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INHIBITION OF DOPAMINE β-HYDROXYLASE BY SOME NEW THIOUREA DERIVATIVES

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Abstract—The inhibition of dopamine β-hydroxylase by thiourea derivatives, *N*-phenyl, *N'*-3-(4H-1,2,4-triazolyl)thiourea and *N*-*n*-butyl,*N'*-3-(4H-1,2,4-triazolyl)thiourea, was studied. Kinetic studies using purified dopamine β-hydroxylase revealed that the inhibition by *N*-phenyl,*N'*-3-(4H-1,2,4-triazolyl)thiourea was of a noncompetitive type with the substrate and of a mixed type with ascorbic acid, one of the cofactors in this reaction. It was also found that the inhibition by *N*-*n*-butyl,*N'*-3-(4H-1,2,4-triazolyl)-thiourea was of a noncompetitive type with both the substrate and ascorbic acid. The inhibition of dopamine β-hydroxylase by *N*-*n*-butyl,*N'*-3-(4H-1,2,4-triazolyl)thiourea was reversed when Cu^{2+} was added to the reaction mixture, indicating that the inhibition by this compound resulted from its metal-chelating activity. On the other hand, the inhibition by *N*-phenyl,*N'*-3-(4H-1,2,4-triazolyl)thiourea was not recovered by the addition of Cu^{2+} to the reaction mixture. When *N*-phenyl,*N'*-3-(4H-1,2,4-triazolyl)-thiourea was administered intraperitoneally to mice, it was demonstrated that the brain level of norepinephrine was lowered.

Dopamine β-hydroxylase (DBH), which catalyzes the conversion of dopamine to norepinephrine (the final step in the biosynthesis of norepinephrine [1]), is a copper-containing enzyme [2, 3], which is inhibited by the analogues of phenylethylamine [4–7] or various chelating agents [8–16]. Johnson *et al.* [17] reported that various aromatic and alkyl thioureas inhibited DBH *in vitro* and that these compounds also changed the levels of catecholamines in mouse and rat brains [18]. Recently we found that thiourea derivatives with a heterocyclic ring were potent inhibitors of DBH. *N*-phenyl, *N'*-3-(4H-1,2,4-triazolyl)thiourea (I) and *N*-*n*-butyl,*N'*-3-(4H-1,2,4-triazolyl)thiourea (II) were the most effective inhibitors among the compounds tested. In this paper, we will report the kinetic data on the inhibition of DBH by these two compounds *in vitro* and the change of the catecholamine content in mouse brain when the inhibitor is used *in vivo*.

MATERIALS AND METHODS

N-Phenyl,*N'*-3-(4H-1,2,4-triazolyl)thiourea (I) and *N*-*n*-butyl,*N'*-3-(4H-1,2,4-triazolyl)thiourea (II) were generously supplied by Eizai Co., Ltd. Tyramine hydrochloride was purchased from Daiichi Kagaku Yakuhin Co., Ltd., and catalase was purchased from Sigma Co., Ltd.

Preparation of DBH. DBH was prepared from bovine adrenal glands according to the method of Friedman and Kaufman [19], which was slightly modified. The bovine adrenal glands were homogenized by 3 vol. of 0·25 M sucrose containing 0·015 M potassium phosphate buffer (pH 6·5), and centrifuged at 700 *g* for 10 min. Then the supernatant was centrifuged at 10,000 *g* for 1 hr. The precipitate was suspended in 0·02 M phosphate buffer (pH 6·5). To the suspension, Nikol OP-10 (detergent) was added to give 1·25% sufficient to dissolve the proteins. The Nikol OP-10-treated suspension was centrifuged at 44,000 *g* for 90 min. The supernatant thus obtained was fractionated with ammonium sulfate. To the supernatant fraction, solid ammonium sulfate was added to give 80% saturation. The precipitate was collected by centrifugation and dissolved in 0·02 M phosphate buffer (pH 6·5), followed by treatment with charcoal. The solution was centrifuged to remove the charcoal, then dialyzed against the same buffer (first ammonium sulfate fraction). The first ammonium sulfate fraction was subjected to the second ammonium sulfate fractionation. The precipitates formed between 25 and 40% ammonium sulfate saturation were collected and dissolved in 0·02 M phosphate buffer (pH 6·5), and then dialyzed overnight against the same buffer (second ammonium sulfate fraction). The dialyzed second ammonium sulfate fraction was applied on a DEAE-cellulose column (1·5 × 45 cm) which was equilibrated with 0·02 M phosphate buffer (pH 6·5). The non-adsorbed protein was washed with 0·02 M phosphate buffer (pH 6·5) and the adsorbed proteins were eluted with a linear salt concentration gradient which was formed from 0·02 and 0·5 M phosphate buffer (pH 6·5). Fractions of 10 ml were collected and fraction numbers 10–12 were used as a partially purified enzyme preparation.

Assay of enzyme activity. DBH was assayed by the method of Van der Schoot *et al.* [20]. Each ml of the reaction mixture contained: potassium phosphate buffer (pH 5·5), 100 μmoles; ascorbic acid, 10 μmoles; fumaric acid, 10 μmoles; tyramine hydrochloride, 10 μmoles; catalase, 200 Sigma units and the partially purified enzyme preparation. The reaction mixture was preincubated with or without inhibitors, which were dissolved in hot ethanol–water (3:7, v/v), for 5 min at 37° without substrate. The reaction was started by adding the substrate and incubating for 15 min at 37° in air. The reaction was terminated by adding

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2 ml of 4 N ammonium hydroxide. The control run was identical to the above procedure but without the incubation. The amount of norsynephrine formed was determined after conversion of norsynephrine to *p*-hydroxybenzaldehyde by adding periodate, and *p*-hydroxybenzaldehyde was determined by measuring the absorbancy at 330 nm. The formation of norsynephrine from tyramine under these conditions proceeded linearly for 20 min, and was also proportional to the amount of enzyme used.

Determination of amine concentration in brain tissue. Animals were sacrificed by decapitation, and the brains were quickly taken out and chilled at 0° in an ice bath. The concentration of catecholamine in brain tissue was determined by the method of Endo and Ogura [21]. Two mouse brains were homogenized with 5 vol. of 0·4 N perchloric acid containing 2 mM EDTA; the homogenates were centrifuged and the supernatant was neutralized with 1 N KOH. The neutralized supernatant solution was applied to a *p*-cellulose column which was equilibrated with 0·01 M sodium phosphate buffer (pH 6·2). Catecholamines adsorbed on the column were eluted by 0·03 M sodium phosphate buffer (pH 6·2). Norepinephrine was determined by the specific trihydroxyindole reaction according to the method of Haggendal [22] with a slight modification. Dopamine was also determined by the specific trihydroxyindole reaction following the method of Anton and Sayre [23] with a slight modification.

RESULTS AND DISCUSSION

The inhibitory effects of two thiourea derivatives, *N*-phenyl,*N'*-3-(4H-1,2,4-triazolyl)thiourea (compound I) and *N*-*n*-butyl,*N'*-3-(4H-1,2,4-triazolyl)-thiourea (compound II), on DBH were investigated. As can be seen in Table 1, DBH was remarkably inhibited by these two thiourea derivatives; the concentrations required for 50 per cent inhibition were about $9{\cdot}2 \times 10^{-7}$ M and $2{\cdot}5 \times 10^{-6}$ M for compounds I and II respectively. These data show that compound I is a more effective inhibitor of DBH than the various aromatic and alkyl thioureas which were reported by Johnson *et al.* [17]. These data show that DBH was much more sensitive to inhibition by heterocyclic thiourea derivatives when the amido group hydrogen was replaced by phenyl (compound I) rather than by *n*-butyl (compound II).

To determine the type of inhibition by these two thiourea derivatives, the kinetics of this reaction were studied. As shown in Fig. 1, Lineweaver-Burk plots showed that the inhibition of DBH by thiourea derivatives (both I and II) was found to be noncompetitive with the substrate, tyramine. K_i values of compounds I and II calculated from these figures were approximately 1×10^{-6} M and $9{\cdot}7 \times 10^{-6}$ M respectively.

It was reported by Johnson *et al.* [17] that DBH was competitively inhibited by *N*-phenyl,*N'*-dimethyl thiourea with the substrate when *N*-phenyl,*N'*-dimethyl thiourea was preincubated for 5 min before addition of the substrate. Moreover, they reported that, when DBH was preincubated with this inhibitor for 15 min before addition of the substrate, the double reciprocal plots were no longer characteristic of competitive inhibition. In contrast to this report, we found the type of inhibition by compound I on DBH to be noncompetitive against tyramine, independent of preincubation with inhibitor (Fig. 2).

Lineweaver–Burk plots for the concentration of ascorbic acid, one of the cofactors of this enzyme reaction, are shown in Fig. 3. This figure shows that the inhibition of compound I is somewhat different from that of compound II, namely the inhibition of compound I seems to be of the mixed type and that of compound II is noncompetitive.

Since DBH is a copper-containing enzyme, it is possible to consider that thiourea derivatives are able

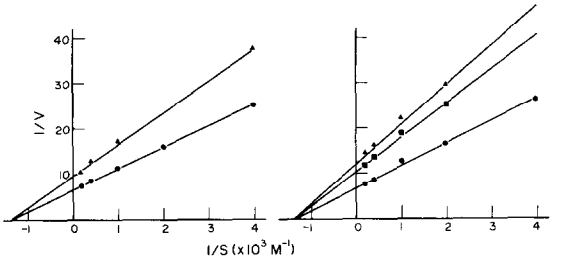
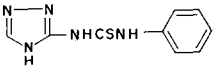
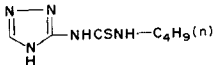


Fig. 1. Lineweaver–Burk plots of tyramine concentration against the rate of norsynephrine formation with and without thiourea derivatives. The assay method of the enzyme is described in Materials and Methods. Right panel: (●——●) without inhibitor; (■——■) with compound I (5×10^{-7} M); and (▲——▲) with compound I (1×10^{-6} M). Left panel: (●——●) without inhibitor; and (▲——▲) with compound II (5×10^{-6} M).

Table 1. Inhibition of dopamine β-hydroxylase by thiourea derivatives*

Compound No.	Inhibitor	% Inhibition Concn (M) 10^{-4}	10^{-5}	10^{-6}	10^{-7}
I			89·0	51·2	13·7
II		97·9	68·0	27·9	

* The enzyme was preincubated for 5 min with inhibitor but without substrate. The reaction mixture was incubated for 15 min at 37°. The enzyme activity was assayed as described in Materials and Methods.

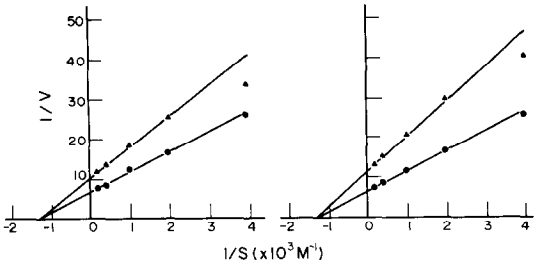


Fig. 2. Inhibition of the β-hydroxylation of tyramine with compound I (5×10^{-7} M). The assay method of the enzyme is described in Materials and Methods. Right panel: without preincubation of compound I and enzyme. Left panel: with preincubation (5 min) of compound I and enzyme. Key: (●——●) without inhibitor; and (▲——▲) with inhibitor.

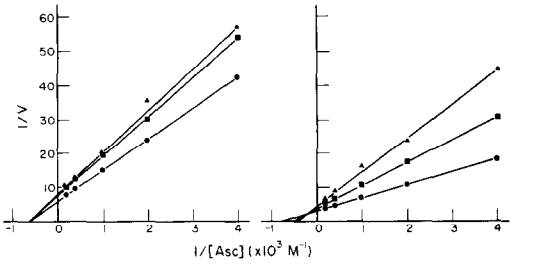


Fig. 3. Lineweaver-Burk plots of ascorbic acid concentration against the rate of hydroxylation with and without thiourea derivatives. The assay method of the enzyme is described in Materials and Methods. Right panel: (●——●) no addition; (■——■) 5×10^{-7} M compound I was added; and (▲——▲) 1×10^{-6} M compound I was added. Left panel: (●——●) no addition; (■——■) 1×10^{-6} M compound II was added; and (▲——▲) 5×10^{-6} M compound II was added.

to act on DBH as chelating agents, for thiourea derivatives, from their structural characteristics, can chelate metal ions. The effects of copper ion on thiourea derivative inhibition of DBH were investigated, and the results are shown in Table 2. As can be seen, the inhibition by 5×10^{-6} M compound II was completely reversed by the addition of 1×10^{-6} M Cu^{2+}. On the other hand, the inhibition by compound II was not reversed by the addition of Cu^{2+}. These data indicate that there may be some difference between the mode of action of compound I and compound II on DBH inhibition. The mechanism of inhibition by compound I cannot be sufficiently explained by its chelating activity, because a simple supplement of Cu^{2+} could not reverse the inhibition.

We investigated the possibility of reversibility of the inhibition by thiourea derivatives on DBH by removing these compounds by dialysis. The enzyme was incubated with thiourea derivatives for 10 min at 37°, then dialyzed against 200 vol. of 0·05 M phosphate buffer, pH 6·5 at 4° for 24 hr. The buffer was changed three times under dialysis. Table 3 shows that the decrease of DBH activity resulting from inhibition by thiourea derivatives was not restored by dialysis.

While DBH is not necessarily the rate-limiting reaction under physiological conditions, effective inhibitors of DBH *in vitro*, such as disulfiram [12] and aromatic and alkyl thiourea [17], have been reported to lower endogenous norepinephrine. Since these experiments *in vitro* suggested that compound I was a potent inhibitor of DBH, the next experiments were performed to see whether compound I could deplete norepinephrine in mouse brain when it was used *in vivo*. Compound I, suspended in 0·25% aqueous methylcellulose just prior to use, was administered to ddI male mice intraperitoneally. Three hr after administration, the levels of catecholamine in the brain were determined. As shown in Table 4, increases of 10 and 25 per cent in the dopamine level over the control level were observed after a single administration, respectively, of 50 or 100 mg/kg of compound I. Table 4 also shows that norepinephrine levels in the brain were decreased to 65 per cent compared to those of the control mice after the administration of compound I, but the rate of decrease of norepinephrine concentration did not depend on the dosage of compound I.

Tyrosine hydroxylase was established as the rate-limiting step of catecholamine biosynthesis [24]; however, a potent inhibitor of DBH *in vivo* has been re-

Table 2. Effect of Cu^{2+} on the inhibition of dopamine β-hydroxylase by thiourea derivatives

Cu^{2+} concn (M)	Dopamine β-hydroxylase* (% of control activity)		
	Enzyme alone	Enzyme plus 5×10^{-7} M compound I	Enzyme plus 5×10^{-6} M compound II
0	100·0	50·6	70·2
1×10^{-5}	70·1	62·0	24·1
5×10^{-6}	94·8	58·6	100·0
1×10^{-6}	98·8	52·4	100·0
1×10^{-7}	105·8	50·2	78·7

* The enzyme was preincubated with thiourea derivatives and Cu^{2+} at 37° for 5 min. The reaction was started by addition of the substrate; the reaction mixture was incubated for 15 min at 37°.

Table 3. Reversal of the inhibition of dopamine β-hydroxylase by thiourea derivatives

	Dopamine β-hydroxylase* (% of control activity)	
	Before dialysis	After dialysis
Control	100·0	95·0
Compound I treated enzyme	23·3	22·0
Compound II treated enzyme	49·5	51·9

* The enzyme was incubated with thiourea derivatives (final inhibitor concentration: compound I, 5×10^{-6} M; compound II, 1×10^{-5} M) for 10 min at 37°, and then dialyzed against 200 vol. of 0·05 M phosphate buffer (pH 6·5) at 4° for 24 hr.

Table 4. Effect of compound I upon mouse brain catecholamines

	Dose (mg/kg)	Time (hr)	Norepinephrine (μg/g ± S.E.M.)	Dopamine (μg/g ± S.E.M.)
Control			0·36 ± 0·01 (3)*	0·84 + 0·02 (3)
Compound	50	3	0·22 + 0·01 (3)†	0·92 + 0·01 (4)†
I	100	3	0·23 + 0·01 (3)†	1·04 + 0·01 (5)†

* Numbers in parentheses show number of determinations.
† Significantly different from control, $P < 0.01$.

ported to reduce endogenous levels of norepinephrine [12]. This fact suggests that a sufficiently effective inhibitor of DBH can regulate norepinephrine biosynthesis *in vivo*. Although, at this time, we cannot exclude other possibilities, it is conceivable that the mechanism responsible for the decrease of the endogenous norepinephrine level and the increase of the dopamine level in mouse brain by the administration of compound I might be due to inhibition of DBH itself. However, it is necessary to measure the DBH activity of the mouse brain directly to establish that, as a result of inhibition of DBH *in vivo*, treatment with compound I results in a decrease in norepinephrine content of the mouse brain.

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