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Inhibition of Rabbit Liver Mitochondrial Monoamine Oxidase by New Hydrazine Derivatives. II. Probable Prosthetic Groups of the Enzyme, and Their Blocking by the Compound 31037-S or 31087-S[1]

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Examinations were performed to ascertain the prosthetic groups of rabbit liver mitochondrial monoamine oxidase. This enzyme was inhibited competitively by 8-hydroxyquinoline sulfate. Neocuproine and α,α'-dipyridyl were weak inhibitors, but diethyldithiocarbamate or ethylenediaminetetraacetate were little effective. Cupric ions were available to reactivate the depressed activity of the 8-hydroxyquinoline-treated enzyme. Since recovery was not observed with ferrous ions, the enzyme appeared to be cuproprotein. The second prosthetic group was likely to be sulfhydryl because *p*-chloromercuribenzoate reacted competitively with the enzyme. Oxidants such as 2-methyl-1,4-naphthoquinone or β-naphthoquinone-4-sulfonate were also strong inhibitors.

Inhibitory effects of 1-benzyl-2-(4,5,6,7-tetrahydro-1,2-benzisoxazo-3-lyl)carbonylhydrazine hydrochloride (31037–S) and 1-benzyl-2-(3-methylisoxazol-5-yl)carbonylhydrazine (31087–S) were counteracted clearly by the simultaneous addition of some metal ions. Cupric ions were especially efficient. An analogous counteraction was observed with cysteine, while methionine was inert in this phenomenon.

The inhibitory action of both hydrazine compounds was likely to be dependent on the blocking of these metal and sulfhydryl groups.

Monoamine oxidase [monoamine; O_2 oxidoreductase (deaminating); EC 1.4.3.4] of rabbit liver mitochondria was depressed remarkably by 1-benzyl-2-(4,5,6,7-tetrahydro-1,2-benzisoxazol-3-yl)carbonyl hydrazine hydrochloride or 1-benzyl-2-(3-methylisoxazol-5-yl) carbonyl hydrazine.[3] The effect was based on the blocking of the enzyme active site. It is therefore likely that the compounds[4] are available as tools to examine the nature of this enzyme.

The present examinations were carried out to elucidate the prosthetic groups which are allowed to react with the hydrazine compounds.

Experimental

Enzyme Assay——A suspension of rabbit liver mitochondria was used as an enzyme material. It was prepared as described before.[3] Monoamine oxidase activity was determined manometrically with tyramine as substrate by using a conventional Warburg apparatus. The method was essentially identical with those of Creasey[5] and the preceding paper.[3] The reaction medium consisted of 1.0 ml of the mitochondrial suspension, 0.2 ml of 0.1 M semicarbazide hydrochloride, 0.2 ml of 0.01 M sodium cyanide, 0.2 ml of 0.1 M sodium phosphate buffer at pH 7.4 and 0.2 ml of tyramine hydrochloride in an adequate concentration. The final volume of the reaction mixture was adjusted to 2.0 ml with distilled water. In the blank test, the substrate was omitted from the above-mentioned reaction medium. Various inhibitors and counter-

1) This study is part of a thesis submitted by the author in partial fulfilment of the requirements for the degree of Doctor of Science from Tohoku University.
2) Location: *Sagisu, Fukushima-ku, Osaka.*
3) Part I: A. Kurosawa, *Chem. Pharm. Bull.* (Tokyo), **17**, 36 (1969).
4) The abbreviations used are 31037–S for 1-benzyl-2-(4,5,6,7-tetrahydro-1,2-benzisoxazol-3-yl)carbonyl hydrazine hydrochloride, and 31087–S for 1-benzyl-2-(3-methylisoxazol-5-yl)carbonyl hydrazine.
5) N.H. Creasey, *Biochem. J.*, **64**, 178 (1956).

acting substances were added to the reaction system by replacing distilled water. The solutions of semicarbazide and cyanide were adjusted to pH 7.4 before use. The center well of a Warburg vessel contained 0.2 ml of 1 M sodium cyanide and a filter paper strip. The enzyme activity was obtained from the initial velocity of oxygen consumption, as μ moles of oxidized tyramine/10 mg of protein/hr. Protein contents of the enzyme materials were determined colorimetrically according to a biuret reaction.[6]

Inhibitors and Counteracting Substances——The hydrazine compounds were dissolved in ethanol and diluted adequately with distilled water. Aqueous solutions of ethanol alone, corresponding to each inhibitor solution, were taken as controls. Neocuproine was dissolved in 0.2 M acetic acid and neutralized before use. Aqueous acetic acids, equivalent to each neocuproine solution, were prepared as controls. Menadione (2-methyl-1,4-naphthoquinone) was pipetted as its acetone solution into the main compartment of a Warburg vessel, evaporated to dryness in nitrogen and then incubated with the reaction medium for 5 min at 37.5°. It was soluble in the mitochondria-containing medium. All of the other reagents were used as their aqueous solutions. Acidic reagents such as cysteine hydrochloride or 8-hydroxyquinoline sulfate were neutralized before use.

UV Spectra of Hydrazine Compounds——Ultraviolet absorption spectra of 31037-S and 31087-S were taken in aqueous ethanol with or without cupric sulfate by using a Beckman Spectrophotometer Model DU. The final concentrations were 1×10^{-5} M for each hydrazine compound and 10^{-4} M for cupric sulfate. When cupric sulfate was used, its aqueous solution (10^{-4} M) was taken as the blank.

Results

If a compound is capable of reacting with an enzyme prosthetic group, it may have an affinity to the substance analogous to the prosthetic group. Finding this substance, therefore, would be valuable in estimating the inhibitor-acting prosthetic group. Various metal ions were found to be counteracting substances against the inhibitory action of 31037-S or 31087-S. Each hydrazine compound was mixed beforehand with each metal ion. The mixture and the substrate were immediately added to the reaction system. As seen in Table I, the enzyme inhibition by the hydrazine compound decreased markedly. Cupric and ferrous ions were especially efficient. Cobalt and nickel ions were also very effective, but magnesium and molybdenum were inert. The metal ions used did not affect normal enzyme activity under the conditions employed. Ultraviolet absorption spectra of the hydrazine compounds were changeable in the presence of cupric ions (Fig. 1). This suggested the complex formation of each hydrazine compound and cupric ions. From these results, some chelating agents were examined for their effects on the monoamine oxidase (Table II). The enzyme activity was depressed markedly by 8-hydroxyquinoline sulfate. Neocuproine and α,α'-dipyridyl were

Table I. Counteraction of Metal Ions on the Monoamine Oxidase Inhibition by 31037-S or 31087-S

Metal salt	Monoamine oxidase inhibition (%) 31037-S added[a]	31087-S added[b]
None	78	80
$CuSO_4$	10	− 2
$FeSO_4$	25	− 3
$CoCl_2$	54	22
$NiCl_2$	44	27
$ZnSO_4$	67	40
$MgSO_4$	72	79
Na_2MoO_4	—	78

Each hydrazine compound (10^{-5} M) was mixed with each metal salts (10^{-3} M) prior to its addition. The mixture (0.2 ml) and tyramine (0.2 ml, 0.04 M) were simultaneously added to the reaction system. The normal enzyme activities in *a*) and *b*) were 19.3 and 16.0 μmoles/10 mg of protein/hr, respectively.

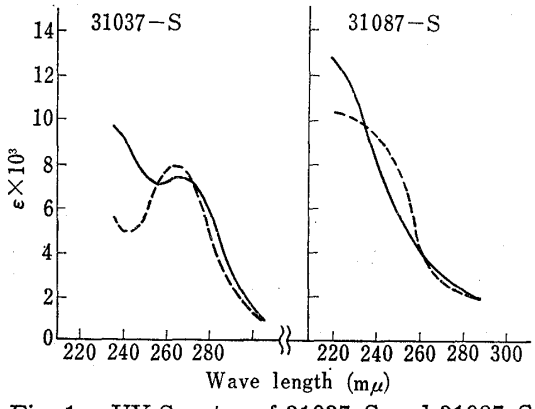

Fig. 1. UV Spectra of 31037-S and 31087-S

The hydrazine compounds were used as their ethanolic aqueous solutions at 10^{-5} M in the presence and absence of 10^{-4} M cupric sulfate (——, control; ------, cupric sulfate added).

6) H.W. Robinson and C.G. Hogden, *J. Biol. Chem.*, **135**, 707 (1940).

Table II. Effects of Chelating Agents on Monoamine Oxidase

Chelating agent	Final conc. (M)	Monoamine oxidase inhibition (%)
8-Hydroxyquinoline sulfate	1×10^{-3}	84
Diethyldithiocarbamate	1×10^{-3}	14
Neocuproine	1×10^{-3}	25
	2×10^{-3}	34
α,α'-Dipyridyl	1×10^{-3}	31
Ethylenediaminetetraacetate	1×10^{-3}	6

The enzyme activity was determined at 4×10^{-3} M of tyramine. Each chelating agent and the substrate were simultaneously added to the reaction system.

weak inhibitors, but little effect was observed with diethyldithiocarbamate and ethylenediaminetetraacetate.

Since 8-hydroxyquinoline acted competitively, it was assumed that heavy metal ions were contained on an active site of the enzyme. To estimate the nature of this metal group, an attempt was made to reactivate the depressed activity of the enzyme. The mitochondrial suspension (25 ml) containing 0.02 M 8-hydroxyquinoline was dialyzed for 6 hr against 1 liter of 0.01 M sodium phosphate buffer (pH 7.4) at 3°. The dialysis was further performed for 20 hr with 1 liter of the fresh phosphate buffer. The control suspension of mitochondria was dialyzed analogously. Each of the dialyzed mitochondria was collected by centrifuging for 10 min at 9000 ***g***, and was resuspended in the fresh phosphate buffer to give the original volume. In the chelator-treated mitochondria, the

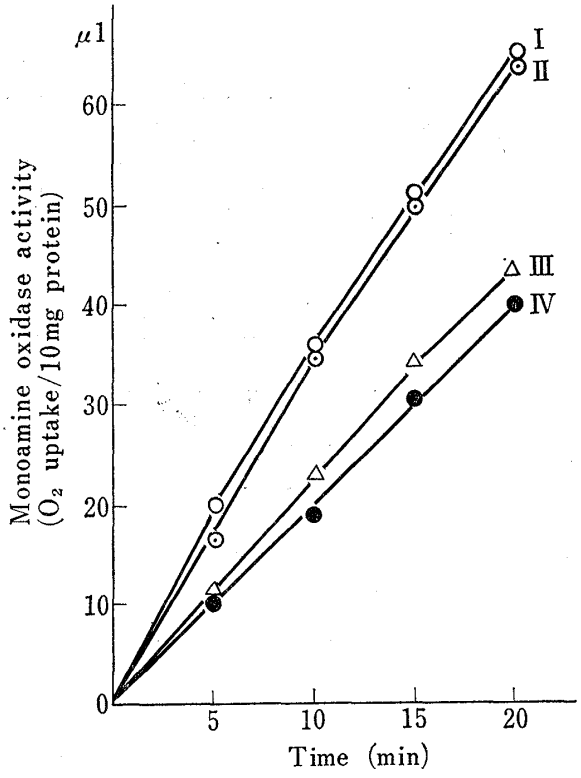

Fig. 2. Reactivation of the 8-Hydroxyquinoline treated Enzyme by the Addition of Cupric Ions

The rabbit liver mitochondrial suspension, containing 0.02 M 8-hydroxyquinoline sulfate, was dialyzed against 0.01 M phosphate buffer (pH 7.4). The dialyzed mitochondria were collected by centrifugation, resuspended in fresh phosphate buffer to give the original volume and tested for their monoamine oxidase activities in the presence or absence of metal ions.
—○— normal
—⊙— treated+3×10^{-4} M $CuSO_4$
—△— treated+3×10^{-4} M $FeSO_4$
—●— treated control

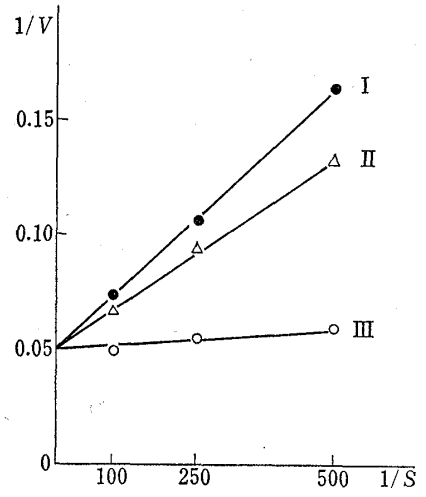

Fig. 3. Inhibition of Monoamine Oxidase by *p*-Chloromercuribenzoate or 8-Hydroxyquinoline

Monoamine oxidase assay was performed with 1.0 ml ofa single batch of the mitochondrial preparation after the simultaneous addition of tyramine and each inhibitor. Velocities are given as μmoles of oxidized tyramine/10 mg of protein/hr, and concentrations of the substrate, as moles/liter.
—●— *p*-chloromercuribenzoate added, at 5×10^{-4} M
—△— 8-hydroxyquinoline sulfate added, at 5×10^{-4} M
—○— control

enzyme activity had been inhibited by about 40%, while it was almost reactivated by the preliminary addition of 3×10^{-4} M cupric sulfate. No recovery was seen in ferrous sulfate under the same experimental conditions (Fig. 2).

Decomposition of hydrogen peroxide by the same enzyme material was also tested manometrically under conditions analogous to those for the monoamine oxidase assay. Instead of tyramine, 5 μmoles of hydrogen peroxide was used as substrate. Oxygen output was followed every min for 7 min. The hydrogen peroxide destruction ability remained in the sample, and was identical with that of the control enzyme material. The above-mentioned increase of oxygen consumption is unlikely to be dependent on the inactivation of the associated catalase. In the normal enzyme, the end value of oxygen consumption was in agreement with that anticipated from the added amount of tyramine even in the presence of cupric ions. This fact contradicts the autocatalytic oxidation of tyramine or its aldehyde product by cupric ions. Moreover, oxygen consumption of the artificial medium, in which only the mitochondrial suspension was replaced with 1 ml of 1% (w/v) bovine albumin, was negligible. The results of Fig. 2 should be taken as the reactivation of the monoamine oxidase itself.

On the other hand, *p*-chloromercuribenzoate was a competitive inhibitor of this enzyme. Some oxidants such as 2-methyl-1,4-naphthoquinone or β-naphthoquinone-4-sulfonate were strong inhibitors. The enzyme inhibition was observed at 72% at 5×10^{-5}M of the former and at 66% at 10^{-4}M of the latter. In the higher concentrations, over 5×10^{-4} M, cupric ions depressed activity of the enzyme. Therefore, sulfhydryl can be assumed to be the second prosthetic group of this enzyme.

Next, *p*-chloromercuribenzoate and 8-hydroxyquinoline were respectively allowed to react with an equal amount of the same enzyme preparation. Each inhibitor and tyramine were simultaneously added to the reaction system. Lineweaver-Burk plots of the enzyme activities are shown in Fig. 3. The inhibition was competitive with both compounds. At 2×10^{-3} M of tyramine, the inhibition degrees were obtained at 64% for *p*-chloromercuribenzoate and at 56% for 8-hydroxyquinoline sulfate, respectively. The meaning of these results are discussed later.

Examinations were further performed to demonstrate the counteraction of a sulfhydryl compound against the inhibitory action of 31037-S or 31087-S. The procedure was analogous to those for metal ions described earlier. The enzyme inhibition was clearly reduced in the presence of cysteine (Table III). Little counteraction was observed in the case of methionine. Results indicate that the counteraction is based on the sulfhydryl group of the cys-

Table III. Counteraction of Cysteine or Methionine on the Monoamine Oxidase Inhibition by Hydrazine Compounds

Hydrazine compound	Amino acid (M)	Monoamine oxidase activity (μmoles/10 mg protein/hr) Hydrazine compound added	Control
31037-S 10^{-6} M	cysteine (10^{-4})	17.1	20.8
	none	5.1	21.5
	methionine (10^{-4})	10.1	15.6
	none	8.0	16.5
31087-S 10^{-6} M	cysteine (10^{-4})	11.4	10.2
	none	5.8	10.8
	methionine (10^{-4})	5.2	10.4
	none	5.8	10.8

The administration of amino acids and hydrazine compounds was analogous to that in Table I. Tyramine was used at 4×10^{-3} M.

teine molecule, and that the hydrazine compounds influence the sulfhydryl prosthetic group of the enzyme.

Discussion

Until recently, flavin,[7] sulfhydryl,[8] and metal ions[7a,9] were assumed to be the prosthetic groups of some mitochondrial monoamine oxidases. Results obtained here also suggested that the rabbit liver enzyme has at least two prosthetic groups of sulfhydryl and heavy metal. Since the enzyme activity was not reduced by dialysis against phosphate buffer, the metal appeared to be combined tightly with the enzyme protein. In the 8-hydroxyquinoline treated enzyme, its depressed activity recovered up to the normal level in the presence of cupric ions. If the enzyme metal group had been blocked by the chelating agent, cupric ions might be available to remove the inhibitor. Some of the added cupric ions might be utilized for the reconstruction of the active group. Under identical conditions, ferrous ions did not cause this reactivation. It is therefore probable that the metal of the enzyme is copper. Copper appeared to play a common and essential role for the oxidative deamination of various amines, since plasma monoamine oxidase[10] and plant diamine oxidase[11] are found to be cuproenzymes. Neocuproine, a chelating agent specific for cuprous copper,[12] was inhibitory to the rabbit liver monoamine oxidase. This fact may also support the presence of copper in the enzyme, although no valency change of copper was demonstrated in the bovine mitochondrial- and plasma-enzymes.[9b,10,13]

Next, an attempt was made to examine whether the two prosthetic groups of sulfhydryl and metal are contained in the same enzyme active site. *para*-Chloromercuribenzoate and 8-hydroxyquinoline were respectively allowed to react with an equal amount of the same enzyme preparation. The inhibition degrees and types of the compounds were examined concurrently. If each of the prosthetic groups is contained in each of two different isozymes, the total of the enzyme inhibition caused by both inhibitors should not be over 100%, unless the effects are not due to allosteric inhibition. As shown in Fig. 3, the effects of 8-hydroxyquinoline and *p*-chloromercuribenzoate were competitive, and the sum of each inhibition degree was obtained at 120% at 2×10^{-3} M of tyramine as substrate. The metal and sulfhydryl groups, therefore, appear to exist in the same enzyme active site.

Inhibitory action of 31037-S or 31087-S was attributable to blocking the active site of this enzyme. Since the inhibitory action was counteracted by cupric ions or cysteine, the inhibitors are considered to have a great affinity to the metal and sulfhydryl prosthetic groups, and to cause the enzyme inhibition by combining with both of the prosthetic groups.

Substituted hydrazines are found to be decomposed by cupric ions with the formation of free radicals.[14] Green[9a] emphasized the striking similarity between the cupric-ion catalyzed decomposition of hydrazines and the enzyme inhibition by the same compounds. He assumed that the enzyme inhibition is based on the free radicals which were formed from the inhibitors by the autocatalytic action of the enzyme metal group. However, the metal in the enzyme active site should be functional only in the state associating with other prosthetic

7) *a*) S. Nara, I. Igaue, B. Gomes, and K.T. Yasunobu, *Biochem. Biophys. Res. Commun.*, **23**, 324 (1966); *b*) I. Igaue, B. Gomes, and K.T. Yasunobu, *ibid.*, **29**, 562 (1967).
8) A.N. Davison, *Biochem. J.*, **67**, 316 (1957); L.B. Klyashtorin and L.I. Gridneva, *Biochemistry* (A translation of *Biokhimiya*, **31**, 716 (1966)).
9) *a*) A.L. Green, *Biochem. Pharmacol.*, **13**, 249 (1964); *b*) S. Nara, B. Gomes, and K.T. Yasunobu, *J. Biol. Chem.*, **241**, 2774 (1966).
10) H. Yamada and K.T. Yasunobu, *J. Biol. Chem.*, **237**, 3077 (1962).
11) J.M. Hill and P.J.G. Mann, *Biochem. J.*, **85**, 198 (1962).
12) E. Frieden, G. Ezell, and Y. Karkhanis, *Federation Proc.*, **16**, 183 (1957).
13) H. Yamada and K.T. Yasunobu, *Biochem. Biophys. Res. Commun.*, **8**, 387 (1962).
14) L.E. Eberson and K. Persson, *J. Med. Pharm. Chem.*, **5**, 738 (1962).

groups. In fact, according to Green himself,[9a] readiness in the decomposition of hydrazine compounds did not always correspond to inhibitory potencies of the same compounds. It is doubtful that the enzyme metal group possess autocatalytic action such as do cupric ions for the inhibitor decomposition.

The preceding results[3] indicated that the binding of 31037-S or 31087-S to the enzyme active site may be similar to that of the substrate. The present results showed that the hydrazine compounds cause the enzyme inhibition by combining with both the sulfhydryl and metal prosthetic groups. Therefore, the metal and sulfhydryl groups of the enzyme are likely to be involved in the binding reaction of these hydrazine compounds.

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