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Studies on Phenylalanine Metabolism by Tracer Techniques. IV.¹⁾ Biotransformation of D- and L-Phenylalanine in Man

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A healthy human female was loaded with D- and L-phenylalanine (Phe)- d_5 (8 mg/kg body weight), and free Phe- d_5 and tyrosine (Tyr)- d_4 , and protein-bound Phe- d_5 in the plasma were assayed by gas chromatography-mass spectrometry with selected ion monitoring. Free Phe- d_5 appeared more rapidly and abundantly in the plasma and disappeared more slowly from the plasma after loading with the D-isomer than after loading with the L-isomer. The maximum concentrations were 1.5 mg/100 ml plasma at 30 min after loading with the D-isomer, and 0.1 mg/100 ml plasma at 2 hr after loading with the L-isomer. The half-lives were 3.3 and 0.5 hr for the D-isomer and the L-isomer, respectively. After loading with the D-isomer, as after loading with the L-isomer, free Tyr- d_4 and protein-bound Phe- d_5 were found in the plasma.

Keywords—D- and L-phenylalanine- d_5 ; deuterium-labeled; gas chromatography-mass spectrometry; metabolism in man; stable isotope

Several D-amino acids, so-called unnatural isomers, serve as sources of essential amino acids in the diet. Only D-methionine and D-phenylalanine (Phe) are available as substitutes for the L-isomers to maintain body protein in man, but other D-amino acids can also be used for growth in the rat. The first use of isotopes to establish the occurrence of inversion of the D-isomer to the L-form was reported by Ratner *et al.*, who added deuterium-, ^{15}N -labeled D-leucine to the stock diet fed to adult rats.³⁾ The L-leucine subsequently isolated from the proteins of the liver and the rest of the carcass contained considerable deuterium, but very little ^{15}N . In this work, we studied the metabolism of D- and L-Phe in man.

Experimental

Chemicals and Equipments—L- and D-Phe- d_5 ⁴⁾ and DL-Tyr- d_4 ¹⁾ were prepared in our laboratory as described previously. Gas chromatography-mass spectroscopy with selected ion monitoring was performed according to the method reported previously.¹⁾

Subject and Methods—A 40-yr-old healthy 47 kg female volunteer was studied. For one week before the investigation the subject received a diet containing 30 g of protein per day and of sufficient calorific value to maintain body weight. The subject was loaded with D- or L-Phe- d_5 (8 mg/kg body weight) plus essential amino acids and 400 kcal of low-protein diet (Table I) at 9 AM after an overnight fast. Nine venous blood samples were obtained (immediately before, and at 15, 30 min, 1, 2, 3, 4, 6 and 8 hr after loading) in heparinized test tubes and centrifuged. At 12 AM and 3 PM the subject received 400 kcal of the same diet as above. Four experiments, two for the D-isomer and two for the L-isomer, were performed at intervals of more than one month.

Free Phe- d_5 and Tyr- d_4 Contents of Plasma—The working procedure is shown in Chart 1. Two 200 μl portions of plasma samples were prepared for double dilution analysis. To one sample, 2 or 10 μg of Phe- d_5 was added. This sample and the other one were deproteinized with 3 volumes of ethanol and centrifuged. The supernatants were applied to cellulose-coated thin-layer plates (Avicel SF, Funakoshi Yakuhin Co. Ltd.) after concentration to about 50 μl under a stream of nitrogen. The plates were developed for 4–5 hr at room temperature with *n*-butanol:acetic acid:water (3:1:1, v/v), then dried in the air. The Phe and Tyr areas were located by comparing the migration with that of authentic reference compounds which were detected with ninhydrin reagent. Phe and Tyr were eluted from the cellulose absorbent with methanol and derivatized to the (N,O)-heptafluorobutyrylmethyl esters (HFB-Me) after removal of the solvent, as des-

cribed previously.¹⁾ A 2–5 μ l portion of the derivative solution was analyzed by GC-MS. The $[M-213]^+$ fragments of m/z 162, 167 of Phe-HFB-Me and m/z 374, 378 of Tyr-HFB-Me were monitored.

Protein-bound Phe- d_5 Content of Plasma—The working procedure is shown in Chart 2. A 100 μ l portion of plasma sample, a precooled 50 μ l aliquot of 0.1 N acetate buffer (pH 4.0) and a 350 μ l aliquot of 60% methanol were placed in a small test tube in a ice-water. After standing in a ice water for 30 min, the test tube was centrifuged in a refrigerated centrifuge at 3000 rpm for 5 min (the precipitate: globulin fraction). A precooled 660 μ l aliquot of methanol was added to the supernatant, and the sample was then centrifuged in the same way as above (the precipitate: albumin fraction). The globulin fraction was washed with a precooled mixture of 50 μ l of 0.1 N acetate buffer (pH 4.0) and 350 μ l of 60% methanol, and the albumin fraction was washed with a precooled mixture of 100 μ l of water and 300 μ l of methanol. The two protein fractions were suspended in 3 ml of water separately. A 0.5 ml aliquot of each was hydrolyzed with 5.5 N HCl at 110° in a sealed tube for 24 hr. The contents of the tubes were evaporated to dryness under reduced pressure, dissolved in a small volume of 70% methanol, chromatographed, derivatized and analyzed in the manner described above.

Results and Discussion

Deuterium-labeled L-Phe has been used in metabolic studies in man by several investigators. Curtius *et al.*⁵⁾ and Trefz *et al.*⁶⁾ administered 200 mg/kg body weight of L-Phe- d_5 (orally) and 30 mg/kg body weight of L-Phe- d_7 (*i.v.*), respectively, to phenylketonurics and healthy controls in order to determine the Phe 4-hydroxylase activity. In the present work, a healthy subject was loaded with 8 mg/kg body weight of D- and L-Phe- d_5 (orally) which corresponds to one-third of the daily Phe requirement,⁷⁾ to avoid disorder of the Phe metabolism as far as possible.

The diets before and after administration of labeled Phe are an important consideration, because the utilization of an amino acid is affected by other amino acids. The subject was given L- or D-Phe- d_5 with 400 kcal of low-protein diet, which corresponds to one-third of the daily energy requirement,⁸⁾ and which contained essential amino acids other than Phe and Tyr according to a provisional amino acid scoring pattern,⁹⁾ plus the dose of labeled Phe (Table I).

TABLE I. Composition of Low-protein Diet and Essential Amino Acids given with Labeled Phe

	g	kcal	Protein g		mg
Pineapple	80	62	0.32	Isoleucine	250
Cornstarch	12	42	0.01	Leucine	440
Sugar	14	54	0.00	Lysine	340
Apple	200	90	0.80	Methionine	220
Margarine	10	72	0.02	Threonine	250
Sugar	20	77	0.00	Tryptophan	60
				Valine	310
		397	1.15		

To determine free Phe- d_0 and - d_5 in plasma by the double dilution method, two plasma samples were prepared and standard Phe- d_5 corresponding to 1/10 to 10 times the amount of Phe- d_5 contained in the sample was added to one of them (Chart 1). The plasma proteins were fractionated according to the method described by Saito and Yoshikawa (Chart 2).¹⁰⁾

In this work, we did not distinguish between D- and L-form Phe- d_5 and Tyr- d_4 in the determination, although the pathway leading to the formation of Tyr- d_4 and protein-bound Phe- d_5 is of interest. It is believed that deamination, followed by reamination to the L-isomer, is the method by which the body utilizes the so-called unnatural isomer of some amino acids. Thus, it is very likely that both Phe- d_5 in the protein fraction and Tyr- d_4 in the free amino acid fraction after loading with D-Phe- d_5 are L-forms.

Chart 1. Assay Procedures for Free Phe- d_5 and Tyr- d_4 in Plasma Chart 2. Assay Procedure for Protein-bound Phe- d_5 in Plasma

The time course of the molar ratio of Phe- d_5 to Phe- d_0 in the free amino acid fraction after loading with D-Phe- d_5 was distinct from that after loading with L-Phe- d_5 : in the former case the molar ratio increased more rapidly and decreased slowly than in the latter case (Fig. 1). The half-life was estimated as the time required for the molar ratio of Phe- d_5 to Phe- d_0 to be reduced to one-half: the values obtained for D-Phe- d_5 were 3.3 and 3.1 hr and those for L-Phe- d_5 were 0.7 and 0.5 hr in two experiments. We reported previously that the half-lives of D-Phe- d_5 and L-Phe- d_5 in rat plasma after *i.v.* injection were 24 and 11.5 min, respectively.¹¹⁾ More remarkable is the incorporation of Phe- d_5 into the plasma protein and the formation of Tyr- d_4 after loading with the D-isomer (Fig. 1).

The time courses of the concentrations of free Phe- d_5 and - d_0 in the plasma, assayed by the double dilution method, are shown in Fig. 2. After loading with D-Phe- d_5 , Phe- d_5 appeared in the plasma rapidly (at 15 min after loading, 0.9 or 1.0 mg/100 ml plasma) and abundantly (maximum concentration: at 30 min after loading, 1.5 or 1.3 mg/100 ml plasma). On the other hand, after loading with the L-isomer, Phe- d_5 appeared in the plasma more slowly and to a lesser extent (maximum concentration: at 1 or 2 hr after loading, 0.4 or 0.1 mg/100 mg plasma).

D-Phe is one of the D-isomers of essential amino acids which can be utilized to an appreciable degree by the adult human for the maintenance of nitrogen equilibrium, but only a limited amount, approximately 0.5 g per day, appears to be utilizable.¹²⁾ The following

Fig. 1. Time Course of Molar Ratios of Phe- d_5 /Phe- d_0 and Tyr- d_4 /Tyr- d_0 in the Plasma after Loading with L- or D-Phe- d_5

(A)–(D); A 40-yr-old healthy 47 kg female. (A), (B); loading with 8 mg/kg body weight of L-Phe- d_5 . (C), (D); loading with 8 mg/kg body weight of D-Phe- d_5 .

○—○; Phe in free amino acid fraction, ○—•—○; Phe in globulin fraction, ○—•—○; Phe in albumin fraction, △—△; Tyr in free amino acid fraction.

Fig. 2. Time Course of Concentrations of Phe- d_5 and Phe- d_0 in the Plasma after Loading with L- or D-Phe- d_5

(A)–(D); same as in Fig. 1.

○—○; Phe- d_0 , ●—●; Phe- d_5 .

explanations have been proposed for the poor utilization of the D-amino acids. 1) The D-isomers may be absorbed at a slower rate from the gastrointestinal tract than the L-isomers. 2) The D-isomers are inverted to the L-forms only slowly. 3) The α -keto acids, which are believed to be the intermediates in the inversion of D-amino acids to the L-forms, are highly reactive and decompose readily. 4) The D-isomers are the more susceptible to urinary excretion. In this experiment Phe- d_5 appeared in the plasma more rapidly and abundantly after loading with the D-isomer (Fig. 2). This does not necessarily suggest quicker absorption of the D-isomer, but may be the result of faster metabolism of the L-isomer in the liver. The time courses of the molar ratios of Phe- d_5 to Phe- d_0 in the plasma proteins and of Tyr- d_4 to Tyr- d_0 in the free amino acid fraction after loading with the D-isomer were similar to those obtained after loading with the L-isomer (Fig. 1). This suggests that D-Phe- d_5 was inverted to its L-form at a reasonably rapid rate, and that decomposition of the presumed α -keto acid intermediate was not excessive under the present conditions.

In conclusion, when a healthy human female was loaded with D- and L-Phe- d_5 (8 mg/kg body weight), free Phe- d_5 appeared more rapidly and abundantly in the plasma and disappeared more slowly from the plasma after loading with the D-isomer than after loading with the L-isomer.

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