[Chem. Pharm. Bull. 17(1) 49—53 (1969)]

UDC 615.214.32.015.11 : 547.334

Inhibition of Rabbit Liver Mitochondrial Monoamine Oxidase by New Hydrazine Derivatives. III.[1] Secondary Action of the Compounds 31037-S and 31087-S on the Preincubated Enzyme[2]

Atsushi Kurosawa

Shionogi Research Laboratory, Shionogi & Co., Ltd.[3]

(Received February 21, 1968)

Additional effects of 1-benzyl-2-(4,5,6,7-tetrahydro-1,2-benzisoxazol-3-yl)carbonylhydrazine hydrochloride (31037–S) and 1-benzyl-2-(3-methylisoxazol-5-yl)carbonylhydrazine (31087–S) were demonstrated in the mitochondrial monoamine oxidase of rabbit liver. The potencies of the compounds were greatly enhanced when the enzyme was preincubated for a long time at 37.5° before the addition of substrate and each hydrazine compound. This phenomenon occurred proportionally to time of the preincubation. Although the potenciated effect was observed analogously with both hydrazine compounds, it was much more remarkable in the case of 31087–S, especially when the enzyme was preincubated with semicarbazide. The preincubation did not influence normal enzyme activity.

The compounds affected competitively the 3 min–preincubated enzyme, while they showed a non–competitive type inhibition in the 10 min–preincubated one. The phenomena observed here were unlikely based on mechanisms such as the transformation of each inhibitor, damage of the mitochondrial structure or the sensitization of the enzyme by cyanide.

As described in the first paper of this series,[4] 1-benzyl-2-(3-methylisoxazol-5-yl)carbonyl hydrazine was a competitive inhibitor of rabbit liver monoamine oxidase [monoamine; O_2 oxidoreductase (deaminating); EC 1.4.3.4]. The compound, however, rarely caused a non–competitive type inhibition of this enzyme. Its allied compound 1-benzyl-2-(4,5,6,7-tetrahydro-1,2-benzisoxazol-3-yl)carbonylhydrazine hydrochloride did not have such a dualistic effect.

During the course of an investigation to elucidate this difference, it was found that both compounds[5] are additionally capable of giving a side effect to the enzyme.

The present report deals with this secondary action of the hydrazine compounds.

Experimental

Enzyme Material——Rabbit liver mitochondria were used as an enzyme material throughout this work. The mitochondrial suspension was prepared as described before.[4] In part of the experiments, a solubilized material of the mitochondria was also used and prepared as follows. Thirty milliliters of the mitochondrial suspension was stirred with 3 ml of 5% (w/v) sodium deoxycholate for 10 min in an ice bath. The material was centrifuged for 15 min at $9000 \times g$, and 20 ml of the supernatant was dialyzed overnight against 2 liters of 0.01M sodium phosphate buffer (pH 7.4) at 3°. The dialysis was repeated twice and then the internal medium was centrifuged for 15 min at $9000 \times g$. One milliliter of the supernatant was used

1) Part II: A. Kurosawa, *Chem. Pharm. Bull.* (Tokyo), **17**, 43 (1969).
2) 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.
3) Location: *Sagisu, Fukushima-ku, Osaka.*
4) A. Kurosawa, *Chem. Pharm. Bull.* (Tokyo), **17**, 36 (1969).
5) The abbreviations used are 31037–S for 1-benzyl-2-(4,5,6,7-tetrahydro-1,2-benzisoxazol-5-yl)carbonylhydrazine hydrochloride, and 31087–S for 1-benzyl-2-(3-methylisoxazol-5-yl)carbonylhydrazine.
6) N.H. Creasey, *Biochem. J.*, **64**, 178 (1956).

for enzyme assay. The material was a clear solution, and its monoamine oxidase activity was about 80% of that of the original sample.

Monoamine Oxidase Assay——On the basis of the previous examinations,[4,6] monoamine oxidase activity was measured at 37.5° in air by following the oxygen uptake during the deamination of tyramine in the presence of 0.01 M semicarbazide and 10^{-3} M sodium cyanide. The enzyme material (1.0 ml), 0.1 M sodium posphate buffer (0.2 ml, pH 7.4), and 0.1 M semicarbazide (0.2 ml), were placed in the main compartment of a two-arm Warburg vessel. One side arm of the vessel contained 0.2 ml of 0.01 M sodium cyanide, and the other contained both tyramine and an inhibitor in adequate concentrations. The semicarbazide and cyanide solutions were adjusted to pH 7.4 before use. Hydrazine derivatives 31037-S and 31087-S were dissolved in ethanol, and were diluted adequately with distilled water. Diluted ethanol solutions, corresponding to each inhibitor solution, were taken as controls. Harmine hydrochloride was used in its aqueous solution. The total volume of the reaction medium was kept to 2.0 ml. For the blank test, the substrate-omitted mixture was prepared. The center well of the vessel contained 0.2 ml of 1 M sodium cyanide and a filter paper strip. The enzyme assay was started by the simultaneous addition of the contents in both side arms, and the activity was obtained as μmoles of oxidized tyramine/10mg of protein/hr. Protein contents of the enzyme materials were determined colorimetrically according to a biuret reaction.[7]

Results

The effectiveness of 31037-S or 31087-S on rabbit liver monoamine oxidase was fairly variable when different mitochondrial preparations were used. Interest in this fact has led to the suggestion that the inhibitory action of these hydrazine compounds may be modified by substances surrounding the enzyme. More direct evidence was obtained by preincubation of the enzyme material. In the Warburg procedure, as is well known, the reaction system must be equilibrated for the bath temperature. When this equilibration period was extended from 3 to 10 min, the enzyme inhibition by the hydrazine compounds was markedly potentiated. This phenomenon was common to both inhibitors, although it was much more marked in the case of 31087-S. In a stable situation, the relative ability to cause the potentiated effect was obtained for 31037-S and 31087-S as the increasing ratio in a percentage of the enzyme inhibition. The ability of 31087-S was 1.5 times greater than that of 31037-S (Table I). Since the normal enzyme activity was little affected by the preincubation, the phenomenon was not directly concerned with the enzyme reaction itself.

Table I. Potentiated Effects of Hydrazine Derivatives on the Preincubated Monoamine Oxidase

Inhibitor	Preincubation of enzyme material (min)	Monoamine oxidase activity (μmoles/10 mg protein/hr)		Inhibition (%)	Rate of inhibition enhancement
		Inhibitor added	Control		
31037-S	3	13.1	15.6	16	1.94
	10	10.2	14.8	31	
31087-S	3	11.8	15.6	24	3.00
	10	4.2	14.8	72	

Rabbit liver mitochondrial suspensions (1 ml each) were incubated with semicarbazide (0.1 M, 0.2 ml) and sodium phosphate buffer (0.1 M, pH 7.4, 0.2 ml) at 37.5° in air for 3 and 10 min, respectively. Then tyramine (0.04 M, 0.2 ml), each inhibitor (10^{-5} M, 0.2 ml) or its control solution, and sodium cyanide (0.01 M, 0.2 ml) were simultaneously added to the above-mentioned medium. Monoamine oxidase activity was obtained by measuring oxygen uptake at 37.5° in air. The rate of inhibition enhancement was calculated as the inhibition (%) of the 10 min-preincubated enzyme/the inhibition (%) of the 3 min-preincubated one.

The potentiated effect was observed progressively with the preincubation time. Figure 1 shows a percentage of the residual enzyme activity against each uninhibited control. In the present examinations, the substrate and each inhibitor were simultaneously added to the

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

reaction system. Therefore, the above-mentioned phenomenon does not imply a cumulative effect of the inhibitors during the preincubation.

To elucidate the exact origin of this potentiated effect, some experiments were carried out. The compound 31087-S was shaken with tyramine for 10 min at 37.5° in air, and then added to the 3 min-preincubated enzyme. The effect was identical with that of the directly added 31087-S (Table II). The compound did not seem to be changeable during the preincubation. The enhancement of the inhibitory action is possibly due to some alterations in the enzyme material.

The potentiated inhibition was also seen in the solubilized mitochondria (Table III). The fact indicates that the alteration is neither due to damage to the semipermeability in mitochondrial membranes nor the elimination of some dialyzable counteracting substances.

Preincubation of the bufferized mitochondria alone similarly resulted in the potentiated effect (Table IV). In this case, semicarbazide, cyanide, tyramine, and each inhibitor were simultaneously added to the preincubated mitochondria. When the preincubation was performed in the presence of semi-

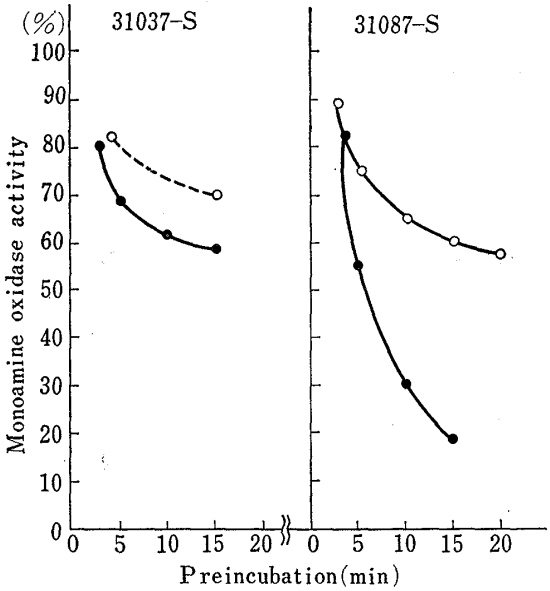

Fig. 1. Effects of Hydrazine Derivatives on the progressively Preincubated Monoamine Oxidase

Mitochondrial suspensions were incubated as described in Table I for different times, and their monoamine oxidase activities were measured analogously after the simultaneous addition of tyramine, cyanide and each hydrazine compound. The enzyme activity was expressed as a percentage of that in each preincubated control. The final concentrations of 31037-S and 31087-S were: —○—, 5×10^{-7} M; —●—, 1×10^{-6} M.

Table II. Inhibitory Action of Preincubated 31087-S on Monoamine Oxidase

Preincubation of enzyme material (min)	Monoamine oxidase activity (μmoles/10 mg protein/hr)		Inhibition (%)
	31087-S added	Control	
3	16.3 (15.7)	22.2	27 (29)
10	10.6	20.0	52

The preincubation of mitochondria and the monoamine oxidase assay were performed identically with that of Table I. The final concentrations of 31087-S and of tyramine were 1×10^{-6} M and 4×10^{-3} M, respectively. Values in parentheses indicate the results obtained by the addition of 31087-S which was preincubated with 0.02 M tyramine for 10 min at 37.5° in air.

Table III. Potentiated Effect of 31087-S on the Monoamine Oxidase in Disintegrated Mitochondria

Preincubation of enzyme material (min)	Monoamine oxidase activity (μmoles/10 mg protein/hr)		Inhibition (%)
	31087-S added	Control	
3	4.0	10.8	63
10	0.6	10.6	94

Mitochondria were solubilized with 0.5% (w/v) sodium deoxycholate, and then the material was dialyzed against 0.01 M sodium phosphate buffer at pH 7.4. Details for the preparation of the enzyme material are presented in the text. The preincubation of the enzyme material and the monoamine oxidase assay were carried out as described in Table I. Tyramine and 31087-S were used at 4×10^{-3} M and 1×10^{-6} M, respectively.

Table IV. Inhibition Enhancement in the Monoamine Oxidase Preincubated with or without Semicarbazide

Inhibitor	Preincubation of enzyme material (System)	(min)	Monoamine oxidase activity (μmoles/10 mg protein/hr) Inhibitor added	Control	Inhibition (%)
31037-S	A	3	8.8	12.1	27
		10	8.1	13.1	37
31087-S	A	3	9.1	12.6	28
		10	7.3	13.0	44
31087-S	B	3	4.6	13.9	67
		10	2.6	14.9	82
Harmine	B	3	8.9	11.8	25
		10	8.6	11.2	23

In System A, mitochondrial suspensions (1 ml each) were preincubated with sodium phosphate buffer (0.01 M, pH 7.4, 0.2 ml) at 37.5° in air for 3 and 10 min, respectively. In System B, the preincubation was performed similarly in the presence of semicarbazide (0.1 M, 0.2 ml). The final reaction system for monoamine oxidase assay was analogous to that in Table I. Concentrations of inhibitors were 1×10^{-6} M for 31037-S or 31087-S, and 5×10^{-6} M for harmine. Tyramine was used at 4×10^{-3} M.

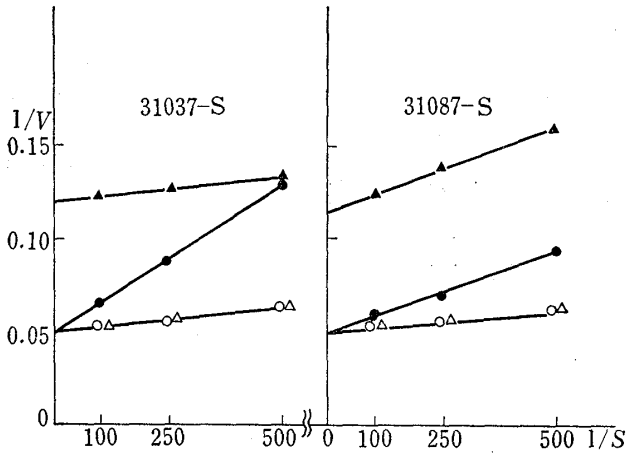

Fig. 2. Lineweaver-Burk Plots on the Monoamine Oxidase Inhibition caused by Hydrazine Derivatives in the Preincubated Mitochondria

Mitochondrial suspensions (1 ml each) were incubated with sodium phosphate buffer (0.1 M, pH 7.4, 0.2 ml) and semicarbazide (0.1 M, 0.2 ml) for 3 (circle) or 10 min (triangle) at 37.5° in air. Hydrazine compound (10^{-5} M, 0.2 ml), tyramine in various concentrations (0.2 ml) and cyanide (0.01 M, 0.2 ml) were added simultaneously. The solid symbols indicate the results obtained by the addition of the inhibitor. The open symbols are their controls. Velocity is given as μmoles of oxidized tyramine/10 mg of protein/hr, and substrate concentration as moles/liter.

carbazide, the effect of 31087-S was further potentiated. Harmine did not cause such an effect even in the presence of semicarbazide.

Alteration in the inhibitory action of the hydrazine compounds was also proved by the following experiment. The bufferized mitochondria were first shaken at 37.5° in the presence of semicarbazide for 3 and 10 min, respectively. The monoamine oxidase activity in these treated mitochondria was determined as usual after the simultaneous addition of tyramine and each inhibitor. Reciprocals of enzyme activities were plotted against reciprocals of tyramine concentrations according to Lineweaver and Burk[8] (Fig. 2). Both hydrazine compounds caused a competitive type inhibition in the 3 min-preincubated mitochondria, whereas they showed a noncompetitive type inhibition in the 10 min-preincubated one. These facts indicate that the compounds are essentially effective on an active site of the enzyme, but they have additional and modified effects on enzyme which has been preincubated for a long time.

8) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

Discussion

The potentiated effects of 31037–S and 31087–S were observed progressively with the equilibration time of the reaction system. This fact first led to the assumption that the phenomenon is based either on the transformation of each inhibitor or the disappearance of some counteracting substances.

Recent works[9] showed that iproniazid and benzylhydrazine are cleaved non–enzymatically to unknown volatile substances, and that the products are powerful inhibitors of monoamine oxidase. Similar findings were demonstrated by Smith, *et al.*[10] If such transformation occurs in 31037–S or 31087–S, it may account for the above–mentioned curious effects. The results of Table II, however, did not support this possibility.

The inhibition enhancement was observed even in the solubilized mitochondria. Permeability of the inhibitors in mitochondrial membranes is not concerned with this phenomenon. The effect is also unlikely to be dependent on the disappearance of dialyzable contaminants in the enzyme material. Some alteration of the enzyme itself or the decreased counteraction of non–dialyzable contaminants should be considered as a probable mechanism.

Effects of iproniazid or other alkylhydrazines on monoamine oxidase are markedly potentiated when the enzyme is shaken in oxygen with cyanide before addition of the inhibitors.[11] In the present tests, however, cyanide was simultaneously added to the reaction system with tyramine and each inhibitor. The results are not analogous to the sensitizing effect of cyanide.

According to Roewer, *et al.*,[12] a high molecular and heat labile component of blood is useful in protecting monoamine oxidase from the inhibitory action of iproniazid. If such a component is included in the present reaction medium, it may serve to explain the potentiated effects of 31037–S and 31087–S. Autooxidation of sulfhydryl residues in various protein contaminants shoud also be considered as a probable mechanism, because these hydrazine compounds are likely to have a high affinity to sulfhydryl substances.[1] To explain only the potentiated effect, these hypotheses may suffice, but it is difficult to account for the alteration of the inhibition type which was observed in the prolonged incubation. One possible explanation is that some conformational changes occurred in the enzyme molecule, and the inhibitors caused additionally allosteric inhibition.

The potentiated effect of 31087–S was more remarkable than that of 31037–S, especially when the enzyme was preincubated with semicarbazide. Therefore, the N-2-acyl groups of the compounds would be involved in the modified inhibition. Moreover, the conformational change of the enzyme might be accelerated in the presence of semicarbazide.

In view of these facts, it is likely that a labile area is present in the molecule of rabbit liver monoamine oxidase even if it is not directly concerned with the enzyme reaction itself.

Acknowledgement The author wishes to express his thanks to Dr. Yasunao Ogawa of this Laboratory for constant guidance in the course of the work, and to Dr. Hideo Kano of this Laboratory for a gift of hydrazine derivatives. Thanks are also due to Dr. Ken'ichi Takeda, Director of this Laboratory, for permission to publish this work. Grateful acknowledgement is made to Professor Kiyoshi Aoki and Assistant Professor Kazuhiko Konishi of Tohoku University for their valuable advice and criticisms.

9) E.S. Mingioli and M. Kory, *Federation Proc.*, **22**, 389 (1963); M. Kory and E.S. Mingioli, *Biochem. Pharmacol.*, **13**, 577 (1964).
10) T.E. Smith, H. Weissbach, and S. Udenfriend, *Biochemistry*, **2**, 746 (1963).
11) A.N. Davison, *Biochem. J.*, **67**, 316 (1957); A.L. Green, *Biochem. Pharmacol.*, **13**, 249 (1964).
12) F. Roewer and E. Werle, *Arch. Exptl. Pathol. Pharmakol.*, **230**, 552 (1957).