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Note

A new assay of monoamine oxidase by gas chromatography

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The enzymatic oxidation of amines in the monoamine oxidase (MAO) reaction is accompanied by the production of ammonia, hydrogen peroxide and the corresponding aldehyde from the amine under consumption of oxygen. It is still required to establish a rapid and specific method for the assay of MAO activity, which is generally applicable to all kinds of substrates. Polarographic measurement of oxygen consumption has been used, but it is not sensitive. Measurement of ammonia formed by colorimetric or fluorometric techniques has also been applied for this purpose [1]. But this method is not applicable for secondary amines such as epinephrine and metanephrine, since ammonia is not formed during the deamination by MAO, and methylamine is produced instead of ammonia. It is necessary, therefore, that the formation of hydrogen peroxide is measured.

Many methods are available for measurement of hydrogen peroxide by enzymatic colorimetry [2] or fluorometry [3] using peroxidase. The colorimetric method is generally not very sensitive. It would be desirable to have a more sensitive method for the determination of hydrogen peroxide in the study of biological systems. Fluorometric determination of hydrogen peroxide formed is sensitive, but is not applicable for catecholamines and serotonin which are preferential substrates for type A MAO [4, 5]. It is known that catalase converts methanol to formaldehyde through the action of hydrogen peroxide formed in this oxidation reaction [6]. This principle was applied to a gas chromatographic (GC) determination of hydrogen peroxide

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[7] and to a colorimetric determination of uric acid in serum and urine with uricase—catalase system [8], but has never been used for the MAO assay.

Pentafluorobenzyloxylamine (PFBOA) hydrochloride has been found to be an excellent reagent for derivatization of low-molecular-weight carbonyl compounds in GC [9, 10]. A condensation reaction of PFBOA and formaldehyde proceeds readily even in an aqueous solution at room temperature. We are not aware of any report dealing with catalase reaction for the determination of MAO activity. The present paper describes a reliable and sensitive GC method for assay of MAO activity by connecting the catalase reaction and the derivatization of formaldehyde with PFBOA.

EXPERIMENTAL

Reagents

PFBOA hydrochloride (melting point 115°C) was synthesized from pentafluorobenzyl bromide (Aldrich, Milwaukee, WI, U.S.A.) and N-hydroxyphthalimide (Tokyo Kasei, Tokyo, Japan) [9]. Iodobenzene was used as an internal standard. A 0.3% hydrogen peroxide solution was prepared by diluting a 30% stock solution of hydrogen peroxide (Merck Sharp and Dohme, Rahway, NJ, U.S.A.) with distilled water. The peroxide concentration was assayed by iodometry. The solution was stable for a month in a refrigerator.

Enzymes

Catalase (hydrogen-peroxide:hydrogen-peroxidase oxidoreductase, EC 1.11.1.6) (270,000 U/ml) was obtained from Boehringer, Mannheim, G.F.R. A stock solution (20,000 U/ml) was prepared by diluting it with distilled water.

For preparations MAO (monoamine:O_2 oxidoreductase, EC 1.4.3.4) male Wistar rats (150—200 g) were decapitated and their livers were homogenated in 6 volumes of pH 7.5, 50 m*M* phosphate buffer. The mitochondrial fraction was prepared by differential centrifugation by the method of Hogeboom et al. [11]. The mitochondria were washed once and resuspended in 50 m*M* potassium phosphate buffer and used as the MAO preparation. The enzyme protein content was estimated by the method of Lowry et al. [12] and was prepared to a concentration of 10 mg/ml.

Apparatus and conditions

A Shimadzu GC-4APE gas chromatograph equipped with a 10-mCi ^{63}Ni electron-capture detector (ECD) and a 2-m glass column packed with 3% XE-60 (Wako Junyaku, Osaka, Japan) on 80—100 mesh Celite 545 (AW DMCS) was used, with a column temperature of 90°C, a detector temperature of 150°C, and an injection temperature of 150°C.

Standard procedure for the assay of MAO activity

To a 10-ml test tube are added 0.25 ml of 8 m*M* substrate, 0.10 ml of 50 m*M* phosphate buffer pH 6.8, 0.20 ml of methanol, 0.10 ml of catalase aqueous solution (20,000 U/ml), 0.25 ml of PFBOA hydrochloride aqueous solution (1 mg/ml), and 0.10 ml of MAO preparation containing an amount of enzyme to give a peak height ratio to the internal standard of between

one and two. The reagents were added in this order and the reaction mixture (total volume 1.0 ml) was shaken for 30 min at 37°C in air. The standard incubation mixture contained 2 m*M* substrate, 20% methanol, 2000 units of catalase and about 30 times as much PFBOA as would be needed for binding 1 μg of formaldehyde formed. After incubation, one drop of 18 *N* sulfuric acid and 1 ml of *n*-hexane containing 3.5 μg of iodobenzene as internal standard were added to the reaction solution, followed by saturation with sodium chloride, and the resulting O-pentafluorobenzyloxime (O-PFBO) was extracted. An aliquot of the extract was diluted 25-fold with *n*-hexane, and 1 μl of the solution was injected on to the column.

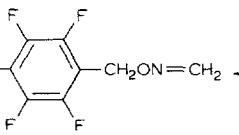
RESULTS AND DISCUSSION

The principle of this method using methanol and catalase is as follows:

$$\text{Substrate} \xrightarrow[O_2,\ H_2O]{\text{MAO}} \text{oxidation product} + NH_3\ (\text{or } RNH_2) + H_2O_2$$

$$H_2O_2 + CH_3OH \xrightarrow{\text{catalase}} HCHO + 2\ H_2O$$

$$HCHO + \text{PFBOA} \longrightarrow \text{O-PFBO} \longrightarrow \text{GC (ECD)}$$



In a preliminary experiment, benzylamine as substrate was incubated with MAO preparation, and then methanol, catalase and PFBOA were added to the reaction solution, and the mixture was allowed to stand for 1 h at room temperature. The resulting O-PFBO derivative of formaldehyde was extracted with *n*-hexane and an aliquot of the extract was subjected to GC. But no peak corresponding to formaldehyde appeared on the gas chromatogram. The cause of the failure was found to be a small amount of catalase existing in the crude MAO preparation used, which had decomposed the hydrogen peroxide formed by MAO reaction before the addition of methanol and catalase.

When methanol and catalase were added to a sample solution containing substrate before starting the MAO reaction, the hydrogen peroxide formed by MAO reaction immediately reacted with methanol to convert it to formaldehyde, and we succeeded in obtaining a sharp peak corresponding to formaldehyde on the gas chromatogram. Thus, a one-step procedure was established to permit the three reactions, MAO reaction, catalase reaction and condensation reaction with PFBOA, simultaneously.

The MAO activities in the rat liver mitochondria were measured according to this standard procedure, using benzylamine or β-phenylethylamine for type B MAO, norepinephrine and serotonin for type A MAO, and dopamine for the two types of MAO. The peak height formed on the gas chromatogram showed a linear relationship with up to 60 min of MAO incubation time, using each of the substrates. The calibration curves of MAO activity against the

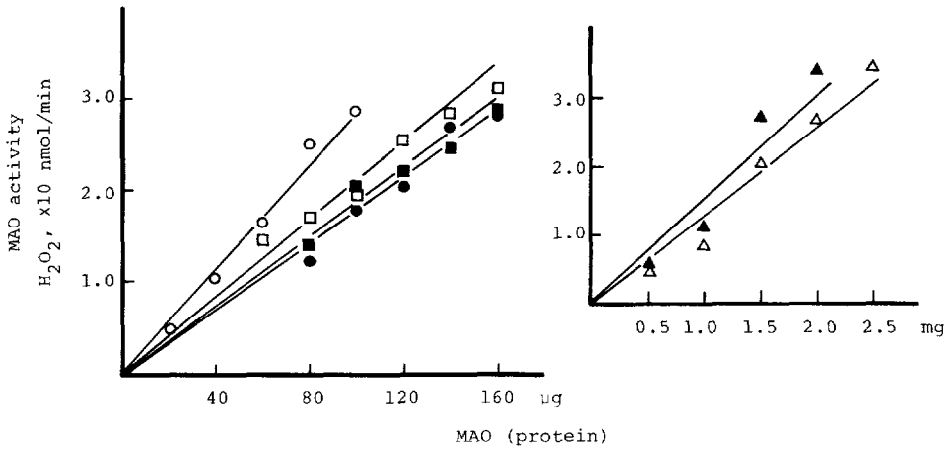


Fig. 1. Relationship between the amount of hydrogen peroxide formed by MAO and the enzyme concentration. Benzylamine (○——○), β-phenylethylamine (●——●), serotonin (□——□), normetanephrine (■——■), norepinephrine (△——△), or dopamine (▲——▲) was used as substrate. Incubations were carried out for 30 min at 37°C with increasing amounts of rat liver mitochondria.

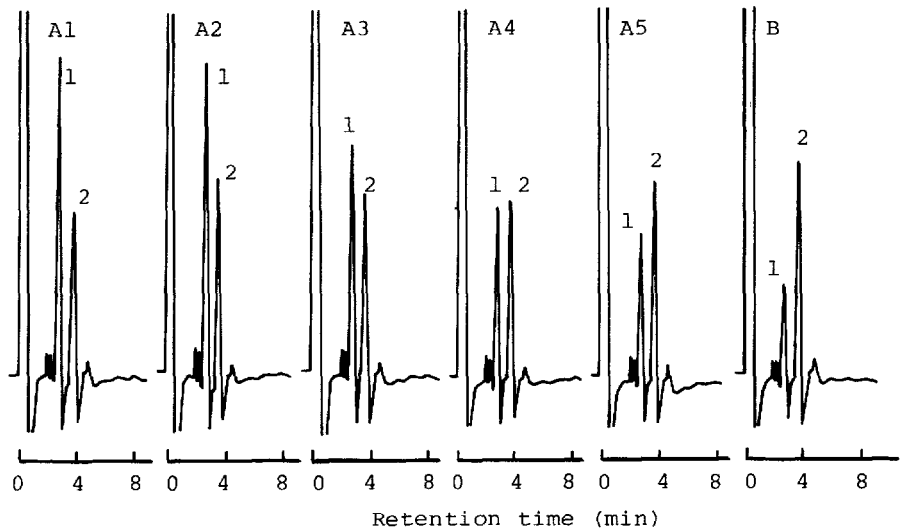


Fig. 2. Typical gas chromatograms obtained by assays using five different MAO concentrations (A1 = 100, A2 = 80, A3 = 60, A4 = 40, A5 = 20 µg of protein; B = no-enzyme blank) and benzylamine as substrate. Peaks: 1 = HCHO-O-PFBO, 2 = internal standard (3.5 µg of iodobenzene). Conditions: 3% XE-60, 2 m, 90°C, ECD.

enzyme concentration are shown in Fig. 1. Linear curves passing through the origin were obtained with all substrates. Fig. 2 illustrates a set of six chromatograms obtained for the calibration curve using benzylamine as substrate. Chromatogram B is of the blank, the product in an incubation without MAO preparation. Iodobenzene was used as a suitable internal standard. The O-PFBO derivative of formaldehyde was very volatile, and the separation could be carried out at a low temperature of 90°C. The O-PFBO derivative was also very sensitive to ECD. The peak in chromatogram A5 (Fig. 2) corresponded

to about 50 ng of hydrogen peroxide formed in the MAO reaction (plus about 50 ng of the blank value in B equals about 100 ng) in the reaction solution, which corresponded to an injection of about 2 pg of formaldehyde.

The precision of the method was determined by five repeated assays on an identical MAO preparation. Standard deviations were 5.9% for benzylamine using 60 μg of protein, 2.9% for β-phenylethylamine using 120 μg of protein, 5.0% for normetanephrine and 4.4% for serotonin using 120 μg of MAO protein. As compared to the substrates mentioned above, the results with dopamine or norepinephrine as substrate were worse in accuracy and sensitivity, probably due to oxidative decomposition by hydrogen peroxide of the catechol ring in the catecholamines. However, it was possible to determine MAO activity using catecholamine or serotonin as substrate. We are now investigating an effective way to protect the catecholamines against oxidation by hydrogen peroxide during MAO incubation.

There is one more problem in the present assay, namely that blank values were found to be slightly high and to correspond to about 50 ng of hydrogen peroxide in the reaction mixture (see B in Fig. 2), so the sensitivity of this MAO assay was about 2 nmol of hydrogen peroxide formed, though the O-PFBO derivative of formaldehyde was extremely sensitive to ECD. The origin of the high peak in the blank is not evident. It may be caused by formaldehyde in air and solutions derived from smoking. As a matter of fact, the blank value was reduced to about half by prohibiting smoking in the laboratory. Reducing blank values is also one of the subjects for a future study.

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