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MECHANISM AND KINETICS OF THE INHIBITION OF DOPAMINE-β-HYDROXYLASE BY 2-MERCAPTOETHYLGUANIDINE*

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Abstract—The inhibition of dopamine-β-hydroxylase (DBH) by 2-mercaptoethylguanidine (MEG) was studied using a partially purified DBH preparation. The MEG-induced inhibition of DBH decreased progressively with increasing Cu^{2+} concentration; equal concentrations of MEG and Cu^{2+} were without effect, supporting the hypothesis that the mechanism of inhibition is through binding of enzymic Cu. It was shown kinetically that MEG inhibits purified DBH by a two-inhibitor molecule interaction. Since MEG inhibits DBH by binding enzymic Cu, two Cu ions must be available at the active site for binding with the two MEG molecules. Several congeners of MEG and other compounds were used to study the structure–activity relationship (SAR) of the MEG inhibition of DBH. The SAR correlated with a hypothetical model of the enzyme active site based on the information obtained from the kinetic studies of the MEG inhibition of DBH. The structure–activity relationship suggested that the intramolecular distance between the anionic site and the site of β-hydroxylation of the substrate, dopamine (DA), was coincident with the intramolecular distance between the positive charge of the inhibitor and the site of binding of the Cu of DBH.

THE ENZYME responsible for the conversion of dopamine (DA) to norepinephrine (NE) has been shown in the adrenal medulla to be present in particles sedimenting with mitochondria; the particles have been isolated and identified as the adrenal medullary granules.[1–3] The enzyme, dopamine-β-hydroxylase [DBH; 3,4-dihydroxyphenylethylamine, ascorbate:oxygen oxidoreductase (hydroxylating), EC 1.14.2.1], has been obtained in a high degree of purity[4,5] and shown to utilize molecular oxygen; ascorbate is required as an electron donor in the hydroxylation reaction.[4] These requirements and the fact that ascorbate is oxidized stoichiometrically during the hydroxylation reaction established DBH as a mixed-function oxidase.[4] Of particular importance was the finding that DBH is a copper-containing enzyme and that the copper undergoes reduction and oxidation during the enzyme-catalyzed hydroxylation reaction;[4,6] two copper ions are reduced (Cu^{2+} to Cu^{+}) for each molecule of ascorbate oxidized.[4] Further, the amount of copper reoxidized is equal to two times the substrate hydroxylated.[4]

The effects of 2-mercaptoethylguanidine (MEG) were previously studied on the uptake of DA and the synthesis of NE by adrenal medullary granules and on norepinephrine synthesis by crude DBH.[7] MEG inhibited NE synthesis in intact medullary

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granules without inhibiting DA uptake, suggesting that MEG may affect DBH directly rather than the uptake mechanism.[7] In addition, studies on crude DBH demonstrated two effects of MEG: an increase in the rate of NE synthesis in the presence of added Cu^{2+} and an inhibition of NE synthesis in the absence of added Cu^{2+}.[7] The nature of the latter effect was resolved by demonstration of the formation of a Cu–MEG complex in pure solutions; thus, the mechanism of inhibition of DBH appeared to be through binding of enzymic Cu.[7]

This report describes the mechanism and kinetics of the MEG inhibition of DBH using a partially purified DBH preparation. An attempt was also made to describe the structure–activity relationship of this inhibition.

METHODS

Preparation of dopamine-β-hydroxylase. DBH was purified by a modification of the procedure of Smith and Kirshner.[8] Cortex-free medulla was dissected from approximately 100 beef adrenal glands (42·5 g of medulla). After lysis of the medullae with water and treatment of the lysate to remove small molecules, the resulting DBH-containing extract was subjected to Sephadex G-200 column chromatography. The most active fractions were pooled and concentrated by dialysis against 50% sucrose in 10 mM phosphate buffer (pH 6·8) ,and the protein was precipitated by the addition of ammonium sulfate to 80 per cent saturation. After centrifugation, the ammonium sulfate precipitate was dissolved in 3 ml of buffer and reapplied to the 2·4 × 45 cm Sephadex G-200 column. Ammonium sulfate was added to 80 per cent saturation to the most active fractions. The partially purified preparation of DBH was stored at 4° as the ammonium sulfate precipitate. Prior to use, an aliquot was centrifuged; the precipitate was dissolved in 20 mM phosphate buffer at pH 6·8 and dialyzed overnight against the same buffer to remove ammonium sulfate.

Dopamine-β-hydroxylase assay. Hydroxylation of DA was measured radiometrically according to the method of Diliberto and DiStefano.[7] The incubation medium used in studies with the partially purified DBH preparation, except where stated otherwise, was as follows: 92·2 mM KH_2PO_4, 5·8 mM K_2HPO_4, 100 mM fumarate, 10·0 mM ascorbate, and catalase (105 units/sample) at pH 6·0. The presence of catalase and these concentrations of ascorbate and fumarate, which were shown to be optimal, indicated a sp. act. for partially purified DBH of 9·39 μmoles of NE formed/30 min/mg of protein with a DA concentration of 10^{-3} M and a pH optimum of approximately 5·5. All drug and substrate solutions were prepared in this medium. The final volume of the reaction mixture was 50 μl. A linear relationship was noted between initial velocity and enzyme concentration, indicating that endogenous inhibitors were not present and that the rate of O_2 diffusion does not limit enzymic activity at these enzyme concentrations.

The enzyme preparation was preincubated at 37° for 5–15 min with and without additions. The incubation was started by the addition of [1-^{14}C]DA and carried out at 37° for 30 min. The reaction was terminated by the addition of 10 μl of 1·2 N perchloric acid containing DA and NE carriers (26 μmoles of each/ml of perchloric acid). After standing on ice for about 1 hr, the sample was centrifuged and an aliquot (20 μl) of the supernatant was taken for assay of catecholamines as their dansyl (5-dimethylaminonaphthalene-1-sulfonyl) derivatives.[7] Dansylated ^{14}C-labeled catecholamines were counted by liquid scintillation spectrometry (Packard Tri-Carb,

model 3380) using 1 ml of methanol to elute the dansylated derivatives from the chromatograms and 10 ml of toluene containing PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-2-(5-phenyloxazolyl)benzene].[7]

Materials. 2-Mercaptoethylguanidine is derived from *S*,2-aminoethylisothiouronium bromide hydrobromide (AET), which undergoes intratransguanylation at pH 7.[9,10] AET, methyl guanidine HCl, and Sephadex G-25 (300 mesh) were obtained from Sigma Chemical Co.; 3-hydroxytyramine HCl (dopamine HCl) and 2-mercaptoethylamine HCl (MEA) from CalBiochem; norepinephrine bitartrate from Nutritional Biochemical Corp.; 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansylCl) from Aldrich Chemical Co.; 3,4-dihydroxyphenyl[1-^{14}C]ethylamine-HBr ([1-^{14}C] dopamine), 6·28 mCi/m-mole, from New England Nuclear; reserpine phosphate (Serpasil) and guanethidine sulfate (Ismelin) from Ciba Pharmaceutical Co.; Sephadex G-200 (40–120 mesh) and Blue Dextran 2000 from Pharmacia Fine Chemicals: diethylaminoethyl cellulose (DEAE cellulose) and SG-81 paper from Whatman Column Chromedia; and disulfiram (Antabuse) from Ayerst Laboratories, Inc.

The congeners of AET, 3-aminopropylisothiouronium bromide HBr and 2-aminoethyl-*N*,*N*′-dimethylisothiouronium bromide HBr, were prepared by Dr. David G. Doherty of the Oak Ridge National Laboratories.

RESULTS

The enzymic activity of the partially purified DBH preparation (PDBH) was greatly increased by the addition of catalase to the incubation medium (Table 1). The

TABLE 1. EFFECTS OF CATALASE ON THE PARTIALLY PURIFIED DBH PREPARATION*

Addition	NE (nmole) 0·12	Fractional activity† (catalase)	Fractional inhibition (MEG)
None			
MEG ($4{\cdot}27 \times 10^{-4}$ M)	0·10		0·17
Catalase (2·5 units/sample)	2·34	19·50	
Catalase (5·0 units/sample)	4·44	37·00	
Catalase (5·0 units/sample) + MEG ($4{\cdot}27 \times 10^{-4}$ M)	0·13		0·97
Catalase (10·0 units/sample)	5·00	41·67	

* The assay was carried out for 30 min at 37° using an incubation medium with the following ingredients: 94·3 mM KH_2PO_4, 5·8 mM K_2HPO_4, 10·0 mM fumarate and 1·0 mM ascorbate at pH 6·0. The [1-^{14}C]DA (6·28 mCi/m-mole) concentration was 3×10^{-4} M.

† The term fractional activity is defined as follows: $a = v_t/v$, where v_t is the treated velocity and v is the untreated velocity.[12]

presence of 2·5 units of catalase/sample increased enzymic activity 20-fold. The addition of 5 and 10 units of catalase/sample increased activity further. Catalase has been shown to protect DBH inactivation by degrading the H_2O_2 formed from the autooxidation of ascorbate.[11] The effects of MEG ($4{\cdot}27 \times 10^{-4}$ M) on PDBH activity in the presence and absence of catalase are also shown in Table 1. In the absence of

catalase, MEG produced a fractional inhibition* of 0·17; the fractional inhibition of MEG in the presence of catalase was 0·97, suggesting that catalase protected MEG from oxidation.

Effect of MEG on the partially purified DBH preparation. The change in fractional inhibition as a function of preincubation time was determined with three concentrations of MEG (Fig. 1). To study rapid changes in the fractional inhibition, samples

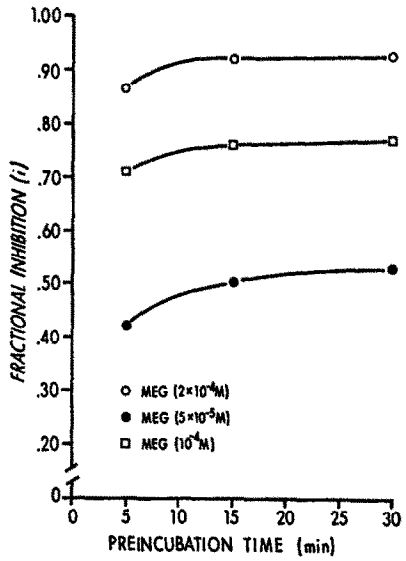

FIG. 1. Change in i as a function of preincubation time with three concentrations of MEG. The PDBH was preincubated with MEG for 5, 15 and 30 min; the reaction was initiated by the addition of [1-^{14}C]DA (10^{-3} M) and allowed to proceed for 5 min.

containing PDBH and MEG were preincubated for 5, 15 and 30 min. The reaction was initiated by the addition of [1-^{14}C]DA and allowed to proceed for a short time period (5 min). Maximal inhibition was produced within 15 min of preincubation at the two highest concentrations of MEG; at the lowest MEG concentration, inhibition was almost complete after 15 min of preincubation and complete after 30 min. The major change in fractional inhibition occurred between 0 and 5 min of preincubation with all concentrations.

Time courses of NE synthesis by PDBH were determined in the presence and absence of MEG ($4{\cdot}27 \times 10^{-5}$ M) (Fig. 2). The character of the curves was unchanged whether or not MEG was present; the inhibitor did not affect the stability of the enzyme.

Changes in the fractional inhibition of PDBH by MEG with dilution are shown in Table 2. MEG was preincubated with PDBH for 30 min at 37°, after which 1:2 and 1:4 dilutions of the mixture with medium were incubated for 5 min. The fractional inhibition decreased from 0·20 with no dilution to 0·06 with a 1:4 dilution, indicating that MEG inhibited DBH reversibly.

* 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.[12]

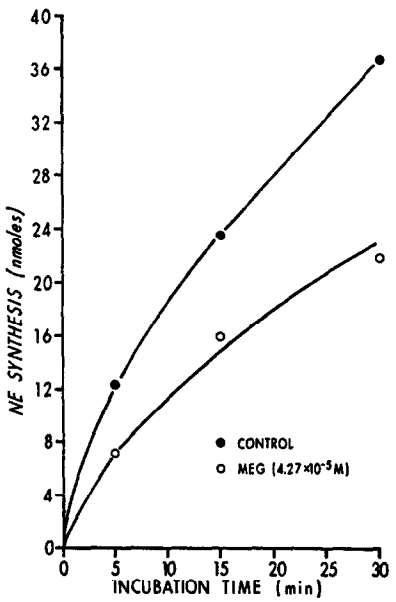

FIG. 2. Time course of NE synthesis in the presence and absence of MEG. [1-^{14}C]DA (10^{-3} M).

The relationship of fractional inhibition as a function of p[MEG]* is shown in Fig. 3. In the situation of a single inhibitor molecule interaction ($E + I \rightleftarrows EI$), the ratio (R) between the inhibitor concentration causing 90 per cent inhibition and 10 per cent inhibition is 81. The value of R for MEG was approximately 30, suggesting that more than one inhibitor is involved in the inhibition.[13] This is also shown by the non-linear plot of $1/i$ vs MEG concentration (Fig. 4a). A plot of $1/i$ vs $1/[I]^2$

TABLE 2. EFFECTS OF DILUTION OF THE PDBH–MEG MIXTURE ON FRACTIONAL INHIBITION*

Dilution	Fractional inhibition
None	0·20
1:2	0·12
1:4	0·06

* MEG was preincubated with the partially purified DBH preparation (PDBH) for 30 min, after which time the mixture was diluted two or four times with medium substrate added and incubated for 5 min. The undiluted concentration of MEG was $1{\cdot}5 \times 10^{-5}$ M. The [1-^{14}]DA concentration was 10^{-3} M.

* p[MEG] = − log[MEG], where [MEG] is the MEG concentration.

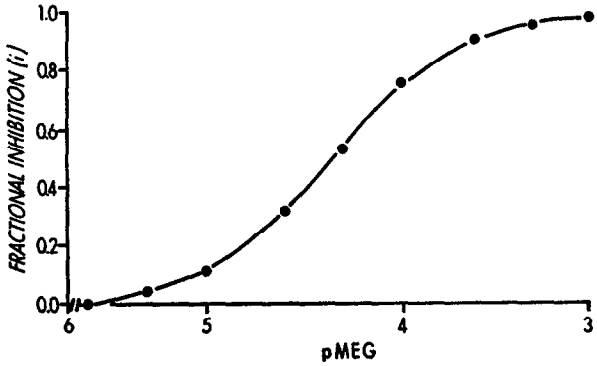

FIG. 3. Plot of i as a function of MEG concentration (p[MEG]). The assay was performed with a [1-^{14}C]DA concentration of 10^{-3} M. Samples were preincubated for 5 min and incubated for 30 min. The curve spans only two orders of magnitude.

gave a straight line, indicating that two inhibitor molecules were involved in the inhibition of DBH by MEG (Fig. 4b).* Since evidence has been presented that MEG inhibits DBH by binding with the Cu of DBH,[7] and kinetics describing a two-inhibitor molecule interaction† suggest that two Cu ions are available at the enzyme active site for binding with MEG.

Reversal of MEG inhibition of DBH activity with Cu^{2+} is shown in Fig. 5. MEG ($4{\cdot}27 \times 10^{-5}$ M) was preincubated with various concentrations of Cu^{2+}. The fractional inhibition (i) of MEG ($4{\cdot}27 \times 10^{-5}$ M) decreased progressively with increasing Cu^{2+} concentration (p[Cu^{2+}]) (Fig. 5). The addition of 3×10^{-5} M Cu^{2+} prevented the inhibition of DBH by MEG. In the absence of MEG, increasing concentrations of Cu^{2+} caused a progressive increase in i.

Several compounds, including a variety of congeners of MEG, were tested to study the structure–activity relationship (SAR) of DBH inhibition (see Fig. 7). All com-

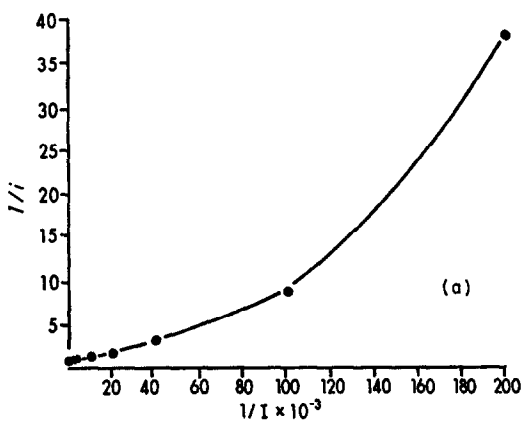

FIG. 4a.

* See the application by Clark[14] of the treatment of Langmuir.[15]

† The term "two-inhibitor molecule interaction" is used to describe the interaction of two inhibitor molecules per active site of the enzyme.

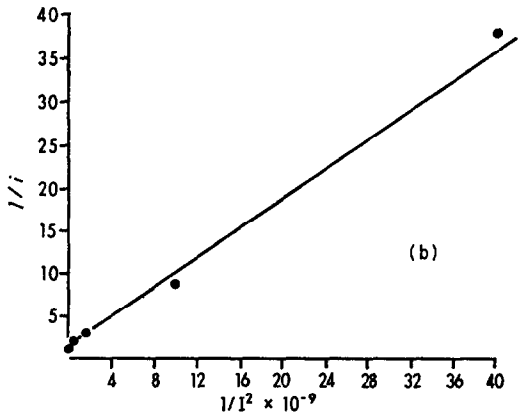

FIG. 4b.

FIG. 4. Plot of $1/i$ vs $1/[I]$ and $1/i$ vs $1/[I]^2$. The data used in the plot of i vs p [MEG] (Fig. 3) were recalculated for the plot of: (a) $1/i$ vs $1/[I]$ where $1/i$ is the reciprocal of the fractional inhibition i and $1/[I]$ is the reciprocal of the inhibitor concentration [MEG] and (b) $1/i$ vs $1/[I]^2$ where $1/i$ is the reciprocal of the fractional inhibition and $1/[I]^2$ is the reciprocal of the square of the inhibitor concentration [MEG].

pounds were introduced at a concentration of $4{\cdot}27 \times 10^{-5}$ M. MEG produced an i of 0·49; two guanidine compounds, methylguanidine and guanethidine, produced insignificant increases in DBH activity as did glutathione. For mercaptoethylamine (MEA) $i = 0{\cdot}72$, and for 3-mercaptopropylguanidine (MPG) $i = 0{\cdot}46$. The *N*-methyl derivative of MPG ($i = 0{\cdot}32$) and the *N*-methyl and *N,N'*-dimethyl derivatives of MEG ($i = 0{\cdot}32$ and 0·19, respectively) were less potent inhibitors than MEG. Thiophenol was the most potent inhibitor of DBH ($i = 0{\cdot}92$) tested; disulfiram, an established inhibitor of DBH,[5] was less potent ($i = 0{\cdot}16$). Finally, reserpine was without effect ($i = 0{\cdot}04$).

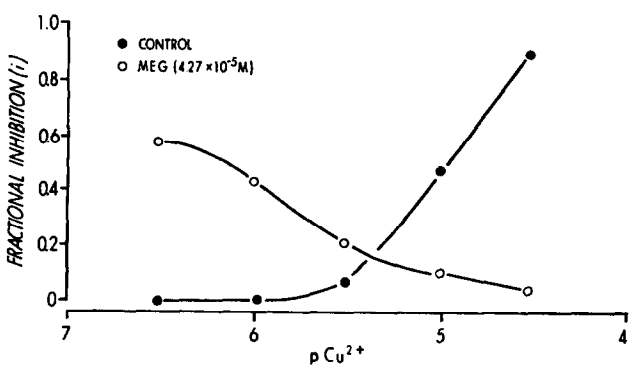

FIG. 5. Fractional inhibition i as a function of the concentration of Cu^{2+} in the presence or absence of MEG. Increasing concentrations of Cu^{2+} reversed the MEG-induced inhibition of DBH. $p[Cu]^{2+} = -\log[Cu^{2+}]$.

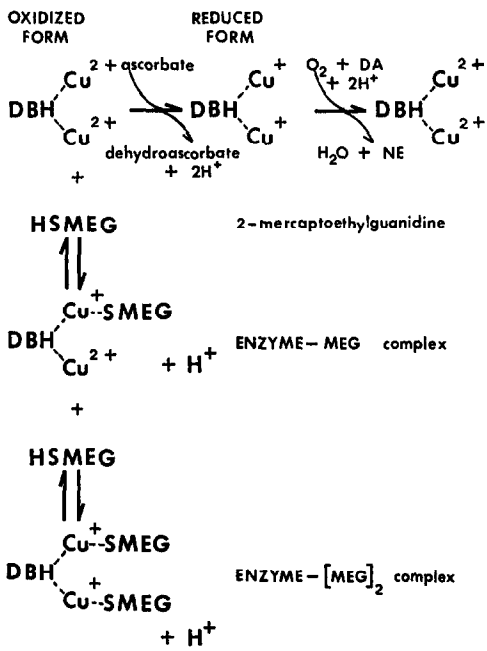

FIG. 6. Model for the inhibition of DBH by MEG. The model illustrates the β-hydroxylation reaction which converts DA to NE and the inhibition of DBH by MEG. MEG inhibits by a two-inhibitor molecule interaction at the enzymic Cu; the presence of one MEG molecule prevents the formation of product ($\beta = 0$). HSMEG, 2-mercaptoethylguanidine.

DISCUSSION

Previous work has demonstrated that MEG decreased NE synthesis in intact granules through some mechanism not involving the DA uptake system. Furthermore, MEG reduced intragranular radioactive NE/DA ratios below control levels; a direct inhibitory effect on DBH was suggested.[7]

It was possible to obtain both stimulation and inhibition of DBH by MEG with a crude DBH preparation. The stimulatory effect could not be explained, but the inhibitory effect appeared to result from binding of the Cu of the enzyme.[7] The crude DBH preparation contained numerous unknown contaminants which may have given misleading information about the behavior of MEG toward DBH. Thus, the present work dealt with the interaction of MEG with a partially purified DBH preparation.

The fractional inhibition of MEG decreased progressively with increasing Cu^{2+} concentration (Fig. 5). Equal concentrations of Cu^{2+} and MEG completely prevented the MEG inhibition of DBH, supporting the hypothesis that the mechanism of inhibition of DBH by MEG is through the binding of the Cu of DBH. Inhibition of DBH by binding of the enzymic Cu has been suggested for several sulfur-containing compounds: diethyldithiocarbamate and disulfiram,[5] aromatic and alkyl thioureas,[16] dimethyldithiocarbamate,[17] D-cysteine, L-cysteine, mercaptoethanol and coenzyme A.[18]

The kinetics describing the inhibition of DBH by MEG indicated that two inhibitor molecules interact with one active site of the enzyme. A simplified model for this type

of interaction assumes noncompetitive inhibition, where the inhibitor does not affect substrate binding.[13] The equation for fractional inhibition (i) in this case is:

$$i = \frac{(1 - \beta)[I'] + ([I']^2/\alpha)}{1 + [I'] + ([I']^2/\alpha)} \tag{1}$$

where α represents the change in inhibitor binding due to the attachment of the first inhibitor molecule, β represents the reduction in the rate of product formation due to the binding of one inhibitor molecule, and $[I']$ the specific concentration of inhibitor $([I]/K_i)$*.[13]

When the interaction of the enzyme with the first inhibitor molecule prevents the formation of product ($\beta = 0$) the following equation results:

$$i = \frac{[I'] + ([I']^2/\alpha)}{1 + [I'] + ([I']^2/\alpha)} \tag{2}$$

When α is low, i.e. when the second inhibitor molecule binds more strongly than the first,† the fractional inhibition (i) approaches the expression:[13]

$$i = \frac{[I]^2}{\alpha K_i^2 + [I]^2}$$

rearranging:

$$\frac{1}{i} = 1 + \alpha K_i^2 \left(\frac{1}{[I]^2}\right) \tag{3}$$

A plot of $1/i$ vs $1/[I]^2$ should be a straight line with a slope of αK_i^2. When the reciprocal of the fractional inhibition ($1/i$) was plotted vs the reciprocal of the square of the MEG concentration ($1/[I]^2$), a linear relationship was observed (Fig. 4b), indicating a two-inhibitor molecule interaction.

Studies of the mechanism of the MEG inhibition of DBH indicated that MEG inhibits DBH by binding with the Cu of the enzyme. Kinetic studies describing a two-inhibitor molecule interaction suggest that two Cu ions are available at the enzyme active site for binding with two MEG molecules.

Friedman and Kaufman[4] demonstrated that two Cu^{2+} are reduced for each ascorbate molecule oxidized and that two Cu^{+} are oxidized for each molecule of product formed. In other words, two Cu ions are present at the enzyme active site involved in the β-hydroxylation of DA. The kinetic studies of the inhibition of DBH by MEG described above agree with the proposal of Friedman and Kaufman that two Cu ions are available at the enzyme active site. A model for the inhibition of DBH by MEG is shown in Fig. 6. The model illustrates the hydroxylation reaction in which DA is converted to NE.[4] Also depicted is the inhibition of DBH by MEG, which indicates that: (1) MEG inhibits by binding enzymic Cu, (2) two MEG molecules are involved in the inhibition, and (3) the presence of one MEG molecule prevents the formation

* $[I]$ is the inhibitor concentration and K_i is the dissociation constant for the enzyme–inhibitor complex.

† For example, the attachment of the first MEG molecule may induce a change in the conformation of the enzyme active site, enabling a greater accessibility of the enzymic Cu for binding with the second molecule of MEG.

of product ($\beta = 0$). The ionic species of Cu that interacts with MEG is not known; the model shows the interaction of MEG with the oxidized form of DBH.

The expression derived above for a two-inhibitor molecule interaction,[13] i.e. $i = [I]^2/(\alpha K_i^2 + [I]^2)$, where $\beta = 0$ and α is low, indicates that the fractional inhibition (i) is a function of the inhibitor concentration and not the enzyme concentration. Therefore, dilution of the enzyme–inhibitor mixture should lead to a decrease in fractional inhibition since the inhibitor concentration is decreased. This is consistent with the results shown in Table 2. The expression also indicates that dilution of the enzyme alone has no effect on the degree of inhibition. Thus the inhibition of NE synthesis by intact granules and by a lysed preparation can be compared, assuming that the intragranular concentration of inhibitor equals the extragranular concentration at the start of incubation.[7]

In conclusion, the inhibition of DBH by MEG, indicating that two Cu ions are present at the enzyme active site, is in agreement with the work of Friedman and Kaufman.[4] A model of the enzyme active site based on the work of Friedman and Kaufman and the results presented here can be postulated (upper portion of Fig. 7). Two points of attachment between DBH and dopamine are depicted: an anionic site which binds the positively charged amino group and an aromatic site which interacts with the π-electrons of the catechol nucleus; ring hydroxyl groups might also function in the binding at this site. The β-carbon of DA is located near the Cu ions which function in the β-hydroxylation of DA to form NE.

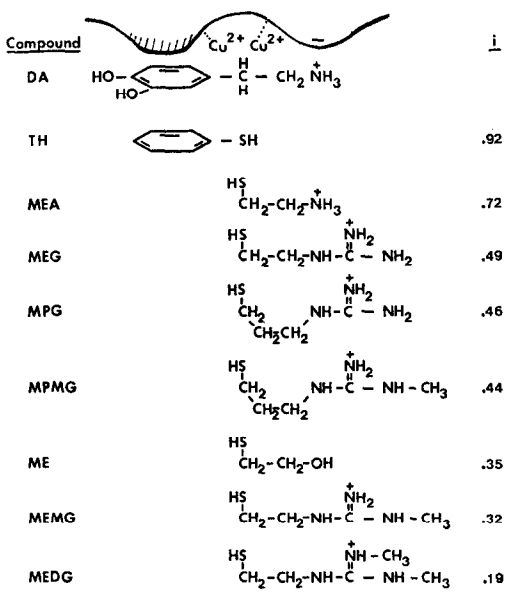

FIG. 7. Structure–activity relationship of the MEG-induced inhibition of DBH with respect to the postulated enzyme active site. The compounds are arranged in decreasing order of their fractional inhibitions (i). DA = dopamine; TH = thiophenol; MEA = 2-mercaptoethylamine; MEG = 2-mercaptoethylguanidine; MPG = 3-mercaptopropylguanidine; MPMG = 3-mercaptopropyl-*N*-methylguanidine: ME = 2-mercaptoethanol; MEMG = 2-mercaptoethyl-*N*-methylguanidine; MEDG = 2-mercaptoethyl-*N*,*N*′-dimethylguanidine.

Several congeners of MEG and other compounds were used to study the structure–activity relationship of the MEG inhibition of DBH. Two guanidines, guanethidine and methylguanidine, had insignificant effects on DBH activity, indicating that the guanido group alone is devoid of inhibitory activity. Of particular interest were the effects of several sulfhydryl compounds. Figure 7 depicts the postulated enzyme active site described above and the structures of several sulfhydryl compounds in decreasing order of their fractional inhibitions (*i*) of PDBH; for simplicity only a single-inhibitor molecule interaction is described. Thiophenol exhibited strong inhibition of PDBH. Its activity can be interpreted according to the model as follows: (1) π-electrons of the aromatic ring could interact with the postulated aromatic site of the enzyme, and (2) an aromatic sulfhydryl compound is more readily ionized than that of an aliphatic sulfhydryl and may have a greater affinity for enzymic Cu. Mercaptoethylamine (MEA) and MEG had a greater fractional inhibition than mercaptoethanol, suggesting that the positive charges of the amino or guanido groups may function in the orientation of the inhibitor molecule at the enzyme active site (Fig. 7). According to the model, the postulated anionic site which functions in the binding of the substrate may aid in the orientation of the inhibitor molecule. Further, the fact that the fractional inhibition of MEG was between the fractional inhibition of MEA and that of mercaptoethanol, an uncharged molecule, indicates that the intramolecular distance between the positive charge of MEG or MEA and the sulfhydryl group affects the degree of inhibition. The fractional inhibition of 3-mercaptopropylguanidine was about the same as that of MEG, which may have resulted from puckering of the 3-carbon chain; thus the intramolecular distance between the guanido and sulfhydryl groups of MEG and 3-mercaptopropylguanidine would be approximately the same. The conformation of a molecule bound to a protein may differ from its conformation in solution. The *N*-methyl derivative of 3-mercaptopropylguanidine was only slightly less inhibitory than the parent compound (Fig. 7), suggesting that the methyl group does not hinder the binding of the inhibitor to DBH. The *N*-methyl and *N,N'*-dimethyl derivatives of MEG, on the other hand, were significantly less inhibitory than MEG, indicating that the presence of methyl groups on the guanido group of MEG hinder the binding of the molecule to DBH. The reason for the difference in response to *N*-methylation between MEG and 3-mercaptopropylguanidine may be that the 3-carbon chain is more flexible than the 2-carbon chain of MEG, and thus the MEG effect may be more easily affected by the presence of *N*-methyl groups.

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