residue, which was purified by chromatography on silica gel, eluting with $CHCl_3$–MeOH, to give pure **7** (400 mg, 91%). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3350, 1460, 1040, 1015. NMR ($CDCl_3$) δ: 4.3—3.7 (2H, m), 3.0 (2H, s, disappeared on adding D_2O), 2.3—1.1 (10H, m, including a triplet at δ 2.0 (J=7 Hz)). Although our synthetic sample did not crystallize (its melting point was reported to be 53.0—54.0° in ref. 3), its identity as *trans*-1,3-diol was firmly established by the presence of a triplet at δ 2.0, which is seen only in this compound, and in no other isomers. This signal was assigned to the methylene protons between the two hydroxyl groups by a decoupling experiment.

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Quantitative Analysis of the Isomers of Hydroxyphenylalanine by High-Performance Liquid Chromatography using a Fluorimetric Detector

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A sensitive high-performance liquid chromatographic technique for the quantitative analysis of the isomers of hydroxyphenylalanine has been developed. The chromatographic conditions were as follows: stainless steel column (2.1×500 mm) packed with Hitachi #3011-C resin; mobile phase, a mixture of equal volumes of 0.025 M sodium acetate and 0.05 M acetic acid; flow rate, 0.8 ml/min; temperature, 45°; detection, fluorimetric detector.

This procedure was found to be suitable for the analysis of 3,4-dihydroxyphenylalanine, *p*-tyrosine, *m*-tyrosine, *o*-tyrosine, and phenylalanine.

Keywords——phenylalanine; *o*-tyrosine; *m*-tyrosine; *p*-tyrosine; 3,4-dihydroxyphenylalanine; fluorescence high-performance liquid chromatography; fluorimetry

In mammals, phenylalanine is in large part metabolized by conversion to tyrosine in the liver. In addition, in the brain and adrenals, phenylalanine is transformed into the catecholamines by way of tyrosine and 3,4-dihydroxyphenylalanine (DOPA).[2] Recently, Tong *et al.*,[3] reported that incubation of phenylalanine-^{14}C with bovine adrenal medulla homogenate in the presence of a pteridine co-factor and a DOPA decarboxylase inhibitor gave rise to three radioactive products which were identified with an amino acid analyzer as tyrosine, *m*-tyrosine and DOPA. However, the phenylalanine peak appears to overlap the *o*-tyrosine peak under their chromatographic conditions. The object of our study was to elucidate the nature of the enzymatic and nonenzymatic hydroxylation system for phenylalanine. In order to study these phenomena quantitatively it is necessary to establish a method for systematic quantitative analysis of hydroxylated phenylalanine compounds. Formerly, paper chromatography[4] and ion-exchange chromatography[5] had been used for this purpose, but a more sensitive method is desirable.

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This paper describes chromatographic conditions for the analysis of phenylalanine and its hydroxylated derivatives by high-performance liquid chromatography (HPLC).

Experimental

Apparatus——A Hitachi 635 high speed liquid chromatograph and a Hitachi 650-10S fluorescence spectrophotometer equipped with a xenon discharge lamp were used.

Materials——Phenylalanine, *o*-tyrosine, *m*-tyrosine, *p*-tyrosine and DOPA were purchased from Sigma Chemical Co., U.S.A., and used without further purification. All other reagents used were of analytical grade.

Chromatographic Conditions——Column packing; Hitachi #3011-C resin was packed in a stainless steel column (2.1 × 500 mm). Column temperature, 45°; mobile phase, a mixture of equal volumes of 0.025 M sodium acetate and 0.05 M acetic acid; flow rate, 0.8 ml/min; fluorimeter sensitivity, 0.1; recorder range, 0.5 V; chart speed, 10 mm/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 *o*-tyrosine, *m*-tytrosine and *p*-tyrosine, and excitation at 280 nm and emission at 318 nm for DOPA, using a fluorescence spectrophotometer equipped with an 18 μl quartz flow cell.

Results and Discussion

Aromatic amino acids are retained on cation-exchange resins to extents that depend on the pK_a values of the compounds, and the hydrogen ion and buffer concentrations of the mobile phase. In this investigation, we employed the weak cation-exchange resin Hitachi #3011-C, which is only suitable for aqueous solutions as a mobile phase. The effects of various buffer concentrations and pH values on the resolution were tested using acetate, citrate and phosphate buffers. Phosphate buffer as a mobile phase gave incomplete separation of DOPA, *p*-tyrosine and *m*-tyrosine. The use of acetate buffer resulted in good resolution. It was found that the best resolution was achieved when the mobile phase consisted of equal volumes of 0.025 M sodium acetate and 0.05 M acetic acid, at pH 4.4. On raising the pH or the concentration of the buffer, the retention time became shorter, and the separation of DOPA, *p*-tyrosine, and *m*-tyrosine became poor.

Although citrate buffer also gave good resolution in the separation of phenylalanine and its hydroxylated compounds, the peak of DOPA overlapped with the strong UV-absorbing background peaks due to metal ion-citrate buffer chelate complex. Therefore, citrate buffer cannot be used in studies on a non-enzymatic hydroxylation system containing metal ions.

The HPLC separation of a synthetic mixture of phenylalanine and its hydroxylated compounds using the acetate buffer described above as a mobile phase, detected by measuring the native fluorescence and UV absorbance, is shown in Fig. 1. The enhancement in sensitivity with the use of the fluorimetric detector compared to the UV detector is obvious. Under these chromatographic conditions, DOPA, *p*-tyrosine, *m*-tyrosine, *o*-tyrosine and phenylalanine were well separated from one another. The retention times

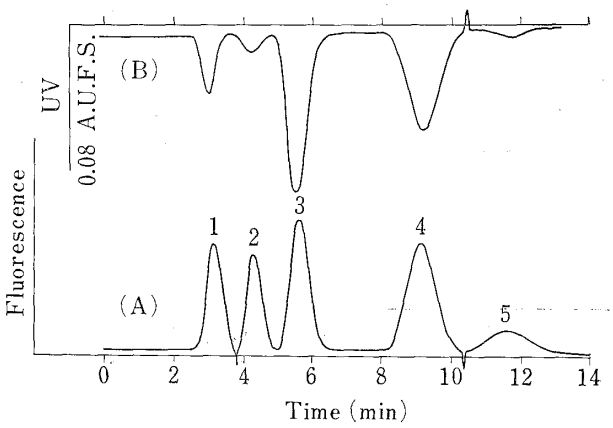

Fig. 1. Chromatogram of Phenylalanine and Its Hydroxylated Derivatives

(A) Injection sample: 50 μl of solution containing 100 ng each of DOPA, *p*-tyrosine, *m*-tyrosine and *o*-tyrosine, and 3 μg of phenylalanine.
For HPLC conditions, see the text.

(B) Injection sample: 50 μl of solution containing 3 μg each of DOPA, *p*-tyrosine, m-tyrosine, *o*-tyrosine and phenylalanine.
HPLC was carried out with an ultraviolet absorbance monitor (260 nm for phenylalanine and 280 nm for DOPA, *o*-tyrosine, *m*-tyrosine and *p*-tyrosine).

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

of DOPA, *p*-tyrosine, *m*-tyrosine, *o*-tyrosine and phenylalanine were 3.2, 4.3, 5.7, 9.2, and 11.5 min, respectively.

Standard curves obtained by plotting the peak area against the amount of substance injected were linear in the range of 100—500 ng/50 μl. The smallest amount that could be determined was 10 ng of hydroxyphenylalanine per injection.

In conclusion, the separation of the isomers of hydroxyphenylalanine by HPLC has advantages over conventional methods in terms of high sensitivity, simplicity and rapidity. The method may be adaptable to studies on phenylalanine metabolites in biological samples.

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Synthesis of Some Cyclic Derivatives of Spermine and Spermidine

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Some cyclic derivatives of spermine and spermidine, 1,5,8,12-tetraazacyclohexadecane **7**, 1,5,9,13-tetraazacycloheptadecane **9**, and 1,5,9-triazacyclotridecane **11** were synthesized as part of our studies on the functions of natural polyamines and the effects of structural modification of the biological activities.

Keywords——synthesis; cyclic spermine; cyclic spermidine; protonation constants; polyamines

The possible biological roles of biogenic polyamines such as spermine **1** and spermidine **2** in numerous growth processes have been scrutinized recently.[2] Promoting effects in DNA replication,[3] stimulatory effects in the synthesis of nucleic acids[4] and proteins,[5] and inhibitory effects in lipid peroxidation[6] have been extensively studied. However, the mechanisms of these functions remain unclear, except for an ealier suggestion[7] that the polyamines interact with nucleic acids, ribosomes, enzymes or lipids as organic polycations, thereby stabilizing higher structures of the macromolecules.

We have now prepared some cyclic derivatives of spermine (trivially called "cyclic spermine"), 1,5,8,12-tetraazacyclohexadecane **7** and 1,5,9,13-tetraazacycloheptadecane **9**, and spermidine, 1,5,9-triazacyclotridecane **11**[8] ("cyclic spermidine"). Our previous studies[9] show-

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