[Chem. Pharm. Bull. 23(10)2362—2368(1975)]

UDC 615.355.011.5 : 577.15.04

Endogenous Inhibitor(s) in Rat Liver of Dopamine β-Hydroxylase

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(Received March 22, 1975)

The addition of both native and boiled 105000 *g* supernatant from rat liver to dopamine β-hydroxylase assay system caused marked decreases in dopamine β-hydroxylase activity, and the degrees of these inhibitions were dose-dependent. When the boiled supernatant was fractionated by using chromatography on a Sephadex G-25 superfine, at least two inhibitory peaks were observed by the addition of each column fraction. The second inhibitory peak (fraction 26) was estimated to have a molecular weight of about 1000—1300. This inhibition was non-competitive with substrate, tyramine and co-substrate, ascorbic acid and completely reversed by the addition of sulfhydryl reactive agents such as N-ethylmaleimide. The second inhibitory peak coincided to the peak of the stimulation of adenyl cyclase of rat brain synaptosomes by the addition of column effluent of a Sephadex G-25 superfine.

The presence of a naturally occurring inhibitor of dopamine β-hydroxylase [DBH: 3,4-dihydroxyphenethylamine, ascorbate-O_2 oxidoreductase (hydroxylating), EC 1.14.2.1] was suggested by the finding that whole homogenates of animal tissues showed little or no DBH activity, although enzyme activity was readily demonstrated after fractionation of these homogenates.[2] Nagatsu, *et al.*[3] found that the inhibition produced by homogenates of rat adrenal gland and brain could be reversed by N-ethylmaleimide (NEM), which reacts with the sulfhydryl groups and that a decrease in the sulfhydryl content of the tissue preparation caused a parallel decrease in the inhibitory activity. Austin, *et al.*[2c] and Chubb, *et al.*[4] have also reported the presence of an endogenous inhibitor of DBH in vas deferens and bovine heart. This report describes the isolation of the inhibitors from homogenates of rat liver by using a Sephadex G-25 column. Although the purification of the inhibitors was preliminary stages, an attempt was also made to investigate the effect of the inhibitory fraction of Sephadex G-25 column on the other sulfhydryl sensitive enzyme such as adenyl cyclase since the inhibition of DBH by this inhibitory fraction was overcomed by the addition of NEM.

Experimental

Preparation of the Native and Boiled Supernatant——All experiments were performed at 0—4° unless otherwise described. Male rats of Sprague-Dawley strain weighing between 200—250 g were utilized in all experiments. Rats were sacrificed by decapitation; livers were quickly removed, perfused with ice-cold 10 mM Tris–HCl buffer (pH 7.4) to remove blood and then homogenized in same buffer in a Waring Blendor (20 sec; two times) and then in a Teflon homogenizer. The resulting homogenates were centrifuged 1000 *g* for 20 min, 10000 *g* for 20 min and 105000 *g* for 60 min, and the supernatant was collected and noted as the native supernatant and heated in a boiling water bath for 5 min. The precipitate so formed was removed by centrifuging at 10000 *g* for 20 min. The supernatant fraction was designated as the boiled supernatant.

Preparation of Dopamine β-Hydroxylase——DBH was purified from beef adrenal glands according to the procedure of Foldes, *et al.*[5] In these studies the enzyme obtained at the stage of diethylaminoethyl (DEAE)-cellulose chromatography elute was used.

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2) *a*) E.Y. Levin, B. Lebenberg, and S. Kaufman, *J. Biol. Chem.*, **235**, 2080 (1960); *b*) L.T. Potter and J. Axelrod, *J. Pharmacol. Exp. Ther.*, **142**, 299 (1963); *c*) L. Austin, B. Livett, and I.W. Chubb, *Circulation Res.*, suppl., **10**, III (1967).
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Assay of Dopamine β-Hydroxylase Activity——Enzyme acitivity was assayed by the following conversion of tyramine to norsynephrine according to the procedure of Van der Schoot, *et al.*[6] Reaction mixture contained in 1.0 ml: pottasium phosphate buffer (pH 5.5) 100 μmoles, ascorbic acid 10 μmoles, fumaric acid 10 μmoles, tyramine hydrochloride 10 μmoles, catalase 200 Sigma unit and the enzyme. The reaction mixture was preincubated at 37° for 5 min. The incubation was started by the addition of substrate and carried out for 15 min at 37° in air. The reaction was terminated by adding 2 ml of 4 N ammonium hydroxide. The amount of norsynephrine formed were determined after conversion of norsynephrine to *p*-hydroxybenzaldehyde by adding periodata, and *p*-hydroxybenzaldehyde was determined by measuring the absorbance at 330 nm. The formation of norsynephrine from tyramine under these conditions proceeded lineally for 20 min and was also proportional to the amount of enzyme used.

Assay of Adenyl Cyclase Activity——Adenyl cyclase activity was determined on the rate of conversion of [^{3}H] ATP to [^{3}H] cyclic AMP by means of a modified method of Krishna, *et al.*[7] The incubation medium (0.6 ml) consisted of 1.0 mM [^{3}H]ATP (10 μCi), 3.3 mM $MgSO_4$, 10 mM NaF, 6.7 mM caffeine and 40 mM Tris–HCl buffer (pH 7.4). In most of the present experiments, incubation was carried out with protein content of about 200 μg per 0.6 ml of the incubation medium.

Protein Determination——The protein contents were determined by the procedure described by Lowry, *et al.*[8] with crystalline bovine serum albumin as standard.

Results

As the native and boiled 105000 *g* supernatant from rat liver inhibited DBH activity on dose dependently as shown in Fig. 1, separation of this heat-stable inhibitor(s) was therefore attempted by a column chromatography (Fig. 2). When fractions were analyzed by measuring

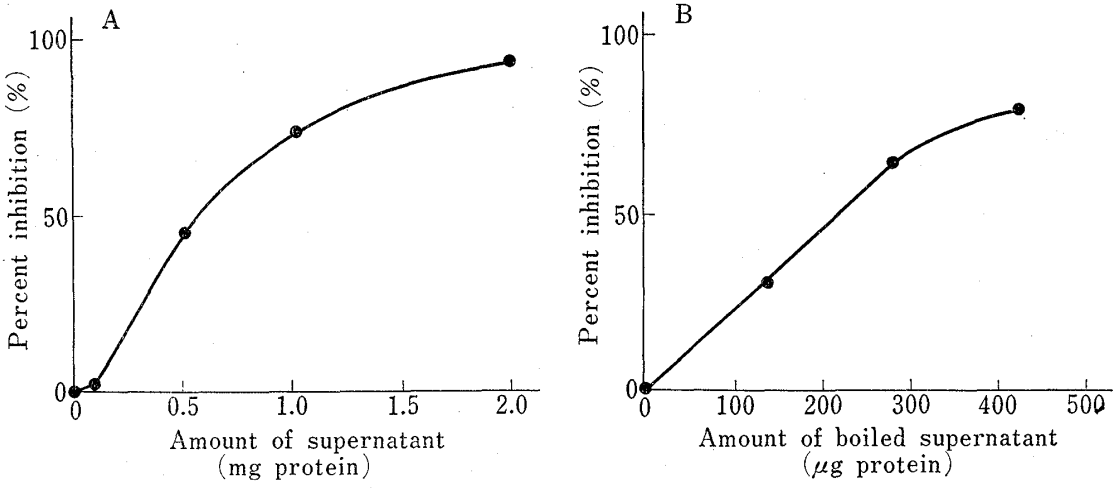

Fig. 1. Effects of the Native (A) and Boiled Supernatant (B) Concentrations on Dopamine β-Hydroxylase Activity

The native and boiled supernatant obtained as described in Experimental were added to incubation medium.

its ability to inhibit DBH activity, at least two inhibitory fractions were observed. At present, although we observed[9] that the inhibitor in the first inhibitory fraction had a molecular weight of about 40000 and was protein or protein associated substance, because its inhibitory activity was abolished by trypsin digestion but we could not refer to this inhibitor in this paper. The inhibitors in the second inhibitory fraction were estimated to have molecular weights of about 1000—1300 by a calibration of this column with glucagon (Mol. Wt. 3500) and Vitamin B_{12} (Mol. Wt. 1350) as markers and by the method of ultrafiltration. Similar

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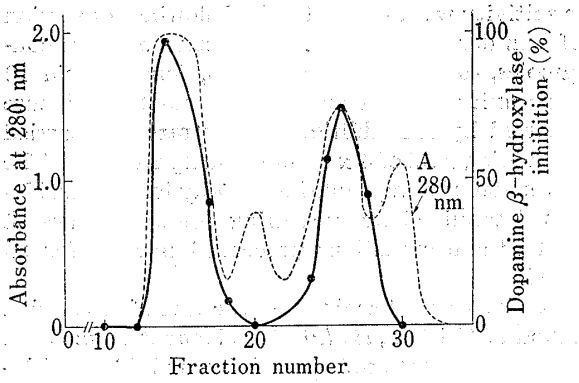

Fig. 2. Chromatography of Dopamine β-Hydroxylase Inhibitors from Rat Liver on a Sephadex G-25 Superfine

The boiled supernatant obtained as described in Experimental was freezed-dried and the resulting material dissolved in water. This material was fractionated by chromatography on a column (1.5×43 cm) of a Sephadex G-25 superfine equilibrated with 50 mM Tris-HCl buffer (pH 7.4). The column was eluted with same buffer at 4° in 2.5 ml of fraction at a flow rate of 10 ml per hour. The inhibitory activity of an aliquot (100 μl) of each fraction was measured as described in Experimental and express ed as per cent inhibition of the control activity (—●—), (·····) absorbance at 280 nm

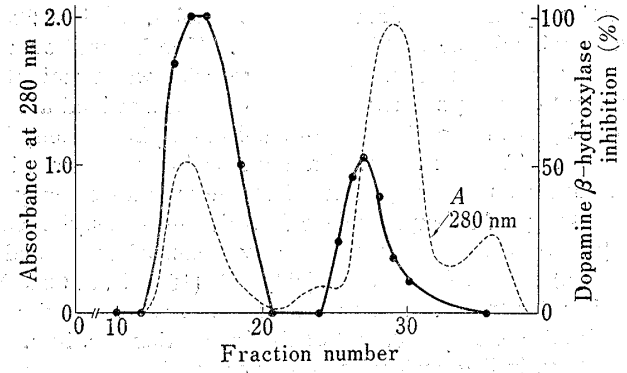

Fig. 3. Chromatography of Dopamine β-Hydroxylase Inhibitors from Bovine Brain on a Sephadex G-25 Superfine

The methods of homogenization and fractionation of the tissue were performed in the same manner for rat liver described in Experimental. Gel filtration on a Sephadex G-25 column was performed in the same manner as in Fig. 2. The inhibitory activity of each fraction was measured as in the legend to Fig. 2. (·····) absorbance at 280 nm

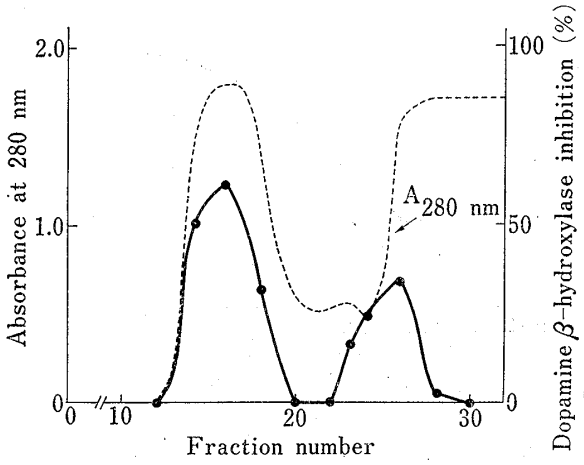

Fig. 4. Chromatography of Dopamine β-Hydroxylase Inhibitors from Bovine Adrenal Medulla on a Sephadex G-25 Superfine

The methods of homogenization and fractionation of the tissue were performed in the same manner for rat liver described in Experimental. Gel filtration on a Sephadex G-25 column was performed in the same manner as in Fig. 2. The inhibitory activity of each fraction was measured as in the legend to Fig. 2. (·····) absorbance at 280 nm

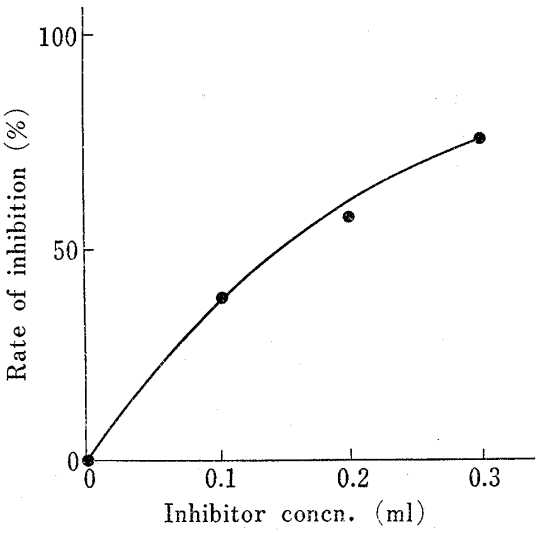

Fig. 5. Effect of Various Concentrations of the Inhibitors on Dopamine β-Hydroxylase

A preparation of the inhibitory fraction from a Sephadex G-25 column (fraction 26) was used as the inhibitors. Results are expressed as per cent inhibition of enzyme activity by the inhibitors.

inhibitory phenomena were also observed in the preparations of bovine brain (Fig. 3) or adrenal medulla (Fig. 4) and rat brain (data not shown), indicating that these inhibitors existed in various tissues of different species. When tissues were homogenized in water instead of Tris-HCl buffer, changes of the inhibitory patterns were not observed.

The relationship between the rate of the inhibition and the concentration of the inhibitors is given in Fig. 5. As the concentration of the inhibitors increased, the extents of the inhibition were proportionally greater.

Norsynephrine formed linearly for the first 15 min period incubation both in the absence and presence of the inhibitors as shown in Fig. 6. The rate of formation of norsynephrine was decreased approximately 50 per cent by the addition of the inhibitors.

To determine the type of inhibition by the inhibitors, kinetical analysis of the reaction was studied. The only parameters which were varied were the concentrations of ascorbate and tyramine. As shown in Fig. 7, Lineweaver-Burk plots exhibited that the inhibition of DBH by the inhibitors was found to be non-competitive with substrate, tyramine and co-substrate, ascorbate.

The possibility that the inhibitors described above are sulfhydryl compound was examined using NEM. As shown in Table I, the inhibition of DBH by the inhibitors was completely reversed by the addition of MEM. However, since sulfhydryl groups are widely distributed in animal tissues and it has been reported that various sulfhydryl compounds such as cysteine,

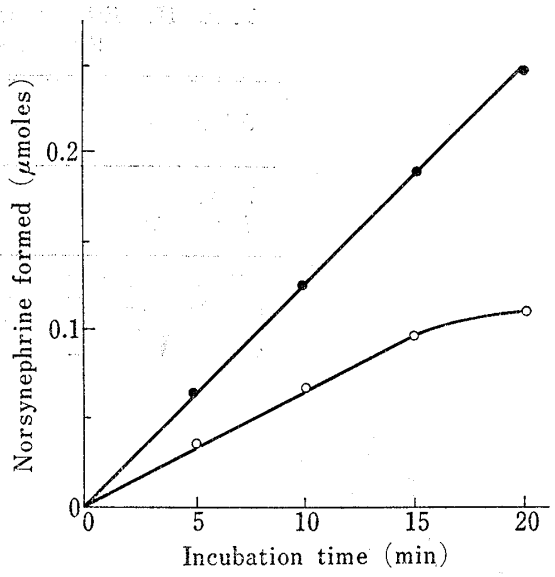

Fig. 6. Norsynephrine Synthesis as a Function of Incubation Time in the Absence (—●—) and Presence of the Inhibitors (—○—)

An aliquot (200 μl) of a preparation of the inhibitory fraction from a Sephadex G-25 column (fraction 26) was added to incubation medium.

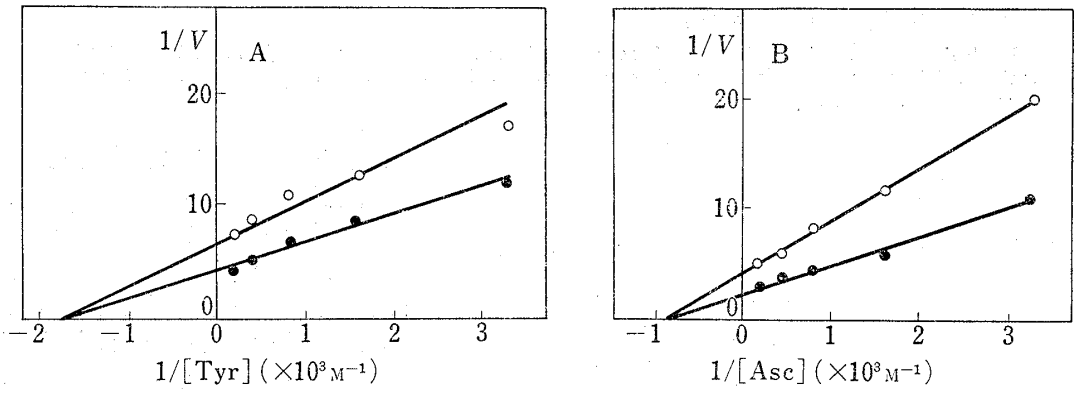

Fig. 7. Lineweaver-Burk Plots of Tyramine (A) and Ascorbic Acid (B) Concentrations Against Rate of Hydroxylating with (—○—) and without the Inhibitors (—●—)

An aliquot (200 μl) of a preparation of the inhibitory fraction from a Sephadex G-25 column (fraction 26) was added to the incubation medium. The velocities are expressed as μmoles of norsynephrine formed per 15 min. Concentrations are expressed in mM.

Table I. Effect of N-Ethylmaleimide on the Inhibition of Dopamine β-Hydroxylase by the Inhibitors

NEM Concentration (M)	Dopamine β-hydroxylase (% of control activity)	
	Enzyme alone	Enzyme + Endogenous inhibitors
0	100	48.1
1×10^{-5}	96.1	62.3
1×10^{-4}	101.4	93.3
1×10^{-3}	107.4	114.0

An aliquot (200 μl) of a preparation of the inhibitory fraction from a Sephadex G-25 column (fraction 26) was added to incubation medium.

Table II. Effect of the Supernatant from Rat Liver on Synaptosomal Adenyl Cyclase

Additions	Adenyl cyclase activity (pmoles cyclic AMP/min/mg protein)
None	50.6 ± 3.3
Supernatant	144.1 ± 5.5

The supernatant obtained as described in Experimental, equivalent to 300 μg of protein, was added to incubation medium. Adenyl cyclase activity was determined as described in Experimental. Data were averages ± S.D. of 3 separate analyses.

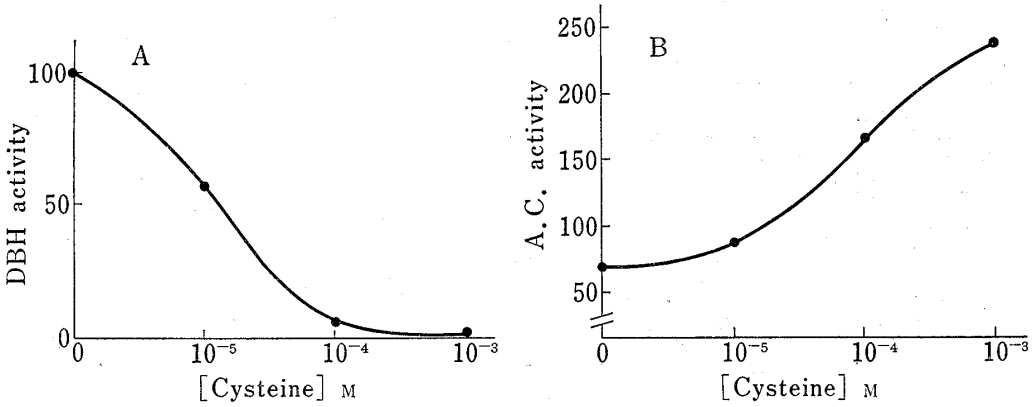

Fig. 8. Effects of Cysteine on Dopamine β-Hydroxylase (A) and Adenyl Cyclase of Rat Brain Synaptosomes (B)

Incubation were carried out with increasing concentrations of cysteine. Dopamine β-hydroxylase and adenyl cyclase activities expressed as per cent inhibition of enzyme activity and pmoles of cyclic AMP formed per min per mg protein, respectively.

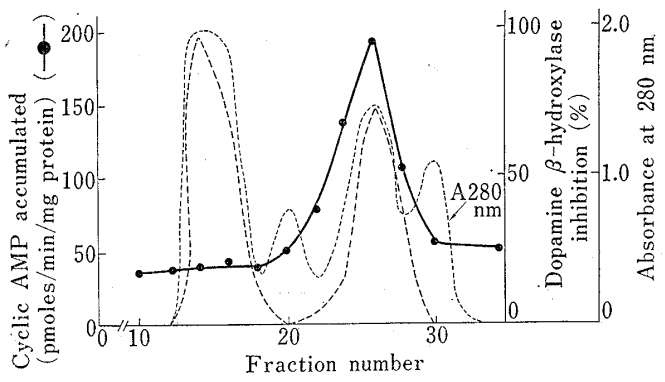

Fig. 9. Chromatography of Adenyl Cyclase Stimulatory Substances on a Sephadex G-25 Superfine

The methods of homogenization and fractionation of rat liver was obtained as described in Experimental and Fig. 2. An aliquot (100 μl) of each fraction was assayed for its ability to activate the synaptosomal adenyl cyclase activity (—●—). The dashed line (---------) represented the inhibitory patterns of dopamine β-hydroxylase by each fraction as described in Fig. 2. (·····) Absorbance at 280 nm.

glutathione and Coenzyme A inhibited DBH activity nonspecifically,[3] it is doubtful that the substances in rat liver given above would act specific inhibitor of DBH. Therefore, we examined the effect of a inhibitory fraction of Sephadex G-25 column on the other sulfhydryl-sensitive enzyme such as adenyl cyclase of rat brain synaptosomes. As shown in Table 2 and Fig. 8, the activity of adenyl cyclase was stimulated by both the boiled supernatant from rat liver and cysteine as well as DBH. When fractions from Sephadex G-25 column were analyzed by measuring its ability to stimulate the adenyl cyclase activity as well as in case of DBH inhibitors shown in Fig. 2, the peak stimulatory fraction coinicides with the second peak inhibitory fraction of DBH as shown in Fig. 9. Contrary to a preparation of DBH, however, we could not investigate the effect of NEM on the stimulation of synaptosomal adenyl cyclase by the stimulatory fraction since the adenyl cyclase in rat brain synaptosomes was inactivated by the addition of NEM.

Discussion

DBH has been reported to be released together with norepinephrine from the sympathetic nerve terminals and from the chromaffin cells of the adrenal medulla,[10] and it has been suggested that the serum DBH originated from the enzyme occurring in the sympathetic nerve system from which it is released together with norepinephrine.[11] It thus appears that DBH exists in the various tissues. The question then arises as to how DBH is regulated. As shown in Figs. 2—4, the inhibitors of DBH were observed in various tissues, suggesting that the native DBH activity may be masked by the presence of these inhibitors. On the other hand, it has been reported that the studies[12,2a] on the intracellular localization of DBH failed to detect significant amounts of this enzyme in the soluble fraction of adrenal glands homogenized in isotonic sucrose because the enzyme activity was almost completely masked by the presence of an inhibitor. Therefore, the purifications of DBH[13,5] and inhibitors[14] have been extensively carried out by various investigators. Duch and Kirshner[14a] demonstrated the presence of sulfhydryl groups in DBH inhibitor from bovine adrenal medulla. However, it has been reported that the inhibitor from bovine heart[14b] contained carbohydrate, organic phosphate, only trace amounts of nitrogen and no sulfur and the inhibition of DBH by this inhibitor was overcomed by Cu^{2+} whereas other sulfhydryl reactive reagents such as NEM were ineffective. These reports indicated that more than one inhibitor of DBH existed in different organ of the same species.

The inhibitors in the second inhibitory fraction seem to be dialyzable and heat-stable substances in nature in that its ability was abolished by treatment with dialysis and was not reduced by treatment with heating at 100° for 5 min (Fig. 1B). Since the purifications of both inhibitors of DBH and activators of adenyl cyclase were still preliminary stages, it is obscure at present that these two substances are identical. However, the results that both DBH and adenyl cyclase were affected by similar fraction of Sephadex G-25 column (Figs. 2,9) and cysteine (Fig. 8) suggest that this inhibitors of DBH in rat liver described in this paper may not be specific for DBH. There is, however, little direct evidence to support this hypothesis.

Evidence has been presented indicating that the biosynthesis of catecholamine may be regulated both tyrosine hydroxylase[15] and by the rate of uptake of dopamine into the catecholamine storage vesicle.[16] Taunger and Hasselbach[17] have demonstrated that sulfhydryl groups are intimately involved in the uptake of catecholamine into the storage vesicle. They suggested that the sulfhydryl nature of the inhibitor isolated from homgenates of bovine adrenal medulla might also function by reacting with the sulfhydryl groups of the vesicle membrane and in this manner could inhibit the uptake of catecholamine in the storage vesicle. However, since the inhibitors described in this paper were originated from liver, it would appear that these inhibitors do not serve to regulate the uptake of dopamine into the storage vesicle. Furthermore, it has been reported by Duch and Kirshner[14a] that the inhibitors isolated from homogenates of bovine adrenal medulla did not affect such uptake mechanism.

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At present, we are under investigations of the purification and the physiological significance of the inhibitors of DBH in rat liver. The available evidence suggests that the inhibitors form a reversible complex with the enzyme through one or more sulfhydryl groups of the inhibitors. The ability of Cu^{2+} (data not shown) and the sulfhydryl-reactive reagent (NEM) (Table I) to readily reverse the inhibition may indicate that the sulfhydryl groups of the inhibitors react with the enzyme-bound Cu^{2+} at the active site of the enzyme.