distilled water. The hydrogen peroxide concentration was determined by iodometry. The solution was stable at least for 1 month in a refrigerator. A derivatizing agent solution was prepared by mixing 0.04 M MA with an equal volume of 0.4 M ammonium acetate.

Catalase solution

Catalase(Type II from bovine liver, EC 1.11.1.6) (270,000U/ml) was obtained from Boehringer(Mannheim, G.F.R.). A test solution(1000U/ml) was prepared by diluting it with distilled water.

Preparation of lutidine derivative standards

3,5-Diacetyl-1,4-dihydrolutidine(HCHO-AA) was prepared by allowing to stand at 60°C for 30 min the reaction mixture of 2 ml of AA, 50 ml of 20% ammonium acetate and 2 ml of 37% formalin. Diethyl-1,4-dihydrolutidine-3,5-dicarboxylate(HCHO-EA) and dimethyl-1,4-dihydrolutidine-3,5-dicarboxylate(HCHO-MA) were prepared in the same way by using EA or MA. The precipitates were filtered off, washed with a small volume of cold water, dried in air overnight and recrystallized from ethanol. HCHO-AA melted over the range 196-198°C. Calculated for $C_{11}H_{15}NO_2$: C, 68.39; H, 7.77; N, 7.25. Found: C, 67.37; H, 7.80; N, 7.15. HCHO-EA melted over the range 191-192°C. Calculated for $C_{13}H_{19}NO_4$: C, 61.64; H, 7.56; N, 5.53. Found: C, 61.87; H, 7.66; N, 5.64. HCHO-MA had a melting point of 231°C. Calculated for $C_{11}H_{15}NO_4$: C, 58.66; H, 6.71; N, 6.22. Found: C, 58.87; H, 6.79; N, 6.20.

Standard procedure

To 1.0 ml of sample solution containing less than 1.5 μ g of hydrogen peroxide in a 10 ml test-tube, 0.5 ml of methanol, 0.5 ml of catalase solution and 2 ml of derivatizing agent solution were added. The solution was shaken and the tube was placed in a water-bath at 37°C for 60 min. After standing for 30 min at a room temperature, the fluorescence intensities were measured at 463 nm with an excitation at 375 nm by a Hitachi 650-60 spectrofluorometer. Blank value was obtained by measuring 1.0 ml of water instead of sample solutions and subtracted from the measured value obtained from the sample solution.

RESULTS AND DISCUSSION

Table 1 Relative fluorescence intensities(RFI) of three lutidine derivatives(10^{-5} M) in 0.1 M ammonium acetate solutions

Lutidine derivative	Excitation(nm)	Emission(nm)	RFI
HCHO-AA	421	515	38.6*
HCHO-EA	360	457	83.3
HCHO-MA	375	463	100.0

* Relative value to that obtained from 10^{-5} M HCHO-MA in 0.1 M ammonium acetate.

Table 2 Relative fluorescence intensities of three lutidine derivatives reached in 60 min at 37°C in solutions of different compositions of derivatizing agent, but with the same initial amount(4×10^{-6} M) of formaldehyde

Concentration of AA, EA or MA(M)		Concentration of ammonium acetate(M)				
		1	0.4	0.1	0.04	0.01
0.01	AA	46*	52	38	11	4
	EA	112	108	83	41	17
	MA	191	130	100	59	6
0.002	AA	45	43	21	11	3
	EA	91	84	42	13	7
	MA	125	102	62	24	1
0.001	AA	41	39	19	9	3
	EA	72	59	23	8	1
	MA	95	61	22	12	1

* Relative value to that obtained from the standard solution containing 0.1 M ammonia and 0.01 M MA.

Under optimum conditions, the fluorometric characters of three lutidine derivatives are summarized in Table 1, where the fluorescence intensities are shown as relative values. HCHO-MA gives the highest fluorescence intensity of the three lutidine derivatives in 0.1 M ammonium acetate solution. The effect of varying the derivatizing agent concentration is shown in Table 2, where the fluorescence intensity is given as a relative value to that obtained with 0.01 M MA and 0.1 M ammonium acetate.

As has already been reported⁴⁾, in case of AA it can be seen that the yield in the reaction expressed as fluorescence intensity falls off much more rapidly with decreasing ammonia concentration than with decreasing AA concentration. But with EA and MA, the yield is effected with both concentrations of ammonia and acetoacetic ester. In most cases, the fluorescence intensities obtained from EA or MA were about twice

those of lutidines obtained from AA, and that obtained from MA was higher than that obtained from EA. The fluorescence of blank solutions became higher with increasing concentration of reagents in the three cases.

Using 1.0 μg of hydrogen peroxide, the effect of the reaction period was investigated through the entire procedure described above. In case of AA, the reaction was sufficient in 30 min to obtain a constant fluorescence intensity. In cases of EA and MA, the fluorescence intensities still slowly continued to increase in 30 min and showed a nearly constant value in 60 min.

In this procedure, the presence of reducing compounds was an important problem, then the possibility of interference by some substances was examined. Each compound of L-ascorbic acid, uric acid, ethylamine, ethylene glycol or acetone was respectively added to the sample solution containing 1.5 μg of hydrogen peroxide at a level of 0.25–5.0 μg and within a range of reducing compound concentrations tested, the measured values were nearly constant.

MA which was found to be the best in regard of fluorescence intensity among one β -diketone and two acetoacetic esters was applied to determination of hydrogen peroxide. A sample solution containing an individual amount of hydrogen peroxide was measured according to the standard procedure and fluorescence intensities were proportional in the range 0.03–1.50 μg of hydrogen peroxide in 1.0 ml of sample solution. Reproducibility of the method was examined by performing 5 replicate analyses of an identical sample solution containing 0.3 $\mu\text{g}/\text{ml}$ of hydrogen peroxide. The coefficient of variation was 1.9%.

From the results obtained above, it is concluded that the proposed method for the determination of traces of hydrogen peroxide in aqueous solution has a higher specificity and gives a reliable determination value. The method seems to be full of promise toward development of a simple, reliable procedure which is generally applicable to all kinds of substrates for determination of monoamine oxidase activity. This method will be also useful for the sensitive determination of other oxidative enzyme activities such as glucose oxidase, uricase and xanthine oxidase, and for the determination of substrates such as glucose, uric acid and hypoxanthine in these enzyme systems.

REFERENCES

- 1) G.G.Guilbault, D.N.Kramer : Anal. Chem., 36, 2494 (1967).
- 2) G.G.Guilbault, D.N.Kramer, E.Hackley : Anal. Chem., 39, 271 (1967).
- 3) P.Nicholls, G.R.Schonbaum : The Enzymes. (P.D.Boyer, H.Lardy, K.Myrbach, eds), vol. 8, p. 147, (1963), (Academic Press, New York).
- 4) T.Nash : Biochem. J., 55, 416 (1953).
- 5) S.Belman : Anal. Chim. Acta, 29, 120 (1963).
- 6) M.Pesez, J.Bartos : Talanta, 14, 1097 (1967).
- 7) N.Kageyama : Clin. Chim. Acta, 31, 421 (1971).

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