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EFFECTS OF 2-MERCAPTOETHYLGUANIDINE AND OTHER COMPOUNDS ON NOREPINEPHRINE SYNTHESIS BY ADRENAL MEDULLARY GRANULES*

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Abstract—The effects of 2-mercaptoethylguanidine and other compounds on dopamine uptake and norepinephrine synthesis by adrenal medullary granules were studied. The effects of these materials on crude dopamine-β-hydroxylase were also tested. A micro-analytical technique was developed which allows the use of the natural precursor, dopamine, in studies of uptake and synthesis by adrenal medullary granules. The conversion of dopamine to norepinephrine by dopamine-β-hydroxylase was also studied with this technique. Catecholamines were assayed as their dansyl (5-dimethylamino-naphthalene-1-sulfonyl) derivatives, thereby increasing greatly the resolution and sensitivity of detection. The effects of 2-mercaptoethylguanidine and reserpine on dopamine uptake and norepinephrine synthesis by medullary granules were compared. Reserpine inhibited norepinephrine synthesis indirectly through inhibition of dopamine uptake. 2-Mercaptoethylguanidine, on the other hand, depressed norepinephrine synthesis in intact granules by a direct inhibitory effect on dopamine-β-hydroxylase rather than on the uptake mechanism. 2-Mercaptoethylguanidine increased the rate of norepinephrine synthesis by crude dopamine-β-hydroxylase in the presence of added Cu^{2+} and inhibited norepinephrine synthesis in the absence of added Cu^{2+}. The nature of the latter effect was resolved by demonstrating the formation of a Cu–2-mercaptoethylguanidine complex in pure solutions. Since dopamine-β-hydroxylase is a Cu-containing enzyme, the mechanism of the 2-mercaptoethylguanidine inhibition of dopamine-β-hydroxylase appears to be through the binding of enzymic Cu.

TISSUES CAN be depleted of catecholamines by a variety of agents. One of the most important is reserpine, which inhibits the uptake of catecholamines at the granule level.[1–3] The decreased substrate within the granules prevents or slows the synthesis of norepinephrine within the granules which, in turn, contributes to the failure to replenish catecholamine stores lost during adrenergic transmission.[1–3]

Other agents may produce catecholamine depletion by inhibiting biosynthetic enzymes, viz. tyrosine hydroxylase[4,5] and dopamine-β-hydroxylase [3,4-dihydroxyphenylethalamine, ascorbate:oxygen oxidoreductase (hydroxylating), EC 1.14.2.1].[6,7]

In a study of the pharmacological effects of 2-mercaptoethylguanidine (MEG), an antiradiation agent, DiStefano and Klahn[8] demonstrated that the norepinephrine levels in the hearts of mice and cats were lowered markedly. Catecholamine depletion by this agent could result from several factors, but since depletion required 3–4 days, the most likely seemed to be blockade of catecholamine uptake at the granule level,

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as with reserpine, or inhibition of enzymes (such as tyrosine hydroxylase and dopamine-β-hydroxylase) involved in the biosynthesis of catecholamines. Tyrosine hydroxylase inhibition by MEG, however, does not correlate with catecholamine depletion (DiStefano, unpublished material).

The present work deals with the effects of MEG and other agents on dopamine uptake and norepinephrine synthesis by adrenal medullary granules. The effects of these compounds on the conversion of dopamine to norepinephrine by crude dopamine-β-hydroxylase was also tested.

METHODS

Preparation of adrenal medullary granules. Bovine adrenal medullary granules were isolated by a modification of the method of Poisner and Trifaró.[9] After Millipore filtration, the filtrate was centrifuged at 10,000 *g* for 10 min and the pellet was washed by gentle swirling with 0·3 M sucrose. The pellet was finally resuspended in 0·3 M sucrose (0·1 ml/g of original medulla). Granule suspensions were stored on ice and used within 3 hr after isolation.

Granule suspensions were standardized by granule wet weight. Ten-μl aliquots of the granule suspension were placed in each of five preweighed microfuge tubes (Beckman) and centrifuged at approximately 12,000 *g* for 4 min. After removal of the supernatants, the tubes were allowed to stand for 10 min and reweighed to determine the granule wet weight.

Catecholamine assay. Catecholamines were assayed as their dansyl (5-dimethylaminonaphthalene-1-sulfonyl) derivatives. Dansyl-catecholamines were prepared by a modification of the method of Diliberto and DiStefano.[10] Thirty μl of dansyl reagent (72 mg/ml of acetone) and 5 μl of 3·5 N Na_2CO_3 were added to the catecholamine solution. After mixing, the reaction was allowed to proceed at ambient temperature for 12–24 hr in the dark. Finally, acetone (110 μl) was added to stop the dansylation reaction and precipitate the Na_2CO_3; centrifugation gave a solution suitable for chromatography.

One hundred μl of the acetone solution was applied to a sheet of Whatman SG 81 paper (20 × 20 cm) at the origin, and cylindrical-ascending chromatograms were developed in benzene–methanol–cyclohexane (88·5:1·5:10·0). Derivatives were visualized by means of a u.v. lamp (maximum emission at 3660 A); the dansylated catecholamines emit a yellow fluorescence.

Quantitation of dansyl-catecholamines. Chromatograms of dansylated ^{14}C-labeled catecholamines were cut into 1 cm sections and counted by liquid scintillation spectrometry (Packard Tri-Carb, model 3380) using 1 ml of methanol to elute the dansylated derivatives and 10 ml of toluene containing PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-2-(5-phenyloxazolyl)benzene]. Quench curves were used to calculate counter efficiencies from external standard ratios.

Background was taken as the average radioactivity of the 1 cm sections above and below the norepinephrine spot on the chromatogram.

Norepinephrine synthesis studies. The incubation medium used for studies with medullary granules had the following composition: 160 mM KCl, 5 mM NaCl, 10 mM TES buffer, 0·5 mM $MgCl_2$, 0·05 mM Na_2EDTA, and 0·5 mM tranylcypromine.[9] Experiments were carried out at pH 7·0. All drug and substrate ([1-^{14}C]dopamine, 6·4 mCi/m-mole) solutions for subsequent addition were also prepared in the medium.

The procedure for granule incubation and subsequent catecholamine assay is as follows. Adrenal medullary granule suspensions were preincubated at 37° for 15 min with or without additions. The incubation was started by the addition of [1-^{14}C]dopamine and carried out at 37° for 60 min. The experiment was terminated by the addition of 1·2 N perchloric acid (10 μl) containing dopamine carrier (4 mg/ml of perchloric acid). The sample, after standing on ice for about 1 hr, was centrifuged and an aliquot (25 μl) of the supernatant was taken for dansylation. After the addition of dansyl reagent (30 μl) and 3·5 N Na_2CO_3 (5 μl) to achieve a pH of approximately 9·4, catecholamines were assayed as previously described.

Dopamine uptake studies. Catecholamine uptake by adrenal medullary granules was studied using the following procedure. After incubation, the granules were centrifuged in the cold at 15,000 *g* for 5 min, and 25 μl of the supernatant (S) was taken for counting. The granules were resuspended in 0·3 M sucrose containing 1 mM Na_2-EDTA and centrifuged again at 15,000 *g* for 5 min; 25 μl of the first wash (W_1) was taken for counting. The procedure was repeated to obtain a second wash (W_2). Finally, the granules were resuspended and lysed with 40 μl of distilled water. Ten μl of 1·2 N $HClO_4$ containing dopamine carrier was added to precipitate protein. This solution was again centrifuged at 15,000 *g* for 5 min and 25 μl of the lysate was taken for counting. The lysate (L) contained the total intragranular radioactivity ([1-^{14}C]-dopamine and [1-^{14}C]norepinephrine); the radioactivity associated with this fraction is a measure of the uptake of [1-^{14}C]dopamine into the granules. The supernatants (S) were analyzed to check for possible variations in substrate addition. The washings (W_1, W_2) were analyzed to determine catecholamine loss from the granules during the washing procedure. The aqueous fractions (S, W_1, W_2 and L) above were assayed for radioactivity by liquid scintillation spectrometry using 10 ml of Bray's solution.[11]

Dopamine uptake and norepinephrine synthesis studies. The procedure for studying the effects of drugs on dopamine uptake and norepinephrine synthesis simultaneously was similar to that described for dopamine uptake. After incubation, the suspension was centrifuged and 25 μl of the supernatant was transferred to another tube to which was added 5 μl of 1·2 N $HClO_4$ with dopamine and norepinephrine carriers. After centrifugation, 25 μl of this solution was assayed for catecholamines by the dansylation procedure. The pellet was resuspended twice in 0·3 M sucrose containing 1 mM Na_2EDTA, and 25 μl of W_1 and W_2 was taken for counting. The pellet was then treated with 50 μl of distilled water, 10 μl of 1·2 N $HClO_4$ containing dopamine and norepinephrine carriers, and centrifuged. Twenty-five μl of the resulting lysate was analyzed for catecholamines as described above.

Preparation of crude dopamine-β-hydroxylase. Medullary granules were isolated as previously described. After washing, granules were lysed in distilled water (0·1 ml/g of original medulla) to obtain a crude dopamine-β-hydroxylase (DBH) preparation. Ten μl of this preparation contained approximately 1·2 mg of medullary granules. The incubation and catecholamine assay procedures were similar to those described for norepinephrine synthesis except that the crude DBH preparation was substituted for the granule suspension. The incubation medium used for the assay of DBH activity was basically similar to that described by Viveros *et al.*[12] and contained the following ingredients: 94·2 mM KH_2PO_4, 5·8 mM K_2HPO_4, 10·0 mM fumarate, 10 μM $CuCl_2$, 5·0 mM ATP, 1·0 mM ascorbate, and 0·5 mM tranylcypromine at pH 6·0. The crude DBH preparation was preincubated in the above medium with or

without drugs for 5 min. The DBH assay was initiated by the addition of [1-^{14}C]-dopamine and carried out for 30 min.

Determination of complexes with Cu and MEG. To demonstrate complexes of 2-mercaptoethylguanidine (MEG) with Cu^{2+}, u.v. spectra were determined by varying the concentration of Cu^{2+} in the presence of fixed concentrations of MEG ($4{\cdot}27 \times 10^{-4}$ M or 10^{-4} M) in potassium phosphate buffer (0·1 M at pH 7·0). The reference cell contained either distilled water or 0·1 M phosphate buffer. All determinations were made on a Perkin–Elmer 202 spectrophotometer.

Statistical methods. Results were treated statistically using Student's *t*-test.[13]

Materials. MEG is derived from *S*,2-aminoethylisothiouronium bromide hydrobromide (AET), which undergoes an intratransguanylation reaction through an unstable intermediate at pH 7·0, forming 2-mercaptoethylguanidine (MEG).[14] The reaction is both instantaneous and independent of concentration.[15] At low pH (2–4), AET is converted to 2-aminothiazoline.[15]. Accordingly, care was taken to add AET only to neutral solutions. Glutathione (reduced form), *p*-hydroxymercuribenzoate sodium, AET and *trans*-2-phenylcyclopropylamine (Tranylcypromine) were obtained from Sigma Chemical Co.; 2-hydroxytyramine-HCl (dopamine) from CalBiochem; epinephrine bitartrate from Nutritional Biochemicals Corp.; 5-dimethylamino-naphthalene-1-sulfonyl chloride (dansyl-Cl) from Aldrich Chemical Co.; 3,4-dihydroxyphenyl[1-^{14}C]ethylamine-HBr([1-^{14}C]dopamine), 6·4 mCi/m-mole from New England Nuclear; reserpine phosphate (Serpasil) and guanethidine sulfate (Ismelin) from Ciba Pharmaceutical Co.

RESULTS

Conversion of dopamine to norepinephrine. Bovine adrenal medullary granules were incubated with [1-^{14}C]dopamine for 60 min. The upper portion of Fig. 1 depicts a chromatogram of dansylated intragranular, extragranular and carrier dopamine (DA),

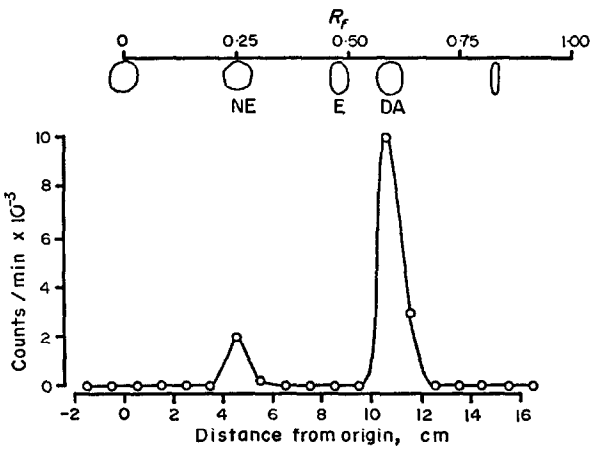

FIG. 1. Chromatographic localization of radioactive dansylated catecholamines from granules incubated with [1-^{14}C]dopamine. The upper portion of the diagram depicts a chromatogram of dansylated dopamine (DA), epinephrine (E), and norepinephrine (NE) obtained from granules incubated with $1{\cdot}5 \times 10^{-4}$ M [1-^{14}C]dopamine (6·4 mCi/m-mole) for 60 min. The lower portion of the diagram is a plot of radioactivity in cpm vs cm from the origin.

and intragranular epinephrine (E) and norepinephrine (NE). A plot of radioactivity in cpm versus cm from the origin is also illustrated in Fig. 1 (bottom). The major peak of radioactivity was coincident with the R_f of dansyl-DA (0·65) and represents both extra- and intragranular DA; the minor peak coincided with dansyl-NE ($R_f = 0{\cdot}25$) and represents newly synthesized NE. There was no corresponding radioactive peak for dansyl-E, indicating that epinephrine is not synthesized in this system. The combined radioactivity recovered as dansyl-DA and dansyl-NE was greater than 95 per cent.

Norepinephrine synthesis by medullary granules. NE synthesis was studied as a function of incubation time (Fig. 2). After a slight lag, the curve rose rapidly initially and subsequently tapered off. The lag in NE synthesis probably resulted from the low initial intragranular concentration of the precursor DA.

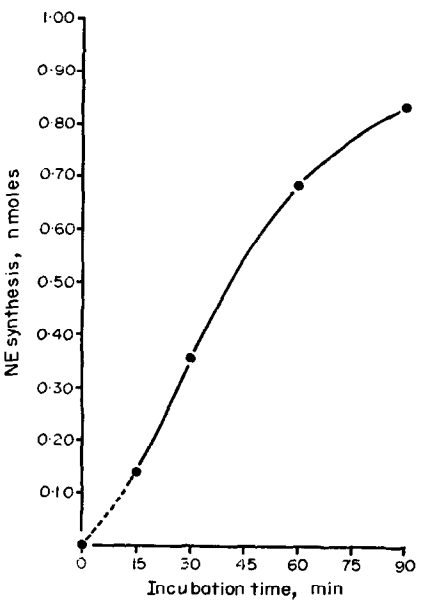

FIG. 2. Time course of norephinephrine synthesis by adrenal medullary granules. The assay was performed with approximately 1·7 mg of granules (wet weight) and $1{\cdot}5 \times 10^{-4}$ M [1-^{14}C]dopamine (6·4 mCi/m-mole). Note the lag (dashed line) in the initial portion of the curve.

The effects of 2-mercaptoethylguanidine (MEG), reserpine and guanethidine on NE synthesis in adrenal medullary granules are shown in Table 1. MEG ($4{\cdot}27 \times 10^{-4}$ M) produced a mean fractional inhibition* of $0{\cdot}31 \pm 0{\cdot}01$ ($N = 4$, $P < 0{\cdot}005$). Reserpine (10^{-5} M) caused a fractional inhibition of 0·62 and guanethidine ($1{\cdot}22 \times 10^{-4}$ M) increased NE synthesis slightly.

Dopamine uptake by medullary granules. DA uptake† (Fig. 3) was rapid initially, with no apparent lag; the rate gradually decreased with time.

* The term fractional inhibition (i) is defined as follows: $i = 1 - a = 1 - (v_i/v)$, where a is the fractional activity, v_i is the inhibited velocity, and v is the uninhibited velocity (Ref. 16).

† DA uptake refers to the total intragranular radioactivity ([1 − ^{14}C]DA + [1 − ^{14}C]NE).

TABLE 1. EFFECTS OF MEG, RESERPINE AND GUANETHIDINE ON NE SYNTHESIS BY MEDULLARY GRANULES*

Addition	N	Mean fractional inhibition ± S. E.
MEG ($4{\cdot}27 \times 10^{-4}$ M)	4	0·31 ± 0·01
Reserpine (10^{-5} M)	1	0·62
Guanethidine ($1{\cdot}22 \times 10^{-4}$ M)	1	−0·06

* The incubations and assays were performed using approximately 1·2 mg of granules (wet weight) and $1{\cdot}5 \times 10^{-4}$ M [1-^{14}C]-dopamine (6·4 mCi/m-mole) as the substrate for 60 min at 37°.

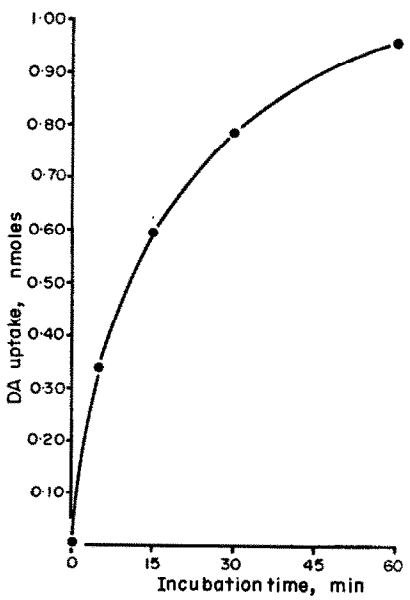

FIG. 3. Time course of [1-^{14}C]dopamine uptake by medullary granules. The incubation was performed with approximately 1·3 mg of granules (wet weight) and $1{\cdot}5 \times 10^{-4}$ M [1-^{14}C]dopamine (6·4 mCi/m-mole). The curve represents the total intragranular radioactivity ([1-^{14}C]dopamine + [1-^{14}C]norepinephrine).

Certain compounds, such as reserpine[3] and *p*-hydroxymercuribenzoate Na (PCMB),[17] inhibit the uptake of catecholamines (CA) into medullary granules. Accordingly, the effects of MEG and glutathione on DA uptake by the granules were compared with those of reserpine and PCMB. MEG ($4{\cdot}27 \times 10^{-4}$ M) decreased DA uptake insignificantly (Table 2); when the concentration was doubled ($8{\cdot}54 \times 10^{-4}$ M), MEG inhibited DA uptake 20 per cent. Reserpine (10^{-5} M) produced a mean inhibition of 56 per cent, while PCMB (10^{-4} M) completely inhibited DA uptake. In the presence of MEG ($4{\cdot}27 \times 10^{-4}$ M), the inhibition of DA uptake by PCMB (10^{-4} M) was incomplete, suggesting that sulfhydryl groups may be involved in the CA transport system. Glutathione, on the other hand, slightly increased DA uptake,

TABLE 2. DOPAMINE UPTAKE BY MEDULLARY GRANULES*

Expt. No.	Addition	DA uptake† (nmoles)	% Inhibition
1	None	0·81	
	MEG ($4{\cdot}27 \times 10^{-4}$ M)	0·77	5
	PCMB (10^{-4} M)	0·00	100
	PCMB (10^{-4} M) + MEG ($4{\cdot}27 \times 10^{-4}$ M)	0·67	16
	Glutathione ($6{\cdot}67 \times 10^{-3}$ M)	0·86	−6
2	None	0·60	
	MEG ($4{\cdot}27 + 10^{-4}$ M)	0·58	3
	MEG ($8{\cdot}54 \times 10^{-4}$ M)	0·48	20
	Reserpine (10^{-5} M)	0·25	59
3	None	0·44	
	Reserpine (10^{-5} M)	0·20	53

* The incubations were carried out for 30 min at 37° using approximately 1·0 mg of granules (wet weight) and $1{\cdot}5 \times 10^{-4}$ M [1-^{14}C]dopamine (6·4 mCi/m-mole). Each experiment was made with a different preparation. Note that even though the controls differ, the per cent of inhibition of DA uptake by MEG and reserpine was the same.

† Calculated from total intragranular radioactivity/NE formed and unchanged intragranular DA.

possibly by protecting the sulfhydryl groups of the transport system from oxidation during incubation.

Dopamine uptake and norepinephrine synthesis by medullary granules. In subsequent experiments simultaneous measurements of DA uptake and NE synthesis were carried out in the presence of MEG and reserpine. Table 3 shows the intragranular

TABLE 3. INTRAGRANULAR DISTRIBUTION OF DA AND NE AFTER TREATMENT WITH MEG AND RESERPINE*

Addition	NE (nmoles)	DA (nmoles)	NE/DA ratio†
None	0·13	0·50	0·26
MEG ($4{\cdot}27 \times 10^{-4}$ M)	0·10	0·48	0·20
MEG ($8{\cdot}54 \times 10^{-4}$ M)	0·08	0·42	·018
Reserpine (10^{-5} M)	0·05	0·19	0·26

* The incubation and assay were performed for 30 min at 37° with approximately 0·7 mg of granules (wet weight) and $1{\cdot}5 \times 10^{-4}$ M [1-^{14}C]dopamine 6·4 mCi/m-mole). Each value represents the mean of three determinations on one preparation.

† Ratio of radioactive NE formed to radioactive intragranular DA.

distribution of NE and DA radioactivity. The ratio of radioactive NE to DA appears in the column on the right. The NE/DA ratio in the presence of reserpine (10^{-4} M) was indistinguishable from the control. Since endogenous intragranular DA has never been demonstrated, and since the intragranular concentration of DA during the experimental period was below the apparent K_m of dopamine-β-hydroxylase, the inhibition of NE synthesis by reserpine must have resulted from inhibition of DA uptake. MEG ($4{\cdot}27 \times 10^{-4}$ M), on the other hand, produced an NE/DA ratio which

was less than the control, suggesting that MEG may inhibit DBH rather than the uptake mechanism. MEG ($8{\cdot}54 \times 10^{-4}$ M) depressed the NE/DA ratio further.

Studies with the crude DBH preparation. MEG appeared to exert its major effect on the enzyme DBH rather than on the uptake mechanism. Therefore, experiments were carried out to determine its effect on DBH activity. The time course of NE synthesis in the presence and absence of MEG ($4{\cdot}27 \times 10^{-4}$ M) was studied using the crude DBH preparation (Fig. 4). NE synthesis by the crude DBH preparation was linear between 10 and 90 min. Surprisingly, MEG produced a striking increase in the rate of

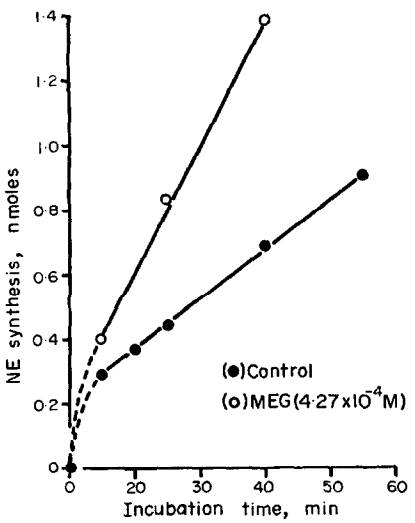

FIG. 4. Norepinephrine synthesis as a function of incubation time with the crude DBH preparation in the presence and absence of 2-mercaptoethylguanidine (MEG). The rate of NE synthesis is rapid initially (dashed line) and linear after 10 min.

NE synthesis. It should be noted that in both cases (with and without MEG) the curves rise rapidly initially. This phenomenon has been described by others[12] but is unexplained.

Incubation of the crude DBH preparation with MEG ($4{\cdot}27 \times 10^{-4}$ M) caused a large increase in enzymic activity, with a mean fractional activity* of $2{\cdot}00 \pm 0{\cdot}10$ (Table 4). Reserpine (10^{-5} M) had no effect.

ATP has been incorporated into studies of DBH activity;[12] the reason for the addition of ATP into the incubation medium is obscure. Consequently, experiments were conducted with and without ATP to determine its effect on the MEG response (Table 5). In the absence of MEG, ATP inhibited NE synthesis markedly (fractional inhibition = 0·65). The effects of MEG on NE synthesis in the presence of ATP showed a fractional activity of 1·49, suggesting that MEG partially reversed the ATP inhibition of DBH. Since ATP did not contribute to the activity of the crude DBH preparation and was in fact inhibitory, it was omitted from the medium in subsequent studies.

* The term fractional activity (a) is defined as follows: $a = v_a/v$, where v_a is the activated velocity and v is the unactivated velocity (Ref. 16).

TABLE 4. EFFECTS OF RESERPINE AND MEG ON THE ACTIVITY OF THE CRUDE DBH PREPARATION*

	N	Mean fractional activity ± S. E.
Control	5	1·00
MEG ($4·27 \times 10^{-4}$ M)	5	2·00 ± 0·10
Control	2	1·00
Reserpine (10^{-5} M)	2	0·96

* The incubation and assay were performed as described in Table 1 except that osmotically lysed granules (crude DBH preparation) were substituted for intact medullary granules.

TABLE 5. EFFECT OF MEG, GLUTATHIONE AND PCMB ON THE ACTIVITY OF CRUDE DBH IN THE PRESENCE OR ABSENCE OF ATP*

Additions	ATP† (5·0 mM)	NE (nmole)	Fractional activity
None	0	0·49	1·00
MEG ($4·27 \times 10^{-4}$ M)	0	0·86	1·76
None	+	0·17	0·35
MEG ($4·27 \times 10^{-4}$ M)	+	0·73	1·49
Glutathione ($6·67 \times 10^{-3}$ M)	+	0·20	0·41
Glutathione ($6·67 \times 10^{-3}$ M) + MEG ($4·27 \times 10^{-4}$ M)	+	0·10	0·20
PCMB (10^{-4} M)	+	0·11	0·22
PCMB (10^{-4} M) + MEG ($4·27 \times 10^{-4}$ M)	+	0·68	1·39

* See Table 4 for details.
† Present (+) or absent (0).

Glutathione ($6·67 \times 10^{-3}$ M) increased NE synthesis slightly; the combination of glutathione and MEG inhibited NE synthesis. PCMB (10^{-4} M) inhibited NE synthesis; the addition of PCMB and MEG increased NE synthesis.

Dopamine-β-hydroxylase is a copper-containing enzyme, and the effects of the various agents may, therefore, be related in some way to binding of some form of Cu. To test the hypothesis that MEG may be reacting with the enzymic Cu,* experiments were performed with the crude DBH preparation in the presence or absence of Cu^{2+}. Several experiments were also performed to determine whether the increase in DBH activity was due to a combination of MEG and Cu^{2+} or to Cu^{2+} or MEG alone (Table 6). The addition of Cu^{2+} (10^{-5} M) to the medium had no effect. MEG (9×10^{-5} M) in the absence of Cu^{2+} also had no effect; however, in the presence of Cu^{2+} (10^{-5} M), MEG (9×10^{-5} M) again caused a marked increase in DBH activity. Thus, only in combination with Cu^{2+} did MEG cause an activation of DBH,

* The terms enzymic Cu, Cu of the enzyme, and Cu of DBH are used because the ionic species of Cu at the active site of the enzyme is not known.

TABLE 6. EFFECT OF MEG ON THE ACTIVITY OF CRUDE DBH IN THE PRESENCE OR ABSENCE OF Cu^{2+}*

Addition	NE (nmole)	Fractional activity
None	0·45	1·00
Cu^{2+} (10^{-5} M)	0·44	0·98
MEG (9×10^{-5} M)	0·43	0·96
MEG (9×10^{-5} M) + Cu^{2+} (10^{-5} M)	0·85	1·89

* See Table 4 for details. ATP was not present in the incubation medium. The data represent the means of two experiments.

and MEG or Cu^{2+} alone had no effect. Curves of fractional activity versus MEG concentration (pMEG)* in the presence or absence of Cu^{2+} (10^{-5} M) are shown in Fig. 5. Each of the curves represents two experiments performed on the crude DBH preparation. In the presence of Cu^{2+} (10^{-5} M), the lower concentrations of MEG increased NE synthesis. However, with increasing concentrations of MEG, DBH activity decreased, reaching control levels at about $1{\cdot}5 \times 10^{-3}$ M MEG and producing a fractional inhibition of 0·64 at $3{\cdot}2 \times 10^{-3}$ M MEG. In the absence of Cu^{2+}, MEG caused a concentration-dependent decline in NE synthesis. These results indicate that the inhibition of NE synthesis observed in intact granules is due to a direct effect on DBH. Furthermore, the same concentration of MEG, $4{\cdot}27 \times 10^{-4}$ M, caused the same degree of inhibition (30 per cent) in both the intact granules and the crude DBH preparation. Finally, the data suggest a mechanism for the inhibition of DBH by MEG, i.e. through the binding of enzymic Cu.

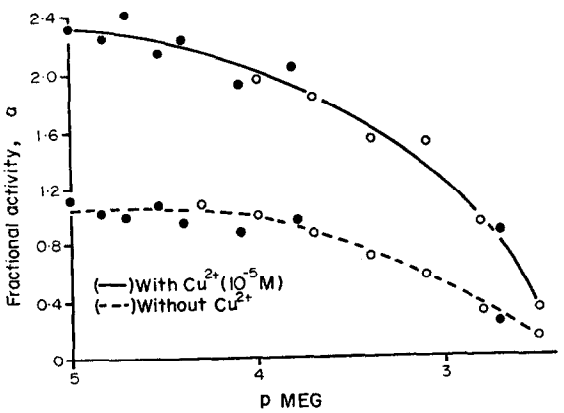

FIG. 5. Fractional activity (*a*) of the crude DBH preparation versus the concentration of MEG (pMEG) in the presence and absence of Cu^{2+}. The incubation medium did not contain ATP. Each curve represents two experiments: ○, first experiment; ●, second experiment. Note the marked stimulation in DBH activity by the lower concentrations of MEG in the presence of Cu^{2+}.

* The term pMEG is defined as follows: pMEG = − log(MEG), where (MEG) is the molar concentration of MEG.

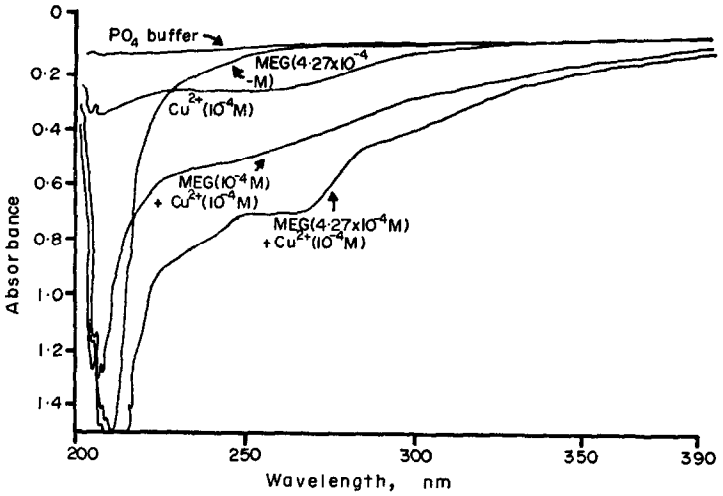

FIG. 6. Ultraviolet spectra of solutions of MEG with and without Cu^{2+}. All solutions were made up in phosphate buffer and the recordings were made with the buffer in the reference cell.

If MEG inhibits NE synthesis by binding the Cu of DBH, a demonstration of an MEG–Cu complex might substantiate this hypothesis. Ultraviolet spectra (Fig. 6) of solutions of MEG ($4{\cdot}27 \times 10^{-4}$ M and 10^{-4} M) and 10^{-4} M Cu^{2+} indicate complex formation, thus strengthening the hypothesis that the mechanism of inhibition of DBH by MEG is through binding of the Cu of DBH.

DISCUSSION

A novel microanalytical method using dansyl-Cl (Diliberto and DiStefano)[10] for the determination of dopamine uptake and/or norepinephrine synthesis by intact medullary granules has been described. The effects of various agents on medullary granules could be studied routinely with the natural precursor of norepinephrine, DA. Most studies of granular catecholamine uptake by other workers have been performed with labeled epinephrine,[3] and DBH activity has been assayed using labeled tyramine.[18] The findings reported here using the natural precursor DA are in agreement with those of others on catecholamine uptake by adrenal medullary granules and on DBH activity with tyramine as the substrate.

DA uptake by adrenal medullary granules decreased with time (Fig. 3). This could have resulted from at least three factors: deterioration of the catecholamine (CA) transport system, saturation of the intragranular catecholamine binding sites, or release of radioactive catecholamines after uptake. The last possibility was discarded, since the supernatant fractions after incubation for 60 min in the presence or absence of MEG or reserpine did not contain [1-^{14}C]NE. In other words, DA taken up and newly synthesized NE remained in the granules for the duration of the incubation. The first and/or the second factor described above may have been involved in the decreased DA uptake.

Several obvious possibilities can be cited for the decline in the rate of NE synthesis with time: (1) deterioration of the catecholamine transport system, decreasing precursor (DA) uptake, resulting in substrate depletion; (2) inactivation of the intragranular enzyme, DBH; (3) limitation of cofactors such as ascorbate; and (4) decrease in the intragranular pO_2 by the utilization of O_2 in the DBH reaction.

Reserpine inhibited NE synthesis in intact medullary granules by blocking the uptake of DA. Experiments reported elsewhere[3] also demonstrated inhibition by reserpine of [^{14}C]E uptake by medullary granules. Decreased NE synthesis due to limitation of DA uptake contributes to the subsequent failure to replenish catecholamine stores lost through physiological release and is probably the mechanism for CA depletion by reserpine.[3,19]

Previous studies of the effects of *p*-hydroxymercuribenzoate Na (PCMB) indicated strong inhibition of granular [^{14}C]E uptake.[17] This compares with the results described here (Table 2), in which complete inhibition of DA uptake was obtained. These observations led to the postulate that sulfhydryl groups are involved in the CA transport system.[17] This hypothesis was substantiated partially by the decreased inhibition produced by PCMB in the presence of MEG, a sulfhydryl compound. The latter may protect the CA transport mechanism by binding the PCMB.

The stimulated DA uptake (Table 2) and the slight increase in NE synthesis caused by glutathione may have resulted from the protection of the sulfhydryl groups of the CA transport system during incubation. At least two mechanisms for this protection are possible: (1) the preferential oxidation during incubation of the sulfhydryl groups of glutathione which would spare the sulfhydryl groups of the CA transport system, or (2) the formation of readily dissociable disulfide bridges between glutathione and the sulfhydryl groups of the CA transport system. These speculations suggest that the increase in NE synthesis by glutathione could have resulted from increased DA uptake.

MEG, on the other hand, inhibited NE synthesis in intact granules (Table 1) without affecting the uptake of [1-^{14}C]dopamine (Table 2). The NE/DA ratio, representing the intragranular distribution of radioactive NE and DA, in the presence of MEG was less than the control. These results suggest that MEG inhibits DBH rather than the uptake mechanism.

Studies with the crude DBH preparation. Since MEG apparently affected DBH directly, studies with DBH were undertaken. Initially, the effects of MEG and several other agents were tested on the activity of a crude DBH preparation after its characterization. The time course in the presence or absence of MEG was linear between 10 and 90 min but did not extrapolate to the origin (Fig. 4). This phenomenon has been reported by others[12] but is unexplained. Perhaps H_2O_2 from the auto-oxidation of ascorbate may have accumulated in the medium prior to incubation. The initial portion of the curve may represent the rapid inactivation of a portion of the DBH by oxidative denaturation. This speculation may have validity since the activity of a partially purified DBH preparation was increased greatly by the addition of catalase to the incubation medium. An alternate explanation for the initial portion of the time course may be differences in behavior of two different fractions of the enzyme. DBH has been shown to exist in membrane-bound and soluble fractions.[12] Perhaps the phenomenon may have been due to the rapid inactivation of only one of these fractions of DBH.

MEG increased markedly the rate of NE synthesis with the crude DBH preparation (Fig. 4). The increase in DBH activity by MEG may have been the result of: (1) an interaction of MEG with endogenous inhibitor(s), (2) a direct effect on the enzyme, or (3) an interaction of MEG with some unknown factor present in the crude DBH preparation.

There is some disagreement as to the localization of the endogenous inhibitor(s) of

DBH in the adrenal medulla. Some workers claim that the endogenous inhibitor(s) is localized within the granules;[20,21] others purport to have demonstrated the presence of this inhibitor in the cytoplasmic fraction of the adrenal medulla.[22] The activities of crude DBH preparations, whether incubated in the presence or absence of Cu^{2+} (10^{-5} M) were indistinguishable; adrenal medullary granule preparations used in the studies reported here did not contain endogenous inhibitor(s) of DBH. Thus, the effects of MEG on the crude DBH preparation probably did not result from an interaction of MEG with endogenous inhibitor(s).

Experiments on the crude DBH preparation with MEG and Cu^{2+} suggested that the MEG–Cu complex may have stimulated the enzyme directly or that MEG may have had an effect on the Cu of DBH. However, in studies to be reported elsewhere, attempts to duplicate the MEG-induced stimulation with a partially purified DBH preparation failed, indicating that the MEG-induced increase in the activity of the crude DBH preparation was not a direct effect on the enzyme. Therefore, the increase in the rate of NE synthesis observed with the crude DBH preparation in the presence of MEG may have resulted from the interaction of MEG with some unknown factor present in the preparation.

In the presence of ATP, MEG caused an increase in DBH activity. The MEG response may have resulted from the formation of an MEG–ATP complex which prevented the high concentration of ATP from complexing the Cu of DBH and the stimulation of DBH by an unknown mechanism, as suggested above.

PCMB inhibited DBH in the crude preparation (Table 5); the addition of MEG reversed the PCMB inhibition and restored DBH activity to a level comparable to that obtained with MEG alone. These results suggest that the sulfhydryl group of MEG is probably not involved in the MEG stimulatory response.

The effects of MEG in the presence of glutathione, PCMB and ATP suggested that the exogenous Cu^{2+} added to the crude preparation was involved in the MEG response. Curves of fractional activity vs pMEG in the presence or absence of Cu^{2+} (Fig. 5) support the role of Cu in the MEG response. The data indicate that MEG inhibits DBH directly and suggest a mechanism for the inhibition of DBH by MEG, i.e. through binding the Cu of the enzyme. This mechanism was supported further by the demonstration of an MEG–Cu complex in pure solutions (Fig. 6).

It was possible to obtain both stimulation and inhibition of DBH by MEG with the crude DBH preparation. The stimulatory effect could not be explained, but the inhibitory effect appears to result from the binding of Cu of the enzyme.

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