Biochemical Pharmacology, 1965, Vol. 14, pp. 837–845. Pergamon Press Ltd., Printed in Great Britain.

INHIBITORS OF PURIFIED BEEF ADRENAL TYROSINE HYDROXYLASE

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(*Received* 9 *November* 1964; *accepted* 30 *November* 1964)

Abstract—Two classes of compounds have been investigated as inhibitors of purified beef adrenal tyrosine hydroxylase. Among the aromatic amino acids tyrosine analogues were found to be most potent, particularly those having an α-methyl or 3-halogen substitution. Two normal metabolites, mono- and diiodo-tyrosine, were found to be very effective inhibitors. Inhibition by the amino acid analogues was shown to be competitive with the substrate. Catechols were also inhibitory, particularly 3,4-dihydroxyphenylpropylacetamide (compound H 22/54). Inhibition by the latter was reversed by cofactor (tetrahydrofolate or $DMPH_4$) but not by tyrosine.

SYMPATHETICALLY innervated tissues have been shown to contain a specific L-tyrosine hydroxylase which catalyzes the conversion of tyrosine to dopa,* the first step in norepinephrine biosynthesis.[1] This enzyme has been purified from beef adrenal medulla and shown to require a tetrahydropteridine† cofactor.[2] Several inhibitors of this enzyme were reported previously.[2] Some were subsequently shown to inhibit tyrosine hydroxylase *in vivo* and to lower endogenous tissue norepinephrine levels in intact animals.[3] The present report is a detailed study of two classes of tyrosine hydroxylase inhibitors, aromatic amino acids and catechols, in which the purified beef adrenal enzyme was used.

MATERIALS

We wish to thank the following for making available certain compounds used in this investigation: Merck Sharp & Dohme, for the α-methyl-tyrosine, α-methyl-dopa (Aldomet), 3-iodo-α-methyl-tyrosine, 3-bromo-α-methyl-tyrosine, 3-chloro-α-methyl-tyrosine, and α-methyl-*m*-tyrosine; Ciba, Basle, for α-methyl-3-chloro-4-methoxyphenylalanine, 3-fluoro-α-methyl-tyrosine and 3-chloro-α-methyl-tyrosine. Psychopharmacology Service Center, National Institute of Mental Health, for 3,4-dihydroxyphenylacetamide; Warner-Lambert Research Institute for 3-chloro-L-tyrosine; Hassle Laboratories, Sweden, for 3,4-dihydroxyphenylpropylacetamide (H 22/54) and related compounds. The decarboxylase inhibitor, *p*-bromo-*m*-hydroxybenzoxyamine, was kindly supplied by Smith, Nephews, Ltd. Other chemicals were obtained from commercial sources.

* Dopa: 3,4-dihydroxyphenylalanine.

† The requirement of mammalian liver phenylalanine hydroxylase for reduced pteridines and the nature of these pteridines is discussed by S. Kaufman in *Oxygenases*, O. Hayaishi, Chap. 4, pp. 129–80, Academic Press, New York (1963).

L-Tyrosine-3,5-^{3}H was obtained both from New England Nuclear Corp. (5,600 mc/mmole) and Nuclear Chicago Corp. (2,000 mc/mmole). 3-Iodo-L-tyrosine-^{131}I and diiodo-L-tyrosine-^{131}I were obtained from Nuclear Chicago and were prepared in the Radiochemical Center, Amersham; α-methyl-DL-tyrosine-^{3}H (8·1 mc/mmole) was a gift from Merck Sharp & Dohme. Each of the radioactive compounds was purified by passage over an alumina column after adjustment to pH 8·4, and the effluents were subsequently filtered through IRC-50 (Na^+) columns, buffered at pH 6·5. The radioactive amino acids were, in some instances, diluted by adding nonradioactive material.

Tetrahydrofolic acid was prepared according to the procedure of O'Dell *et al.*,[4] and stored *in vacuo* at −20°. 2-Amino-4-hydroxy-6,7-dimethylpteridine (DMP) was kindly supplied by Dr. L. Ellenbogen of Lederle Laboratories and reduced to the corresponding tetrahydropteridine ($DMPH_4$) according to the procedure of Pohland *et al.*[5] and stored at −20°. The latter part of the studies was carried out with $DMPH_4$ purchased from Calbiochem.

Tyrosine hydroxylase was prepared from beef adrenal medulla according to the procedure of Nagatsu *et al.*[2] The partially purified enzyme was suspended in 0·005 M KPO_4 buffer, pH 7·5 ,containing 5×10^{-3} M mercaptoethanol, divided into 2-ml portions and stored at −20°.

METHODS

When studying the effects of inhibitors, enzyme activity was assayed with 3,5-ditritiotyrosine as substrate and release of tritium into water was measured as described by Nagatsu *et al.*[6] Incubation mixtures were essentially the same as described previously,[2] except that the substrate concentration was reduced to 5×10^{-5} M, which is just barely saturating. To a test tube were added 50 mμmoles L-tyrosine containing 1 to 2×10^5 cpm of tyrosine-3,5-^{3}H, 5 μmoles tetrahydrofolate, 0·5 μmole $FeSO_4$ $(NH_4)_2SO_4$, 100 μmoles mercaptoethanol, 0·1 μmole *p*-bromo-*m*-hydroxybenzoxyamine, 200 μmoles 1 M acetate buffer (pH 6·0), enzyme in 0·1- to 0·2-ml volume/(0·5 to 1 mg protein), and water to 1·0 ml. The compounds tested for inhibition were preincubated for 5 min with the reaction mixture before addition of substrate, and were then incubated for an additional 15 min. Studies on the mechanism of inhibition of the various compounds were carried out without the preincubation step.

When investigating the effects of catechol analogues as inhibitors, $DMPH_4$ (1×10^{-3} M) was substituted for tetrahydrofolate and Fe^{++} in order to avoid the formation of complexes between added Fe^{++} and the catechols. However, at low concentrations of the catechol derivative, compound H 22/54, it was possible to use Fe^{++} and tetrahydrofolate to study the inhibition.

Hydroxylation of α-methyl-tyrosine was measured by fluorometric assay of the enzymatically formed α-methyl-dopa after adsorption onto alumina and elution with 0·3 M acetic acid as for dopa.[2] α-Methyl-dopa was identified by descending paper chromatography in *n*-butanol:acetic acid:H_2O (100:25:25). The radioactivity of the product was measured in a strip counter and carrier α-methyl-dopa and α-methyl-dopamine* were visualized by treating the paper with 0·25% ferricyanide and ammonia vapor.

* Dopamine: 3,4-dihydroxyphenethylamine.

3-Hydroxylation of 3-iodotyrosine-^{131}I and 3,5-diiodotyrosine-^{131}I was followed by measuring the appearance of radioactive ^{131}I. Separation of the iodotyrosines from the released radioactive iodide was accomplished with a Dowex-50 H^+ column as described for separation of tyrosine-3,5-^{3}H from tritium-labeled water.[6] Incubations were for 45 min under conditions as described above. Radioactivity was measured in a liquid scintillation counter.

RESULTS

Inhibition by amino acid derivatives

Two classes of inhibitors are described in this report, aromatic amino acids and catechols. Almost all phenylalanine and tyrosine analogues were found to be inhibitory to some degree. In every instance the D-amino acid was far less active. Some of the more interesting of these compounds are listed in Table 1. It may be noted that

TABLE 1. AMINO ACID ANALOGUES AS INHIBITORS OF TYROSINE HYDROXYLASE

Compound	Concentration required for 50% inhibition* (M)	K_I (M)
L-Tryptophan	$>1 \times 10^{-3}$	
L-Phenylalanine	2×10^{-4}	
D-Phenylalanine	$>1 \times 10^{-3}$	
3-Iodo-L-tyrosine	5×10^{-7}	$3{\cdot}9 \times 10^{-7}$
3-Chloro-L-tyrosine	1×10^{-5}	
3-Fluoro-DL-tyrosine	1×10^{-3}	
3,5-Diiodo-L-tyrosine	2×10^{-5}	$9{\cdot}3 \times 10^{-6}$
3,5-Dibromo-L-tyrosine	5×10^{-4}	
α-Methyl-L-tyrosine	$2{\cdot}5 \times 10^{-5}$	$1{\cdot}7 \times 10^{-5}$
α-Methyl-D-tyrosine	$>1 \times 10^{-3}$	
α-Methyl-*m*-DL-tyrosine	$>1 \times 10^{-3}$	
α-Methyl-DL-phenylalanine	8×10^{-5}	
3-Iodo-α-methyl-DL-tyrosine	3×10^{-7}	$1{\cdot}8 \times 10^{-7}$
3-Bromo-α-methyl-DL-tyrosine	$1{\cdot}5 \times 10^{-6}$	
3-Chloro-α-methyl-DL-tyrosine	5×10^{-6}	
3-Fluoro-α-methyl-DL-tyrosine	2×10^{-4}	
3-Chloro-4-methoxy-α-methyl-DL-phenylalanine	5×10^{-4}	

Each compound was preincubated with the reaction mixture for 5 min before addition of L-tyrosine (5×10^{-5} M) as substrate.

* Data represent a range of 45–55% inhibition. Where $>1 \times 10^{-3}$ M concentrations are indicated inhibition was less than 20% with 1×10^{-3} M inhibitor.

the α-methyl-amino acids are more potent than the unmethylated analogues. In addition a marked increase in activity is attained on substituting a halogen atom at the 3-position of the benzene ring. Thus the naturally occurring compounds, mono- and diiodo-tyrosine, are potent inhibitors. Even more potent are the α-methyl-3-halogenated tyrosines so that α-methyl-3-iodo-DL-tyrosine produces 50 per cent inhibition at 3×10^{-7} M. Assuming the D compound to be inactive, then the inhibition by the L-isomer is 50 per cent at $1{\cdot}5 \times 10^{-7}$ M. The relative activities of the 3-substituted α-methyl-tyrosine analogues were I > Br > Cl > H > F.

It was shown previously that the inhibition produced with α-methyl-L-tyrosine is competitive with substrate.[2] It was found subsequently that aromatic amino acids

generally inhibit by competition with tyrosine. This is shown in Fig. 1 for two of the more potent inhibitors, 3-iodotyrosine and 3-iodo-α-methyl-tyrosine. K_i values for some of the inhibitors are also shown in Table 1.

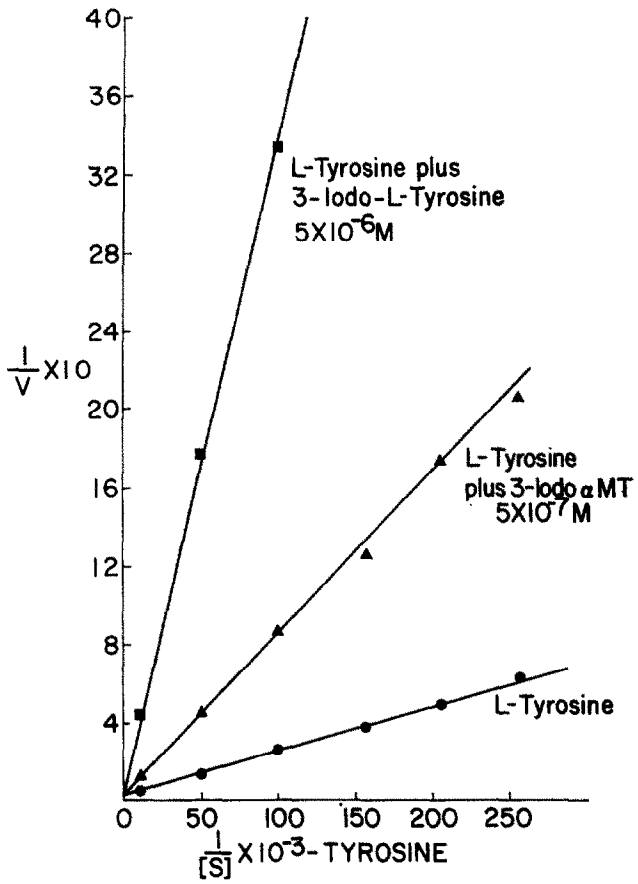


FIG. 1. Inhibition of tyrosine hydroxylase by 3-iodotyrosine and α-methyl-3-iodotyrosine. Activity was measured in the standard incubation mixture with substrate concentrations ranging from 1×10^{-4} M to $3{\cdot}9 \times 10^{-6}$ M.

Inhibition by catechol derivatives

The catechol amino acids listed in Table 2 were not very potent inhibitors, but here too the L-isomers[9] were more active. As shown in Table 2 almost all catechols produced inhibition. However, 3,4-dihydroxy phenylpropylacetamide (H 22/54) was a highly potent inhibitor. On further investigation of this compound and its analogues (Table 3) it was found that it was the most potent of its class. Substitution of smaller or larger groups for the propyl group reduced activity as did any other type of change. Compound H 22/54 was studied as a representative catechol inhibitor. As shown in Fig. 2a the inhibition was not competitive with tyrosine. However, the inhibition was reversed

upon addition of either tetrahydrofolate or $DMPH_4$. On further investigation the tetrahydropteridines and catechols were shown to be competitive (Fig. 2b). The possibility that the catechol inhibition was due to complexing the iron is not likely, since compound H 22/54 at 10^{-5} M inhibited just as well in the presence of 10^{-4} M Fe^{++}.

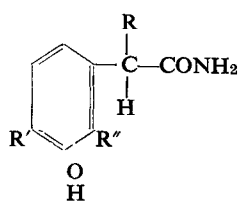
TABLE 2. CATECHOL ANALOGUES AS INHIBITORS OF TYROSINE HYDROXYLASE

Compound	Concentration for 50% inhibition* (M)
Catechol	5×10^{-3}
L-Norepinephrine	1×10^{-3}
3,4-Dihydroxyphenylacetamide	1×10^{-3}
3,4-Dihydroxyphenylpropylacetamide (H-22/54)	2×10^{-5}
3,4-Dihydroxy-L-phenylalanine	2×10^{-3}
3,4-Dihydroxy-D-phenylalanine	$>4 \times 10^{-3}$
α-Methyl-3,4-dihydroxy-L-phenylalanine (Aldomet)	$1{\cdot}5 \times 10^{-3}$
α-Methyl-3,4-dihydroxy-D-phenylalanine	$>8 \times 10^{-3}$

Each compound was preincubated with the reaction mixture for 5 min before addition of L-tyrosine (5×10^{-5} M) as substrate.

* Data represents a range of 45–55% inhibition.

TABLE 3. THE EFFECT OF VARIOUS SUBSTITUENTS ON INHIBITORY ACTIVITY OF CATECHOLS



R	R′	R″	Relative inhibition
H	OH	H	1
CH_3	OH	H	0
C_2H_5	OH	H	50
n-C_3H_7	OH	H	100
iso-C_3H_7	OH	H	48
n-C_4H_9	OH	H	76
iso-C_4H_9	OH	H	70
OH	OH	H	2
OCH_3	OH	H	6
OC_2H_5	OH	H	4
n-C_3H_7	H	OH	6
H	H	H	8

Compound H 22/54 (R = n-C_3H_7; R′ = OH and R″ = H) is arbitrarily taken as 100. Incubations were as described in Table 2. The compounds in which R represents a substituent other than hydrogen are optically active. The racemic mixture was used in each case.

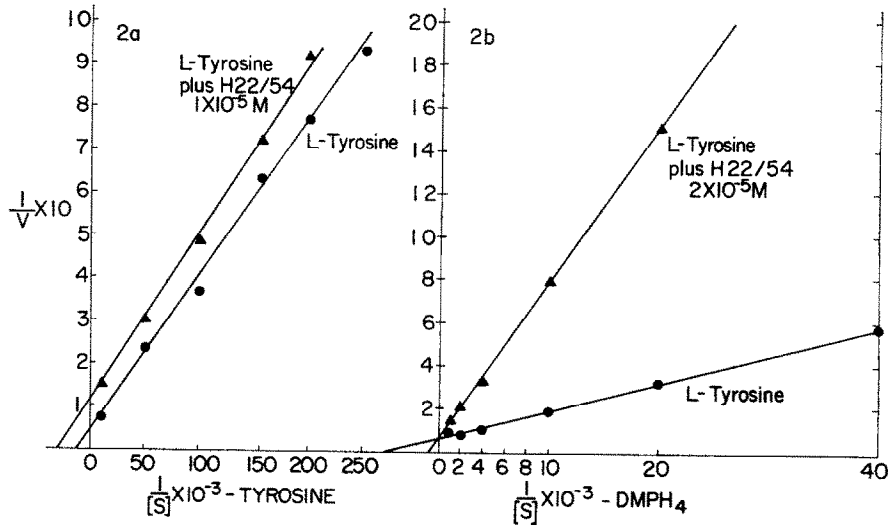


FIG. 2. The effect of 3,4-dihydroxyphenylpropylacetamide (H 22/54) on the hydroxylation of L-tyrosine. In 2a tyrosine concentrations were varied from 1×10^{-4} M to 4×10^{-6} M, in the standard incubation mixture. In 2b tyrosine was kept at 5×10^{-5} M and the cofactor, dimethyltetrahydropteridine ($DMPH_4$), was varied from 1×10^{-3} M to $2{\cdot}5 \times 10^{-5}$ M.

Amino acid derivatives as substrates

The question arose as to whether any of the inhibitory amino acids was also a substrate of tyrosine hydroxylase. By using radioactive α-methyl-DL-tyrosine a small but significant amount of a catechol product was formed on incubation with the enzyme (Table 4). With larger amounts of enzyme and substrate it was possible to obtain enough of the product to identify it as α-methyl-dopa by paper chromatography (Fig. 3).

TABLE 4. HYDROXYLATION OF TYROSINE AND α-METHYL-TYROSINE BY TYROSINE HYDROXYLASE

Substrate		Dopa or α-methyl-dopa formed	
		(cpm)	(mμmoles)
Tyrosine + enzyme		12,678	23·8
Tyrosine − enzyme		361	
α-Methyl-tyrosine + enzyme	(a)	1,363	1·3
	(b)	1,186	1·0
α-Methyl-tyrosine − enzyme		531	

L-tyrosine-^{3}H, 80,000 cpm and 1×10^{-4} M.
DL-α-Methyl-tyrosine-^{3}H, 203,000 cpm and 2×10^{-4} M.
Incubation was for 45 min at 37°.

The availability of ^{131}I-labeled mono- and diiodo-tyrosine made it possible to determine whether they were also substrates through a displacement of the ring halogen. In this case liberation of radioactive inorganic iodide was used as an index of activity, and comparisons were made with the tritium released from 3,5-tritio-tyrosine. As shown in Table 5, the radioactive iodide released enzymatically was negligible when either mono- or diiodo-tyrosine was used as substrate.

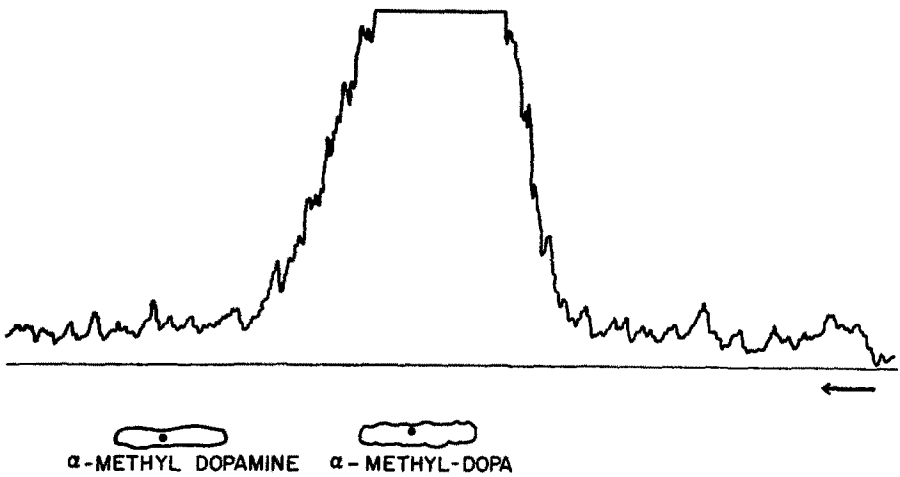


FIG. 3. Paper chromatography of apparent α-methyl-dopa isolated from an incubation mixture containing L-α-methyl-tyrosine as substrate. Radioactivity was measured with a Vanguard strip counter. Chromatograms were run overnight in a descending manner and the solvent (*n*-butanol:acetic acid:H_2O; 100:25:25) was permitted to run off the paper. α-Methyl-dopa migrated 15·5 cm, and α-methyl-dopamine 23 cm.

TABLE 5. ATTEMPT TO USE IODOTYROSINES AS SUBSTRATES OF TYROSINE HYDROXYLASE

Substrate	Amount incubated		^3H or ^{131}I released	^3H or ^{131}I released
	(M)	(cpm)	(cpm)	(mμmoles)
Experiment 1				
3-^{3}H-tyrosine	10^{-4}	103,500	27,206	26.3
3-^{131}I-tyrosine	10^{-4}	440,717	181	0·05
3,5-di-^{131}I-tyrosine	10^{-4}	462,164	457	0·1
Experiment 2				
3-^{3}H-tyrosine	7×10^{-7}	66,500	58,000*	0·55*
	10^{-4}	66,500	21,181	31·8
	10^{-3}	66,500	2,118*	31·8*
3-^{131}I-tyrosine	7×10^{-7}	475,000	469	0·00
	10^{-3}	475,000	369	0·46
3,5-di-^{131}I-tyrosine	7×10^{-7}	486,000	310	0·00
	10^{-3}	486,000	0	0·00
Experiment 3				
3-^{3}H-tyrosine	10^{-6}	92,600	69,000*	0·64*
	10^{-4}	92,600	24,458	26·4
3-^{131}I-tyrosine	2×10^{-7}	145,000	160	0·00
	1×10^{-6}	727,000	820	0·00
3,5-di-^{131}I-tyrosine	2×10^{-7}	147,000	0	0·00
	1×10^{-6}	733,000	500	0·00

* Extrapolated values from Lineweaver-Burk plot.
Incubations were at 37° for 30 min.
^3H or ^{131}I was measured in the effluent after passage through a Dowex-50 H^+ column as described under Methods. The values are corrected for the small amount of radioactive ^3H or ^{131}I displaced in the absence of enzyme. This generally amounted to several hundred counts/min.

DISCUSSION

Tyrosine hydroxylase appears to be the rate-limiting step in norepinephrine biosynthesis.[7] Inhibitors of this enzyme will therefore be of interest from the standpoint of blocking activity of the sympathetic nervous system. Information concerning the mechanism of inhibition is of more than theoretical interest in that it may help in explaining *in vivo* actions of these compounds.

The inhibition produced by mono- and diiodo-tyrosine may have clinical significance. Patients with a defect in the dehalogenase enzyme, which metabolizes the iodotyrosines, accumulate these iodinated amino acids in the body.[8] The tissue concentrations obtained appear to be sufficient to inhibit tyrosine hydroxylase and, in fact, administration of monoiodotyrosine to animals has already been shown to lower tissue norepinephrine levels.[9] Some of the pharmacological effects produced by the iodotyrosines are similar to those observed with other inhibitors of tyrosine hydroxylase.[10] It is conceivable that the interaction of iodotyrosines with tyrosine hydroxylase may represent some regulatory mechanism. However, its significance is not apparent.

The findings with α-methyl-tyrosine are of interest, since the compound is apparently a competitive substrate having a low K_m and a very low V_{max}. Although it is a poor substrate, this activity must be considered in explaining the *in vivo* actions of α-methyl-tyrosine, since the hydroxylated product, α-methyl-dopa, also lowers tissue levels of norepinephrine by virtue of decarboxylation to α-methyl-dopamine and oxidation to the norepinephrine displacing agent, α-methyl-norepinephrine.[11, 12] It appears, however, that formation of related α-methyl catecholamines does not occur to a significant extent *in vivo*.[3] In the perfused guinea pig heart no labeled α-methyl catecholamines were found after perfusing large amounts of radioactive α-methyl-tyrosine.[13]

The inhibition produced by the catechol derivatives is of interest, since it represents a case of end-product inhibition. It remains to be seen, however, whether this has physiologic significance. Of all the catechol derivatives investigated, compound H 22/54 was by far the most potent and, as is apparent from Table 3, the inhibitory activity is markedly lowered by any change in structure. Since the inhibition by compound H 22/54 is not reversed by substrate but by the pteridine cofactor, one would expect the catechol to inhibit other pteridine-requiring enzymes. This is apparently so. Carlsson *et al.*[14] reported preliminary observations on the inhibition of liver phenylalanine hydroxylase. More recently Burkard *et al.*[15] confirmed the inhibition of phenylalanine hydroxylation by compound H 22/54 and other catechols. They also reported evidence for inhibition of tryptophan hydroxylation. Lovenberg *et al.*[16] were able to demonstrate inhibition of a soluble tryptophan hydroxylase from mast cells by compound H 22/54 and α-methyl-dopa, the former being much more potent. It would appear, therefore, that catechols are general inhibitors of mammalian aromatic hydroxylases. It will be interesting to see whether catechols inhibit other kinds of pteridine enzymes and whether they affect hydroxylases that utilize cofactors other than pteridines.

It should be noted (Table 1) that L-tryptophan does not inhibit the purified tyrosine hydroxylase. At first glance this would appear inconsistent with a previous report from this laboratory[2] which indicated that inhibition by tryptophan was significant. However, inhibition by tryptophan has been noted only with the particulate enzyme and may signify competition for active uptake of tyrosine by the particle. Bagchi and

McGeer[17] have also reported that tryptophan inhibits tyrosine hydroxylation by brain particles.

Some of the compounds reported here also inhibit tyrosine hydroxylase activity *in vivo*, and with continued administration these compounds markedly lower tissue levels of norepinephrine.[10] Concomitantly the animals become sedated and their tyramine and norepinephrine pressor responses are lowered. The availability of a number of agents that block norepinephrine synthesis *in vivo* should provide the pharmacologist with useful tools for elucidating the actions of the sympathetic nervous system and of other drugs that interact with it.

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