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Formation of *m*-Tyrosine and *o*-Tyrosine from L-Phenylalanine in Various Tissues of Rats

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The hydroxylation of L-phenylalanine was investigated in subcellular fractions of various organs of rat under aerobic conditions. L-Phenylalanine was metabolized to *p*-, *m*-, and *o*-tyrosine in the 2000 × *g* homogenates of liver, kidney, brain and adrenal of male rats. The three metabolites in the incubation mixture were determined by high-performance liquid chromatography. This hydroxylation reaction occurred only in the postmicrosomal fraction of the organs mentioned above but not in the microsomal fractions, and required a pteridine as a cofactor.

The hydroxylation of phenylalanine was inhibited by the addition of *p*-chlorophenylalanine and α-methyltyrosine, which are specific inhibitors of phenylalanine hydroxylase and tyrosine hydroxylase, respectively. On the other hand, superoxide dismutase and potassium iodide, which are known scavengers of the superoxide radical and hydroxyl radical, had no significant effect on the hydroxylation. These results indicate that the hydroxylation of phenylalanine to *m*- and *o*-tyrosines is caused mainly by phenylalanine hydroxylase and tyrosine hydroxylase.

Keywords——phenylalanine; *p*-tyrosine; *m*-tyrosine; *o*-tyrosine; rat; fluorescence high-performance liquid chromatography

The hydroxylation of phenylalanine to tyrosine (*p*-tyrosine) by phenylalanine hydroxylase is an important metabolic pathway.[1] In addition, a number of *meta*- and *ortho*-substituted phenols such as hydroxyphenylacetic acid and hydroxymandelic acid of endogenous origin have been found in human urine.[2] Fell *et al.*, Hoskins and Greenway, and Petitclerc *et al.* reported that these compounds might all be derived from the metabolism of a precursor, *m*-hydroxyphenylalanine (*m*-tyrosine) or *o*-hydroxyphenylalanine (*o*-tyrosine).[3] Thus, it seems probable that *m*-tyrosine and *o*-tyrosine are formed in mammals. The first indication that the formation of *m*-tyrosine might be associated with the hydroxylation of phenylalanine came from a study by Tong *et al.*, in which phenylalanine was treated with bovine adrenal medulla homogenate, and the formation of *m*-tyrosine was found to be prevented by the tyrosine hydroxylase inhibitor, α-methyltyrosine.[4] However, the formation of *o*-tyrosine in mammalian tissues has not been clarified as yet.

In the present paper, we show that *o*-tyrosine, besides *m*- and *p*-tyrosine, is formed *in vitro* in various tissues of the rat, and that the formation of the tyrosine isomers is caused mainly by phenylalanine and tyrosine hydroxylases. A preliminary communication has already appeared.[5]

Experimental

Materials——L-Phenylalanine, L-3,4-dihydroxyphenylalanine (DOPA), L-*p*-tyrosine, DL-*m*-tyrosine, DL-*o*-tyrosine, α-methyl-DL-*p*-tyrosine, catalase (bovine liver) and superoxide dismutase (bovine blood) were obtained from Sigma Chemicals Co. (St. Louis, U.S.A.), D-phenylalanine from the Peptide Institute Inc. (Osaka), 6,7-dimethyl-5,6,7,8-tetrahydropterine ($DMPH_4$), 6-methyl-5,6,7,8-tetrahydropterine (MPH_4) and 2-mercaptoethanol from Nakarai Chemicals, Ltd. (Kyoto), potassium iodide from Wako Pure Chemical Industries (Osaka), and DL-*p*-chlorophenylalanine from Tokyo Kasei Kogyo Co. (Tokyo). All other chemicals used were of the highest purity

commercially available.

Apparatus——A Hitachi 638-50 high-speed liquid chromatograph and a Hitachi 650-10S fluorimetric detector were used.

Chromatographic Conditions——Cosmosil C_{18} was packed in a 150 × 4.6 mm i.d. stainless steel column; mobile phase, 1% acetic acid containing 1% sodium chloride; flow rate, 0.8 ml/min; chart speed, 5 mm/min; detection, excitation at 275 nm and emission at 305 nm.

Preparation of Supernatant——Male Wistar rats (150—200 g) were killed and the brain, heart, lung, spleen, liver, kidney and adrenal were removed and immediately washed with 0.25 M sucrose. The tissues were homogenized in two volumes (brain, heart, lung, spleen, liver and kidney) or twenty volumes (adrenal) of 0.25 M sucrose solution under cooling. The homogenate was centrifuged at 2000 × *g* for 20 min to yield supernatant. The supernatant fluid was used as tissue homogenate. The supernatant fluid was further ultracentrifuged at 105000 × *g* for 60 min, and the precipitate (microsomal fraction) was separated from the supernatant (postmicrosomal fraction).

Hydroxylation——The reaction mixture contained 8 μmol of phenylalanine, 0.6 μmol of $DMPH_4$, 20 μmol of 2-mercaptoethanol and 100 μmol of citrate buffer (pH 6.0) in a total volume of 2.0 ml. The mixture was incubated in air with shaking in a water bath at 37 °C, and the reaction was terminated by the addition of 0.5 ml of 1.0 M trichloroacetic acid. The reaction mixture was centrifuged at 12000 × *g* for 10 min at 0—4 °C. After filtration through a 0.22-μm filter, an aliquot (50 μl) of the supernatant was directly injected into the high-performance liquid chromatograph (HPLC).

Assay of Phenylalanine Hydroxylase and Tyrosine Hydroxylase Activities——Phenylalanine hydroxylase activity was assayed by the method of Bailey *et al.*[6] and one unit of phenylalanine hydroxylase is defined as the amount that catalyzes the formation of 0.1 μmol of *p*-tyrosine in 1 min. Tyrosine hydroxylase activity was determined by a modification of the method of Nagatsu *et al.*[7] The reaction mixture contained 1 μmol of *p*-tyrosine, 1 μmol of MPH_4, 100 μmol of 2-mercaptoethanol, 100 μg of catalase and 200 μmol of acetate buffer (pH 6.0) in a total volume of 1.0 ml. Incubation was done at 37 °C for 10 min, and the reaction was stopped by adding 1.0 ml of 0.5 M perchloric acid. After centrifugation, an aliquot (50 μl) of the supernatant was subjected to HPLC (excitation at 280 nm and emission at 318 nm). One unit of tyrosine hydroxylase is defined as the amount that catalyzes the formation of 0.1 μmol of DOPA in 1 min. Protein concentrations were estimated by the method of Lowry *et al.* using bovine serum albumin as a standard.[8]

Results and Discussion

Hydroxylation of L-Phenylalanine by Various Rat Tissue Homogenates

Rat brain homogenate was added to citrate buffer (pH 6.0) containing phenylalanine to examine the hydroxylation of phenylalanine. A typical chromatographic pattern of the reaction mixture is shown in Fig. 1. *p*-, *m*- and *o*-tyrosines were identified on the basis of the retention times. Furthermore, the assignment of peaks 1—3 in Fig. 1 to *p*-, *m*- and *o*-tyrosines, respectively, was verified by ion-exchange chromatographic analysis. As shown in Fig. 2, *m*-, *p*-, and *o*-tyrosine emerged from this system at 108, 112, and 116 min, respectively. The retention time of each peak was identical with that of a corresponding authentic sample.

The hydroxylation of phenylalanine by various rat tissue homogenates was examined. The results are summarized in Table I. The hydroxylation of phenylalanine was induced by liver, kidney, brain and adrenal homogenates, and yielded *p*-, *m*- and *o*-tyrosines. No significant hydroxylation reaction occurred on omission of phenylalanine and/or homogenate from the complete system. Traces of hydroxylation products were induced by spleen homogenate, but not by those from the lung and heart. In addition, the amount of each tyrosine detected when D-phenylalanine was added as a substrate was found to be less than 10% of that formed from L-phenylalanine. This result indicates that D-phenylalanine is not active as a substrate. The hydroxylation of phenylalanine was examined in more detail using the postmicrosomal and microsomal fractions.

Hydroxylation of Phenylalanine Induced by Postmicrosomal Fraction

Table II shows the results after a 30 min incubation. The hydroxylation reaction of phenylalanine, especially the formation of *p*-tyrosine by liver and kidney postmicrosomal fractions, was accelerated by the addition of $DMPH_4$. The data in Table II demonstrate the absolute requirement for $DMPH_4$ in the case of liver and kidney postmicrosomal fractions.

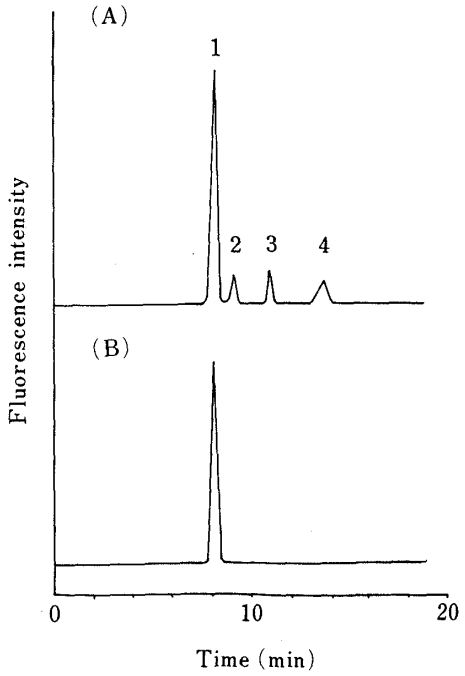

Fig. 1. High-Performance Liquid Chromatogram of the Reaction Mixture

(A) Injection sample: After reaction of phenylalanine with rat brain homogenate for 30 min.
(B) Injection sample: Same as (A), except for phenylalanine.
Fifty μl of the sample prepared as described in the text was subjected to HPLC. Peaks: 1 = *p*-tyrosine; 2 = *m*-tyrosine; 3 = *o*-tyrosine; 4 = phenylalanine.

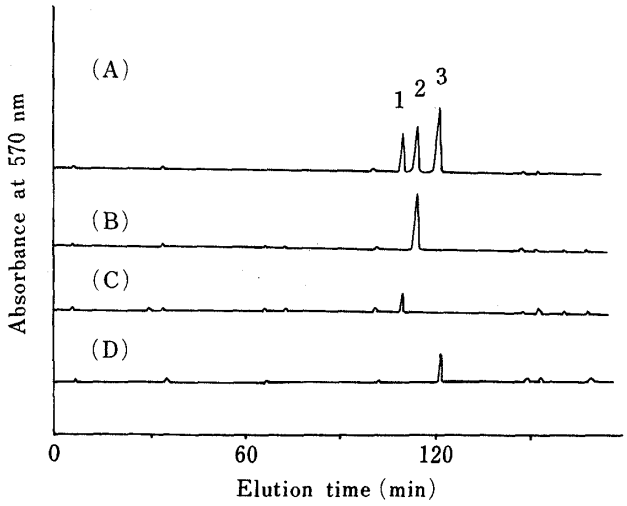

Fig. 2. Ion-Exchange Chromatogram of Tyrosine Isomer Fractions Eluted from HPLC

The *p*- (B), *m*- (C) and *o*- (D) tyrosine fractions collected, and authentic samples of tyrosine isomers (A) were subjected to ion-exchange chromatography.
The analyses were performed with a Hitachi 835 amino acid analyzer, using MCI-Buffer (Mitsubishi Chemical Industries Ltd., Tokyo) for physiological fluid analysis as the eluent. Peaks: 1 = *m*-tyrosine; 2 = *p*-tyrosine; 3 = *o*-tyrosine.

TABLE I. The Hydroxylation of Phenylalanine by Various Rat Tissue Homogenates

Tissue	Tyrosines formed (μg/2.5 ml)		
	p-	*m*-	*o*-
Liver	2.48	0.94	1.25
Kidney	2.35	0.46	0.45
Brain	0.43	0.32	0.33
Adrenal	0.19	0.17	0.23
Spleen	Trace	Trace	Trace
Heart	0.0	—[a)]	—
Lung	0.0	—	—

a) Not detected. The reaction mixture contained 8 μmol of phenylalanine and 100 μmol of citrate buffer (pH 6.0) in a total volume of 2.0 ml.

The boiled postmicrosomal fraction was no longer active for the hydroxylation of phenylalanine. With the microsomal fractions, no formation of hydroxyphenylalanines was observed. The time course of the hydroxylation of phenylalanine in the reaction mixtures during the first 45 min is shown in Table III. The amounts of accumulated *m*- and *o*-tyrosines were maximum at 30 min and thereafter declined. The amount of *p*-tyrosine with the kidney fraction increased with time, whereas with the liver, brain and adrenal fractions, it reached a maximum at 30 min.

TABLE II. The Formation of *p*-, *m*-, and *o*-Tyrosine from Phenylalanine Using the Postmicrosomal Fractions of Various Tissues of Rat

System	Liver			Kidney			Brain			Adrenal		
	p-	*m*-	*o*-	*p*-	*m*-	*o*-	*p*-	*m*-	*o*-	*p*-	*m*-	*o*-
Complete system	76.63	2.92	3.16	30.38	3.90	4.87	1.16	1.26	1.35	0.57	0.66	1.00
$-DMPH_4$	3.39	1.13	1.50	2.54	0.48	0.71	0.41	0.42	0.65	0.37	0.37	0.47
+Boiled enzyme[a]	0.0	0.02	0.02	0.0	0.01	0.02	0.0	0.01	0.03	0.0	0.01	0.04

Values are expressed in μg/2.5 ml. *a*) Heated for 5 min at 100 °C.

TABLE III. Time Course of the Hydroxylation of Phenylalanine by Postmicrosomal Fractions of Various Tissues of Rat

Reaction time (min)	Liver			Kidney			Brain			Adrenal		
	p-	*m*-	*o*-	*p*-	*m*-	*o*-	*p*-	*m*-	*o*-	*p*-	*m*-	*o*-
10	44.72	2.45	2.65	11.42	3.71	4.01	0.61	0.69	0.78	0.36	0.52	0.66
20	69.94	2.62	2.96	25.32	3.76	4.29	0.76	0.82	0.90	0.46	0.57	0.76
30	76.63	2.92	3.16	30.38	3.90	4.87	1.16	1.26	1.35	0.57	0.66	1.00
45	66.55	2.71	3.01	46.19	3.22	3.84	1.09	0.97	1.07	0.45	0.48	0.90

Values are expressed in μg/2.5 ml.

TABLE IV. Effects of Various Substances on the Hydroxylation of Phenylalanine (A) and Enzyme Activities (B) Induced by Postmicrosomal Fractions

(A)

Substance added	Liver			Kidney			Brain			Adrenal		
	p-	*m*-	*o*-	*p*-	*m*-	*o*-	*p*-	*m*-	*o*-	*p*-	*m*-	*o*-
Complete system	76.63	2.92	3.16	30.38	3.90	4.87	1.16	1.26	1.35	0.57	0.66	1.00
+*p*-Chlorophenylalanine (5 μmol)	26.93	1.59	1.64	6.68	1.99	2.24	1.02	1.34	1.57	0.53	0.56	0.92
+α-Methyltyrosine (2 μmol)	72.21	3.11	3.46	30.13	2.06	2.45	0.18	0.01	0.02	0.04	0.01	0.03
+Superoxide dismutase (25 μg/ml)	71.69	2.65	3.13	27.70	4.61	5.18	1.22	1.34	1.57	0.79	0.73	1.02
+Potassium iodide (10 μmol)	67.89	2.29	2.64	24.26	3.73	4.04	1.01	1.12	1.60	0.37	0.48	0.78

(B)

Substance added	Phenylalanine hydroxylase (Relative activity % of control)		Tyrosine hydroxylase (Relative activity % of control)		
	Liver	Kidney	Brain	Adrenal	Kidney
Control	100 (0.47)[a]	100 (0.04)	100 (7×10^{-4})	100 (5×10^{-4})	100 (5×10^{-4})
+*p*-Chlorophenylalanine	26	20	98	104	96
+α-Methyltyrosine	109	104	60	50	48
+Superoxide dismutase	102	96	105	106	98
+Potassium iodide	89	88	98	88	93

a) Enzyme activity (unit/ml).

TABLE V. The Activities of Phenylalanine Hydroxylase and Tyrosine Hydroxylase in the Postmicrosomal Fractions of Various Tissues of Rat

Tissue	Phenylalanine hydroxylase (unit/g wet weight)	Tyrosine hydroxylase (unit/g wet weight)
Liver	1.87	—
Kidney	0.27	0.010
Brain	—	0.014
Adrenal	—	0.102

Effectors of the Hydroxylation of Phenylalanine

To investigate the mechanism of the hydroxylation of phenylalanine by the postmicrosomal fraction, the following experiments were performed.

Effects of Superoxide Dismutase and Potassium Iodide——Superoxide anion is known to be produced in normal biological processes[9]; moreover, hydroxyl radicals have been detected as end products in a number of systems that are capable of generating superoxide anions.[10] In a previous paper,[11] we reported on the mechanism of nonenzymatic hydroxylation of phenylalanine by active oxygen, and suggested that hydroxyl radical was responsible for the hydroxylation of phenylalanine to yield *p*-, *m*- and *o*-tyrosines. The effects of scavengers of the superoxide anion[12] and hydroxyl radical[13] on the hydroxylation of phenylalanine induced by the postmicrosomal fraction were examined. The results are summarized in Table IV. Superoxide dismutase and potassium iodide, employed as scavengers of superoxide anion and hydroxyl radical, did not significantly affect the hydroxylation of phenylalanine, or the phenylalanine hydroxylase and tyrosine hydroxylase activities. These results suggest that active oxygen species play no part in the hydroxylation reaction.

Effects of *p*-Chlorophenylalanine and α-Methyltyrosine——It was shown previously that the phenylalanine hydroxylase reaction was markedly inhibited by the addition of *p*-chlorophenylalanine,[14] and the tyrosine hydroxylase reaction was markedly suppressed by the addition of α-methyltyrosine.[15] Thus, the effects of *p*-chlorophenylalanine and α-methyltyrosine on the hydroxylation of phenylalanine were examined (Table IV). The addition of *p*-chlorophenylalanine to the reaction system resulted in decreased formation of tyrosines from phenylalanine by the liver and kidney fractions, and the phenylalanine hydroxylase activity was markedly inhibited. However, the hydroxylation of phenylalanine by the adrenal and brain postmicrosomal fractions was not affected by the addition of *p*-chlorophenylalanine. The addition of α-methyltyrosine to the reaction system reduced the rate of tyrosine formation induced by the adrenal and brain postmicrosomal fractions, but the formation of *p*-tyrosine induced by the kidney fraction was not affected. The adrenal and brain postmicrosomal fractions do not contain phenylalanine hydroxylase.[16] Previously, Ikeda *et al.* reported the conversion of phenylalanine to *p*-tyrosine induced by bovine adrenal tyrosine hydroxylase.[17] In addition, Nagatsu *et al.* reported the presence of tyrosine hydroxylase in the dog kidney.[18] In this study the activities of phenylalanine hydroxylase and tyrosine hydroxylase were examined in various organs of the rats; the results are summarized in Table V. The distributions of the enzyme activities were as follows: phenylalanine hydroxylase was found in the liver and the kidney; the tyrosine hydroxylase level was highest in the adrenal gland but was also detectable in the brain and the kidney. These results imply that the hydroxylation of phenylalanine to *p*-, *m*- and *o*-tyrosines is caused by phenylalanine hydroxylase and tyrosine hydroxylase. Further studies using rats on the hydroxylation of phenylalanine *in vivo* and on the production of hydroxyphenylalanines are in progress.

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