Biochemical Pharmacology, Vol. 17, pp. 255–264. Pergamon Press. 1968. Printed in Great Britain

ENDOGENOUS INHIBITOR(S) IN ADRENAL MEDULLA OF DOPAMINE-β-HYDROXYLASE*

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(*Received* 29 *May* 1967; *accepted* 3 *August* 1967)

Abstract—The addition of Cu^{2+} ions to various subcellular fractions of the adrenal medulla caused marked increases in dopamine-β-hydroxylase activity. Further studies with Cu^{2+} and other sulfhydryl-reactive reagents such as Hg^{2+}, Ag^{1+}, *p*-hydroxymercuribenzoate, and N-ethylmaleimide indicate that the increased enzymatic activity was due to the inactivation of one or more endogenous sulfhydryl compounds. The addition of Ca^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, and Ni^{2+} to the various fractions of the medulla had no effect on dopamine-β-hydroxylase activity. The dopamine-β-hydroxylase activity in the subcellular fractions measured with and without the addition of Cu^{2+} confirms previous reports that the enzyme is largely localized in the catecholamine storage vesicles and also indicates that the enzyme is more easily solubilized than was previously thought.

PREVIOUS reports[1–3] on the subcellular localization of dopamine-β-hydroxylase in the bovine adrenal medulla have indicated that this enzyme is tightly bound to the membranes of the catecholamine-storage vesicles. Levin *et al.*[3] solubilized the enzyme by extracting the water-washed storage vesicle membranes with a non-ionic detergent. During the subsequent purification, these authors found an increase in the total enzyme activity of their preparation and proposed that this increase in activity was due to the removal of an inhibitor. These observations were confirmed in our laboratory[4] and it was also found that the pH optimum of the solubilized enzyme was 5·5, in contrast to the pH optimum of 7·5 for the enzymatic activity of the intact storage vesicles isolated in isotonic sucrose. It was further observed[5] that the enzyme activity of the lysed storage vesicles was only about 25 per cent of that of the intact storage vesicles when measured under identical conditions at pH 7·4.

In preliminary studies of dopamine-β-hydroxylase activity in adrenal glands of the cat, we could not detect significant levels of enzyme activity in lysed storage vesicles at pH 7·5, whereas the activity of the lysed vesicles at pH 5·5 was only a small fraction of the activity of the intact vesicles measured at pH 7·5. Little or no enzyme activity was detected either at pH 5·5 or 7·5 in the supernatant solutions after removal of the particulate fraction by centrifugation at 25,000 *g* for 20 min.

Dopamine-β-hydroxylase is a copper-containing protein, and it has been shown that during the enzymatic β-hydroxylation the copper undergoes oxidation and reduction[6,7].

*This work was supported by Grants AM 05427 from the National Institutes of Health GB 5182 from the National Science Foundation, and by a Grant from the American Medical Association Education and Research Foundation.

†Special Research Fellow, Institute of Physiology, Faculty of Medicine, University of Chile, Santiago.
‡Career Development Awardee, National Institutes of Health, 5K3 GM 15184.

The addition of copper ions at a final concentration of 2×10^{-4} M inhibited the purified enzyme 60–80 per cent[7]. However, the data reported here show that at lower concentrations Cu^{2+} caused a marked increase of the enzyme activity of the lysed granules and led to the detection of large amounts of enzyme activity in the supernatant fractions. These observations prompted further studies on the distribution of dopamine-β-hydroxylase in adrenal homogenates and the role of Cu^{2+} in stimulating this enzyme activity.

EXPERIMENTAL PROCEDURE

Cat adrenal glands. The adrenal glands from two cats were removed, washed in cold 0·30 M sucrose, blotted dry, and weighed. The combined glands were homogenized in 10 vol. ice-cold 0·30 M sucrose and divided into two equal aliquots. Both aliquots were centrifuged at 25,000 *g* for 20 min and the supernatants combined. This fraction is called the sucrose supernatant. One of the pellets was resuspended in a glass homogenizer with a teflon pestle in fresh 0·30 M sucrose to give a suspension containing the material from 100 mg fresh tissue per ml. The other pellet was resuspended in the same manner in cold distilled water. These fractions are designated sucrose particles and water particles respectively.

Bovine adrenal glands. Fresh bovine adrenal glands were obtained from a local abattoir. Each adrenal medulla was dissected from the cortex, washed in 0·3 M sucrose, blotted dry, and weighed. The medullas were homogenized in 7–8 vol. 0·3 M sucrose in a conical Potter–Elvehjem glass homogenizer and fractionated as shown in Fig. 1. The particulate fractions were resuspended in sucrose or water such that 1 ml of the suspension contained the material prepared from 400–500 mg fresh tissue.

Assay of dopamine-β-hydroxylase. Dopamine-β-hydroxylase was assayed essentially as described by Friedman and Kaufman[8] with uniformly labeled H^3-tyramine as

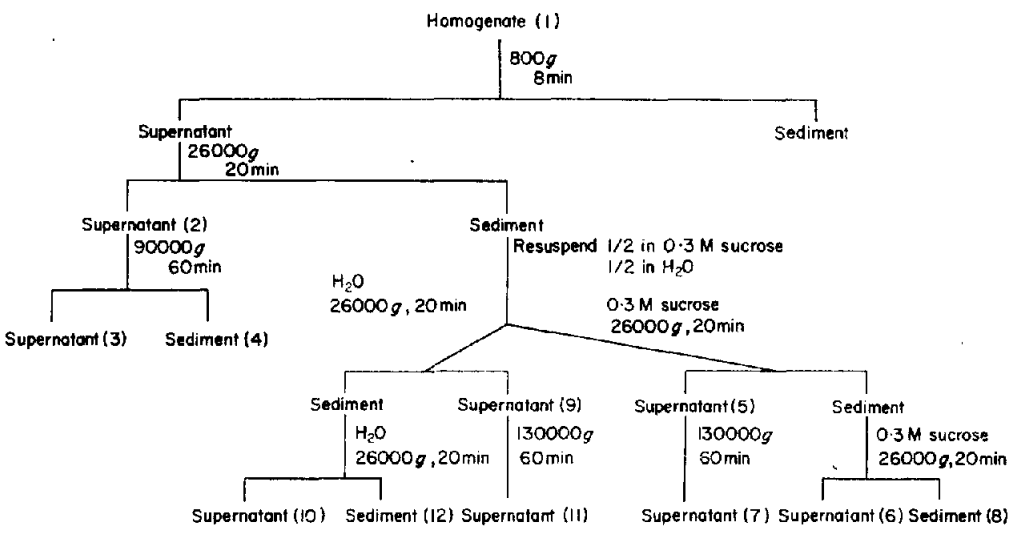

FIG. 1. Differential centrifugation of bovine adrenal medulla homogenates. The adrenal medullas were homogenized in 7–8 vol. 0·3 M sucrose. The numbers in parentheses refer to the fractions in Table 2.

substrate. Each reaction vessel contained in micromoles: phosphate buffer, pH 7·5 (for the sucrose particles) or pH 5·5 (for the water particles and sucrose supernatant), 100; ATP, 5; ascorbate, 1; fumarate, 10; $MgCl_2$, 5; tranylcypromine, 5; H^3-tyramine, 0·005 (sp. act., 63,000 cpm/mμmole); and 0·1 or 0·2 ml of the various fractions corresponding to 20–50 mg fresh tissue. The final volume was 1·0 ml. Incubations were carried out for 15 min with bovine adrenal preparations and for 30 min with cat adrenal glands, at 37° under 100% O_2 in a shaking water bath. Under these conditions, the reaction was linear for 15 min and approximately linear for 30 min. The reaction was stopped by the addition of 1·0 ml of 0·8 N perchloric acid and the octopamine formed was determined by oxidation with periodate as described by Friedman and Kaufman[8]. Blanks containing water instead of the tissue preparation were carried through the entire procedure. The blank controls generally gave about 100 cpm (counts per minute of about 80 above this level, uncorrected for dilutions, generally corresponded to 0·1 mμmole of product).

Ion-exchange chromatography. Octopamine was separated from tyramine by chromatography on Dowex-50 X4 (200–400 mesh), NH_4^+ form. The reaction mixture was deproteinized with 0·4 N perchloric acid. After neutralization with KOH and removal of the $KClO_4$, an aliquot of the solution was placed on a 0·6 × 3 cm column of Dowex-50–NH_4^+. The column was washed with water until the optical absorbance and radioactivity were near blank values. The column was then eluted with 0·4 N HCl at a flow rate of 0·5 ml/min. One-ml samples were collected and monitored at 280 mμ for octopamine and tyramine. The radioactivity in the fractions was measured in a liquid scintillation spectrometer.

RESULTS

The stimulatory effect of Cu^{2+} on the dopamine-β-hydroxylase activity of the sucrose particles, water particles, and sucrose supernatant of cat adrenal glands is shown in Table 1. The optimum Cu^{2+} concentration differed for the various fractions and from

TABLE 1. EFFECTS OF Cu^{2+} ON DOPAMINE-β-HYDROXYLASE OF CAT ADRENAL GLANDS*

		Cu^{2+} (μM)					
		0	10	25	50	75	100
Fraction		Octopamine (mμ mole/100 mg tissue/hr)					
Sucrose Particles	a	622	792	970	768	310	
	b	250		650	371	179	
	c	237		559	235	83	
	d	705	766	1006	408		
Water Particles	a	55	80	1077	2952	3357	
	b	51		1244	1242	857	
	c	35		1277	1297	890	
	d	50		495	992	866	
Sucrose supernatant	a	118	365	1819	2474	1972	
	b	0			1548	1329	879
	c	0		890	1953	2126	
	d	35			1993	2119	1825

*The assays were performed as described in the Methods section with a 30-min incubation time; a, b, c, and d refer to different preparations.

preparation to preparation, but only over a narrow range. The activity of the sucrose particles was stimulated, at most, 2½-fold, whereas the activity of the water particles was stimulated 10- to 20-fold and the activity of the sucrose supernatant was stimulated a minimum of 20-fold. These data suggested that Cu^{2+} ions were either activating the enzyme directly or increasing the activity of the enzyme by removing an inhibitor. After removal of the Cu^{2+} from the enzyme, Friedman and Kaufman[8] were able to recover 40 per cent of the original activity by adding Cu^{2+} at a final concentration of 50 μM. They also showed that, after inhibition of the enzyme with diethyldithiocarbamate, complete activity could be restored by addition of 1 μM Cu^{2+} to the dialyzed enzyme.

Effect of Cu^{2+} on dopamine-β-hydroxylate activity of bovine adrenal homogenates. To obtain further information on the role of Cu^{2+}, additional studies were carried out with preparations from bovine adrenal medulla. For simplicity in the ensuing presentation, it will be assumed that Cu^{2+} exerts its stimulatory effect by interfering with an endogenous inhibitor(s), and evidence will be presented in a later section to support this view.

The stimulatory effects of Cu^{2+} on dopamine-β-hydroxylase activity of subcellular fractions of bovine adrenal medulla are shown in Table 2. Cu^{2+} markedly stimulated

TABLE 2. DOPAMINE-β-HYDROXYLASE IN HOMOGENATES OF BOVINE ADRENAL MEDULLA*

		Octopamine formed (mμmoles/100 mg medulla/hr)	
		$-Cu^{2+}$	$+Cu^{2+}$
1.	Homogenate	3·6	65·1
2.	26,000 *g* Supernatant	0·9	14·1
3.	90,000 *g* Supernatant of 2	0·5	11·5
4.	90,000 *g* Sediment of 2	7·5	2·1
5.	First sucrose wash	3·3	0·6
6.	Second sucrose wash	2·4	0·2
7.	130,000 *g* Supernatant of 2	1·9	0·3
8.	Sucrose particles	7·7	8·7
8A†	Sucrose particles	47·0	8·4
9.	First water extract	21·2	1·3
10.	Second water extract	7·8	1·0
11.	130,000 *g* Supernatant of 9	21·5	2·9
12.	Water particles	4·7	20·4

*Fractions were assayed as described in Methods. All samples were initially assayed at a Cu^{2+} concentration of 10 μM. Those fractions which were stimulated by Cu^{2+} were reassayed at Cu^{2+} concentrations of 5, 10, and 50 μM. Maximal stimulation was obtained with 10 μM Cu^{2+} in the supernatant fractions and at 5 μM in the particulate fractions. The specific activities are defined as mμmoles of octopamine formed/100 mg medulla/hr and refer to the amounts of fresh tissue from which each of the fractions was prepared. The fraction numbers refer to Fig. 1.

†Fraction 8 was diluted 1:3 before assay.

the activity in fractions 1, 2, 3, and 12, and either inhibited or had little effect on the other fractions. However, as shown in 8A of Table 2 and in Fig. 2, whether one obtains inhibition or stimulation depends to a large extent upon the dilution of the preparation. When fractions 3 and 12, which contained the equivalent of 190 and 480 mg tissue/ml,

respectively, were diluted as shown in Fig. 2 and assayed in the presence of a constant amount of Cu^{2+}, the specific activities decreased precipitously and then levelled off to a constant value of $\frac{1}{3}$ to $\frac{1}{5}$ of the maximum activity. When these fractions were diluted and assayed in the absence of Cu^{2+}, there was a rapid increase in the specific activity to almost the same maximum levels obtained with the undiluted preparations measured

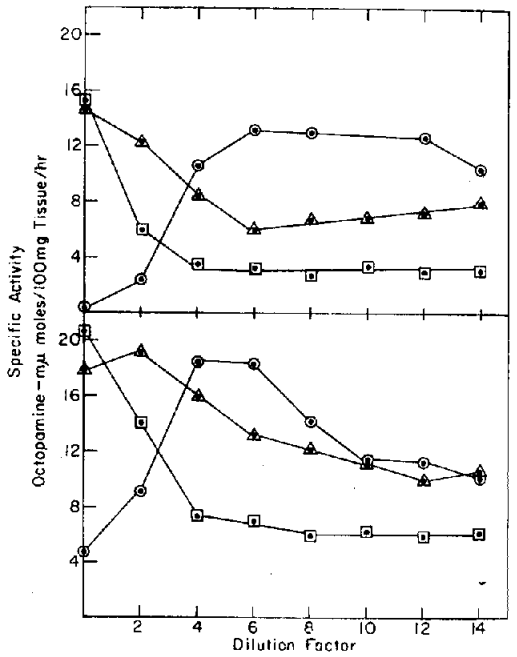

FIG. 2. Effect of dilution on enzyme activity. Upper curves, soluble enzyme, fraction 3, Table 2; lower curves, particulate enzyme, fraction 12, Table 2. One ml of fraction 12, undiluted, contained material from 480 mg adrenal medulla; one ml of fraction 3, undiluted, contained material from 190 mg medulla. In each assay, 0·1 ml of the undiluted and diluted fractions were used. Incubations were as described in Methods. Each reaction vesicle contained 5 μM Cu^{2+} for fraction 12 and 10 μM Cu^{2+} for fraction 3, ⊡——⊡; no Cu^{2+} added, ○——○. The undiluted samples contained 5 and 10 μM Cu^{2+}, respectively, for fractions 12 and 3; this sample was then diluted as indicated so that the ratio of Cu^{2+} to protein remained constant, △——△.

in the presence of Cu^{2+}. Upon further dilution, the specific activities remained constant and then slowly decreased. When both the Cu^{2+} and the enzyme preparation were diluted so that their relative concentrations remained constant, the initial decrease in specific activities was slower than that observed in the presence of constant amounts of Cu^{2+}, and upon further dilution the specific activities approached those which had no added Cu^{2+}. Those fractions, 7 and 11, which were initially inhibited by Cu^{2+}, showed only a slow decrease in specific activity upon dilution when assayed in the absence of Cu^{2+}.

The data in Table 2 are the maximal activities measured. In those fractions which were stimulated by Cu^{2+} (fractions 1, 2, 3, and 12) as well as in those which were not stimulated by Cu^{2+}, with the exception of fraction 8, maximal specific activities were obtained with the undiluted fractions. However, because the activities were not linear with enzyme concentrations, the reported specific activities may not be an

accurate measurement of the real enzyme content of these fractions. Nevertheless, the total activities of the combined fractions isolated in 0·3 M sucrose (fractions 2, 5, 6, and 8A) or of those isolated and washed with water (fractions 2, 9, 10, and 12) are, within experimental error, the same as the maximal specific activity measured in the whole homogenate. In two other preparations a similar distribution of enzyme activity was found.

Identification of the reaction product.

Because of the marked stimulation by Cu^{2+} it was necessary to ascertain whether the product formed was octopamine or the product of some other reaction. In the routine assay, octopamine is oxidized at alkaline pH to *p*-hydroxybenzaldehyde which, after acidification, is extracted into toluene and assayed for radioactivity. When the incubation mixtures were not oxidized with periodate, but merely acidified and extracted, no significant amounts of radioactivity were obtained in the toluene extracts. Thus the routine assay itself provided evidence that hydroxylation had occurred at the β-position.

Direct identification of the product was obtained by a combination of ion-exchange and filter paper chromatography. An aliquot of fraction 3 (Table 2) was dialyzed to remove catecholamines and incubated with uniformly labeled H^3-tyramine in the presence and absence of Cu^{2+}. A control containing Cu^{2+} and all the other components of the incubation mixtures except fraction 3 was incubated simultaneously. After deproteination, carrier octopamine and tyramine were added and the reaction mixtures chromatographed on Dowex-50 as described in Methods. Fig. 3 shows the elution pattern of radioactivity, octopamine and tyramine. The radioactive fractions in the peak corresponding to octopamine were pooled, concentrated, and chromatographed on Whatman No. 1 filter paper using butanol saturated with 1 N HCl. Octopamine was located by spraying the paper with diazotized *p*-nitroaniline followed by Na_2CO_3[9]. The paper was cut into strips and the radioactivity measured in a liquid scintillation spectrometer. Ninety-three per cent of the radioactivity on the chromatogram corresponded to the location of octopamine.

Inhibition of purified dopamine-β-hydroxylase

The data of Table 2 indicated that Cu^{2+} stimulated enzyme activity by removing an inhibitor. This was confirmed by the experiments reported in Table 3. Purified dopamine-β-hydroxylase was inhibited by "native" and by boiled fraction 3, and the inhibition was reversed by Cu^{2+} at a concentration of 10 μM.

The amounts of octopamine formed when purified dopamine-β-hydroxylase and fraction 3 were incubated together were more than the sum of the amounts formed when the enzyme preparations were incubated separately. This could be due to a variety of factors, among them increased stability of the enzyme in the presence of larger amounts of protein, or the presence of some small molecules which additionally stimulate the enzyme. The decrease in specific activity noted in Fig. 2 may also be due to dilution below effective levels of some component which stimulates the enzymatic reaction.

Effect of sulfhydryl reagents

In addition to Cu^{2+}, a variety of other reagents which can react with sulfhydryl groups can reverse the endogenous inhibitor of dopamine-β-hydroxylase (Table 4).

Of the metal ions tested, Cu^{2+} appeared to be the most effective. In all cases the metal ions exhibited a dual effect—at the lower concentrations they inactivated the inhibitor and at higher concentrations they inhibited the enzyme. *p*-Chloromercuribenzoate and *N*-ethylmaleimide were effective only after a preincubation period. In these studies a 10-min preincubation period was used.

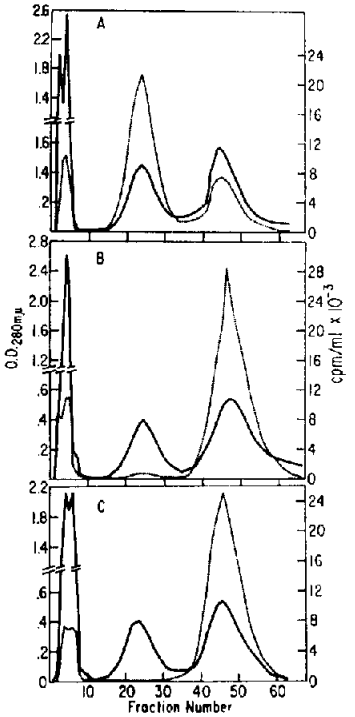

FIG. 3. Separation of octopamine, tyramine, and radioactive reaction products by ion-exchange chromatography. The enzyme preparation was the dialyzed fraction 3 of Table 2. The incubation conditions were as described in Methods with an incubation time of 1 hr. (A) 10 μM Cu^{2+} added to incubation mixture; (B) no Cu^{2+} added; (C) no enzyme. Solid lines = absorbance; broken lines = radioactivity. From right to left the peaks are tyramine, octopamine, and miscellaneous unabsorbed material.

TABLE 3. INHIBITION OF PURIFIED DOPAMINE-β-HYDROXYLASE BY ADRENAL EXTRACTS*

	Octopamine formed	
	$-Cu^{2+}$	$+Cu^{2+}$
Dopamine-β-hydroxylase	1·74	0·40
90,000 *g* Supernatant (fraction 3)	0·05	3·20
Fraction 3, boiled 5 min	0·0	0·0
Dopamine-β-hydroxylase + fraction 3	0·22	5·92
Dopamine-β-hydroxylase + boiled fraction 3	0·20	1·63

*The 90,000 *g* supernatant (fraction 3) was the same as in Table 2. Cu^{2+}, where indicated, was present at a final concentration of 10 μM. The purified dopamine-β-hydroxylase was prepared as described by Smith *et al.*,[10] with further purification by passage through Sephadex G-200.[8] Equal amounts (0·1 ml corresponding to 30 mg fresh tissue) of boiled and "native" supernatants were used. Incubations were as described in Methods.

In addition to the compounds shown in Table 4, Ca^{2+}. Mn^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, and Cu^{1+} were tested for their ability to inactivate the inhibitor. None of these ions except Cu^{1+} had any significant effect. Cu^{1+} at a concentration of 10μM was only about 10–15 per cent as effective as Cu^{2+}.

TABLE 4. EFFECT OF SULFHYDRYL REACTIVE REAGENTS ON DOPAMINE-β-HYDROXYLASE ACTIVITY*

Reagents	Concentration (μM)			
	5	10	50	100
	Octopamine formed (mμmole/100 mg medulla/hr)			
$CuSO_4$	8·6	14·2	4·2	3·0
$AgNO_3$	0·7	1·9	17·2	9·0
$HgCl_2$	4·6	11·5	10·9	10·6
p-Chloromercuribenzoate†	0·3	14·9	15·2	3·4
N-ethylmaleimide†	0·3	1·4	2·9	11·2
Iodoacetate†	0·2	0·2	0·4	0·4
None	0·3			

*Incubations were as described in Methods. The enzyme preparation was fraction 3, described in Table 2.

†Preincubated for 10 min at 37° before addition of substrate.

Effect of sulfhydryl compounds

The effects of cysteine, glutathione, and mercaptoethanol on dopamine-β-hydroxylase are reported in Table 5. Rather high concentrations of these compounds were

TABLE 5. EFFECT OF SULFHYDRYL COMPOUNDS ON DOPAMINE-β-HYDROXYLASE ACTIVITY*

Addition		Octopamine (mμmoles/hr)
None		2·20
Endogenous inhibitor(s)		0·76
Cysteine	10^{-3}	0
	10^{-4}	2.20
	10^{-5}	2·64
Glutathione	10^{-3}	1·24
	10^{-4}	3·12
	10^{-5}	1·92
Mercaptoethanol	10^{-3}	1·52
	10^{-4}	3·52
	10^{-5}	2·52

*The enzymatic assays were performed as described in Methods. The enzyme preparation was purified dopamine-β-hydroxylase described in Table 3. The endogenous inhibitor was prepared by precipitating the protein of fraction 2, Table 2, with 0·4 N PCA and redissolving the precipitate in 0·01 M phosphate buffer, pH 6·5. This fraction contained the inhibitor and had no enzyme activity. The amount of inhibitor added was equivalent to 20 mg fresh tissue.

required for effective inhibition of the enzyme. At lower concentrations, these compounds enhanced the enzymatic activity. Data on the free amino acid content of the adrenal gland do not appear to be available. However, from the report of Tallan *et al.*[11] on the free amino acid content of various tissues of the cat, and assuming that the adrenal gland has the same content of cysteine and glutathione as those tissues containing the highest concentrations of these compounds, one might reasonably expect the concentration of cysteine plus cystine to be about 1 mg/100 g tissue and that of glutathione to be about 120 mg/100 g. Thus, in our incubation mixtures, we would expect a minimum of 10^{-5} to 10^{-6} M endogenous cysteine and 10^{-4}–10^{-5} M endogenous glutathione, concentrations which are below effective inhibitory levels.

The effect of Cu^{2+} on reversal of the inhibition by cysteine is shown in Table 6. When cysteine was added by itself to the reaction mixture, Cu^{2+}, at a concentration

TABLE 6. THE EFFECT OF Cu^{2+} ON CYSTEINE-INHIBITED DOPAMINE-β-HYDROXYLASE ACTIVITY*

Additions	$<Cu^{2+}$	$+Cu^{2+}$
None	2·52	1·64
Cysteine (5×10^{-4} M)	1·28	1·56
Cysteine (1×10^{-3} M)	0·0	0·0
Endogenous inhibitor(s)	0·48	3·96
Endogenous inhibitor(s) plus cysteine (5×10^{-4} M)	0·08	2·28
Endogenous inhibitor plus cysteine (1×10^{-3} M)	0·01	1·16

*The enzymatic assays were performed as described in Methods. The purified dopamine-β-hydroxylase was prepared as described in Table 3. The endogenous inhibitor was prepared by heating fraction 2, Table 2, for 10 min in a boiling water bath. The inhibitor was added in an amount equivalent to 20 mg fresh tissue. Cu^{2+}, where indicated, was present at a concentration of 10 μM.

which effectively reversed the endogenous inhibitor did not reverse the inhibitory effects of cysteine. However, when cysteine was added together with the boiled fraction containing the endogenous inhibitor, Cu^{2+} appeared to reverse partially the inhibition due to cysteine.

DISCUSSION

Previous studies on the intracellular localization of dopamine-β-hydroxylase[1–3] 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. The data of Table 2 are still consistent with the previous finding[1–3] that dopamine-β-hydroxylase is attached to the membranes of the catecholamine storage vesicles, but it now appears that it is more readily washed out of the membranes than was previously thought. In the preparation described in Table 2, 22 per cent of the total enzyme activity was recovered in the initial 26,000 *g* supernatant fraction and 80 per cent of this enzyme activity remained in solution after centrifugation at 90,000 *g* for 60 min. When the first 26,000 *g* sediment of the homogenate was washed twice with 0·3 M sucrose, an additional 9 per cent of the total activity was recovered in the supernatant fraction and 70 per cent of the total homogenate activity was recovered in the particulate fraction. However, when the 26,000 *g* sediment was suspended and washed in distilled water,

45 per cent of the total homogenate activity was recovered in the soluble fraction and 21 per cent in the particulate fraction. The partition of the enzyme between "soluble" and "particulate" fractions depends to a large extent upon the rigor of homogenization and washing, but the data presented here and earlier[1,2] clearly indicate that, in the intact cell, dopamine-β-hydroxylase is a constituent of the catecholamine storage vesicles.

The intracellular localization of the inhibitor is less clear. The data of Table 2 show that the inhibitor is present in the soluble fraction of the initial homogenate and in the washed particles, but washing the particles with water did not appear to remove the inhibitor even though two-thirds of the enzyme activity was solubilized. The available data do not enable one to distinguish among three alternative possibilities for the distribution of the inhibitor in the intact cell: (1) the inhibitor is entirely in the cytoplasm and upon homogenization some of the inhibitor becomes bound to the enzyme; (2) the inhibitor is bound to the particulate enzyme and upon homogenization the inhibitor as well as some of the enzyme becomes solubilized; (3) the inhibitor is partly free in the cytoplasm and partially bound to the enzyme.

At present, work is in progress to isolate and identify the endogenous inhibitor(s) and to evaluate its physiological role. The results reported here suggest that the inhibitor may play a role in regulating noradrenaline synthesis in the adrenal medulla. The inhibitor is stable to boiling for 10 min and is inactivated by sulfhydryl reagents. Dialysis of fraction 3 indicates that the inhibitor is only slowly dialyzable in the presence of the enayme or that it is slowly inactivated. When boiled fraction 3 was dialyzed, the inhibitor could not be detected in the dialysate, in the solution in the dialysis bag, or in a combination of the two.

The available evidence suggests that the inhibitor forms a reversible complex with the enzyme through one or more sulfhydryl groups of the inhibitor. The ability of Cu^{2+} and other sulfhydryl-reactive reagents to readily reverse the inhibition may indicate that the sulfhydryl group(s) of the inhibitor reacts with the enzyme-bound Cu^{2+} at the active site of the enzyme.[8]

REFERENCES

1. N. KIRSHNER, *J. biol. Chem.* **226**, 821 (1957).
2. N. KIRSHNER, *Pharmac. Rev.* **11**, 350 (1959).
3. E. Y. LEVIN, B. LEVENBERG and S. KAUFMAN, *J. biol. Chem.* **235**, 2080 (1960).
4. W. J. SMITH, Doctoral dissertation, Duke University (1963).
5. N. KIRSHNER, *J. biol. Chem.* **237**, 2311 (1962).
6. S. FRIEDMAN and S. KAUFMAN, *J. biol. Chem.* **240**, PC552 (1965).
7. M. GOLDSTEIN, E. LAUBER and M. R. MCKEREGHAN, *J. biol. Chem.* **240**, 2066 (1965).
8. S. FRIEDMAN and S. KAUFMAN, *J. biol. Chem.* **240**, 4763 (1965).
9. H. G. BRAY, W. V. THORPE and K. WHITE, *Biochem. J.* **46**, 271 (1950).
10. W. J. SMITH and N. KIRSHNER *Molec. Pharmac.* **3**, 52 (1967).
11. H. H. TALLAN, S. MOORE and W. H. STEIN, *J. biol. Chem.* **211**, 927 (1954).

Addendum—While this manuscript was in preparation, related work by two others was brought to our attention. C. R. Crevelling (*Studies on dopamine-β-oxidase*, doctoral thesis, George Washington University, Washington, D.C., 1962) studied the endogenous inhibitors in the adrenal gland and brain and found the inhibitor to be unstable and partially protected by the presence of protein. T. Nagatsu (in *Biological and Chemical Aspects of Oxygenases* (Eds. K. E. Bloch and O. Hayaishi), p. 273. Maruzen, Tokyo (1966) reported that dopamine-β-hydroxylase was inhibited by cysteine, glutathione, coenzyme A, and mercaptoethanol and that *N*-ethylmaleimide inactivated the endogenous inhibitors from adrenal glands and brain.