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Studies on Phenylalanine Metabolism by Use of Tracer Techniques. II.[1] Measurement of Distribution of L- and D-Phenylalanine with Their Deuterated Species

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The technique of mass fragmentography was applied to the determination of deuterated L- and D-phenylalanine loaded in animals. Method I: L-Phe-d_5 or D-Phe-d_5 is injected into the rat intravenously and the labeled compound in the plasma is measured at appropriate intervals with L-Phe-d_8 as an internal standard. Method II: Equimolar mixture of L-Phe-d_8 and D-Phe-d_5 is injected into the rat intravenously and the isomers in the plasma are measured simultaneously by the double dilution method. The values obtained by the two different methods agreed well. In the distribution, however, a large difference between L- and D-forms was recognized.

Keywords——gas chromatography/mass spectrometry (GC–MS); tracer; L-phenylalanine; D-phenylalanine; metabolism

Recent developments in the equipments and techniques for gas chromatography/mass spectrometry (GC–MS) prompted this preliminary study to devise method for the research into D-amino acid metabolism by the use of the metabolites labeled with stable isotopes.

We reported the synthesis and method of determination of L- and D-phenylalanine (Phe) (arom. -d_5) previously.[1] In the present paper, the distribution of the labeled L- and D-forms injected into the rat intravenously was measured with commercial L-Phe-d_8. We describe also the isotope effect of the Phe-d_8 derivative on GC and the stability of the deuterium-labels on Phe-d_8 *in vivo*. This method may be extended to human experimentation.

Experimental

Materials——L-Phe (U-^{14}C) was obtained from the Radiochemical Centre Amersham, England. L- and D-Phe (arom. -d_5) were synthesized from benzaldehyde (arom. -d_5). L-Phe-d_8 (98 atom % *d*) was obtained from Merck Sharp and Dohme, Canada Limited.

Animals and Loading——The experiments were carried out on male Wistar rats (Sankyo Lab. Co. Ltd.) weighing 140—150 g, anesthetized with pentobarbital. L- or D-Phe-d_5 (Method I) or mixture of L-Phe-d_8 and D-Phe-d_5 (Method II) (0.05 mg in 0.2 ml of distilled water/100 g body weight respectively) were injected through the external iliac vein. At appropriate intervals, blood samples were drawn from the abdominal aorta with heparinized injectors.

Preparation of Samples——A plasma sample (1.0 ml) was deproteinized with alcohol (3.0 ml) and centrifuged. In Method I, 0.003 μmol of L-Phe-d_8 as an internal standard was added to an aliquot (1.0 ml) of the supernatant. In Method II, 0.003 μmol of L-Phe-d_8 was added to one aliquot (1.0 ml) of the supernatant, while to another aliquot (1.0 ml) no addition was made for double dilution analysis. These were evaporated to a small volume and chromatographed on a cellulose-coated plate (Abisel SF, Funakoshi Yakuhin Co. Ltd.). The solvent system used was *n*-butanol–acetic acid–water (4: 2: 1, v/v). The zone corresponding to Phe was located by comparing its migration with the authentic reference compound which was detected with ninhydrin reagent. Phe was eluted with methanol.

Derivatization——The samples prepared in this manner were derivatized to enaminemethyl esters as described by W.E. Pereira *et al.*[3] (Chart 1, 2).

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1) Part I: S. Tokuhisa, H. Yoshikawa, S. Ichihara, and S. Baba, *Radioisotopes* (Tokyo), **26**, 630 (1977).
2) Location: *a) Komagome 3-24-3, Toshima-ku, Tokyo, 170, Japan*; *b) Horinouchi 1432-1, Hachiōji-shi, Tokyo, 192-03, Japan.*
3) W.E. Pereira, V.A. Bacon, Y. Hoyano, R. Summons, and A.M. Duffield, *Clin. Biochem.*, **6**, 300 (1973).

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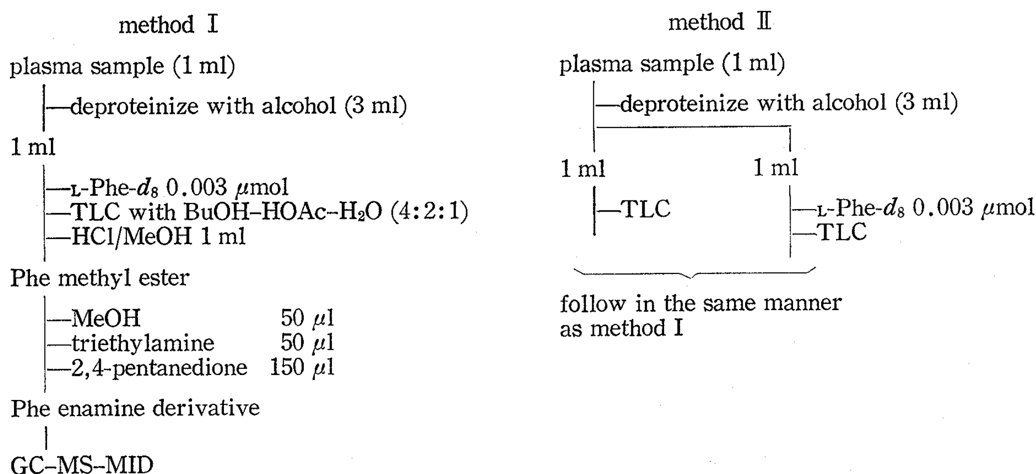

Chart 1. Experimental Procedure

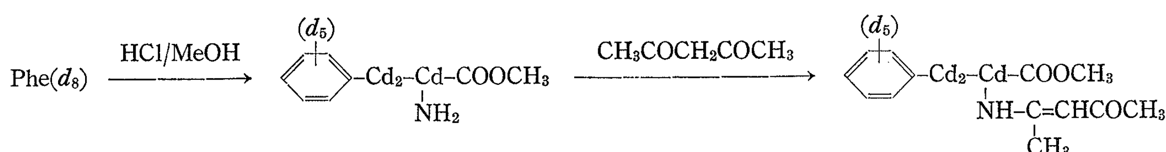

Chart 2. Preparation of Phe-d_8 Enaminemethyl Ester

GC-MS Method——GC-MS was performed with a Shimadzu LKB-9000 equipped with multiple ion detector (MID) and two-pen recorder. The glass column, length 2 m, was packed with 1.7% OV-1, and the determination was carried out under the following conditions: flush heater, 250°; column, 220°; separator, 280°; ion sourse, 310°. Ionization energy was 20 eV with a He gas flow of 20 ml/min.

Results and Discussion

The mass spectrometric behavior of labeled compounds which should be clarified before the study on the metabolism was investigated as enaminemethyl esters,[1] because we chose those as the derivatives for determination.[1] The mass spectra of L-Phe-d_8 enaminemethyl ester are shown in Fig. 1. The peaks at m/e 269, 210 and 171 correspond to M^+, ($M^+-COOCH_3$) and ($M^+-CH_2C_6H_5$), respectively. In the following work, M^+ was used although it constitutes only about 13% of the total ions.

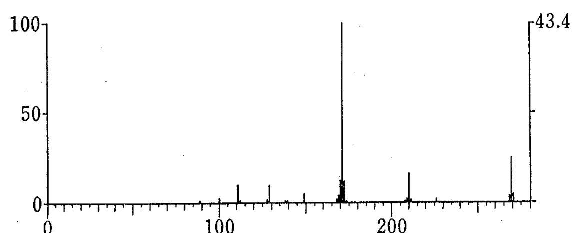

Fig. 1. Mass Spectrum of L-Phe-d_8 Enaminemethyl Ester

In the previous report, we described that the retention time of the d_5-derivative was shorter than that of the d_0-derivative.[1] In this experiment the enaminemethyl esters were prepared from an equimolar mixture of L-Phe-d_5 and L-Phe-d_8, and mass fragmentography of each M^+ was done with a visicorder (Fig. 2). When the retention time for the d_5-derivative was 2 min, that of d_8-derivative was shorter by about 0.4 sec. This result shows that MID is essential for their quantitative determination.

The calibration curves in the range where the molar ratios of Phe-d_5/Phe-d_0 and Phe-d_8/Phe-d_0 are 1/10—1/300 showed good linearity (Fig. 3).

The amount of SI tracer to be loaded should be carefully chosen so as to permit preciseness in the determination and at the same time to preserve physiological conditions. L-Phe-(U-^{14}C) (1.2×10^7 dpm, 0.37 mg Phe in 0.2 ml of water/100 g body weight) was injected

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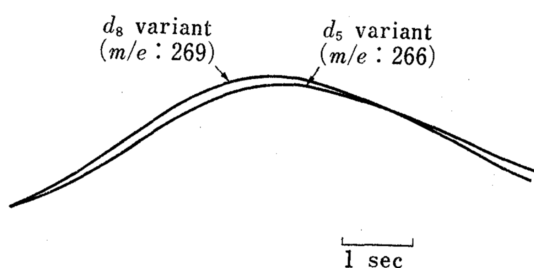

Fig. 2. Isotope Effect of L-Phe-d_5 and L-Phe-d_8 Enaminemethyl Esters on GC

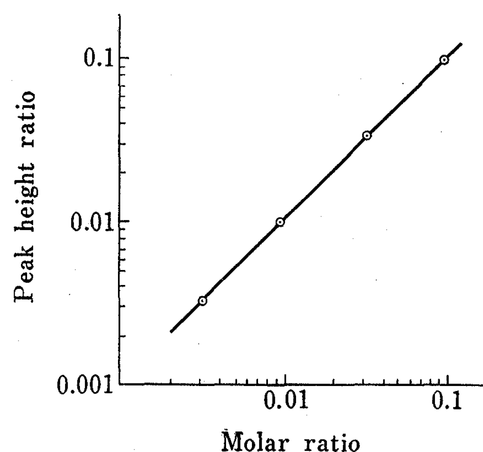

Fig. 3. Relation between Molar Ratio and Peak Height Ratio of Phe-d_5/Phe-d_0

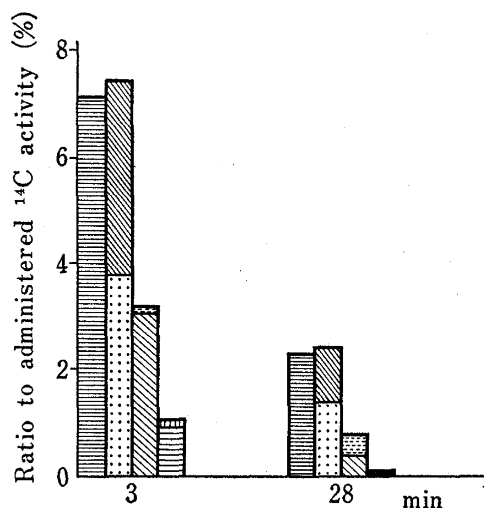

Fig. 4. Distribution of ^{14}C Activity in Rat Blood following an *i.v.* Injection of L-Phe (U-^{14}C)

whole blood, cells, plasma, Pro, non-Pro, Tyr, Phe.
1.2×10^7 dpm, 0.37 mg Phe/100 g body weight.

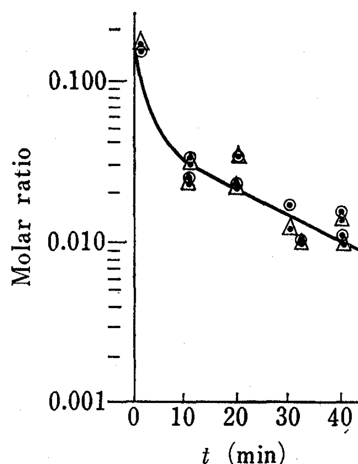

Fig. 5. Molar Ratio (Phe-d/Phe) in Rat Plasma after an *i.v.* Injection of L-Phe-d_5+L-Phe-d_8 (1 : 1)

⊙ L-Phe-d_5/L-Phe-d_0, △ L-Phe-d_8/L-Phe-d_0.

through the rat caudal vein. Three and 28 min after the injection, blood samples were drawn from the abdominal aorta with heparinized injectors, centrifuged, deproteinized with alcohol and separated with thin-layer chromatography (TLC). ^{14}C-Activities distributed in each part were measured with a liquid scintillation counter (Fig. 4). About 0.1% of the activity injected into rats remained as Phe in whole body plasma after 28 min and we determined the amount of SI tracer to be loaded as 0.05 mg/100 g body weight.

In the metabolic studies, compounds that suffer neither loss nor "scrambling" of the labels must be used. Though Baba and Horie showed that the tritium-labels on the aromatic ring were stable *in vivo* with *l*-ephedrine,[4] the stability of the labels on the side chain of Phe was not proved. When an equimolar mixture of L-Phe-d_8 and L-Phe-d_5 was injected into rats intravenously, no difference in the distribution rate observed between d_5 and d_8 isomers (Fig. 5), then we concluded that the deuterium labels on the side chain as well as on the aromatic ring of Phe were stable.

4) S. Baba and M. Horie, *Yakugaku Zasshi*, **94**, 779 (1974).

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We have developed two rapid, specific and reliable methods for determination of the distribution rate of labeled L- and D-Phe for the characterization of the metabolism of L- and D-form. Method I: L-Phe-d_5 or D-Phe-d_5 is injected into rats and the isomers in the plasma are measured with L-Phe-d_8 as an internal standard. Method II: An equimolar mixture of L-Phe-d_8 and D-Phe-d_5 is injected into rats and the isomers in the plasma are determined simultaneously by the double dilution method. Method II is more desirable than Method I, because it is not affected by individual differences of the animals. The results are shown in Fig. 6. Models have been used to describe and to interpret a set of data obtained by experiment. According to the curves–blood level *versus* time–, our experimental data seem to fit a "open two compartment model".[5] In the model, the "drug" entering the body does not instantly distribute between the blood and those other body fluids or tissues which it eventually reaches. The body fluids or tissues which instantly equilibrate with the circulatory system comprise the "central compartment". Those body fluids or tissues into which the "drug" distributes slowly comprise the "peripheral compartment". The distribution volumes of these compartments were calculated according to the equation commonly used (Table I).[5a] It became clear that the volumes of the central compartments for L- and D-Phe correspond to the volume of extracellular fluid, *i.e.*, interstitial fluid and blood plasma *etc.*

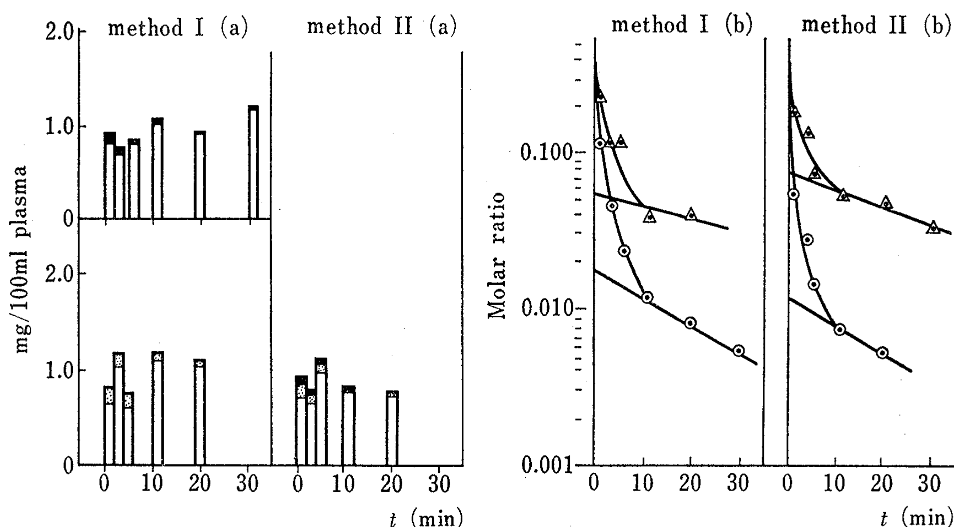

Fig. 6. Phe Concentration (a) and Molar Ratio (b) in Rat Plasma after an *i.v.* Injection of Deuterium-labeled Phe. Method I: load with L-Phe-d_5 or D-Phe-d_5, Method II: load with L-Phe-d_8+D-Phe-d_5

Method I (a) □ L-d_0, ■ L-d_5, ▨ D-d_5. Method II (a) □ L-d_0, ■ L-d_8, ▨ D-d_5. Method I (b) ⊙ L-d_5/d_0, △ D-d_5/d_0. Method II (b) ⊙ L-d_8/d_0, △ D-d_5/d_0.

Table I. Apparent Volume of Distribution of L-Phe and D-Phe

	L-Phe	D-Phe
Central compartment	14 ml	14 ml
Peripheral compartment	380 ml	83 ml

Per 100 g body weight of rat.

5) W.A. Ritschel, "Handbook of Basic Pharmacokinetics," Drug Intelligence Publications, Hamilton, Illinois, 1976; *a*) p. 180 $Cp=B \cdot e^{-\beta t}+A \cdot e^{-\alpha t}$

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Qualitative comparisons of the L- and D-forms of the essential amino acids indicate that, in some instances both forms can be used for tissue synthesis or repair, but that in other instances only the L-form will be utilized for either purpose.[6] Phe belongs to the former group. Prior to the use of D-amino acid, it must be converted to the L-form, *i.e.*, it is deaminized with loss of asymmetry, then reaminized to the L-form. On the other hand, it is recognized that D-isomers are more readily excreted into urine than L-isomers, probably because of a lower kidney threshold for D-amino acids. Hence the disappearance of deuterium-labeled D-Phe seems to be mainly due to deamination and excretion into urine. The series of enzyme systems for deaminizing D-amino acids oxidatively are contained in liver and kidney of all vertebrates. The distribution of D-Phe did not decrease in the rats of which liver was injured by the administration of CCl_4.

6) A.A. Albanese (ed.), "Protein and Amino Acid Nutrition," Academic Press, New York and London, 1959.

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