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Inhibition of Rabbit Liver Mitochondrial Monoamine Oxidase by New Hydrazine Derivatives. I. Active Moieties of the Compounds 31037-S and 31087-S[1]

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The effects of hydrazine compounds on rabbit liver mitochondrial monoamine oxidase were examined using a manometric method. Two hydrazine derivatives, *i.e.*, 1-benzyl-2-(4,5,6,7-tetrahydro-1,2-benzisoxazol-3-yl)carbonyl hydrazine hydrochloride (31037–S) and 1-benzyl-2-(3-methylisoxazol-5-yl)carbonyl hydrazine (31087–S) were especially powerful inhibitors of this enzyme. They acted competitively with the enzyme and were more potent than iproniazid. When each inhibitor was allowed to react with the enzyme in the absence of substrate, its inhibitory action was irreversible and much enhanced. Cyanide and oxygen promoted the inhibitory action of both compounds. In comparison with the potencies of some allied compounds, it was suggested that an active part of 31037–S or 31087–S is a benzylhydrazine moiety, and that it directly influences the enzyme active site.

Monoamine oxidase[3] [monoamine; O_2 oxidoreductase (deaminating); EC 1.4.3.4] is observed predominantly in the mitochondrial fraction of mammalian tissues. As the enzyme is readily capable of oxidizing pressor amines such as serotonin or tyramine, it is believed to play a leading role in the metabolism of these physiologically active amines. While complete purification of the mitochondrial enzyme has never been achieved recent works[4] have shown that its partial purification is feasible. The exact nature of the enzyme itself is still obscure.

On the other hand, since the discovery of iproniazid,[5] considerable attention has been paid to alkyl- or arylalkyl-hydrazines as characteristic inhibitors of monoamine oxidase. They are widely used for physiological or pharmacological studies of this enzyme. However, little is known about the mechanism of their inhibitory reaction.

In this work, 1-benzyl-2-(4,5,6,7-tetrahydro-1,2-benzisoxazol-3-yl) carbonyl hydrazine hydrochloride and 1-benzyl-2-(3-methylisoxazol-5-yl) carbonyl hydrazine were examined for their inhibitory effects on rabbit liver mitochondrial monoamine oxidase, and were concurrently utilized as tools to examine the enzyme properties.

The present report is the first paper of this series and is concerned with the essential structure of the compounds[6] causing enzyme inhibition.

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) A.N. Davison, *Physiol. Rev.*, **38**, 729 (1958); H. Blaschko, "The Enzymes," 2nd ed., Vol. 8, ed. by P.D. Boyer, H. Lardy, and K. Myrbäck, Academic Press Inc., New York, N.Y., 1963, pp. 337—351.
4) L.B. Klyashtorin, L.I. Gridneva, and V.Z. Gorkin, *Biochemistry* (A translation of *Biokhimiya*), **31**, 143 (1966); T. Nagatsu, *J. Biochem.* (Tokyo), **59**, 606 (1966); S. Nara, B. Gomes, and K.T. Yasunobu, *J. Biol. Chem.*, **241**, 2774 (1966).
5) E.A. Zeller, J. Barsky, J.R. Fouts, W.F. Kirchheimer, and L.S. van Orden, *Experientia*, **8**, 349 (1952); E.A. Zeller and J. Barsky, *Proc. Soc. Exptl. Biol. Med.*, **81**, 459 (1952).
6) 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. Both derivatives and the other numbering compounds were synthesized by Dr. Hideo Kano and his coworkers of this Laboratory.

Experimental

Enzyme Material——Male and female rabbits, weighing about 2 kg, were sacrificed by exsanguination. The livers were promptly removed, washed with ice cold saline, and then homogenized for 5 min with 5 volumes of 0.25 M sucrose in an ice bath by using a Potter–Elvehjem glass homogenizer. The sucrose solution was initially adjusted to pH 7.4 with 0.01 M sodium phosphate buffer. The mitochondrial fraction was separated from the above-mentioned homogenate according to the method of Hogeboom,[7] and was suspended in 0.01 M sodium phosphate buffer at pH 7.4. One milliliter of this suspension contained mitochondria corresponding to 1 g of tissue. A part of the mitochondrial fraction was also prepared from the liver of Wistar strain rats weighing about 120 g. These mitochondrial suspensions were frozen immediately and stored in dryice in a vacuum bottle until use. After melting at room temperature and stirring well, 1 ml of the suspension was used for enzyme assay.

To reduce the individual differences among the animals in the enzyme activity, equal parts of mitochondrial suspensions were mixed. Comparison of the enzyme activity with a different substrate, or of its inhibition with various inhibitors, was always performed on a single batch of the mitochondrial mixture. Average values were obtained as the results of duplicate experiments.

Monoamine Oxidase Assay——The enzyme activity was determined monometrically by using a conventional Warburg apparatus. The method was essentially identical with that of Creasey.[8] The standard reaction medium consisted of the mitochondrial suspension (1 ml), 0.1 M sodium phosphate buffer at pH 7.4 (0.2 ml), 0.1 M semicarbazide hydrochloride (0.2 ml), 0.01 M sodium cyanide (0.2 ml), substrate in an adequate concentration (0.2 ml), and an inhibitor or its control solution (0.2 ml). The final volume of the reaction media was constantly kept to 2.0 ml. Tyramine hydrochloride and serotonin creatinine sulfate were used as substrates. The substrate-omitted medium was prepared for the blank test. Both solutions of semicarbazide and cyanide were adjusted to pH 7.4 before use. The center well of the reaction vessel contained 0.2 ml of 1 M sodium cyanide and a filter paper strip. The vessels were shaken in a 37.5° water-bath at a rate of 130 cycles/min. After 5 min of temperature equilibration, the reaction was started by the addition of substrate from a side arm. Oxygen uptake was measured every 5 min for 30 min in air. The monoamine oxidase activity was obtained from the initial velocity as μmoles of oxidized substrate/10 mg of protein/hr. Protein content in the enzyme material was determined colorimetrically according to Robinson, *et al.*[9] with bovine serum albumin as standard.

Decomposition of Hydrogen Peroxide in the Reaction System——This ability was estimated manometrically under conditions identical to those for the monoamine oxidase assay, in which the substrate was replaced by 5 μmoles of hydrogen peroxide. Oxygen output in the reaction system was measured every 1 min during a period of 7 min.

Inhibitor——The compounds 31037-S, 31087-S, and their relating derivatives were dissolved in a small amount of ethanol. The solutions were diluted adequately with distilled water before use. Aqueous ethanol solutions corresponding to each inhibitor solution were taken as controls. Harmine hydrochloride, iproniazid phosphate, isoniazid and amphetamine sulfate (*dl*-α-methylphenethylamine sulfate) were used as their aqueous solutions.

Results

A) General Properties of Rabbit Liver Mitochondrial Monoamine Oxidase

Monoamine oxidase catalyzes the following reaction.

$$R\text{–}CH_2\text{–}NH_2 + H_2O + O_2 \longrightarrow R\text{–}CHO + NH_3 + H_2O_2 \qquad (I)$$

If the mitochondrial preparation is used as an enzyme material, the hydrogen peroxide formed is readily decomposed to water and molecular oxygen by the associated catalase. The overall reaction is written as equation (II) and it can be determined by measuring oxygen consumption.

$$2R\text{–}CH_2\text{–}NH_2 + O_2 \longrightarrow 2R\text{–}CHO + 2NH_3 \qquad (II)$$

From this reaction scheme, it can be anticipated that one atom of oxygen is apparently consumed for the oxidation of each molecule of substrate. However, when a crude enzyme material

7) G.H. Hogeboom, "Methods in Enzymology," Vol. 1, ed. by S.P. Colowick and N.O. Kaplan, Academic Press Inc., New York, N.Y., 1955, pp. 16—19.
8) N.H. Creasey, *Biochem. J.*, **64**, 178 (1956).
9) H.W. Robinson and C.G. Hogden, *J. Biol. Chem.*, **135**, 707 (1940).

is used, the observed oxygen uptake is not always identical with the theoretical value. The difference is probably due to the further oxidation of an aldehyde, that is, the primary deamination product of the substrate. In a washed mitochondrial preparation of rat liver, Creasey[8] concluded that a bufferized reaction mixture containing 10^{-2}M semicarbazide and 10^{-3}M cyanide is suitable for monoamine oxidase assay. This reaction mixture was also suitable for the manometric determination of the rabbit liver enzyme. The oxygen uptake for tyramine oxidation was in agreement with the theoretical value for equation (II), linear with time in the initial phase of the reaction, and directly proportional to the amount of the mitochondria. The catalase activity associating with the mitochondrial suspension was maintained sufficiently in this reaction medium. The cyanide in the reaction medium was available to prevent the oxidation of ethanol in each inhibitor solution.

The apparent properties of rabbit liver monoamine oxidase were virtually analogous to those of the mitochondrial monoamine oxidases in other animal tissues. The optimum pH for the reaction ranged from 7.4 to 7.6. It was obtained by carrying out a series of activity measurements at different pH's in 0.01M sodium phosphate buffer. At pH values lower than 7.0, activity was reduced. In deep freeze, the enzyme was stable for at least one month, while half its activity was lost by heating for 5 min at 50°. The enzyme was insoluble and could not be extracted with distilled water, 0.2M sodium bicarbonate or 0.01M sodium phosphate buffer at pH 5.3 or 8.0. Its clear solution was obtained by the treatment with 0.5% (w/v) sodium deoxycholate followed by dialysis against 0.01M phosphate buffer at pH 7.4. The addition of ammonium sulfate to this clear solution between 10 and 30% saturation brought out an insoluble sediment containing about 60% of the total enzyme activity in the original solution. Further purification was unsuccessful. Some mitochondrial enzymes such as succinic dehydrogenase (EC 1.3.99.1) can be solubilized with *n*-butanol treatment.[10] The rabbit liver monoamine oxidase, however, lost activity with the treatment of *n*-butanol (10%, v/v). Lipid components of the mitochondria may be involved in the activity of this enzyme.

Tyramine and serotonin were good substrates. They were likely to be attacked by the same enzyme because the oxidation of the former was retarded in the presence of the latter. Such a phenomenon was observed analogously in the rat liver enzyme. Results are presented in Table I.

Table I. Oxidation of Mixed Substrates by Monoamine Oxidase

Enzyme material	Substrate	Final conc. (M)	Enzyme activity[a]
Rabbit–liver mitochondria	a) tyramine	5×10^{-3}	21.7
	b) serotonin	5×10^{-3}	4.9
	c) mixture of a), b)		12.8
Rat–liver mitochondria	a) tyramine	3×10^{-3}	5.0
	b) serotonin	3×10^{-3}	3.5
	c) mixture of a), b)		2.4

a) μmoles of oxidized substrate/10 mg of protein/hr

Michaelis constants (K_m) of both rabbit and rat liver enzymes were calculated for tyramine and serotonin by means of Lineweaver and Burk.[11] The values for tyramine were not very variable in either enzyme. The value for serotonin, however, was markedly small in the rabbit liver enzyme (Table II). In comparison with the maximum velocity (V_{max}), the rabbit liver enzyme showed the strongest activity with tyramine as substrate. This fact was noticed as a characteristic property of the rabbit liver enzyme, and it was useful in the present examination

10) R.K. Morton, *Nature*, **166**, 1092 (1950).
11) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

since the effects of the inhibitors could be observed in the more selected condition for tyramine oxidation. From these results, tyramine was exclusively used as the substrate for further examinations.

Table II. The Maximum Reaction Velocity (V_{max}) and Michaelis Constant (K_m) for Monoamine Oxidase

Enzyme material	Substrate	V_{max}	K_m
Rabbit–liver mitochondria	tyramine	19.7	5×10^{-4}
	serotonin	3.3	4×10^{-3}
Rat–liver mitochondria	tyramine	3.4	5×10^{-4}
	serotonin	3.0	3×10^{-4}

The V_{max} and the K_m are expressed in μmoles of oxidized substrate/10 mg of protein/hr, and moles/liter, respectively. The values were calculated by means of Lineweaver and Burk.

B) Inhibitory Action of Hydrazine Derivatives

Previous investigators[12] reported that the effects of alkylhydrazines on monoamine oxidase are markedly enhanced in the cyanide–containing medium, and that the potentiated and irreversible inhibition is observed if the inhibitors are allowed to react with the enzyme for different length of time in oxygen before the addition of substrate. The present study was also designed to ascertain whether such characteristic phenomena could be observed in the enzyme inhibition by 31037–S or 31087–S.

Cyanide was certainly effective in increasing the inhibitory action of both compounds (Table III). The potentiated effects were observed analogously in the preliminary addition

Table III. Enhancement of Monoamine Oxidase Inhibition by Cyanide

Inhibitor	Conc. (M)	Monoamine oxidase inhibition (%) Cyanide absent[a]	Standard medium
Iproniazid	1×10^{-4}	33	89
Harmine	5×10^{-5}	38	41
31037–S	5×10^{-7}	17	31
31087–S	1×10^{-6}	10	33

a) The cyanide in the standard reaction medium was replaced with distilled water.
Each inhibitor was allowed to react with the rabbit liver mitochondria for 5 min at 37.5° in the presence or absence of cyanide, and then tyramine (4×10^{-3} M) was added to the reaction system.
normal enzyme activity, 16.5 μmoles/10 mg of protein/hr

Table IV. Enhancement of Monoamine Oxidase Inhibition by the Preliminary Addition of Some Inhibitors

Inhibitor	Final conc. (M)	Monoamine oxidase inhibition (%) A	B
Iproniazid	5×10^{-5}	31	9
Harmine	1×10^{-4}	56	55
31037–S	5×10^{-5}	86	56
31087–S	5×10^{-5}	79	43

Rabbit liver mitochondria were incubated with each inhibitor for 5 min at 37.5° in air prior to the addition of 3×10^{-3} M tyramine (A). In B, the inhibitors were added simultaneously with tyramine. The normal enzyme activity of the material was obtained by 18.0 μmoles/10 mg of protein/hr.

12) A.N. Davison, *Biochem. J.*, **67**, 316 (1957); A.L. Green, *Biochem. Pharmacol.*, **13**, 249 (1964).

of the inhibitors, that is, the enzyme was incubated with each inhibitor for 5 min at 37.5° in air, and then tyramine was added to the reaction system for the enzyme assay. The resulting effects were more remarkable than those obtained with the simultaneous addition of tyramine and each inhibitor (Table IV). The inhibitory enhancement could not be observed with harmine. The phenomenon seemed to be specific for the effects of alkyl- or arylalkyl-hydrazines.

Under aerobic or anaerobic conditions, the enzyme was allowed to react with 31037–S or 31087–S for 5 min at 37.5° in the presence of cyanide. The enzyme activity was then determined as usual by tipping the substrate. When nitrogen was used, the vessels were flushed with air after the addition of the substrate. It was found that oxygen promoted the inhibitory action of these compounds (Table V). Thus, 31037–S and 31087–S are very similar in their effects to iproniazid.

Table V. Potentiated Effects of Hydrazine Derivatives under the Aerobic Condition

Inhibitor	Final conc. (M)	Monoamine oxidase inhibition (%) Preincubation	
		In air	In nitrogen
31037–S	2.5×10^{-5}	48	26
31087–S	2.5×10^{-5}	58	15

Rabbit liver mitochondria were preincubated with each inhibitor for 5 min at 37.5° in air or in nitrogen before the addition of tyramine (4×10^{-3} M). When nitrogen was used, the vessels were flushed with air after the addition of the substrate. The normal enzyme activity was 12.2 μmoles/10 mg of protein/hr.

Irreversibility of 31037–S or 31087–S was observed in the following experiment. Freshly prepared mitochondria of rabbit liver were exposed to an inhibitor for 10 min at 37.5° in air. The treated mitochondria were washed once with 0.25 M sucrose solution and resuspended in the original volume of 0.01 M sodium phosphate buffer at pH 7.4. The washing procedure was performed analogously for the control mitochondria. The residual activities were expressed as a percentage of the control. In the samples treated with hydrazine compounds, complete reactivation was not observed (Table VI). The effect of harmine was eliminated fully in the washed mitochondria. The result agreed with the view that harmine is a reversible inhibitor of monoamine oxidase.[13]

Table VI. Reversibility of Monoamine Oxidase Inhibition

Inhibitor	Final oonc. (M)	Monoamine oxidase activity (%) Mitochondria	
		In situ	Washed
Iproniazid	5×10^{-6}	22	62
Harmine	5×10^{-5}	68	105
31037–S	5×10^{-7}	20	55
31087–S	2×10^{-6}	37	63

Rabbit liver mitochondria were incubated with each inhibitor for 10 min at 37.5°. After washing with 0.25 M sucrose, the mitochondria were supplied for enzyme assay. Residual enzyme activities of the treated mitochondria were expressed as a percentage of the untreated control.

In Fig. 1, reciprocals of reaction velocities, with and without 31037–S, are plotted against reciprocals of tyramine concentrations. The intersection on the $1/v$ axis indicates that inhibition is competitive. Under the same conditions, iproniazid, harmine, and 31087–S were

13) S. Udenfriend, B. Witkop, B.G. Redfield, and H. Weissbach, *Biochem. Pharmacol.*, **1**, 160 (1958).

also competitive inhibitors. The last compound, however, rarely caused a non-competitive type inhibition.

Examinations were further performed to elucidate the active moiety of 31037-S or 31087-S. The potencies of both hydrazine derivatives were compared with those of some related compounds. Results were obtained in a fixed condition by using a single batch of the mitochondria. Data are summarized in Table VII. Among the compounds used, 31037-S was the most powerful inhibitor, and 31087-S ranked next. N-1-Benzyl substituted derivatives were more potent than N-1-isopropyl compounds. The conjugation of other groups to the benzyl substituent diminished the potency of each original compound. Such a tendency can be observed in the effects of 31076-S, 31122-S and 31090-S. All of the inhibitors acted competitively to the enzyme, and non-alkylated hydrazide, *i. e.*, isoniazid, was inactive. The decomposition of hydrogen peroxide in the reaction media was not influenced by the hydrazine inhibitors. Five μmoles of hydrogen peroxide was destroyed completely within 2 min. In the presence of each inhibitor, the ability was identical with that of the control. The added amount of hydro-

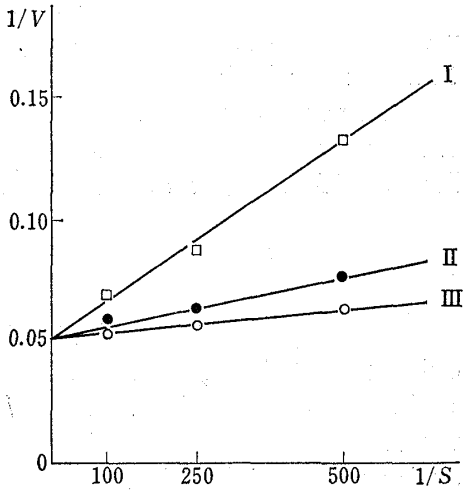
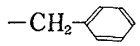
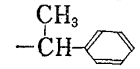
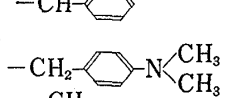
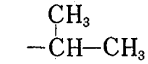
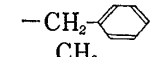
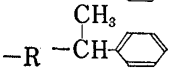
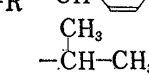
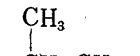


Fig. 1. Competitive Inhibition of Rabbit Liver Mitochondrial Monoamine Oxidase by 31037-S

The enzyme activity was obtained by the simultaneous addition of tyramine and 31037-S. Velocities are given as μmoles of oxidized tyramine/10 mg of protein/hr, and substrate concentrations, as moles/liter. Concentrations of 31037-S are 5×10^{-7} M (—□—), 1×10^{-7} M (—●—) and none (—○—).

Table VII. Inhibitory Effects of Some Hydrazine Compounds on Rabbit Liver Mitochondrial Monoamine Oxidase

Compound	R-Substituent	Monoamine oxidase inhibition (%) 1×10^{-4} M	5×10^{-5} M
31037-S		81	68
31076-S		62	60
31122-S		42	27
31075-S		19	13
31087-S		79	48
31090-S		70	20
31088-S		54	20
Iproniazid		17(32)	6
Isoniazid	—H	5(−1)	−6

Results were obtained at 3.3×10^{-3} M of tyramine by using a single batch of the mitochondrial suspension. Each compound and the substrate were simultaneously added to the reaction system. Values in parentheses are the results obtained in the enzyme which was allowed to react with the compound for 5 min at 37.5° in air. The normal enzyme activity was 19.7 μmoles /10 mg of protein/hr.

gen peroxide corresponds to that formed during 30 min of the monoamine oxidase reaction under the conditions employed. Therefore, the reduced oxygen consumption should be taken as the inhibition of the monoamine oxidase itself.

On the other hand, amphetamine did not serve as a substrate of this enzyme. When it was added to the reaction system at 10^{-3}M, the enzyme activity was depressed competitively. Its inhibition degree was obtained at 17% with 4×10^{-3}M of tyramine as substrate.

Discussion

Although 31037–S and 31087–S were more potent inhibitors of rabbit liver monoamine oxidase than iproniazid, their properties in inhibitory action were analogous to those of various alkylhydrazines. The effects were accelerated by cyanide and oxygen. When the compounds were allowed to react with the enzyme in the absence of substrate, they showed an irreversible inhibition. To explain such curious effects of alkylhydrazines, some investigators[12] assumed that the enzyme inhibition is caused by the oxidized substances which are possibly formed from inhibitors at an active site on the enzyme itself. Since hydrazine compounds are not very stable, it is probable that the compounds are transformed to irreversible inhibitors in the reaction medium. However, with a crude enzyme material such as mitochondria, the transformation should not be attributed only to the oxidative action of the enzyme itself. Multiple oxido–reduction systems or other contaminants of mitochondria may also be concerned with this mechanism. In fact, Smith, *et al.*[14] demonstrated that iproniazid can be cleaved non–enzymatically to a potent inhibitor if it is incubated with an enzyme material in the presence of oxygen and cyanide. The potentiated and irreversible effects of these alkylhydrazines must be re–examined in the future by using a purified enzyme preparation.

The potencies of the hydrazine compounds used were mainly dependent on the structure of N-1-substituents. Differences of N-2-acyl groups in the compounds did not cause a remarkable alteration in the enzyme inhibition. A non–alkylated hydrazide was inert. These facts prove that N-1-alkyl or arylalkyl groups are involved in the inhibitory reaction. Amphetamine was even less efficient than 31037–S or 31087–S. The hydrazine moiety of the compounds is also required to cause the violent effects described before. An active part of 31037–S or 31087–S is likely to be a benzylhydrazine moiety. The moiety will be capable of reacting with an active site of the enzyme, because both hydrazine compounds were competitive inhibitors. In the present experiments, the substrate and each hydrazine compound were simultaneously added to the reaction system. Therefore, the inhibition degree appears to reflect an affinity of each compound to the enzyme active site. From this point of view it was noted that the most active moiety is benzylhydrazine and its molecular sequence is very similar to tyramines. It is great interest in connection with the fact that tyramine was a characteristically good substrate of this enzyme. The binding of 31037–S or 31087–S with the enzyme active site may be analogous to that of the substrate.

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14) T.E. Smith, H. Weissbach, and S. Udenfriend, *Biochemistry*, 2, 746 (1963).