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SUBSTRATES AND INHIBITORS OF DOPAMINE-β-OXIDASE

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SUMMARY

The enzyme dopamine-β-oxidase not only effects the hydroxylation of dopamine to norepinephrine, but accepts as substrates a wide variety of phenethylamine derivatives such as epinine, *m*-tyramine and their branched α-methyl derivatives, *m*-methoxytyramine, which is converted to normetanephrine, 3,5-dimethoxytyramine, and to some extent even mescaline. The fact that many well-known sympathomimetic drugs are good substrates for the enzyme raises the possibility that compounds such as amphetamine, paredrinol, paradrine and α-methyl-*m*-tyramine owe some of their activity to the corresponding metabolites which are all derivatives of ephedrine. The non-specificity of dopamine-β-oxidase prompted a search for competitive or specific inhibitors. Benzylhydrazine, isosteric with phenethylamine, inhibited the enzyme strongly at concentrations of 10^{-5} *M*. The therapeutic and clinical implications of this observation are pointed out.

INTRODUCTION

Substrates and inhibitors of dopamine-β-oxidase

The enzyme, dopamine-β-oxidase, which catalyzes the final step in the conversion of tyrosine to norepinephrine, has now been purified from beef adrenals and many of its requirements have been ascertained[1]:

Fig. 1. tyrosine → DOPA → dopamine → norepinephrine.

The enzyme has also been demonstrated in brain[2] and is presumably distributed throughout sympathetically innervated tissues. In a previous report from this laboratory[3] it was shown that the enzyme is not specific for dopamine but catalyzes equally well the conversion of tyramine to norsynephrine. Subsequent studies[4–6] have shown that several other phenethylamines also serve as substrates of the enzyme.

The present study was undertaken to evaluate more fully the substrate requirements of purified dopamine-β-oxidase, gain an understanding of the mechanism of the hydroxylation reaction and to assist in the design of specific inhibitors of this enzyme. The relative non-specificity of the enzyme for β-phenethylamines suggested

the study of compounds isosteric with respect to the side chain. It has now been found that compounds of the type, $C_6H_5CH_2—X—NH_2$ are potent inhibitors of dopamine-β-oxidase.

Many of the known drugs which previously have been implicated in the pharmacology of the sympathetic nervous system have also been evaluated as substrates and inhibitors of this enzyme.

METHODS

Enzyme activity was measured in an incubation mixture containing the following components (in μmoles): potassium phosphate buffer (pH 5.5), 100; ascorbic acid, 10; fumaric acid, 12; tyramine hydrochloride, 10; enough crystalline catalase to give maximal stimulation of the reaction rate*, and 20–40 μg of the purified enzyme. The final volume was adjusted to 1.0 ml and the mixture was incubated for 10–30 min in an open tube at 36°. The enzyme activity was a linear function of both time and enzyme concentration. Relative activities of other substrates were determined by comparison with tyramine in simultaneous control incubations using the same amount of enzyme. With poor substrates larger amounts of enzyme were used at the incubation time was extended to 60 min. The reaction was stopped by the addition of 0.1 ml of 3.0 *M* trichloroacetic acid and a 1.0-ml aliquot of the acidified reaction mixture** was transferred to a Dowex-50-8X, H^+ column (3 × 0.25 cm). The column was washed with 10 ml of water and the amines eluted with 3.0 ml of 3 *N* ammonium hydroxide.

The norsynephrine formed from tyramine was assayed on an aliquot of the column eluate by periodate oxidation and measurement of the absorbancy of the *p*-hydroxybenzaldehyde formed[7]. To 1.0 ml of the column eluate was added 0.3 ml of a 2% solution of sodium periodate and the oxidation allowed to proceed for 4 min; 0.3 ml of a 10% solution of sodium metabisulfite was then added to reduce the excess periodate. The absorbancy of this solution was measured at 330 mμ. The recovery of authentic samples of norsynephrine carried through the entire isolation and assay was 95–100%. Similar assays based on periodate cleavage were performed whenever possible on the products formed from other substrates (see Table I).

Products obtained from noncatecholic substrates were prepared for chromatography by evaporation of the column eluate under a stream of nitrogen and dissolution of the residue in 0.1 ml absolute alcohol. The alcoholic solutions were centrifuged to separate undissolved salts, and an aliquot of the supernatant was spotted on Whatman No. 1 paper for chromatography. Catechol products were prepared by elution of the column with 10 ml of 2 *N* hydrochloric acid followed by lyophilization to remove the hydrochloric acid. The residue was taken up in acid methanol for chromatography. The products of periodate cleavage were prepared for chromatography by extraction of the resulting aldehyde or ketone into chloroform. The carbonyl compounds were identified by thin-layer chromatography on silica-gel G

* The catalase solution used in these experiments was prepared by making a saturated aqueous solution of crystalline bovine catalase. Aliquots from 0.005 to 0.02 were found to give maximal activity.

** No visible precipitate formed with this amount of enzyme upon addition of trichloroacetic acid and centrifugation was not required.

TABLE I

PROPERTIES OF SUBSTRATES AND PRODUCTS

Solvent systems: (1) butanol–acetic acid–water (120 : 30 : 50); (2) methanol–butanol–benzene–water (2 : 1 : 1 : 1); (3) *sec.* butanol–formic acid–water (75 : 15 : 10); (4) Thin layer chromatograph, silica gel-G; ethanol–dioxane–ammonium hydroxide (1 : 8 : 1); (5) phenol–0.1 *N* HCl, saturated with SO_2, 80 g/20 ml. (6) Thin layer chromatograph, silica gel-G; benzene–ethyl acetate (3 : 1) and for 3,4,5-trimethoxybenzaldehyde benzene–ethyl acetate (9 : 1). Spray reagent 2% 2,4-dinitrophenylhydrazine in ethanol. Color tests: ninhydrin spray; 0.2% ninhydrin in acetone containing 2% pyridine. DPNA, diazotized *p*-nitroaniline.

Amine substrates and products	*R_F values in solvent system*					*Color tests*		*Aldehyde formed on periodate cleavage*	*R_F values (b)*		
	1	*2*	*3*	*4*	*5*	*Ninhydrin*	*DPNA*		*Authentic*	*Found*	$\lambda_{max.}$
Tyramine	0.71	0.74				Blue	Purple				
Norsynephrine	0.61	0.68				Brown	Red	*p*-Hydroxybenzaldehyde	0.61	0.60	332
Paredrine	0.78	0.79				Yellow					
p-Hydroxynorephedrine	0.70	0.75				Purple		*p*-Hydroxybenzaldehyde	0.61	0.60	332
3-Methoxytyramine	—	0.67									
Normetanephrine	0.36	0.59					Purple	Vanillin	0.57	0.55	348
Mescaline	0.68					Blue					
β-Hydroxymescaline	0.55					Purple		3,4,5-Trimethoxybenzaldehyde	0.74	0.71	
Phenethylamine	0.70					Blue					
Phenylethanolamine	0.65					Russet		Benzaldehyde			
N-methyltyramine	—										
Synephrine	0.48						Red	*p*-Hydroxybenzaldehyde	0.61	0.60	332
m-Tyramine											
m-Hydroxyphenylethanolamine								*m*-Hydroxybenzaldehyde	0.77	0.74	364
3-Methoxy-6-hydroxy-tyramine			0.33								
6-Hydroxynormetanephrine			0.15								
4-Methoxy-*m*-tyramine											
4-Methoxyaramine								Iso-vanillin	0.48	0.47	—
3,5-Dimethoxytyramine											
3,5-Dimethoxyphenylethanolamine								Syringaldehyde	0.56	0.55	370
α-Phenylpropylamine				0.58		Red					
				0.33		Red					
p-Hydroxyphenylpropylamine				0.47		Blue					
				0.28		Blue					
Dopamine	0.41				0.71		Purple				
Norepinephrine	0.23				0.29		Pink				
Epinine	0.45						Blue				
Epinephrine	0.37						Pink				
α-Methyldopamine	0.60				0.54		Purple				
Cobefrine	0.50				0.35		Yellow				
β-Methyltyramine								*p*-Hydroxyacetophenone	0.43	0.40	324

(Brinkmann Instruments Inc.) and by vapor phase chromatography on a 3-ft column of 1.5% SE-30 on 100–140 mesh chromasorb W (Applied Science Laboratories) at temperatures from 90–150°. The latter proved particularly useful for the qualitative and quantitative assay of benzaldehyde, *p*-methoxybenzaldehyde, 3,4-dimethoxybenzaldehyde and 3,4,5-trimethoxybenzaldehyde.

Catechol amines were assayed directly on an aliquot of the acidified incubation mixture by the trihydroxyindole method of VON EULER AND FLODING[8], adapted to the Aminco-Bowman spectrophotofluorometer.

Inhibitors were evaluated with the standard reaction mixture using tyramine as substrate. The mixture, without tyramine, was preincubated for 10 min with the inhibitor. Tyramine was then added and the mixture was incubated for another 20 min. Appropriate blanks containing inhibitors and no tyramine were carried through this procedure. Since the enzyme activity deteriorated by about 10–15% during the 10-min preincubation, even in the absence of inhibitors, comparisons were made with controls which were also preincubated 10 min before the addition of substrate.

MATERIALS

Dopamine, norepinephrine, epinephrine, tyramine, *N*,*N*-dimethyltyramine (hordenine), phenethylamine, phenethanolamine, tryptamine, serotonin, DL-tyrosine and *p*-hydroxyphenylacetic acid were purchased from Calbiochem; *m*-tyramine from Cyclo Chemical Corp., 3,4-dimethoxyphenethylamine and syringaldehyde from Aldrich Chemical Co., isovanillin from K & K Laboratories, the other aldehydes, *p*-hydroxyacetophenone, *p*-hydroxyphenylethane and ethylbenzene from Eastman Organic Chemicals. We wish to thank the following for making available important compounds: Burroughs-Welcome Laboratories for epinine; the Sterling-Winthrop Research Institute for α-methyldopamine, DL-cobefrine, 3-methoxytyramine, normetanephrine, 4-methoxy-*m*-tyramine, and 3,5-dimethoxytyramine; the Upjohn Corp. for β-methyltyramine; Hoffmann-La Roche & Co. for mescaline and β-hydroxymescaline; Lilly Research Laboratories for γ-phenylpropylamine; Smith, Kline and French Laboratories for DL-α-methyltyramine; and Merck, Sharpe and Dohme for α-methyl-*m*-tyramine.

The following compounds were synthesized according to standard methods: *o*-tyramine[9], *N*-methyltyramine[10], 3-methoxy-α-methyltyramine[11], 3-methoxy-6-hydroxytyramine[12], 6-hydroxynormetanephrine[13], *p*-methoxyphenethylamine[14], *p*-hydroxy-γ-phenylpropylamine[15], *p*-aminophenethylamine[16], DL-tyrosine ethyl ester[17], *N*-acetyltyramine[18], *p*-hydroxyphenylethanol (tyrosol)[16], and *p*-hydroxyphenylmethylcarbinol[19].

Dopamine-β-oxidase was prepared from fresh bovine adrenal glands according to the method of LEVIN, LEVENBERG AND KAUFMAN[1]. The fractions eluted from calcium phosphate gel between 0.05 and 0.1 *M* phosphate buffer were pooled, divided into 5-ml portions, and stored at −20°. The enzyme activity was stable at this temperature for several months. Aliquots containing 20–80 μg of protein, with a specific activity of approx. 37 mμmoles/μg of protein/10 min, were added to the standard incubation mixture.

RESULTS

Substrate studies

A variety of compounds were tested as substrates of dopamine-β-oxidase. Table I summarizes the criteria used for identification of the β-hydroxylated products. Comparison of the relative activities of the various substrates with that of tyramine is shown in Table II. It must be pointed out that the comparisons were made at pH 5.5, the optimum pH for conversion of tyramine to norsynephrine. The relative activities would be expected to differ at other pH values. For example, the pH optimum for dopamine is 6.2.

TABLE II

RELATIVE ACTIVITY OF SUBSTRATES OF DOPAMINE-β-OXIDASE

Relative activity is based on *p*-tyramine which was arbitrarily taken as 100. Values reported as + indicate that the expected product was formed but that the amounts were too small to permit quantitation with available methods.

Substrate	*Relative activity*
p-Tyramine	100
m-Tyramine	60–80
o-Tyramine	0
Dopamine	85–90
Epinine	25
N-Methyltyramine	25
N,*N*-Dimethyltyramine	0
α-Methyltyramine	60–70
α-Methyl-*m*-tyramine	50–70
α-Methyldopamine	50–60
β-Methyltyramine	5–10
3-Methoxytyramine	50–70
3-Methoxy-α-methyltyramine	5–10
4-Methoxy-*m*-tyramine	+
3-Methoxy-6-hydroxytyramine	0
3,5-Dimethoxytyramine	25–30
3,4,5-Trimethoxy-β-phenethylamine (mescaline)	2–5
p-Amino-β-phenethylamine	0
β-Phenethylamine	10–15
p-Methoxyphenethylamine	2–5
3,4-Dimethoxyphenethylamine	2–5
γ-Phenylpropylamine	+
p-Hydroxy-γ-phenylpropylamine	++
p-Hydroxy-β-phenethanol	0
p-Hydroxy-β-phenylacetic acid	0
Ethylbenzene	0
p-Hydroxy-β-phenylathene	0
p-Tyrosine	0
p-Tyrosine ethyl ester	0
N-Acetyltyramine	0
Tryptamine	0
5-Hydroxytryptamine (serotonin)	0

The unsubstituted parent compound, phenethylamine, is a substrate. Substitution of hydroxyl groups on the ring, in the *meta* or *para* position or both, increases the activity several fold. By contrast, ring methoxy groups decrease activity. It is of

interest, however, that mescaline which is a trimethoxy-β-phenethylamine is oxidized to β-hydroxymescaline. 3-Methoxytyramine which is a normal metabolite of dopamine is readily converted to normetanephrine. Substitution on the aromatic nucleus by an amino group abolishes all activity.

With respect to substitutions on the phenethylamine side chain, it is apparent that methylation of the nitrogen diminishes activity, the *N,N*-dimethyl compound being totally inactive. Substitution of methyl groups on the α position of the side chain lowers activity less than substitution on the β position but compounds with such substitution are still hydroxylated by the enzyme. Lengthening the side chain by one carbon to a propylamine group does not abolish all activity. However, the products of the phenylpropylamones were not identified nor were the results quantified since standards were not available. Nevertheless, incubation of the phenylpropylamines consistantly produced small amounts of products with properties consistent with those of the expected oxidation products. For example, the product formed from *p*-hydroxyphenylpropylamine was a phenolic primary amine which was stable to the action of periodate, suggesting that the product formed was γ-hydroxy-γ-*p*-hydroxyphenylpropylamine.

The side chain apparently must be basic, since of all the compounds tested only primary and secondary amines were substrates. However, tyrosine ethyl ester which is also basic was not a substrate. The only other aromatically substituted ethyl amines studied were indolethylamines. However tryptamine and serotonin were apparently not substrates. Ethylbenzene which is oxidized *in vivo* on the β carbon to yield phenylmethylcarbinol[20] was not a substrate of dopamine-β-oxidase. This would suggest the presence of another enzyme which can carry out β-oxidation.

Inhibitor studies

The relative lack of specificity of dopamine-β-oxidase makes available many compounds which can inhibit oxidation of the natural substrate by competing as substrates for the same enzyme. Many compounds in Table II were indeed shown to inhibit oxidation of dopamine and tyramine. Results of a kinetic study of the effects of dopamine on tyramine oxidation are shown in Fig. II. Values for the K_m and K_i of dopamine were found to be $5.8 \cdot 10^{-3}$ and $6.0 \cdot 10^{-3}$ when dopamine was used as an inhibitor of norsynephrine formation showing that this is a competitive inhibition.

The lack of specificity of the enzyme also suggests that many related compounds which are not substrates might have an appreciable affinity for the enzyme and thereby act as inhibitors. Thus, tryptamine and serotonin produce some inhibition at 10^{-3} *M*. Whether this has any physiological or pharmacological significance is yet to be determined. The data in Table III show that none of the drugs and chemical agents which have been implicated previously in the pharmacology of the sympathetic nervous system have much activity below 10^{-3} *M*. Thus, it is questionable whether guanethidine, TM-10 or bretyllium owe their action through inhibition of dopamine-β-oxidase.

Another class of compounds which suggested themselves as possible inhibitors were analogues having a side chain isosteric with ethylamine, $C_6H_5—CH_2—X—NH_2$, where a carbon atom could be replaced by hetero-atoms such as nitrogen, oxygen,

TABLE III

COMPOUNDS TESTED AS INHIBITORS OF DOPAMINE-β-OXIDASE

Each compound was preincubated for 10 min before addition of tyramine ($5 \cdot 10^{-3}$ M) as substrate. Percent inhibition was calculated from the decrease in the formation of norsynephrine from tyramine in the presence of an inhibitor. α-Methyl-DOPA, α-methyl-*o*-tyrosine and α-methyl-*m*-tyrosine were donated by Merck Sharp & Dohme. Benzylhydrazine and 1-phenyl-2-hydrazinopropane were donated by the Warner-Lambert Research Institute. Imipramine and desmethylimipramine were donated by Geigy Research Laboratories. 2,6-Xylyl ether bromide (TM-10) was donated by Dr. RICHARD CROUT. The remaining compounds were obtained from commercial sources.

Compound	*Concentration (M)*	*Inhibition (%)*
α-Methyl-DOPA	10^{-3}	0
α-Methyltyrosine	10^{-3}	5
α-Methyl-*o*-tyrosine	10^{-3}	10
α-Methyl-*m*-tyrosine	10^{-3}	25
α-Methyl-*m*-tyrosine	10^{-4}	12
α-Methyl-*m*-tyramine	$5 \cdot 10^{-3}$	20
α-Methyl-*m*-tyramine	10^{-3}	0
Imipramine	10^{-3}	5
Desmethylimipramine	10^{-2}	20
Desmethylimipramine	$5 \cdot 10^{-3}$	16
Desmethylimipramine	10^{-3}	6
Guanethidine	10^{-3}	0
Bretylium	10^{-3}	5
Reserpine	10^{-3}	0
Harmaline	10^{-3}	0
1-Phenyl-2-hydrazinoethane	10^{-3}	30
1-Phenyl-2-hydrazinopropane (JB-516)	10^{-3}	17
2,6-Xylyl ether bromide (TM10)	10^{-3}	0
Hordenine	10^{-3}	0
Tryptamine	10^{-3}	17
Serotonin	10^{-3}	57
Adrenalone	10^{-3}	50
Benzylhydrazine	10^{-3}	71
	$5 \cdot 10^{-4}$	65
	10^{-4}	50
	10^{-5}	42

sulphur, etc. The first compound of this type to become available for detailed study was benzylhydrazine. As shown in Table III benzylhydrazine inhibited appreciably at concentrations as low as 10^{-5} M. The 2 other hydrazines studied, phenethylhydrazine and phenylisopropylhydrazine, had only a fraction of the activity indicating that the phenethylamine isostere has a much greater affinity for the enzyme.

DISCUSSION

It is apparent that the action of dopamine-β-oxidase is not limited to dopamine but that this enzyme catalyzes the hydroxylation of many amines derived from phenethylamine and substituted or branched derivatives. The non-specific character of this enzyme has implications concerning the biosynthesis and metabolism of normally occurring amines. Preliminary studies in this laboratory indicate that tyramine can serve as an alternate precursor for the biosynthesis of norsynephrine as shown below:

$$\text{Tyrosine} \xrightarrow[\text{L-amino acid decarboxylase}]{\text{aromatic}} \text{tyramine} \longrightarrow \text{norsynephrine} \longrightarrow \text{norepinephrine}$$

The significance of this remains to be evaluated. Another interesting possibility is suggested by the demonstration that 3-methoxytyramine can be hydroxylated by dopamine-β-oxidase to normetanephrine. It has been shown that large amounts of 3-methoxytyramine are excreted in human urine[21]. If 3-methoxytyramine is also present in tissues its hydroxylation might substantially contribute to the excretion of normetanephrine, which heretofore has been assumed to represent metabolized norepinephrine.

Of great pharmacological interest is the finding that many well-known sympathomimetic drugs are excellent substrates of dopamine-β-oxidase and that the resultant products are in many instances also well known drugs, with higher activity. Several such pairs of drugs are shown in Table IV. It may well be that at least some

TABLE VI

DRUGS WHICH ARE SUBSTRATES OF DOPAMINE-β-OXIDASE AND THEIR CORRESPONDING HYDROXYLATED PRODUCTS

Without β-hydroxy moiety	*With β-hydroxy moiety*
Tyramine	Norsynephrine
N-Methyl-β-(*p*-hydroxyphenyl)ethylamine	Synephrine
α-Methyl-*m*-tyramine	Aramine (*m*-hydroxynorephedrine)
Paradrine (β-(*p*-hydroxyphenyl)isopropylamine)	*p*-Hydroxynorephedrine
Paredrinol (*N*-methyl-β-(*p*-hydroxyphenyl)-isopropylamine)	*p*-Hydroxyephedrine
Amphetamine	Norephedrine
Methedrine (*N*-methyl-β-phenylisopropylamine)	Ephedrine
Dopamine	Norepinephrine
Epinine	Epinephrine
α-Methyldopamine	Cobefrine (β-hydroxy-β-(3,4-dihydroxy-phenyl)-isopropylamine)
Mescaline	β-Hydroxymescaline

of the activity of the parant drug is, in each case, due to the oxidized product formed in sympathetic nervous tissue by dopamine-β-oxidase. Tyramine yields norsynephrine, which may mediate some of the actions of the parent compound. CARLSSON[22] has reported the appearance of appreciable amounts of aramine in brain following administration of the precursor α-methylmetatyramine. More recently GESSA *et al.*[23] have shown that aramine is many times more effective as a norepinephrine-releasing agent compared to the unoxidized precursor, α-methylmetatyramine. It may be desirable to reevaluate the various activities *in vitro* of these known drugs in order to determine to what extent dopamine-β-oxidase action is responsible for pharmacological activity observed *in vivo*.

The realization of the non-specificity of dopamine-β-oxidase encourages a screening program *in vitro* for compounds with affinity for the enzyme. From such a program one would expect to find potent enzyme inhibitors some of which may be sufficiently active *in vivo*. As shown in Fig. 1 the production of norepinephrine from

tyrosine involves 3 enzymic steps. Although potent decarboxylase inhibitors are now available, they do not appear to be capable of blocking effectively endogenous norepinephrine formation, because the powerful enzyme, aromatic L-amino acid decarboxylase, will not easily be inhibited to the point where decarboxylation becomes the rate-limiting step in the over-all reaction[24]. By contrast dopamine-β-oxidase is a less active enzyme and may conceivably become rate-limiting with an adequate inhibitor. Benzylhydrazine which was arrived at through a consideration of phenethylamine isosteres is a good inhibitor and may be active *in vivo*. Several compounds which appear to inhibit norepinephrine synthesis *in vivo*, according to COSTA *et al.*[25], are indeed phenethylamine isosteres, and we have shown that they are very potent inhibitors of dopamine-β-oxidase *in vitro*. Since completion of the present studies a large number of inhibitors of this type have become available, many of them much more active than benzylhydrazine.

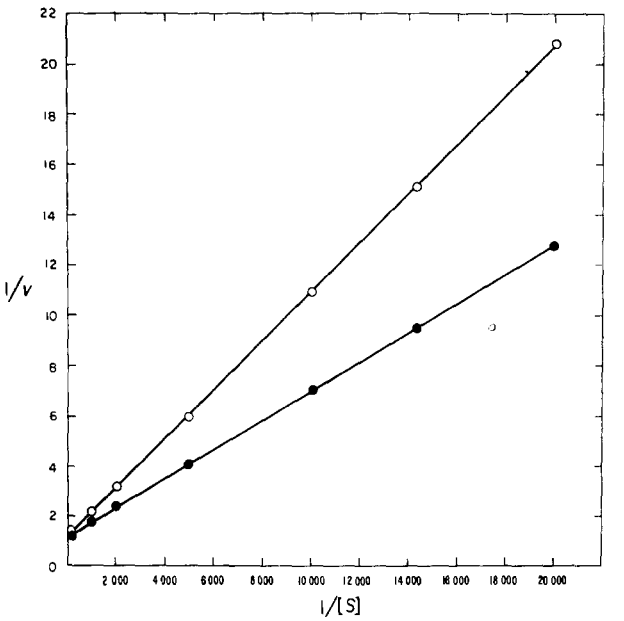

Fig. 2. The effect of a competitive substrate on the conversion of tyramine to norsynephrine. Activity was measured in the standard incubation mixture with substrate concentrations from $5 \cdot 10^{-5}$ *M* to $5 \cdot 10^{-3}$ *M* and plotted according to the Lineweaver-Burk method. ●—●, the reciprocal plot with tyramine as substrate; ○—○, with tyramine in the presence of $2.5 \cdot 10^{-3}$ *M* dopamine.

It should be pointed out that hydrazines are highly reactive substances so that many other effects *in vivo* should be expected such as pyridoxal phosphate antagonism or monoamine oxidase inhibition. Nevertheless, it already appears that the inhibitory action of benzylhydrazine on dopamine-β-oxidase is more marked than the other actions. It is hoped that other phenethylamine isosteres can be found which will be relatively specific inhibitors of this enzyme. Regardless of the therapeutic and clinical implications it should be possible at this point to utilize benzylhydrazine as a laboratory tool to investigate the feasibility of enzyme inhibition *in vivo* and to determine its biochemical and pharmacological consequences.

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