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Studies on the Formation of 3,4-Dihydroxyphenylalanine, *m*-Tyrosine and *o*-Tyrosine from L-Phenylalanine by Rat Liver and Adrenal

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Incubation of L-phenylalanine with the soluble fractions of liver and adrenal in the presence of a pterine cofactor, 2-mercaptoethanol and catalase gave rise to a hydroxylated compound which was identified as 3,4-dihydroxyphenylalanine (DOPA), as well as *p*-, *m*- and *o*-tyrosines, on the basis of reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection. Chromatographic peaks were identified on the basis of their retention behavior and the assignment of the DOPA peak of HPLC was verified by ion-exchange chromatographic analysis.

The formation of DOPA from L-phenylalanine by purified phenylalanine hydroxylase [EC 1.14.16.1] and tyrosine hydroxylase [EC 1.14.16.2] from rat liver and adrenal, respectively, was observed. The hydroxylation of L-phenylalanine to *m*- and *o*-tyrosines was also found to be catalyzed by the purified phenylalanine and tyrosine hydroxylases. In addition, the hydroxylation of L-*p*-tyrosine to DOPA was caused by the purified phenylalanine hydroxylase.

Keywords——rat; liver; adrenal; phenylalanine; 3,4-dihydroxyphenylalanine; *m*-tyrosine; *o*-tyrosine; phenylalanine hydroxylase; tyrosine hydroxylase; fluorescence HPLC

It is well known that phenylalanine is metabolized largely by conversion to tyrosine (*p*-tyrosine) in mammals.[1] Many authors have reported the presence of *m*- and *o*-substituted phenol derivatives of tyramine,[2-6] octopamine,[7,8] and hydroxymandelic acid[9,10] of endogenous origin in human urine and adrenal gland, and it has been demonstrated that these compounds are derived from *m*-hydroxyphenylalanine (*m*-tyrosine) and *o*-hydroxyphenylalanine (*o*-tyrosine).[11,12] These findings suggest that *m*- and *o*-tyrosines may be formed in mammals. In fact, *m*- and *o*-tyrosines have been detected in rat and human serum.[13,14] 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.[15] In addition, Ikeda *et al.* reported that *p*-tyrosine and 3,4-dihydroxyphenylalanine (DOPA) were formed from L-phenylalanine by the purified tyrosine hydroxylase from bovine adrenal.[16,17] Recently, we have reported the formation of *m*- and *o*-tyrosines from L-phenylalanine *in vitro* by various tissues of the rat, and have suggested that the conversion of phenylalanine to *m*- and *o*-tyrosines may be catalyzed by phenylalanine hydroxylase and tyrosine hydroxylase.[18] However, the formation of DOPA from L-phenylalanine by rat liver phenylalanine hydroxylase has not yet been described, and no detailed study on the hydroxylation of L-phenylalanine to *m*- and *o*-tyrosines has been done.

In this report, we show that the formation of DOPA, besides *m*- and *o*-tyrosines, from L-phenylalanine also occurs in liver and adrenal preparations of rats, and that the hydroxylation of phenylalanine to *m*- and *o*-tyrosines and DOPA is catalyzed by liver phenylalanine hydroxylase and adrenal tyrosine hydroxylase of rat.

Experimental

Materials——L-Phenylalanine, L-*p*-tyrosine, D-*p*-tyrosine, DL-*m*-tyrosine, DL-*o*-tyrosine, L-DOPA, catalase (bovine liver), 6-methyl-5,6,7,8-tetrahydropterine (MPH_4) and 6,7-dimethyl-5,6,7,8-tetrahydropterine ($DMPH_4$) were obtained from Sigma Chemicals Co. (St. Louis, U.S.A.), and D-phenylalanine from the Peptide Institute Inc. (Osaka). All other chemicals used were of the highest purity commercially available.

Animal——Male Wistar rats, initially weighing 150—200 g each were used. Before the experiments, the animals were starved overnight but allowed to drink water freely. The rats were killed and the liver and adrenal were removed. The tissues were homogenized in four volumes (liver) or eighty volumes (adrenal) of 50 mM Tris–HCl buffer (pH 7.3) containing 0.32 M sucrose. The homogenates were centrifuged at $12000 \times g$ for 20 min to yield the supernatants. The supernatant fluids were further ultracentrifuged at $105000 \times g$ for 60 min, and the soluble fractions were obtained.

Hydroxylation——The reaction mixture contained 4 μmol of phenylalanine or 50 nmol of *p*-tyrosine or 50 nmol of *m*-tyrosine, 0.3 μmol of $DMPH_4$, 10 μmol of 2-mercaptoethanol, 5 μg of catalase and 25 μmol of Tris–HCl buffer (pH 7.4) in a total volume of 1 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 M trichloroacetic acid. The reaction mixture was centrifuged at $12000 \times g$ for 10 min at 0—4 °C. After filtration through a 0.22-μm filter, an aliquot (100 μl) of the supernatant was subjected to high-performance liquid chromatography (HPLC).

Measurement——(i) HPLC: The mobile phase was 0.1 M phosphate buffer (pH 2.0). The other chromatographic conditions were as described in our previous paper.[14]

(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 analysis.

Assay of Phenylalanine Hydroxylase and Tyrosine Hydroxylase Activities——The reaction mixture contained 0.2 μmol of phenylalanine or of *p*-tyrosine, 0.5 μmol of MPH_4, 50 μmol of 2-mercaptoethanol, 5 μg of catalase and 50 μmol of Tris–HCl buffer (pH 7.4) in a total volume of 1 ml. Incubation was done at 37 °C for 10 min.

Method I: The reaction was stopped by adding 0.5 ml of 6% trichloroacetic acid. After centrifugation, an aliquot (10 μl) of the supernatant was subjected to HPLC (excitation at 275 nm and emission at 305 nm).

Method II: The DOPA formed through enzymatic reaction was extracted by the method of Nagatsu *et al.*[19] The eluate (100 μl) was injected into an HPLC instrument (excitation at 280 nm and emission at 318 nm). One unit of phenylalanine hydroxylase or tyrosine hydroxylase is defined as the amount that catalyzes the formation of 1 nmol of *p*-tyrosine or DOPA in 1 min, respectively. The protein concentrations were determined by the method of Bradford with bovine serum albumin as a standard.[20]

Purification of Phenylalanine and Tyrosine Hydroxylases——Phenylalanine and tyrosine hydroxylases were prepared from rat liver by the method of Shiman *et al.*[21] and from rat adrenal by the method of Nagatsu *et al.*[19] The specific activities of the final preparations of phenylalanine and tyrosine hydroxylases were approximately 1120 and 138 unit/mg, respectively. Each purified phenylalanine hydroxylase and tyrosine hydroxylase gave a single protein band on 10% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.[22]

Results

The Formation of DOPA from L-Phenylalanine by the Soluble Fractions of Liver and Adrenal

When L-phenylalanine was incubated with the soluble fractions of liver and adrenal in the presence of a pterine cofactor, 2-mercaptoethanol and catalase, four hydroxylated products (DOPA, *p*-, *m*- and *o*-tyrosines) were formed. Figure 1 shows a typical chromatographic pattern of the reaction mixture. Although a large amount of *p*-tyrosine of endogenous origin existed in the soluble fractions, there were no detectable amounts of *m*- and *o*-tyrosines and DOPA. The peak of DOPA was identified by two methods of chromatography. First, the peak of DOPA was identified on the basis of HPLC retention behavior and co-injection with the reference compound. Then, the effluent corresponding to the peak was collected, and subjected to ion-exchange chromatography using an amino acid analyzer. As shown in Fig. 2, DOPA emerged from this system at 106 min. The retention time of the peak was identical with that of a corresponding authentic sample. The assignment of peaks 2—4 in Fig. 1 to *p*-, *m*- and *o*-tyrosines, respectively, was already made on the basis of ion-exchange chromatographic analysis.[18] These chromatographic analyses indicated the presence of a significant amount of DOPA, besides *p*-, *m*- and *o*-tyrosines, in the reaction mixture.

The hydroxylation of phenylalanine by the soluble fractions of liver and adrenal is summarized in Table I. The formation of DOPA, besides tyrosines, from phenylalanine by the

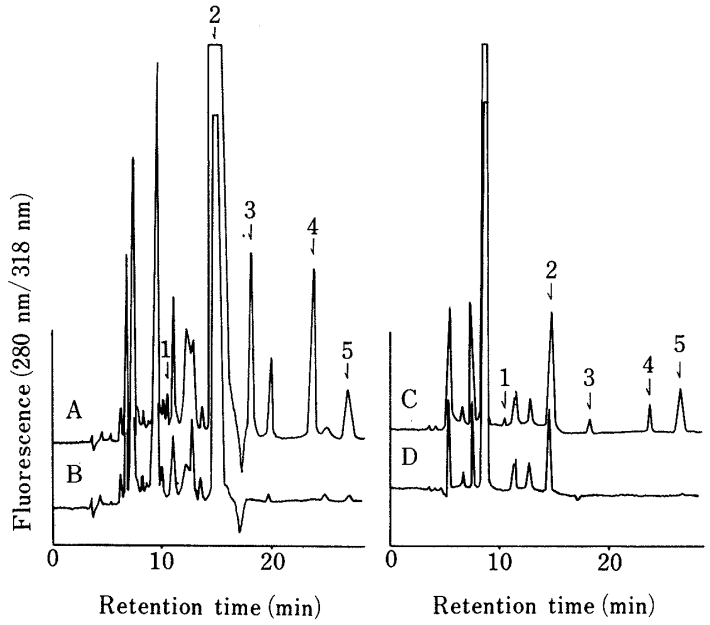

Fig. 1. High-Performance Liquid Chromatogram of the Reaction Mixture

(A) Injection sample: after reaction of L-phenylalanine with the soluble fraction of liver, pterine cofactor, 2-mercaptoethanol and catalase for 30 min under the conditions described in the text.

(B) Injection sample: same as A, except for L-phenylalanine.

(C) Injection sample: after reaction of L-phenylalanine with the soluble fraction of adrenal, pterine cofactor, 2-mercaptoethanol and catalase for 30 min under the conditions described in the text.

(D) Injection sample: same as C, except for L-phenylalanine. A 100 μl aliquot was used for HPLC.

Peaks: 1 = DOPA; 2 = *p*-tyrosine; 3 = *m*-tyrosine; 4 = *o*-tyrosine; 5 = phenylalanine.

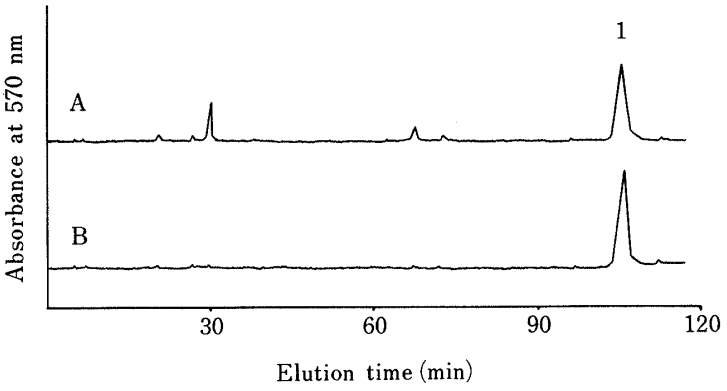

Fig. 2. Ion-Exchange Chromatogram of DOPA Fraction Eluted from HPLC

The DOPA (A) fraction collected, and an authentic sample of DOPA (B) were subjected to ion-exchange chromatography.

Peak: 1 = DOPA.

soluble fractions of liver and adrenal occurred in the presence of $DMPH_4$. The amount of DOPA formed by the adrenal per mg protein was approximately 12 times that by the liver. In addition, when L-*p*-tyrosine was incubated with the soluble fraction of liver in the presence of $DMPH_4$, 2-mercaptoethanol and catalase, the formation of DOPA occurred (data not shown). We previously reported that phenylalanine hydroxylase was found in the liver but not in the adrenal, and tyrosine hydroxylase was found in the adrenal but not in the liver.[18] The formation of DOPA from phenylalanine or from *p*-tyrosine was greatly reduced if $DMPH_4$

TABLE I. The Formation of *p*-Tyrosine, *m*-Tyrosine, *o*-Tyrosine and DOPA from L-Phenylalanine by the Soluble Fractions of Liver and Adrenal

Tissue	Substrate	Substance	Hydroxyphenylalanines formed (nmol)			
			p-Tyrosine	*m*-Tyrosine	*o*-Tyrosine	DOPA
Liver	L-Phenylalanine	$+DMPH_4$	240.62	0.95	0.80	0.04 (0.005)[a]
		$-DMPH_4$	1.66	0.08	0.08	0
Adrenal	D-Phenylalanine	$+DMPH_4$	6.62	0.10	0.09	0
	L-Phenylalanine	$+DMPH_4$	0.14	0.06	0.06	0.01 (0.06)[a]
		$-DMPH_4$	0.02	0.02	0.01	0
	D-Phenylalanine	$+DMPH_4$	0.03	0	0	0

The reaction mixtures contained the liver soluble fraction or the adrenal soluble fraction, 4 μmol of phenylalanine, 0.3 μmol of $DMPH_4$, 10 μmol of 2-mercaptoethanol, 5 μg of catalase and 25 μmol of Tris–HCl buffer (pH 7.4) in a total volume of 1 ml. Incubation was carried out at 37 °C for 30 min. The specific activities of phenylalanine hydroxylase of the liver soluble fraction and tyrosine hydroxylase of the adrenal soluble fraction used were approximately 5.54 and 0.094 unit/mg protein. The reaction mixtures contained 43.6 units of phenylalanine hydroxylase activity or 0.015 unit of tyrosine hydroxylase activity. *a*) ( ): nmol per mg protein.

TABLE II. The Formation of *p*-Tyrosine, *m*-Tyrosine, *o*-Tyrosine and DOPA by the Purified Phenylalanine Hydroxylase and Tyrosine Hydroxylase

Enzyme	Substrate	Hydroxyphenylalanines formed (nmol)			
		p-Tyrosine	*m*-Tyrosine	*o*-Tyrosine	DOPA
Phenylalanine hydroxylase	L-Phenylalanine	61.26	0.22	0.26	0.02
	D-Phenylalanine	4.42	0	0	0
Tyrosine hydroxylase	L-Phenylalanine	0.22	0.19	0.26	0.01
	D-Phenylalanine	0	0	0	0

Enzyme	Substrate	DOPA (nmol)
Phenylalanine hydroxylase	L-*p*-Tyrosine	0.02
	D-*p*-Tyrosine	0
	DL-*m*-Tyrosine	0.14
Tyrosine hydroxylase	L-*p*-Tyrosine	0.09
	D-*p*-Tyrosine	0
	DL-*m*-Tyrosine	0.02

The reaction mixtures contained 11.2 units of phenylalanine hydroxylase activity or 0.067 unit of tyrosine hydroxylase activity, 4 μmol of phenylalanine or 50 nmol of *p*-tyrosine or 50 nmol of *m*-tyrosine, 0.3 μmol of $DMPH_4$, 10 μmol of 2-mercaptoethanol, 5 μg of catalase and 25 μmol of Tris–HCl buffer (pH 7.4) in a total volume of 1 ml. Incubation was carried out at 37 °C for 30 min. The specific activities of the final preparations of phenylalanine hydroxylase and tyrosine hydroxylase used were approximately 1120 and 138 unit/mg protein, respectively.

was omitted from the reaction mixture. It is well known that $DMPH_4$ is a coenzyme of phenylalanine and tyrosine hydroxylases.[23,24] The findings may suggest that the hydroxylation of L-phenylalanine to DOPA was catalyzed by phenylalanine hydroxylase and tyrosine hydroxylase, and that of L-*p*-tyrosine to DOPA was also catalyzed by phenylalanine hydroxylase. However, the formation of DOPA and *m*- and *o*-tyrosines from L-phenylalanine by the enzymes has not been elucidated in detail. Thus, we investigated the formation of DOPA and *m*- and *o*-tyrosines from phenylalanine by using the purified phenylalanine hydroxylase and tyrosine hydroxylase.

The Formation of DOPA, *m*-Tyrosine and *o*-Tyrosine from L-Phenylalanine by the Purified Phenylalanine Hydroxylase and Tyrosine Hydroxylase

To identify the products formed by the reaction of phenylalanine, *p*-tyrosine and *m*-

tyrosine with the purified enzymes in the presence of $DMPH_4$, 2-mercaptoethanol and catalase, the reaction mixtures were subjected to HPLC. The results are summarized in Table II. The formation of DOPA from L-phenylalanine was observed by the purified phenylalanine hydroxylase and tyrosine hydroxylase. Our observation that DOPA was formed from L-phenylalanine by the action of tyrosine hydroxylase from rat adrenal agrees with the results of experiments using the purified tyrosine hydroxylase from bovine adrenal.[16,17] In addition, the formation of DOPA from L-*p*-tyrosine was catalyzed by phenylalanine hydroxylase. On the other hand, when D-phenylalanine and D-*p*-tyrosine were added as substrates, the formation of DOPA was not detected. The result indicates that the enzymes are specific for L-phenylalanine and L-*p*-tyrosine. When DL-*m*-tyrosine was added as a substrate, the formation of DOPA was observed. This result is consistent with a report that L-*m*-tyrosine was converted to DOPA by a partially purified rat liver phenylalanine hydroxylase and beef adrenal medulla tyrosine hydroxylase.[25]

In addition, as we would expect, the hydroxylation of phenylalanine to *m*- and *o*-tyrosines was catalyzed by the phenylalanine hydroxylase and tyrosine hydroxylase. No significant hydroxylation reaction occurred on omission of $DMPH_4$ from the complete system. These results suggest that *m*- and *o*-tyrosines are formed from L-phenylalanine, and the conversion is catalyzed by phenylalanine and tyrosine hydroxylases.

Discussion

In the present study, we examined the hydroxylation of L-phenylalanine by the soluble fractions of liver and adrenal and the purified phenylalanine and tyrosine hydroxylases, and obtained additional information on the formation of DOPA, *m*- and *o*-tyrosines from L-phenylalanine.

The formation of DOPA, besides *m*- and *o*-tyrosines, from L-phenylalanine by the soluble fractions of liver and adrenal was observed. In the case of liver and adrenal, the formation of DOPA, *m*- and *o*-tyrosines was not observed by the use of 0.2 μmol/ml of L-phenylalanine. The result indicates that the formation of DOPA, *m*- and *o*-tyrosines is influenced by the amount of L-phenylalanine in the reaction mixure. The amount of DOPA formed by the adrenal per mg protein was approximately 12 times that by the liver. The results suggest that the ability of the adrenal to form DOPA from L-phenylalanine is higher than that of the liver. The formation of DOPA, *m*- and *o*-tyrosines from L-phenylalanine was also catalyzed by the purified phenylalanine hydroxylase and tyrosine hydroxylase. We calculated the amounts of tyrosines (*m*-+*o*-) formed per activity unit of phenylalanine and tyrosine hydroxylases with the crude and the purified enzyme preparations. The results are listed in Table III. If phenylalanine and tyrosine hydroxylases were the sole enzymes catalyzing phenylalanine to *m*- and *o*-tyrosines, the amounts of tyrosines formed per enzyme activity unit should remain constant between the crude and the purified enzyme preparations. It was found that the amounts of tyrosines (*m*-+*o*-) formed per activity unit of the enzymes were similar

TABLE III. The Relationship between the Activities of Hydroxylases and the Formation of Tyrosines

Enzyme	Enzyme source	Tyrosines (*m*-+*o*-) formed/enzyme activity[a] (nmol/unit)	Enzyme	Enzyme source	Tyrosines (*m*-+*o*-) formed/enzyme activity[a] (nmol/unit)
Phenylalanine hydroxylase	Crude[b]	0.040	Tyrosine hydroxylase	Crude[b]	8.0
	Purified	0.043		Purified	6.7

a) The values were calculated on the basis of the data of Tables I and II. *b*) The soluble fractions were used.

with the crude and the purified enzymes. This result demonstrates that no other enzyme catalyzing the conversion of phenylalanine to *m*- and *o*-tyrosines exists besides phenylalanine and tyrosine hydroxylases. From the present results, it was concluded that the formation of *m*- and *o*-tyrosines and DOPA from phenylalanine was catalyzed by phenylalanine and tyrosine hydroxylases of rat liver and adrenal, respectively. In addition, the hydroxylation of L-*p*-tyrosine to DOPA was also observed by the soluble fraction of liver and the purified phenylalanine hydroxylase, suggesting that L-*p*-tyrosine is also a substrate for rat liver phenylalanine hydroxylase.

As shown in Table III, a remarkable difference in the rate of formation of tyrosines (*m*-+*o*-) was observed between phenylalanine and tyrosine hydroxylases. That is, the amounts of tyrosines (*m*-+*o*-) formed per activity unit of the crude and purified tyrosine hydroxylases were 200 and 156 times, respectively, greater than those of the crude and purified phenylalanine hydroxylases. We previously reported that the serum concentrations of *m*- and *o*-tyrosines were significantly reduced by the administration of α-methyltyrosine, which is an inhibitor of tyrosine hydroxylase, and the inhibition of phenylalanine hydroxylase activity by the administration of *p*-chlorophenylalanine and ethionine resulted in a marked increase of the serum concentration of *m*- and *o*-tyrosines.[26] These results might suggest that tyrosine hydroxylase was implicated mainly in the *in vivo* conversion of phenylalanine to *m*- and *o*-tyrosines. The present results (Table III) may also support the above considerations.

Recently, Ibrahim *et al.* reported that the concentration of *m*-octopamine in urine of neuroblastoma patients was 3- to 5-fold higher than that of normal subjects.[27] In addition, *m*-hydroxymandelic acid in urine of phenylketonuria patients was significantly increased after the administration of a dose of *m*-tyrosine.[28] It was shown in the previous paper that the concentrations of *m*- and *o*-tyrosines were influenced by the amount of phenylalanine in the serum.[26] It is, therefore, of interest to examine the serum concentrations of *m*- and *o*-tyrosines in patients with various diseases.

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