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Comparison of halogenated phenylalanine analogues as inhibitors as particle-bound and soluble tyrosine hydroxylase*

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THE CONVERSION of tyrosine to 3,4-dihydroxyphenylalanine by the enzyme, tyrosine hydroxylase appears to be the rate-determining step in the biosynthesis of norepinephrine.[1] Thus this enzyme is the focal point for possible physiological and pharmacological control of norepinephrine synthesis.

A relatively large number of compounds have been shown to be inhibitors of tyrosine hydroxylase.[2–5] These generally fall into two classes, aromatic amino acid analogues which compete with tyrosine and catecholamines which compete with the pteridine cofactor. Studies with tyrosine analogues have indicated that: 1) α-methyl-amino acids are more potent than unmethylated analogues, and 2) a marked increase in activity occurs on substituting a halogen at the 3-position of the benzene ring.[2,3,6] The relative activities of the 3-substituted α-methyl-tyrosines were: I> Br> Cl> H> F.[2] Although a relatively large number of phenylalanine derivatives have been studied, the structural requirements of phenylalanine analogues for inhibition of tyrosine hydroxylase are not clear.

We report in this paper the results of studies with a series of iodinated phenylalanine analogues and α-methyl derivatives of 3-halogenated phenylalanines. The activity of these compounds as inhibitors of partially purified tyrosine hydroxylase and particle-bound tyrosine hydroxylase was studied.

All compounds investigated, except 4-fluorophenylalanine and DL-α-methylphenylalanine, were synthesized in the laboratory of Dr. Raymond Counsell, Department of Medicinal Chemistry, University of Michigan. The *p*-fluorophenylalanine was purchased from Mann Laboratories. The DL-α-methylphenylalanine was generously supplied by Merck, Sharp & Dohme Research Laboratory. The pteridine cofactor, 2-amino-4-hydroxy-6,7-dimethyltetrahydroperidine ($DMPH_4$), was purchased from Cal-Biochem. L-Tyrosine-3,5-^{3}H was obtained from New England Nuclear Corp. All other chemicals were obtained from commercial sources. Bovine adrenals were purchased from Pel-Freeze Biologicals, Rogers, Ark. They arrived frozen and were stored at $-20°$ until used.

Tyrosine hydroxylase was prepared from bovine adrenal medulla according to the procedure of Nagatsu *et al.*[7] The enzyme was purified through the first $(NH_4)_2SO_4$ precipitation. The partially purified enzyme was dissolved in 0·005 M potassium phosphate buffer, pH 7·0, containing 5×10^{-3} M mercaptoethanol. Enzyme was stored at $-20°$.

Tyrosine hydroxylase was assayed by measuring the release of tritium into water from 3,5-ditritio-tyrosine, as described by Nagatsu *et al.*[8] Incubation mixtures contained 50 mμmoles L-tyrosine that contained 1 to 2×10^5 cpm of tyrosine-3,5-^{3}H, 2 μmoles $DMPH_4$, 200 μmoles potassium phosphate buffer, pH 6·0, enzyme (1·0–2·0 mg protein) and water to 1·0 ml. The reaction was stopped after 10 min with 0·05 ml of glacial acetic acid. The amount of product formed versus time was linear for 15 min and the amount of product formed versus protein concentration was linear up to 4 mg protein. The compounds tested for inhibition were dissolved in water. In some cases diluted HCl was added until the compound dissolved. When this was necessary, a control incubation was run in which an equivalent amount of HCl was added. In no case did the HCl control differ significantly from the usual control assay.

The particle-bound tyrosine hydroxylase used in this study was prepared from bovine adrenal medulla as follows. Adrenal medulla was homogenized in 4 vol. of 0·25 M sucrose (20% homogenate). The homogenate was strained through one layer of surgical gauze and centrifuged at 500 *g* for 10 min (Spinco model L-2-65, 65 head). The resulting precipitate was suspended in 0·25 M sucrose, homogenized and centrifuged again at 600 *g* for 10 min. The wash was combined with the supernatant from the first 600 *g* sedimentation. The combined supernatant was centrifuged at 20,000 *g* for 20 min. The precipitate was suspended in 0·25 M sucrose and centrifuged again at 20,000 *g* for 20 min. The 20,000 *g* particulate fraction was suspended in 0·25 M sucrose, layered on top of 1·6 M sucrose

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and centrifuged at 100,000 g for 60 min as described by Smith and Winkler.[9] The tyrosine hydroxylase activity remained at the top of the tube. The top fraction was removed, suspended in 0·25 M sucrose and sedimented at 100,000 g for 20 min. The sediment was suspended in 0·005 M potassium phosphate buffer, pH 7·0, containing 5×10^{-3} M mercaptoethanol and stored at $-20°$.

Protein was determined by the method of Lowry *et al.*[10].

The inhibition of tyrosine hydroxylase by various phenylalanine analogues is shown in Table 1.

TABLE 1. INHIBITION OF SOLUBLE TYROSINE HYDROXYLASE BY PHENYLALANINE ANALOGUES

Compound*	Concn (M)	Inhibition (%)
L-Phenylalanine	2×10^{-4}	57; 57; 49
DL-α-Methylphenylalanine	2×10^{-4}	45; 46
DL-3-Iodophenylalanine	2×10^{-4}	68; 70; 66
L-3-Iodophenylalanine	1×10^{-4}	78; 78
DL-4-Iodophenylalanine	2×10^{-4}	0
	1×10^{-3}	0
DL-2-Iodophenylalanine	2×10^{-4}	0
DL-4-Fluorophenylalanine	2×10^{-4}	22; 37
DL-α-Methyl-3-iodophenylalanine	2×10^{-4}	96; 95
	1×10^{-4}	86
	2×10^{-5}	55
DL-α-Methyl-3-bromophenylalanine	2×10^{-4}	75; 78
DL-α-Methyl-3-chlorophenylalanine	2×10^{-4}	45; 51
DL-α-Methyl-3-fluorophenylalanine	2×10^{-4}	19;27
DL-3-Iodophenylglycine	2×10^{-4}	0
DL-4-(3-Iodophenyl)-2-methyl-2-amino-butanoic acid	2×10^{-4}	0

* Each compound was tested in an incubation mixture that contained 50 mμmoles L-tyrosine. Each value is the average of duplicate incubations.

DL-3-Iodophenylalanine at 2×10^{-4} M shows considerable inhibitory activity, while DL-2-iodophenylalanine and DL-4-iodophenylalanine are not inhibitory at that concentration or at a concentration of 1×10^{-3} M. The substitution of a methyl group on the α-carbon increases the inhibitor activity of DL-3-iodophenylalanine. L-3-Iodophenylalanine is about twice as active as DL-3-iodo-

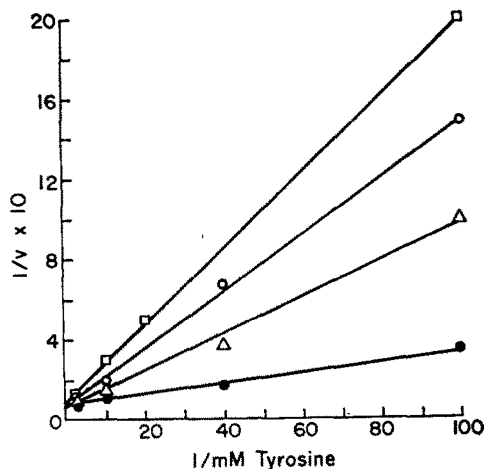

FIG. 1. Inhibition of tyrosine hydroxylase. Phenylanine, 2×10^{-4} M (□—□); 3-iodophenylalanine 2×10^{-4} M (○—○); α-methyl-3-iodophenylalanine, 2×10^{-5} M, (▲—▲); activity without inhibition (●—●).

phenylalanine. The series of 3-halogenated-α-methylphenylalanines show decreasing inhibitory activity from I to F. The extension of the aliphatic chain by one carbon eliminates the inhibitory activity. The shortening of the chain by one carbon also eliminates the inhibitory activity. The *N*-acetyl derivatives of the 3-halogenated-α-methylphenylalanines are also inactive as inhibitors.

The inhibition of soluble tyrosine hydroxylase by DL-3-iodophenylalanine and DL-α-methyl-3-iodophenylalanine is competitive with substrate (Fig. 1). We also find the inhibition by phenylalanine to be competitive with tyrosine. The inhibition by α-methyl-3-iodophenylalanine is noncompetitive with the cofactor $DMPH_4$.

Many of the compounds that showed inhibitory activity with soluble tyrosine hydroxylase were tested as inhibitors for particle-bound tyrosine hydroxylase activity. Phenylalanine and the various phenylalanine analogues were inhibitors of the particulate system, but their activity was in all cases half that observed with the soluble enzyme (Table 2). DL-Norepinephrine, on the other hand, inhibited both preparations to the same extent (30 per cent at 1×10^{-3} M).

TABLE 2. INHIBITION OF PARTICLE-BOUND TYROSINE HYDROXYLASE BY PHENYLALANINE ANALOGUES*

Compound	Concn (M)	Inhibition (%)
L-Phenylalanine	2×10^{-4}	15; 21; 30
DL-α-Methyl-3-iodophenylalanine	2×10^{-5}	25; 26; 30
DL-α-Methyl-3-fluorophenylalanine	2×10^{-4}	10; 0
DL-α-Methyl-3-chlorophenylalanine	2×10^{-4}	25; 24
DL-α-Methyl-3-bromophenylalanine	2×10^{-4}	47; 50
DL-3-Iodophenylalanine	2×10^{-4}	34; 34
L-3-Iodophenylalanine	1×10^{-4}	46
DL-α-Methylphenylalanine	2×10^{-4}	24

* See Table 1 for explanations.

We have investigated the structural requirements of phenylalanine analogues for inhibitory activity of tyrosine hydroxylase. We observed, as have others[2, 3, 5] that phenylalanine itself has considerable inhibitory activity. The addition of iodine to the 2- or the 4-position eliminates the inhibitory activity, while iodination at the 3-position enhances the inhibitory activity. The substitution of a methyl group at the α-carbon leads to an increase in the inhibitory activity for both phenylalanine and 3-iodophenylalanine, a result consistent with work reported on tyrosine analogues.[2] Phenylalanine analogues are not as active as the corresponding tyrosine analogues. Thus, DL-α-methyl-3-iodophenylalanine gives 55 per cent inhibition at 2×10^{-4} M, while DL-α-methyl-3-iodotyrosine inhibits 50 per cent at 3×10^{-7} M.[2] However, substitution of F or I at the 4-position lowers the inhibitory activity below that of phenylalanine. Substitution of I at the 2-position also eliminates the inhibitory activity. The relative activity for the 3-halogenated-α-methylphenylalanines are I $>$ Br $>$ Cl $>$ F. These are the same as those reported for 3-halogenated-α-methyltyrosine analogues.[2] A free amino group is required at a distance one carbon from the ring, since DL-3-iodophenylglycine and DL-4-(3-iodophenyl)-2-methyl-2-amino butanoic acid are both inactive as was the DL-*N*-acetyl-3-iodophenylalanine.

We have found the inhibition of phenylalanine to be competitive with substrate, in agreement with Ikeda *et al.*[11] but in disagreement with the results of Saari *et al.*[3] The inhibition by 3-iodophenylalanine and α-methyl-3-iodophenylalanine is also competitive with substrate, This does not agree with the conclusion of Saari *et al.*[3] that an unhindered 4-hydroxyl group is necessary for competitive inhibition with substrate.

The inhibitory activity of the 3-halogenated-α-methylphenylalanine analogues with particle-bound tyrosine hydroxylase falls in the same relative order as that observed with the soluble enzyme. However, the inhibition of the particle-bound enzyme was about half that observed with the soluble enzyme. This may be caused by a poor permeability of the particle for the phenylalanine analogues, but the fact that the particles were frozen and thawed prior to their use and that norepinephrine had the same activity with particle-bound enzyme and soluble enzyme would argue against this possi-

bility. It would seem more likely that the difference in activity resulted from a difference in the immediate environment around the enzyme. These differences between the soluble enzyme and the particle-bound enzyme also indicate that care should be taken in the interpretation of results when whole homogenates are used for the testing of tyrosine hydroxylase inhibitors.

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Metabolism of 7-hydroxymethyl-12-methylbenz(a)anthracene-12-^{14}C *in vitro**

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IT IS NOW well established from the experiments of Huggins *et al.*[1–3] and Dao and Tanaka[4,5] that pretreatment with any of several polycyclic hydrocarbons protects rats from adrenal necrosis and mammary cancer induced by DMBA.* There is also evidence[6,7] that the proximal necrotic agent is the 7-hydroxymethyl derivative of DMBA (7-OHM-12-MBA) and that treatment of rats with certain polycyclic hydrocarbons alters the metabolism of DMBA by hepatic tissue from side-chain to ring hydroxylation.[8,9] It was therefore considered of interest to study the metabolism of 7-OHM-12-MBA by rat liver microsomes and also adrenal tissue and to determine the effect of compounds which protect against adrenal necrosis on the metabolism of this biologically active metabolite of DMBA.

Mature (55–70 days old) male hooded or Sprague–Dawley rats, with free access to food (Purina Labena) and water, were used. The polycyclic hydrocarbons (10 mg) dissolved in sesame oil (1 ml) by gentle heating were administered by stomach tube or by intraperitoneal injection 24 hr before killing the animals.

Radioactive 7-hydroxymethyl-12-methylbenz(a)anthracene (7-OHM-12-MBA-12-^{14}C) was prepared by the method of Boyland and Sims[10] from DMBA-12-^{14}C (0·2 mc in 5·5 mg), obtained from the Radiochemical Centre, Amersham, and diluted with 150 mg "cold" DMBA. The acetoxy derivative, however, was not isolated but hydrolyzed by refluxing with 2% (w/v) methanolic KOH before

* The following abbreviations are used: DMBA, 7,12-dimethylbenz(a)anthracene; 7-OHM-12-MBA, 7-hydroxymethyl-12-methylbenz(a)anthracene; 12-OHM-7-MBA, 12-hydroxymethyl-7-methylbenz(a)anthracene; 7,12-DiOHM-BA, 7,12-dihydroxymethylbenz(a)anthracene; MC, 3-methylcholanthrene.