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In Vivo Studies on the Formation of *m*-Tyrosine and *o*-Tyrosine from L-Phenylalanine in Rats

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L-Phenylalanine was administered intraperitoneally, intravenously, intramuscularly and orally to rats, and its metabolites were separated and identified by high-performance liquid chromatography (HPLC) with fluorescence detection. Chromatographic peaks were identified on the basis of their retention behavior and the assignment of peaks of HPLC was verified by ion-exchange chromatographic analysis. The three metabolites *p*-, *m*- and *o*-tyrosines were formed *in vivo* after phenylalanine administration. The extents of formation of *m*- and *o*-tyrosines were significantly increased by the administration of L-phenylalanine. However, the concentrations of *m*- and *o*-tyrosines in the serum were reduced by the administration of α-methyltyrosine, which is an inhibitor of tyrosine hydroxylase. On the other hand, the inhibition of phenylalanine hydroxylase activity by the administration of *p*-chlorophenylalanine and ethionine resulted in a marked increase of the concentration of phenylalanine in the serum, and the formation of *m*- and *o*-tyrosines was also significantly increased. These results suggest that the formation of *m*- and *o*-tyrosines from phenylalanine is caused mainly by tyrosine hydroxylase, but not by phenylalanine hydroxylase.

Keywords——rat; phenylalanine; *p*-tyrosine; *m*-tyrosine; *o*-tyrosine; fluorescence HPLC; ion-exchange chromatography; tyrosine hydroxylase

L-Phenylalanine is metabolized largely by conversion to tyrosine (*p*-tyrosine) in mammals.[1] In addition, many authors have reported the presence of *m*-tyramine[2,3] and *o*-tyramine[4-6] as constituents of human urine, and it has been demonstrated that all these compounds are derived from *m*-hydroxyphenylalanine (*m*-tyrosine) and *o*-hydroxyphenylalanine (*o*-tyrosine) in the human body.[7-9] These findings suggest that *m*- and *o*-tyrosines may be formed in mammals. In fact, *m*- and *o*-tyrosines have been detected in rat serum.[10] The first indication that the formation of *m*-tyrosine is associated with the hydroxylation of L-phenylalanine *in vitro* came from the study of Tong *et al.*,[11] in which phenylalanine was treated with bovine adrenal medulla homogenate. Recently, we also reported the formation of *m*- and *o*-tyrosines from L-phenylalanine *in vitro* by rat.[12,13]

In this study, we examined the *in vivo* formation of *m*-, *o*- and *p*-tyrosines after administration of L-phenylalanine, an the effect of inhibitors of phenylalanine and tyrosine hydroxylases on the formation of *m*- and *o*-tyrosines. A preliminary communication has already appeared.[10]

Experimental

Materials——L-Phenylalanine, L-3,4-dihydroxyphenylalanine (DOPA), L-*p*-tyrosine, DL-*m*-tyrosine, DL-*o*-tyrosine and α-methyl-DL-*p*-tyrosine were obtained from Sigma Chemicals Co. (St. Louis, U.S.A.), D-phenylalanine from the Peptide Institute Inc. (Osaka), 6-methyl-5,6,7,8-tetrahydropterin (MPH_4) and 2-mercaptoethanol from Nakarai Chemicals, Ltd. (Kyoto), DL-ethionine 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.

Animals and Treatments——Male Wistar rats, initially weighing 150—200 g each were used. Before the experiment, the animals were starved overnight but were allowed to drink water freely. L-Phenylalanine (80 mg/kg) in saline was administered intraperitoneally (*i.p.*), intravenously (*i.v.*), intramuscularly (*i.m.*) or *per os* (*p.o.*) to the rats, while control rats were treated with an equal volume of saline. Phenylalanine hydroxylase activity is inhibited by the administration of *p*-chlorophenylalanine and ethionine,[14] and tyrosine hydroxylase activity is suppressed by the administration of α-methyltyrosine.[15] The rats were administered *i.p.* *p*-chlorophenylalanine (720 mg/kg) and ethionine (80 mg/kg) and/or α-methyltyrosine (250 mg/kg). The serum of the rats was obtained 24 h after dosing. Furthermore, L-phenylalanine (80 mg/kg) in saline was administered *i.p.*, *i.v.*, *i.m.* or *p.o.* to the rats. Blood samples were collected from the abdominal aorta at the designated intervals after the treatment and centrifuged at 3000 rpm for 5 min to obtain serum. A 2 ml sample of the serum was deproteinized by the addition of 0.5 ml of 1.0 M trichloroacetic acid. The mixture was centrifuged at 12000 × *g* for 10 min at 0—4 °C. After filtration through a 0.22-μm filter, an aliquot of the supernatant was directly injected into a high-performance liquid chromatograph (HPLC) equipped with a fluorescence detector. The liver, kidney, brain and adrenal were dissected out rapidly, washed in ice-cold 0.25 M sucrose, blotted dry and stored at −20 °C until required for enzyme assay.

Measurements——(i) HPLC: A Hitachi 638-50 high-speed liquid chromatograph and a Hitachi 650-10S fluorimetric detector were used in series with a stationary phase of Cosmosil ODS (Nakarai Chemicals, Ltd., Kyoto) packed in 250 × 4.6 mm i.d. stainless steel tubing. The eluent used was 1% acetic acid containing 1% sodium chloride at a flow rate of 0.8 ml/min. The fluorescence was monitored with excitation at 258 nm and emission at 288 nm for phenylalanine, excitation at 275 nm and emission at 305 nm for *p*-, *m*- and *o*-tyrosines, and excitation at 280 nm and emission at 318 nm for DOPA. All separations were carried out at room temperature.

(ii) Amino Acid Analyzer: A Hitachi 835 amino acid analyzer was used. The eluent used was MCI-Buffer (Mitsubishi Chemical Industries Ltd., Tokyo) for physiological fluid analysis.

Assay of Enzyme Activities——The liver, kidney, brain and adrenal were each homogenized in two volumes (liver, kidney and brain) or twenty volumes (adrenal) of 0.25 M sucrose solution under cooling. The homogenate was centrifuged at 12000 × *g* for 20 min to yield the supernatant. The supernatant fluid was further ultracentrifuged at 105000 × *g* for 60 min, and the supernatant fraction was used for the enzyme assay.

(i) Phenylalanine Hydroxylase Activity: The enzyme activity was assayed by the method of Bailey *et al.*[16] 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.

(ii) Tyrosine Hydroxylase Activity: The enzyme activity was determined by a modification of the method of Nagatsu *et al.*[17] 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 with 1.0 ml of 0.5 M perchloric acid. After centrifugation, an aliquot (50 μl) of the supernatant was subjected to HPLC. One unit of tyrosine hydroxylase is defined as the amount that catalyzes the formation of 1 nmol of DOPA in 1 min.

Results

Serum Concentration of *p*-, *m*- and *o*-Tyrosines after Administration of L-Phenylalanine

Figure 1 shows the chromatograms obtained from control serum (B) and a serum sample at 15 min after *i.p.* administration of phenylalanine (A). The peaks of *p*-, *m*- and *o*-tyrosines were identified by two methods of chromatography. First, the peaks of the tyrosine isomers were identified judging from their HPLC retention behavior and co-injection with the reference compounds. Then, the effluent corresponding to each peak was collected, and subjected to ion-exchange chromatography using an amino acid analyzer. As shown in Fig. 2, *m*-, *p*- and *o*-tyrosines 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.

Figure 3 shows the time courses of the formation of *p*-, *m*- and *o*-tyrosines in the serum after administration of L-phenylalanine. Before administration, the concentrations of the compounds were: *p*-tyrosine, 12.5 ± 1.6 μg/ml; *m*-tyrosine, 3.3 ± 1.9 ng/ml; *o*-tyrosine, 3.6 ± 1.8 ng/ml[10]; phenylalanine, 8.9 ± 1.8 μg/ml. The concentrations of *m*- and *o*-tyrosines as well as *p*-tyrosine in the serum were significantly increased by the administration of phenylalanine. The maximum level of phenylalanine was observed at 7.5—15 min after the administration. The concentration of *p*-tyrosine in the serum was maximum at 7.5 min after *i.v.*, but at 15 min after *i.p.*, *p.o.* or *i.m.* administration. Furthermore, the concentrations of *m*- and *o*-tyrosines reached a maximum at around 7.5 to 15 min after administration, and

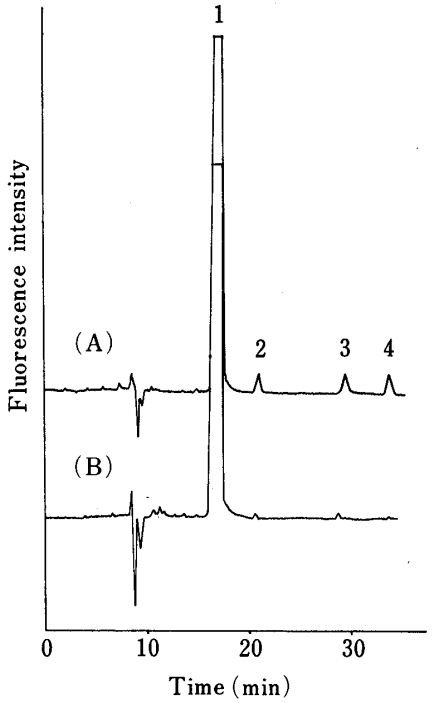

Fig. 1. HPLC Profiles of Rat Serum

(A) Injection sample: serum sample obtained from a rat 15 min after intraperitoneal administration of L-phenylalanine (80 mg/kg). A 50 μl portion was used for HPLC.

(B) Injection sample: serum sample obtained from a control rat. A 100 μl portion was used for HPLC.

Peaks: 1 = *p*-tyrosine; 2 = *m*-tyrosine; 3 = *o*-tyrosine; 4 = phenylalanine.

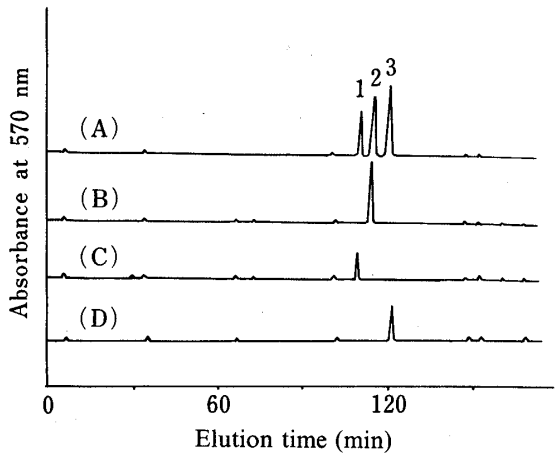

Fig. 2. Ion-Exchange Chromatogram of Tyrosine Isomer Fractions Eluted from HPLC

The *p*- (B), *m*- (C) and *o*- (D) tyrosine fractions collected, and authentic samples of tyrosine isomers (A) were subjected to ion-exchange chromatography.

Peaks: 1 = *m*-tyrosine; 2 = *p*-tyrosine; 3 = *o*-tyrosine.

thereafter returned to their normal levels. At a given time (*i.v.*, 7.5 min; *i.p.* and *p.o.*, 15 min) after the administration of a larger dose of L-phenylalanine, the amounts of tyrosine isomers were measured. The results are summarized in Table I. The serum concentrations of *p*-, *m*- and *o*-tyrosines were significantly increased for *i.p.* (400 mg/kg), *i.v.* and *p.o.* (100 mg/kg) administration compared to those seen after the administration of 80 mg/kg phenylalanine. In addition, D-phenylalanine (80 mg/kg) was administered *i.p.* to the rats and the serum was subjected to chromatography after 7.5, 15 and 30 min. The amounts of tyrosine isomers were found to be less than 10% of those formed from L-phenylalanine. The above results suggest that D-phenylalanine is not active as a substrate, and the formation of *m*- and *o*-tyrosines is caused mainly by an enzymatic reaction.

Effect of Administration of *p*-Chlorophenylalanine, Ethionine and α-Methyltyrosine

The effect of *p*-chlorophenylalanine, ethionine and α-methyltyrosine on the enzyme activities of phenylananine hydroxylase and tyrosine hydroxylase in various organs of the rat are shown in Table II. The distribution of the enzyme activities was as follows: phenylalanine hydroxylase was detected in the liver and kidney; tyrosine hydroxylase was present in the adrenal, brain and kidney.[13] Phenylalanine hydroxylase activity was inhibited by the administration of *p*-chlorophenylalanine and ethionine, and tyrosine hydroxylase activity was suppressed by the administration of α-methyltyrosine.

The effect of *p*-chlorophenylalanine, ethionine and α-methyltyrosine on the formation of tyrosine isomers from phenylalanine is shown in Table III. It was observed that basal levels of *m*-tyrosine and *o*-tyrosine showed approximately 70% reductions at 24 h after the administration of α-methyltyrosine, which is an inhibitor of tyrosine hydroxylase, whereas the

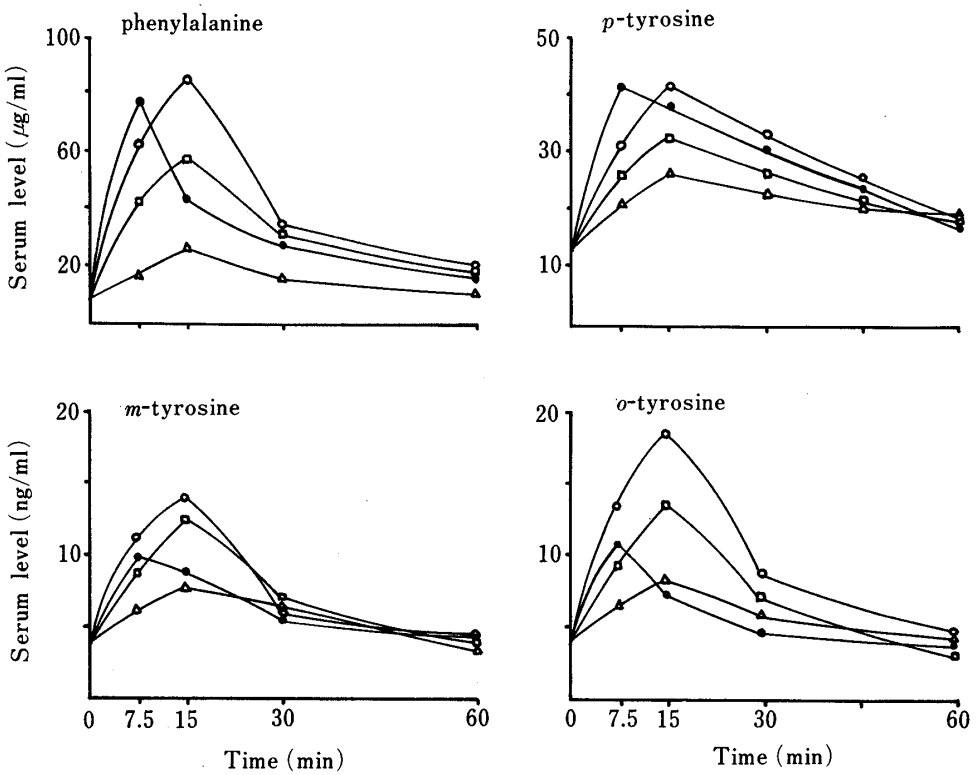

Fig. 3. Serum Levels of Phenylalanine and Tyrosine Isomers after Administration of L-Phenylalanine (80 mg/kg) to Rats

—○—, intraperitoneal; —●—, intravenous; —□—, intramuscular; —△—, peroral. Each point represents the mean of 3 animals.

TABLE I. Concentrations of Tyrosine Isomers in the Serum after Intraperitoneal, *Per os* or Intravenous Administration of L-Phenylalanine to Rats

	p-Tyrosine (μg/ml)	*m*-Tyrosine (ng/ml)	*o*-Tyrosine (ng/ml)
Control	12.5	3.3	3.6
i.p.	52.5 (40.2)[a)]	27.8 (13.3)[a)]	36.6 (18.3)[a)]
p.o.	34.4 (26.5)[a)]	8.6 (7.2)[a)]	9.3 (8.1)[a)]
i.v.	42.5 (39.5)[a)]	10.4 (8.5)[a)]	12.6 (10.7)[a)]

L-Phenylalanine was administered intraperitoneally (400 mg/kg), *per os* (100 mg/kg) or intravenously (100 mg/kg). When L-phenylalanine was given *p.o.* or *i.p.*, measurement was done at 15 min after administration, and when it was given *i.v.*, measurement was done at 7.5 min after administration. Each value represents the mean of 3 animals. *a*) The dose of L-phenylalanine (80 mg/kg).

TABLE II. Activity Changes of Phenylalanine and Tyrosine Hydroxylases in Various Tissues by Administration of the Hydroxylase Inhibitors to Rats

Inhibitor	Phenylalanine hydroxylase (unit/g wet weight)		Tyrosine hydroxylase (unit/g wet weight)		
	Liver	Kidney	Adrenal	Brain	Kidney
Control (no inhibitor)	1.92	0.28	10.15	1.40	1.01
α-Methyltyrosine	1.96	0.27	2.54	0.65	0.42
p-Chlorophenylalanine, ethionine	0.50	0.11	10.25	1.36	0.96

The inhibitors were administered intraperitoneally to rats and the enzyme activities were examined at 24 h after dosing. Each value represents the mean of three experiments.

TABLE III. Serum Phenylalanine, *p*-, *m*- and *o*-Tyrosine Concentrations after Administration of Inhibitors of Tyrosine and Phenylalanine Hydroxylases to Rats

		Concentration			
		p-Tyrosine (μg/ml)	*m*-Tyrosine (ng/ml)	*o*-Tyrosine (ng/ml)	Phenylalanine (μg/ml)
Control		12.5	3.3	3.6	8.9
α-Methyltyrosine		15.1	0.5	1.2	8.2
p-Chlorophenylalanine, ethionine		12.8	15.5	15.8	105.9
Phenylalanine	*i.v.*	39.5	8.5	10.7	78.3
	i.p.	40.2	13.3	18.3	84.9
	p.o.	26.5	7.2	8.1	24.5
	i.m.	30.5	12.4	13.8	58.7
α-Methyltyrosine + phenylalanine	*i.v.*	35.8	2.6	2.8	37.7
	i.p.	49.4	1.5	2.3	30.4
	p.o.	44.3	1.8	1.5	18.3
	i.m.	37.9	2.3	2.6	45.7
p-Chlorophenylalanine, ethionine + phenylalanine	*i.v.*	15.2	18.5	23.7	128.0
	i.p.	14.9	19.7	25.6	138.5
	p.o.	14.4	16.7	18.3	120.4
	i.m.	14.1	17.1	19.3	143.1

The rats were treated *i.p.* with *p*-chlorophenylalanine (720 mg/kg), ethionine (80 mg/kg) and α-methyltyrosine (250 mg/kg), and were used for 24 h after dosing. L-Phenylalanine was administered at the dose of 80 mg/kg. Rats given L-phenylalanine *i.p.*, *p.o.* or *i.m.* were measured at 15 min after administration, and those given it *i.v.* were measured at 7.5 min after administration. Each value represents the mean of 3 animals.

concentrations of phenylalanine and *p*-tyrosine in the serum were not affected. In addition, the inhibition of phenylalanine hydroxylase activity by the administration of *p*-chlorophenylalanine and ethionine caused a marked increase in the concentrations of phenylalanine, *m*- and *o*-tyrosines in the serum, though the concentration of *p*-tyrosine was not affected.

The formation of *p*-, *m*- and *o*-tyrosines in the serum after the administration of phenylalanine was significantly increased. However, in α-methyltyrosine-treated rats, the formation of *m*- and *o*-tyrosines was decreased after the administration of phenylalanine, though the concentration of *p*-tyrosine in the serum was not decreased. On the other hand, in *p*-chlorophenylalanine and ethionine-treated rats, the formation of *m*- and *o*-tyrosines was further increased by the administration of phenylalanine, whereas the concentration of *p*-tyrosine was markedly reduced.

Discussion

In the present study, we examined the formation of *m*- and *o*-tyrosines from L-phenylalanine *in vivo* and the mechanism of the hydroxylation of phenylalanine to tyrosine isomers, and obtained additional information on the metabolic fate of L-phenylalanine in mammals.

After intraperitoneal, intravenous, intramuscular and oral administration of L-phenylalanine to rats, the serum concentrations of *m*- and *o*-tyrosines were higher than those in control serum, and the concentrations of *m*- and *o*-tyrosines in the serum were significantly increased after the administration of a larger dose of L-phenylalanine. These results suggest that *m*- and *o*-tyrosines are formed directly from L-phenylalanine, and are not of dietary origin.

The administration of D-phenylalanine to the rats did not increase the concentrations of *m*- and *o*-tyrosines in the serum, indicating that the formation of *m*- and *o*-tyrosines is due to enzymatic reaction. Previously, D'Iorio *et al.* reported that *m*-tyrosine was formed from L-phenylalanine by a bovine adrenal medulla preparation *in vitro*, and that *m*-tyrosine was formed by the action of tyrosine hydroxylase.[18] In addition, Ikeda *et al.* reported that the conversion of L-phenylalanine to *p*-tyrosine was induced by bovine adrenal tyrosine hydroxylase.[19] We reported that L-phenylalanine was hydroxylated to *m*- and *o*-tyrosines in the soluble fraction of liver, kidney, brain and adrenal of rats, and that the formation of *m*- and *o*-tyrosines from phenylalanine is caused mainly by enzymatic reaction.[13] Our present results indicate that the concentrations of *m*- and *o*-tyrosines in the serum after the administration of phenylalanine were significantly increased. In addition, when phenylalanine hydroxylase activity was inhibited, the concentration of phenylalanine showed a marked increase in the serum, and the *m*- and *o*-tyrosine levels were also increased. Furthermore, in the phenylalanine hydroxylase inhibitor-treated rats, the formation of *m*- and *o*-tyrosines was further increase after the administration of phenylalanine. These results indicated that the administration of L-phenylalanine to rats stimulated the formation of *m*- and *o*-tyrosines with a concomitant increase in the level of phenylalanine in the serum. The experimental results described above suggest that the increment *m*- and *o*-tyrosines were not caused by the action of phenylalanine hydroxylase. In contrast, the basal *m*- and *o*-tyrosine levels were reduced by the administration of tyrosine hydroxylase inhibitor. Moreover, in the tyrosine hydroxylase inhibitor-treated rats, the concentrations of *m*- and *o*-tyrosines were suppressed after the administration of phenylalanine. Therefore, it is concluded that the formation of *m*- and *o*-tyrosines from phenylalanine *in vivo* is caused mainly by tyrosine hydroxylase, but not by phenylalanine hydroxylase.

No significant change occurred in the basal *p*-tyrosine level after the administration of the phenylalanine and tyrosine hydroxylase inhibitors (Table III). When the rats were starved continuously for 3 d and the concentrations of phenylalanine and *p*-tyrosine in the serum were examined, the basal phenylalanine level was reduced to less than 30% of that in control rats, whereas the basal *p*-tyrosine level was maintained for 1 d, followed by a slight decline over the next 2 d. Moreover, long-term (3 d) administration of α-methyltyrosine to the rats had little effect on the basal *p*-tyrosine level. In contrast, administration of phenylalanine to rats tended to increase the serum concentration of *p*-tyrosine, which thereafter returned to the normal level (Fig. 3). In addition, in α-methyltyrosine-treated rats, the *p*-tyrosine level in the serum was increased after the administration of phenylalanine (Table III). The experimental results described above suggest that the basal *p*-tyrosine level was influenced by the amount of phenylalanine in the serum, but it seems reasonable to assume that the *p*-tyrosine in the serum is controlled by an enzymatic mechanism.

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