[Chem. Pharm. Bull. 24(4) 698—704 (1976)]

UDC 547.238.09 : 577.158.04

Effects of Hydroxylamine on Mitochondrial Monoamine Oxidase in Rat Liver

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(Received July 4, 1975)

The report here is the study as for the effects of NH_2OH on the enzymic reaction of monoamine oxidase (MAO) using rat liver mitochondria.

In the short-term reaction (1 min) by MAO, NH_2OH decreased the MAO activity by either tyramine or benzylamine as substrate. In the long-term reaction (60 min), there was an increased activity in the MAO reaction by tyramine and an decreased one in that by benzylamine under various concentrations of NH_2OH. In the long-term reaction, *K*m-values for tyramine and benzylamine were $1.82 \times 10^{-3.0}$M and $2.50 \times 10^{-2.0}$M, respectively, which were consistent with those in the presence of NH_2OH. Pursuing the changes of MAO activities by various concentrations of NH_2OH using the tyramine substrate at intervals of 1, 10, 30, and 60 min, there was a decrease of MAO activity accompanying an increased concentration of NH_2OH after 1 min period, a partial increase after 10 min, and an entire increase in extensive concentrations of NH_2OH after 30 min. In the other substrates such as tryptamine, amylamine, hexylamine, and β-phenylethylamine, the effects of NH_2OH on MAO were similar to those in tyramine. In the butylamine substrate, NH_2OH caused as inhibitory effects as shown in the benzylamine substrate. These results suggest the existence of a complex of MAO enzymes corresponding to monoamine substrates. The multiplicity of MAO enzyme is further discussed in the text.

Monoamine oxidase (MAO; EC 1.4.3.4) localizing in mitochondrial outer membranes[2] and covalent binding with flavin adenine dinucleotide (FAD)[3] has been considered as a multiform enzyme because of its broad substrate specificities.[4] In addition, this multiple form of MAO has been reported to be dependent on different molecular weights by physicochemical techniques.[5]

On the assumption that MAO acts biologically in the form of a collective complex of different amine oxidases corresponding to each amine substrate,[6] the authors are now in the stage of teleological studies on multiple behavior which is resulted from the interaction between the collectives. From this point of view, experiments were carried out as follows: (a) Kinetic study was applied to the two enzymic reactions of MAO with tyramine and benzylamine as well as the effect of hydroxylamine (NH_2OH) on their MAO reactions: (b) Changes in MAO activity were estimated in course of time with various concentrations of NH_2OH using tyramine

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5) *a*) M.B.H. Youdim and M. Sandler, *Biochem. J.*, **105**, 43p (1967); *b*) G.G.S. Collins and J. Southgate, *ibid.*, **117**, 38p (1970); *c*) H.C. Kim and A. D'lorio, *Can. J. Biochem.*, **46**, 295 (1968); *d*) B. Gomes, I. Igaue, H.G. Kloepfer, and K.T. Yasunobu, *Arch. Biochem. Biophys.*, **132**, 16 (1969); *e*) V.Z. Gorkin, *Experientia*, **25**, 1142 (1969); *f*) M.B.H. Youdim and G.G.S. Collins, *Eur. J. Biochem.*, **18**, 73 (1971); *g*) M.B.H. Youdim, *Brit. Med. Bull.*, **29**, 120 (1973).
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as substrate: (c) The effects of NH_2OH on MAO activity were observed also by the other substrates.

Materials and Methods

Preparation of Mitochondrial Fraction[2a,4b]——Experiments were carried out according to the following method. Liver mitochondrial fractions were prepared from adult Wistar rats of both sexes weighing 200—300 g. A total of 300 g of the livers was chopped after weighing in wet and was then added to a solution of 0.25M sucrose—0.01M tris(hydroxymethyl)aminomethane HCl buffer (sucrose—Tris buffer, pH 7.5) in the proportion of 1 to 3. The homogenate was obtained by means of both homogenizers of Potter–Elvehjem and Waring blender, suspended by adding sucrose—Tris buffer in the final proportion of 1 to 9, and then the fibrous substances were discarded by filtration of gauze. The solution in suspension was centrifuged first at $15000\times g$ for 30 min in order to remove microsomes and especially blood from its mixture. The precipitate was washed with sucrose—Tris buffer and centrifuged at $15000\times g$ for 30 min. After addition of sucrose—Tris buffer (1: 1) to the precipitate containing nuclei and mitochondria, the mixture was centrifuged at $15000\times g$ for 30 min. The precipitate was washed and centrifuged. The precipitate being suspended with a small amount of sucrose—Tris buffer, the suspension was layered on 0.35M sucrose—0.01M Tris buffer solution (pH 7.5) and centrifuged at $900\times g$ for 5 min. After the sucrose concentration in molar of the supernatant was adjusted to 0.25M, centrifugation was again performed at $15000\times g$ for 30 min. The precipitate was washed and centrifuged. This precipitate was suspended with sucrose—Tris buffer in the proportion of 1 to 1. This enzymatically active preparation using tyramine as substrate was in consequence subjected to experiment for a reaction by MAO as mitochondrial enzyme. All steps of the procedures mentioned above were performed at 0—4°.

Assay of Enzyme Activity——In preparing each solution of reagent, all chemicals except NH_2OH were calculated as free bases. The short-term r action by the enzyme was measured by means of oxygen electrode and the long-term reaction by Warburg manometer. In the former method, substrate was added to the enzyme solution after equilibrium was reached and measurement was made over a period of 1 min. In the latter method, activity in reaction was recorded every 10 min after adding substrate to the 20 min-preincubated enzyme solution and regarded as the reaction of 60 min period between 10 to 70 min. The short-term reaction of a period of 1 min was measured at 43° and the long-term reaction of a period of 60 min at 38°. In order to correlate the substrate amount in the long-term reaction with that in the short-term reaction, the substrate concentrations were used as follows: tyramine $10^{-1.0}$M (used for the short-term reaction) and $10^{-1.23}$M (used for the long-term reaction), benzylamine; $10^{-1.0}$M and $10^{-1.0}$M, tryptamine; $10^{-1.49}$M and $10^{-1.35}$M, butyl amine; $10^{-2.0}$M and $10^{-0.5}$M, amylamine; $10^{-1.0}$M and $10^{-0.5}$M, hexylamine; $10^{-3.0}$M and $10^{-0.5}$M, β-phenylethylamine; $10^{-2.5}$M and $10^{-2.5}$M. The reaction mixture was a total volume of 3.0 ml. The detailed technique has been reported elsewhere.[7] Values in activity of MAO enzyme were given as the rate of oxygen uptake of μl/ml of enzyme preparation for 60 min in both experimental systems in reaction.

Results

Effects of NH_2OH on the Enzymic Reaction of MAO

Study was carried out on the effects of NH_2OH to the respective reactions of MAO with tyramine, benzylamine, tryptamine, butylamine, amylamine, hexylamine, and β-phenylethylamine (Fig. 1a and b). From the results of both reactions of MAO with tyramine and benzylamine, these reaction curves were found to be similarly inhibitory patterns in the short-term reaction, whereas in the long-term reaction there was a markedly increased activation in the pattern by tyramine and reversely a typical inhibition in benzylamine. Namely, in the short-term reaction, the activity curves for tyramine and benzylamine showed the inhibitory rates of 50% when the NH_2OH concentrations were $10^{-0.7}$M and $10^{-2.3}$M, respectively. In the long-term reaction, MAO activity by tyramine was an increased value in maximum at a $10^{-1.5}$M-concentration of NH_2OH whereas the inhibitory rate for benzylamine was 50% at a $10^{-2.9}$M-concentration of NH_2OH.

As shown in Fig. 1a and b, there were reverse phenomena in the two reactions of MAO with tyramine and benzylamine under NH_2OH. Almost the same activity curves with that

7) K. Kamijo, G.B. Koelle, and H.H. Wagner, *J. Pharmacol. Exptl. Therap.*, **117**, 213 (1956); S. Sho, H. Kinemuchi, N. Shimizu, E. Nabatame, Y. Toyoshima, and K. Kamijo, *Showa Med. J.*, **27**, 932 (1967).

for tyramine, with which NH_2OH produced an inhibitory pattern in the short-term reaction and remarkably increased values in the long-term reaction, were obtained by using the other substrates such as tryptamine, amylamine, hexylamine, and β-phenylethylamine, in respective reactions with which the inhibitory rates of 50% were obtained by the NH_2OH concentrations of $10^{-0.6}$M, $10^{-1.4}$M, $10^{-0.7}$M, and $10^{-0.6}$M, in the short-term reaction, and for which activity values in maximum resulted from the NH_2OH concentrations of $10^{-1.5}$M, $10^{-1.5}$M, $10^{-0.9}$M, and $10^{-0.9}$M, respectively, in the long-term reaction.

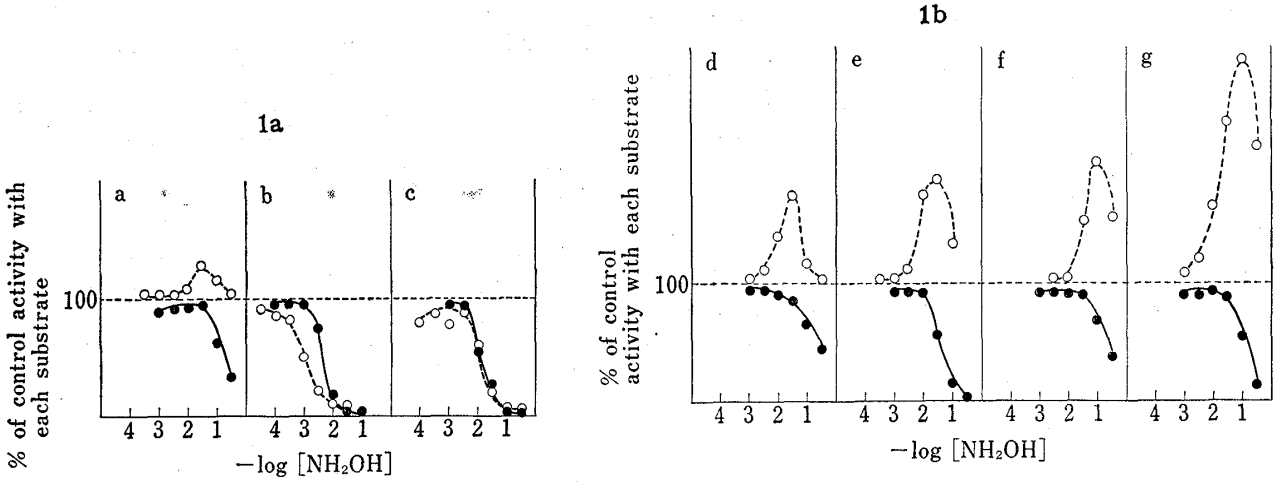

Fig. 1a. Effects of NH_2OH on the Short- and Long-Term Reactions of MAO with a Given Amount of Each Substrate

Fig. 1b. Effects of NH_2OH on the Short- and Long-Term Reactions of MAO with a Given Amount of Each Substrate

Measurements in the short- and long-term reactions were done for 1 min (—●—) and 60 min (--○--), respectively. The amounts of tryptamine (*a*), benzylamine (*b*), butylamine (*c*), tyramine (*d*), amylamine (*e*), β-phenylethylamine (*f*), and hexylamine (*g*), were $10^{-1.49}$ M, $10^{-1.0}$ M, $10^{-2.0}$ M, $10^{-1.0}$ M, $10^{-1.0}$ M, $10^{-2.5}$ M, and $10^{-3.0}$ M, respectively, in the short-term reaction, in addition to $10^{-1.35}$ M, $10^{-1.0}$ M, $10^{-0.5}$ M, $10^{-1.23}$ M, $10^{-0.5}$ M, $10^{-2.5}$ M, and $10^{-0.5}$ M, respectively, in the long-term reaction. Details are described in the text.

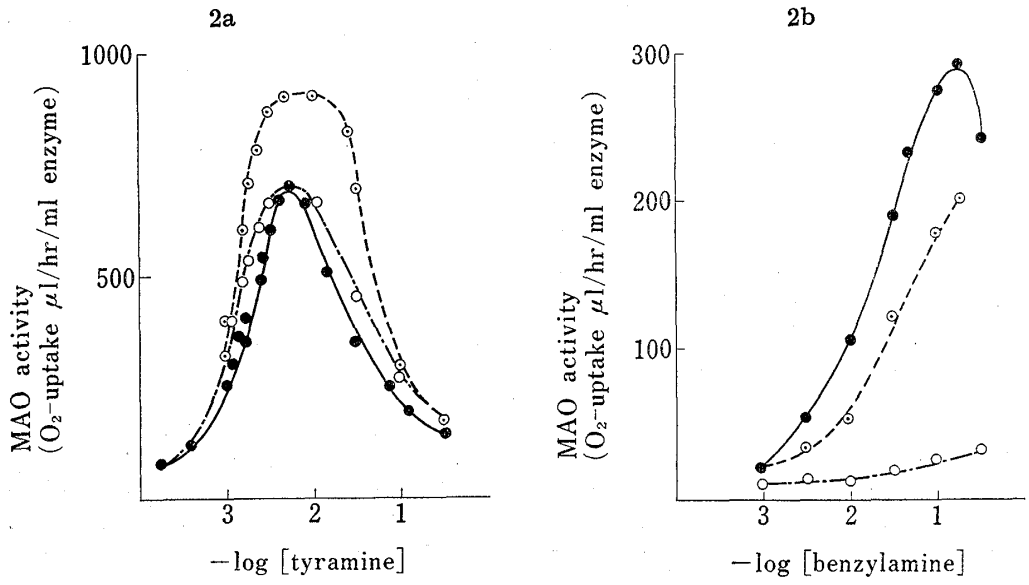

Fig. 2a. Effects of NH_2OH on the Long-Term Reaction of MAO with Various Concentrations of Tyramine

Fig. 2b. Effects of NH_2OH on the Long-Term Reaction of MAO with Various Concentrations of Benzylamine

Measurements were done for 60 min. The activity curves were obtained in the presence of the NH_2OH concentrations of $10^{-1.5}$ M (—⊙—), $10^{-1.0}$ M (—○—) (Fig. 2a), $10^{-3.0}$ M (--⊙--), and $10^{-2.5}$ M (—○—) (Fig. 2b). The control curves for tyramine and benzylamine show the activities of MAO without NH_2OH (—●—). Details are described in the text.

On the other hand, the activity curve for butylamine was similar to that for benzylamine with which MAO activities were inhibited in both the systems in reaction and showed the inhibitory rate of 50% at a $10^{-1.8}$M-concentration of NH_2OH.

Km-Values of MAO for Tyramine and Benzylamine

From the study on the effects of NH_2OH to the reaction of MAO with tyramine in the long-term reaction (Fig. 2a), the NH_2OH concentrations of $10^{-1.5}$M and $10^{-1.0}$M were found to apparently increase MAO activity by the tyramine substrate ranging from $10^{-3.0}$M to $10^{-1.0}$M. A $10^{-1.0}$M-concentration of NH_2OH caused no change in the maximum value of MAO activity by a $10^{-2.3}$M-concentration of tyramine, but a $10^{-1.5}$M-concentration of NH_2OH markedly influenced the MAO reaction in which an increased activity was obtained by the tyramine substrate ranging from $10^{-3.0}$M to $10^{-1.0}$M on which the increments of activities were constant at the range of $10^{-2.3}$M to $10^{-2.0}$M.

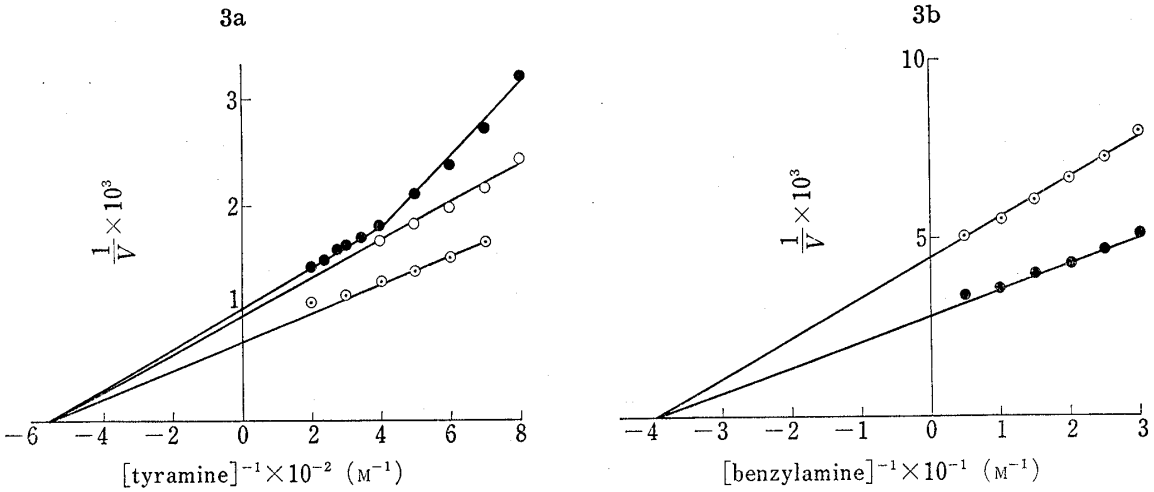

Fig. 3a. Double-Reciplocal Plots of Degradation of Tyramine by Oxidative Deamination of MAO in the Presence of NH_2OH

Fig. 3b. Double-Reciplocal Plots of Degradation of Benzylamine by Oxidative Deamination of MAO in the Presence of NH_2OH

Measurements were made for 60 min by means of Warburg manometer (see Meterials and Methods). The MAO activities were obtained by degradations of tyramine and benzylamine in the presence of the NH_2OH concentrations of $10^{-1.5}$ M (—⊙—), $10^{-1.0}$ M (—○—) (Fig. 3a), and $10^{-3.0}$ M (—⊙—) (Fig. 3b), and without NH_2OH (—●—).

Expressing with the double-reciprocal plots for the reaction of MAO with tyramine (Fig. 3a), the plots showed a linear curve on which slopes were low at a concentration of $2.5\times10^{-3.0}$M ($=4\times10^{2.0}$M as 1/S) or more and high below its concentration. This was consistent with the reciprocal point on the abscissa plotted in the presence of NH_2OH, the *K*m of which was a value of $1.82\times10^{-3.0}$M.

Subsequently, studying on the effects of NH_2OH to the activity curve for benzylamine with MAO in the long-term reaction (Fig. 2b), a $10^{-3.0}$M-concentration of NH_2OH produced the partially inhibited reaction of MAO enzyme, which was completely inhibited with NH_2OH of $10^{-2.5}$M. Furthermore, the reciprocal plots for the reaction of MAO with benzylamine were linear and this plot on the abscissa was in agreement with that in the presence of NH_2OH, the *K*m of which was thus a value of $2.50\times10^{-2.0}$M (Fig. 3b).

The Short-Term Enzymic Reaction of MAO in the Presence of NH_2OH

(a) Tyramine as substrate (Fig. 4a). The short-term activity curve for tyramine with MAO showed a maximum peak at a concentration of $10^{-1.0}$M and a broad shoulder at the range in concentration from $10^{-3.0}$M to $10^{-1.5}$M, without NH_2OH. Activities in MAO by various concentrations of tyramine were under the slight effect of NH_2OH of $10^{-1.5}$M whereas they were markedly inhibited by a $10^{-1.0}$M-concentration of NH_2OH.

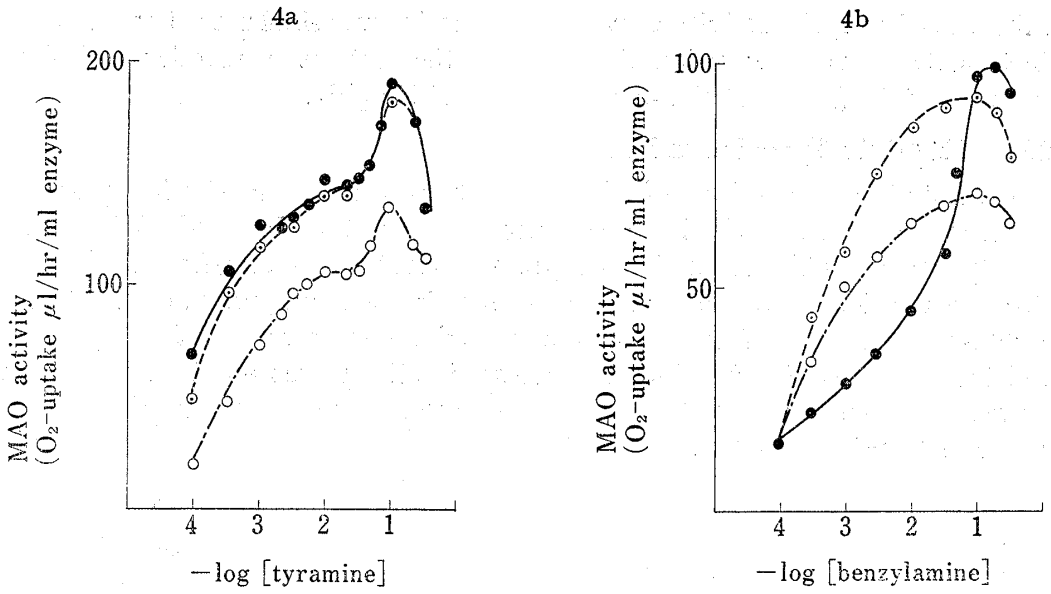

Fig. 4a. Effects of NH_2OH on the Short-Term Reaction of MAO with Various Concentrations of Tyramine

Fig. 4b. Effects of NH_2OH on the Short-Term Reaction of MAO with Various Concentrations of Benzylamine

Measurements were done for 1 min. The activity curves were obtained in the presence of the NH_2OH concentrations of $10^{-1.5}$ M (--⊙--), $10^{-1.0}$ M (—○—) (Fig. 4a), $10^{-3.0}$ M (--⊙--), and $10^{-2.5}$ M (—○—) (Fig. 4b). The control curves for tyramine and benzylamine show the activities of MAO without NH_2OH (—●—). Details are described in the text.

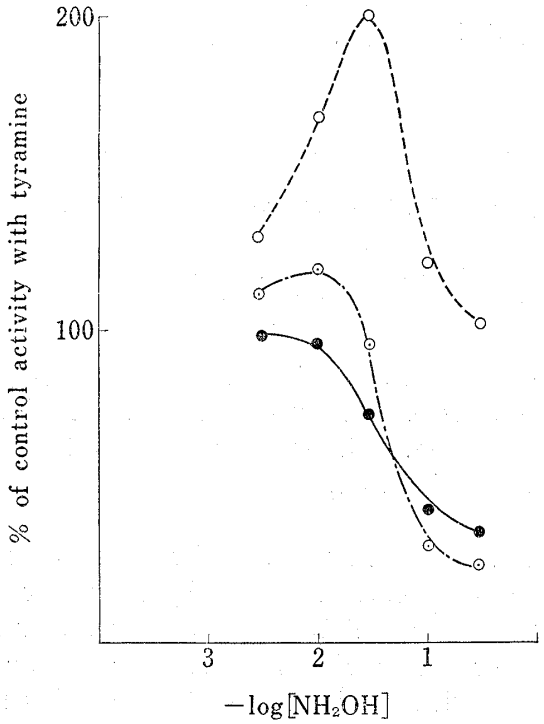

Fig. 5. Time-Course of the Enzymic Reaction of MAO with Tyramine in the Presence of NH_2OH

The activity after 1 min (—●—) was determined by means of oxygen electrode and the ones after 10 min (—⊙—) and 30 min (--○--) by Warburg manometer. A $10^{-1.23}$ M-concentration of tyramine was used in both experimental systems in reaction. All experiments were carried out at 38°. Further informations can be found in the text.

(b) Benzylamine as substrate (Fig. 4b). NH_2OH, which brought about apparently different changes in the short- and long-term reactions of MAO with various concentrations of benzylamine, produced the maximum value of activity even either of the NH_2OH concentrations of $10^{-3.0}$M and $10^{-2.5}$M when the concentration of benzylamine was $10^{-1.0}$M, and subsequently it caused an inhibitory effect which inhibitory rate was much higher at a $10^{-2.5}$M concentration of NH_2OH compared with that in $10^{-3.0}$M when the benzylamine concentrations were $10^{-1.0}$M or more. However, NH_2OH, at both concentrations of $10^{-3.0}$M and $10^{-2.5}$M, caused a further increase in the activities which occurred at the benzylamine concentrations of $10^{-1.5}$M or less. The benzylamine concentrations at which NH_2OH brought about a significant increment in MAO activity were in between $10^{-3.5}$M and $10^{-1.5}$M.

Time-Course of the Activity of MAO by Tyramine Substrate in the Presence of Various Concentrations of NH_2OH, Using Warburg Manometric Method

A large amount of enzyme was used in order to lessen an error in activity and measurement

was done for 30 min after addition of substrate (Fig. 5). Since the reaction pattern obtained after 5 min by means of Warburg manometric method was similar to that after 1 min using oxygen electrode, the amount of the substrate used in the experimental system of Warburg manometric method was applied to that of oxygen electrode, particularly in order to correlate both the reactions by means of two differential apparatus, as possible. Experiments were performed at 38° in both experimental systems in reaction.

In the reaction of 1 min period, the activity of MAO by tyramine increased its inhibitory rate with an increased concentration of NH_2OH. The same pattern was obtained in the reaction of 10 min period to which there was a tendency to increase much more activity than that in the reaction of 1 min period, but these activities were greatly inhibited by high concentrations of NH_2OH. In the reaction of 30 min period, besides, the activity of MAO by tyramine was extremely enhanced with an increased concentration of NH_2OH and the amount of a rise of the activity was in maximum at a $10^{-1.5}$M-concentration of NH_2OH.

Discussion

As reported previously,[8] the presence of NH_2OH brought about the increased activity of MAO even in rat brain, and similarly in the present study activation under NH_2OH was observed in the long-term reaction. However, comparing the short- and long-term reactions of MAO with either of tyramine or benzylamine, NH_2OH resulted in entirely reverse effects (Fig. 2 and 4). It is of interest, in the short-term reaction, that NH_2OH decreased the activity of MAO by an extensive concentration of tyramine whereas it increased the activity by benzylamine ranging in concentration from $10^{-3.5}$M to $10^{-1.5}$M. There were likewise different phenomena in both the long-term reactions of MAO with tyramine and benzylamine under NH_2OH. Namely, in the MAO reaction by tyramine a $10^{-1.5}$M-concentration of NH_2OH enhanced increasingly the activity of MAO, but in the MAO reaction by benzylamine a $10^{-3.0}$M-concentration of NH_2OH reversely reduced it. Furthermore, the *K*m-values of MAO for tyramine and benzylamine were $1.82\times10^{-3.0}$M and $2.50\times10^{-2.0}$M, respectively (Fig. 3).

Multiple behaviors ascribing to the MAO complex are supported from the results that the two reactions of MAO by which tyramine and benzylamine were degraded in the presence of NH_2OH were different from each other compared with the patterns of activity in the one mutual short-term reactions, in the other mutual long-term reactions, and in both their experimental systems in reaction, and that both substrates resulted in different values of *K*m irrespective of addition of NH_2OH. Such a complex or multienzyme complex, according to Frieden,[9] was defined to be what at least one of the interacting enzyme is influenced by the other or what binding of enzyme proteins having different activities is unable to be easily isolated. But, assuming that MAO is a collection of variously functioning enzymes with each substrate,[6] or that the counteracted phenomena which occurred in the short- and long-term reactions by each substrate, *i.e.*, the reactions of activation and inhibition, depend on conformational changes in MAO enzyme,[5f,10] in addition to these experiments it is much more necessary that the multiplicity of the reaction mechanism is evidenced on the basis of the simultaneous use of two or more substrates and subsequently the multiplicity of MAO makes it necessary to demonstrate whether it depends on an catalytic action or on an regulatory action or whether the active site in some enzyme subunit transforms into a regulatory site of other enzyme in a complex.

There are, besides, similar studies as to the effect of $NaNO_2$, instead of NH_2OH, on MAO.[4c,8] These indicate that MAO activity under $NaNO_2$ varies according to different

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substrates and which shows the same phenomena of activation and inhibition as described above.

In the MAO reaction by tyramine, NH_2OH reduced the activity of MAO in the short-term reaction whereas it increasingly reinforced the activity in the long-term reaction. From studying on continuity of changes in between the inhibition and activation reactions, it was found that there were significant increases in activity at low concentrations of NH_2OH ($10^{-2.5}$M to $10^{-3.0}$M) within 10 min, but inhibitory effects were meantime obtained by high concentrations of NH_2OH ($10^{-1.5}$M$<$) as well as those in the reaction of 1 min period. Consequently, the enzymic reaction of MAO was showed to follow time-dependent activity under an optical concentration of NH_2OH. The inhibition mechanism of the MAO reaction by hydrazine compounds was suggested to be degradation of the inhibitor by oxidative dehydrazination of MAO.[11] According as such hydrazine inhibitors compete with amine substrate,[11] our kinetic experiments can indicate that the other time-dependent phenomenon, activation in the long-term reaction, may be the result of no reaction of oxidative degradation by MAO to NH_2OH.

It is considered that such diverse phenomena in activity as activation and inhibition, about which MAO brought by a given drug, may occur on the basis of the multiplicity of MAO. Considering one side of a phenomenon of the activation in the enzyme, the coexistence of some regulators by which enzyme activity may be controlled, is probable from teleological functions *in vivo*. As regulators in MAO enzyme, it is possible to give end-products,[12a,b] related enzymes,[4c,6,8b,12c,d] substrates,[4a,c,12d,e] trace amounts of low molecular compounds,[4b,12f] and membrane permeability,[12g] and thus it is assumed that an activation of an enzyme may occur indirectly by the effects of drugs to such regulators.

In conclusion, from the above results for MAO activity obtained in the presence of NH_2OH, particularly in the long-term reaction by tyramine, it is noted that a extremely increased rate of degradation of the substrate by MAO embossed enzymatically the peculiarity of NH_2OH under enzyme. That is to say, NH_2OH promotes further an excess of consumption in oxygen, entirely causes the disappearance of the sigmoidicity in MAO activity obtained by low concentrations of tyramine ($10^{-2.3}$M$>$), and then makes the activity change into the michaelis type in curve which brought about time-dependently alterable activity under an optical concentration of NH_2OH.

The present results were only a help to explain the multiplicity of MAO enzyme and the phenomena attributable to the enzyme complex. However, regarding the above suggestions, further informations should be obtained by means of treatment of detergents, denature by temperature, and modification of the hydrogen ion concentration, in order to study whether the partially denatured MAO produces disappearance in the effects of NH_2OH, as described above.

11) B.V. Clineschmidt and A. Horita, *Biochem. Pharmacol.*, **18**, 1011 (1969).

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