BBA 65601

INHIBITION OF DOPAMINE β-HYDROXYLASE BY SULFHYDRYL COMPOUNDS AND THE NATURE OF THE NATURAL INHIBITORS

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(Received January 31st, 1967)

SUMMARY

1. Dopamine β-hydroxylase (3,4-dihydroxyphenylethylamine, ascorbate:O_2 oxidoreductase (hydroxylating), EC 1.14.2.1) was found to be inhibited by various sulfhydryl compounds such as cysteine, glutathione, coenzyme A and mercaptoethanol. D-Cysteine was equally as effective an inhibitor as L-cysteine. Cystine did not inhibit the enzyme. *N*-Ethylmaleimide, which reacts with sulfhydryl groups, completely reversed the inhibition by cysteine. Preincubation of the enzyme with cysteine had no effect on inhibition. The inhibition produced by cysteine was reversed by dialysis. The extent of inhibition was not related to the concentration of substrate, ascorbate or fumarate. The inhibition of dopamine β-hydroxylase by cysteine seems to be the result of a chelation of the copper atom at the active site of the enzyme.

2. The inhibition of the enzyme by natural inhibitors of brain and adrenal medulla was completely protected by the addition of *N*-ethylmaleimide. Decrease in sulfhydryl contents of the tissue preparation caused a parallel decrease in the inhibitory activity for the enzyme. The results indicate that the natural inhibitors of dopamine β-hydroxylase are sulfhydryl compounds.

INTRODUCTION

Dopamine β-hydroxylase (3,4-dihydroxyphenylethylamine, ascorbate:O_2 oxidoreductase (hydroxylating), EC 1.14.2.1), which catalyzes the conversion of dopamine to norepinephrine, was solubilized and partially purified from bovine adrenal medulla by LEVIN, LEVENBERG AND KAUFMAN[1]. The enzyme was obtained in essentially homogeneous form and shown to be a copper protein by FRIEDMAN AND KAUFMAN[2]. In their first report[1], they described a marked gain in units of enzyme activity during the purification procedure, and suggested that physiological inhibitors and regulators limit the reaction rate in the intact gland. CREVELING[3] (in UDENFRIEND's laboratory) studied the endogenous inhibitors in adrenal gland and brain, and found that the inhibitor in brain is partially dialyzable and very unstable. He suggested that glutathione may be the endogenous inhibitor.

In the course of our work on the inhibition of dopamine β-hydroxylase by various natural substances, sulfhydryl compounds were found to be inhibitors of the enzyme. The mechanism of this inhibition and the evidence that the endogenous inhibitors are sulfhydryl compounds are described in the present communication. A preliminary report was presented at the United States–Japan Symposium on Oxygenases[4].

MATERIALS AND METHODS

Beef adrenals were obtained fresh, packed in ice, from the slaughterhouse. Dopamine β-hydroxylase was purified from bovine adrenal medulla by the method of FRIEDMAN AND KAUFMAN[2]. Most experiments were carried out on the eluate from calcium phosphate gel (5–30 μg of enzyme). The reaction mixture for the enzymic assay (final vol., 1.0 ml) contained (in μmoles): fumarate, 10; potassium phosphate (pH 6.5), 300; substrate (tyramine or dopamine), 10; ascorbate, 10; an appropriate amount of the enzyme; and enough catalase to give maximum stimulation. The reaction mixture was incubated for 30 min at 37°.

The enzymic activity was measured by two methods. (1) When tyramine was substrate, the conversion of tyramine to norsynephrine was followed according to the spectrophotometric procedure of CREVELING *et al.*[5]. The reaction was stopped by the addition of 0.2 ml of 3 M trichloroacetic acid. As a control, the reaction mixture without tyramine was incubated at the same time, and the substrate was added after stopping the reaction. Centrifugation was not required when pure sulfhydryl compounds were added as inhibitors. When crude tissue preparations were added as natural inhibitors, the acidified reaction mixture was centrifuged, and the supernatant was used. Tyramine and norsynephrine were adsorbed on an Amberlite IR-CG-120, H^+ column and eluted with NH_4OH. The norsynephrine formed from tyramine was assayed on an aliquot of the column eluate by periodate oxidation and measurement of $A_{330\ m\mu}$ of the *p*-hydroxybenzaldehyde formed. (2) When dopamine was used as substrate, the reaction was stopped by the addition of 0.2 ml of 0.6 M trichloroacetic acid. A 10-μl aliquot of the acidified reaction mixture was added with 1.99 ml of 1 M acetate buffer (pH 6.5). Norepinephrine formed from dopamine was assayed according to the fluorometric procedure of VON EULER AND FLODING[6] by an Aminco–Bowman spectrophotofluorometer.

To investigate the natural inhibitors of dopamine β-hydroxylase, rat organs and bovine adrenal medulla were used. The organs were homogenized with 9 vol. of 0.25 M sucrose, and the homogenate was diluted to the concentration required to produce 40–70% inhibition. Chromaffin granules in bovine adrenal medulla were isolated by the differential centrifugation method of HILLARP[7].

When chromaffin granules of bovine adrenal medulla, or proteins solubilized from the granules by the use of a detergent Cutscum, were used as natural inhibitors, these semipurified preparations showed small endogenous enzymic activities. These activities were measured separately, and the inhibition rate (%) was calculated as follows:

$$\left[1 - \frac{\text{The activity of purified enzyme } \textit{plus} \text{ the adrenal preparation}}{\text{The activity of purified enzyme } \textit{plus} \text{ the endogenous activity of the adrenal preparation}}\right] \times 100$$

Protein was measured by the method of LOWRY *et al.*[8]. Sulfhydryl groups were meas-

ured by amperometric titration[9]. The titration mixture was prepared by mixing 4.0 ml of 1.0 M Tris with 3.4 ml of 1.0 M HNO_3 and 0.3 ml of 1.0 M KCl. After the addition of 0.5 ml of 10% tissue homogenate, the solution was made up to 60 ml and titrated. The titrating solution ($1 \cdot 10^{-3}$ M $AgNO_3$) was added from a microburette. Catalase activity was measured by spectrophotometric methods[10].

RESULTS

Inhibition of dopamine β-hydroxylase by sulfhydryl compounds

Various sulfhydryl compounds, such as cysteine, glutathione, coenzyme A and mercaptoethanol, inhibited dopamine β-hydroxylase as shown in Table I. All sulfhydryl compounds produced 100% inhibition at $1 \cdot 10^{-3}$ M concentration, 50% inhibition at $1 \cdot 10^{-4}$ M concentration. At 10^{-5} M concentration the inhibition was not significant. Cystine did not inhibit the enzyme at all at 10^{-3} M concentration. As shown in Table I, D-cysteine was equally as effective an inhibitor as L-cysteine. The inhibition by sulfhydryl compounds was not due to the inactivation of the enzyme by H_2O_2

TABLE I

INHIBITION OF DOPAMINE β-HYDROXYLASE BY SULFHYDRYL COMPOUNDS

Compound	*Concn. (M)*	*% of control activity*
— (control)	—	100
L-Cysteine	$1 \cdot 10^{-2}$	0
	$1 \cdot 10^{-3}$	1
	$5 \cdot 10^{-4}$	4
	$2 \cdot 10^{-4}$	20
	$1 \cdot 10^{-4}$	50
	$7 \cdot 10^{-5}$	58
	$5 \cdot 10^{-5}$	71
	$2 \cdot 10^{-5}$	82
	$1 \cdot 10^{-5}$	99
D-Cysteine	$1 \cdot 10^{-3}$	3
	$2 \cdot 10^{-4}$	25
	$1 \cdot 10^{-4}$	59
	$5 \cdot 10^{-5}$	82
	$1 \cdot 10^{-5}$	98
Glutathione	$1 \cdot 10^{-2}$	0
	$1 \cdot 10^{-3}$	6
	$5 \cdot 10^{-4}$	17
	$2 \cdot 10^{-4}$	49
	$1 \cdot 10^{-4}$	71
	$5 \cdot 10^{-5}$	83
Mercaptoethanol	$1 \cdot 10^{-2}$	0
	$1 \cdot 10^{-3}$	4
	$5 \cdot 10^{-4}$	6
	$2 \cdot 10^{-4}$	16
	$1 \cdot 10^{-4}$	20
	$7 \cdot 10^{-5}$	29
	$5 \cdot 10^{-5}$	41
	$2 \cdot 10^{-5}$	50
	$1 \cdot 10^{-5}$	61
Coenzyme A	$5 \cdot 10^{-4}$	28

(ref. 11) which is produced by non-enzymic autoxidation of sulfhydryl compounds. All the incubations were carried out in the presence of catalase. Neither cysteine nor glutathione inhibited catalase at a concentration of 10^{-3} M. Moreover, increasing the catalase concentration in the reaction mixture did not affect the extent of the inhibition produced by cysteine. As reported by LEVIN, LEVENBERG AND KAUFMAN[1], neither cysteine nor glutathione had activity as cofactor.

Cysteine inhibited the enzymic formation of norepinephrine from dopamine to the same extent as that of norsynephrine from tyramine. At a concentration of $2 \cdot 10^{-4}$ M, the conversion of dopamine to norepinephrine was inhibited by 74%, and that of tyramine to norsynephrine by 73%.

TABLE II

PROTECTION OF THE INHIBITORY EFFECT OF CYSTEINE BY *N*-ETHYLMALEIMIDE

Cysteine was preincubated with *N*-ethylmaleimide at 37° for 25 min. Following this, complete reaction mixture was added, and the reaction was continued at 37° for 30 min.

Addition	*Concn. (M)*	*% of control activity*
— (control)	—	100
Cysteine	$1 \cdot 10^{-3}$	3
Cysteine *plus* *N*-ethylmaleimide	$1 \cdot 10^{-3}$	
	$1 \cdot 10^{-3}$	61
Cysteine *plus* *N*-ethylmaleimide	$1 \cdot 10^{-3}$	
	$2.5 \cdot 10^{-3}$	93

Protection of the inhibition of cysteine by N-ethylmaleimide

N-Ethylmaleimide, which reacts with the sulfhydryl group[12], protected the inhibition by cysteine (Table II). An excess (2.5-fold) of *N*-ethylmaleimide was necessary for the complete recovery of the inhibition.

Reversibility of cysteine inhibition

Preincubation of the enzyme with cysteine in the presence of catalase did not affect the extent of the inhibition. The inhibition produced by cysteine was reversed

TABLE III

REVERSAL OF CYSTEINE INHIBITION OF DOPAMINE β-HYDROXYLASE BY DIALYSIS

Enzyme was incubated with cysteine ($1 \cdot 10^{-2}$ M) in the presence of catalase for 10 min at 37°. One aliquot was then removed for measurement of enzyme activity (final cysteine concentration, $2 \cdot 10^{-4}$ M), and other aliquots were dialyzed against 1000 vol. of 0.05 M phosphate buffer (pH 6.5) at 5° for 2 h. Buffer was changed at 1 h.

	% of control activity	
	Before dialysis	*After dialysis*
Control	100	45
Cysteine-treated enzyme	47	73

by dialysis in the presence of catalase against phosphate buffer (Table III). The presence of cysteine protected the inactivation of the enzyme during the dialysis. Following dialysis, therefore, the enzyme treated with cysteine was more active than the dialyzed control.

Cysteine inhibition was reversed by the addition of Cu^{2+}. As shown in Table IV,

TABLE IV

REVERSAL OF CYSTEINE INHIBITION OF DOPAMINE β-HYDROXYLASE BY Cu^{2+}

L-Cysteine and Cu^{2+} were preincubated at 22° for 10 min. Complete incubation mixture was then added, and the reaction was carried out at 37° for 30 min.

Concn. Cu^{2+} (M)	*% of control activity*		
	Enzyme alone	*Enzyme plus $2 \cdot 10^{-4}$ M cysteine*	*Enzyme plus $1 \cdot 10^{-4}$ M cysteine*
0	100	22	50
$1 \cdot 10^{-5}$	21	84	80
$5 \cdot 10^{-6}$	36	78	85
$1 \cdot 10^{-6}$	87	40	73
$1 \cdot 10^{-7}$	100	28	59

maximum reversibility of inhibition by $2 \cdot 10^{-4}$ M cysteine was 84% with $1 \cdot 10^{-5}$ M Cu^{2+}, and that by $1 \cdot 10^{-4}$ M cysteine was 85% with $5 \cdot 10^{-6}$ M Cu^{2+}.

Kinetic studies on cysteine inhibition

The kinetics of cysteine inhibition were studied by using LINEWEAVER–BURK plots[13]. As shown in Fig. 1, cysteine inhibition was non-competitive with a co-substrate, ascorbate. The inhibition of cysteine was of the non-competitive type with a substrate, tyramine, as shown in Fig. 2. The extent of cysteine inhibition did not depend significantly on fumarate concentration between $1 \cdot 10^{-2}$ and $1 \cdot 10^{-5}$ M.

Reversal of the inhibition of endogenous inhibitors in rat brain or bovine adrenal medulla by N-ethylmaleimide

Distributions of endogenous inhibitors of dopamine β-hydroxylase in rat organs (adrenal gland, brain, spleen, heart, liver, kidney, lung, and muscle) and in subcellular fractions of bovine adrenal medulla were examined as described in MATERIALS AND METHODS. The inhibitory activity was found in every organ of rat and every subcellular fraction of bovine adrenal medulla, without correlation with norepinephrine concentration. Chromaffin granules of bovine adrenal medulla, where the enzyme is localized, were isolated by means of sucrose density-gradient centrifugation, and washed with sucrose 4 times. The particles were then treated with a detergent Cutscum and dialyzed. It was found that some part of the inhibitory activity was dialyzable but easily lost the activity during dialysis. The inhibitory activity was also observed in the soluble protein fraction. When soluble proteins were fractionated by DEAE-cellulose, the inhibitory activity was observed only in the enzyme protein fraction.

Preliminary experiments on the properties of natural inhibitors in rat brain and

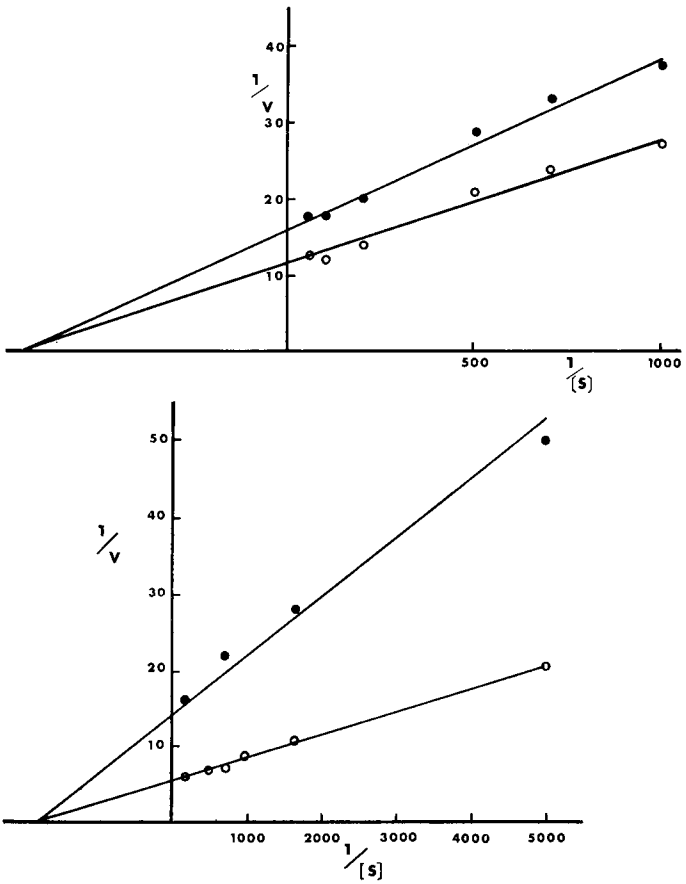

Fig. 1. LINEWEAVER–BURK plot of ascorbic acid concentration against rate of hydroxylation with and without L-cysteine, $1 \cdot 10^{-4}$ M. L-Cysteine and ascorbic acid were added simultaneously. Incubation was for 30 min. The assay was carried out as described in MATERIALS AND METHODS. The velocities are expressed as μmoles of norsynephrine formed per 30 min. The substrate concentration is expressed in moles. ○—○, enzyme alone; ●—●, enzyme with $1 \cdot 10^{-4}$ M L-cysteine.

Fig. 2. LINEWEAVER–BURK plot of tyramine concentration against rate of hydroxylation with and without L-cysteine, $1 \cdot 10^{-4}$ M. L-Cysteine and tyramine were added simultaneously. Incubation was for 30 min. The assay was carried out as described in MATERIALS AND METHODS. The velocities are expressed as μmoles of norsynephrine formed per 30 min. The substrate concentration is expressed in moles. ○—○, enzyme alone; ●—●, enzyme with $1 \cdot 10^{-4}$ M L-cysteine.

in bovine adrenal medulla suggested that the inhibitors are unstable and lose activity by oxidation. The possibility that the endogenous inhibitors are sulfhydryl compounds was examined by using *N*-ethylmaleimide. As shown in Table V, the inhibition by the homogenate of brain and adrenal medulla was completely reversed by the addition of *N*-ethylmaleimide.

Concentration of sulfhydryl groups in brain and adrenal medulla homogenates and the degree of inhibition of dopamine β-hydroxylase

Concentrations of sulfhydryl groups in the homogenates of adrenal medulla and

TABLE V

N-ETHYLMALEIMIDE REVERSAL OF DOPAMINE β-HYDROXYLASE INHIBITION BY BRAIN AND ADRENAL MEDULLA HOMOGENATES

Diluted sucrose homogenate equivalent to 1 mg of tissue was preincubated with *N*-ethylmaleimide ($2.5 \cdot 10^{-3}$ M) for 10 min at 25°. A complete reaction mixture for dopamine β-hydroxylase was then added, and the incubation was continued for a further 30 min at 37°. Sulfhydryl contents in the homogenates, added as natural inhibitors with or without the preincubation with *N*-ethylmaleimide, were measured by amperometric titration[9] as described in MATERIALS AND METHODS and expressed as the final concentration in the incubation mixture.

Natural inhibitor added		*N-Ethylmaleimide*	
		(−)	*(+)*
—	Sulfhydryl contents (M)	0	0
	% of control activity	100	102
Brain homogenate	Sulfhydryl contents (M)	$1.2 \cdot 10^{-5}$	$1.8 \cdot 10^{-6}$
	% of control activity	19	98
Adrenal medulla homogenate	Sulfhydryl contents (M)	$2.0 \cdot 10^{-5}$	$2.5 \cdot 10^{-6}$
	% of control activity	46	99

brain, which were added as natural inhibitors, were measured by amperometric titration[9]. As shown in Table V, the decrease in the concentration of sulfhydryl groups by the preincubation of the homogenate with *N*-ethylmaleimide caused a parallel decrease in the inhibitory activity of dopamine β-hydroxylase.

Measurement of dopamine β-hydroxylase activity in adrenal medulla homogenate in the presence of N-ethylmaleimide

Because of the presence of natural inhibitors, the activity of dopamine β-hydroxylase of crude preparations such as homogenates and particles can be measured only by sensitive radioassay. It was found that the activity in the bovine adrenal

TABLE VI

ASSAY OF DOPAMINE β-HYDROXYLASE ACTIVITY IN THE HOMOGENATE OF BOVINE ADRENAL MEDULLA BY THE ADDITION OF *N*-ETHYLMALEIMIDE

The incubation mixture contained 0.25 M sucrose homogenate (4.1 mg bovine adrenal medulla), 300 μmoles potassium phosphate (pH 6.5), 2.5 μmoles *N*-ethylmaleimide, 0.3 μmole harmaline, 10 μmoles fumarate, 10 μmoles ascorbate, 10 μmoles tyramine, 20 μl of saturated catalase solution, and water to 1.0 ml. Incubation was carried out for 50 min at 37° in a metabolic shaker. The reaction was stopped by the addition of 0.2 ml of 3 M trichloroacetic acid. As a control, the reaction mixture without tyramine was incubated at the same time, and the substrate was added after stopping the reaction. Norsynephrine was measured as described in MATERIALS AND METHODS.

N-Ethyl-maleimide	*Enzymic activity*		
	Absorbance (−blank value)	*Norsynephrine*	
		mμmoles	*μmoles/h per g*
(−)	0.011	1.6	0.5
(+)	0.494	70.6	20.6

medulla homogenate could be measured by a less sensitive spectrophotometric method by including *N*-ethylmaleimide ($2.5 \cdot 10^{-3}$ M) and harmaline (a monoamine oxidase inhibitor) ($3 \cdot 10^{-4}$ M) in the reaction mixture. One example of such assay is shown in Table VI. Since a small amount of adrenal medulla (about 4 mg) was enough for the measurement, the increase in absorbance of the blank by catecholamines contained in the homogenate was less than 0.3 and did not interfere in the assay.

DISCUSSION

The experimental results suggest that the inhibition of dopamine β-hydroxylase by sulfhydryl compounds is due to chelation of the copper atom at the active site of the enzyme. Cysteine inhibition was reversed by dialysis (Table III), and the preincubation had no effect on inhibition. This inhibition, therefore, seems to be the result of a chelation *in situ* rather than a removal of the metal from the apoenzyme. This is different from the inhibition by such chelating agents as diethyldithiocarbamate and KCN. FRIEDMAN AND KAUFMAN[2] showed that diethyldithiocarbamate or KCN removed copper from the enzyme.

It has been reported that tyrosinase, a copper enzyme, was inhibited by sulfhydryl compounds[14]. AGUS, COX AND GRIFFIN[15] recently reported that cysteine inhibits alkaline phosphatase by chelation of the zinc atom at the active site of the enzyme. The mechanism of inhibition of dopamine β-hydroxylase by sulfhydryl compounds seems to be similar to that of the cysteine inhibition of AGUS, COX AND GRIFFIN[15].

Since sulfhydryl groups are widely distributed in animal tissues, it is conceivable that sulfhydryl compounds inhibit dopamine β-hydroxylase activity *in vivo*. The presence of endogenous inhibitors was described by several authors[1,3]. The reversal of the inhibitory effect of a crude preparation of adrenal medulla and brain by *N*-ethylmaleimide (Table V) indicates that natural inhibitors are sulfhydryl compounds. Measurement of sulfhydryl groups in the preparations added as natural inhibitors further supports this possibility. Decrease in sulfhydryl groups in the homogenate by preincubation or by the addition of *N*-ethylmaleimide caused a parallel decrease in the inhibitory activity. A part of the natural inhibitors of bovine adrenal medulla was dialyzable but very unstable: it may be that the inhibitory activity was lost by oxidation. However, the non-dialyzable part of the inhibitors may be proteins or associated with proteins, and it is conceivable that protein sulfydryl groups can be a part of the natural inhibitors. Cysteine inhibited by about 80% at $2 \cdot 10^{-4}$ M, whereas adrenal medulla or brain homogenate inhibited by about 80% at a sulfhydryl concentration of $2 \cdot 10^{-5}$ M. This suggests that natural inhibitors are more effective than cysteine or glutathione. To recover the inhibitory effect of natural inhibitors by *N*-ethylmaleimide, it was not necessary to block all the sulfhydryl groups. When the sulfhydryl concentration was decreased to about $2 \cdot 10^{-6}$ M, the inhibitory effects were completely reversed. This may indicate that there is a fraction of sulfhydryl groups available as natural inhibitors. OGURA AND SEIJI[16] reported that both acid-soluble sulfhydryl groups and protein-bound sulfhydryl groups can regulate melanoma tyrosinase.

It is interesting that activity of dopamine β-hydroxylase in bovine adrenal medulla homogenate appeared on the addition of *N*-ethylmaleimide. A value assayed by this method was 20.6 μmoles/60 min per g of adrenal medulla. This value is compatible with that reported by LEVIN, LEVENBERG AND KAUFMAN[1] after they had re-

moved the endogenous inhibitor by purification of the enzyme (13.3–26.6 μmoles/60 min per g of adrenal medulla).

It is still not clear whether such natural inhibitors of dopamine β-hydroxylase have any physiological significance. It has been established that tyrosine hydroxylase is the rate-limiting step in the biosynthesis of norepinephrine[17] and regulates its biosynthesis[18]. A preliminary experiment showed that intraperitoneal injection of glutathione (625 mg/kg) into a rat caused about 50% decrease in norepinephrine in brain, heart and spleen. However, injection of glutathione at a dose of 330 mg/kg had no effect on the endogenous concentration of norepinephrine. This suggests the possibility that norepinephrine biosynthesis can be inhibited by sulfhydryl compounds *in vivo*. This problem remains for further investigation.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the Ministry of Education, Japan. The Aminco-Bowman spectrophotofluorometer was purchased by United States Public Health Service Research Grant No. 7. Ro 5 TW-00219-01A1 to T. NAGATSU. The authors are grateful to Dr. D. E. WOLF (Merck Sharp and Dohme Research Laboratories, Rahway) for gifts of norsynephrine and Cutscum. The authors' thanks are also due to Dr. M. TADA and Mrs. M. TADA (Aichi-Cancer Center Research Institute, Nagoya) for permitting the use of their apparatus.

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