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Studies on Separation of Amino Acids and Related Compounds. III. Separation of Diastereomers of Leucyl Dipeptides by Ion-Exchange Chromatography

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A series of experiments on a column chromatography of Dowex 50 with ammonium acetate solvent systems as eluent was carried out in order to find optimal conditions for the separation of the diastereomers of leucylalanine, leucylvaline, leucylleucine and leucyltyrosine. The effects of pH concentration of the salt and alcohols in the solvent systems for the separation of the diastereomers were studied. Among many conditions tested, the change of pH in the solvent system afforded most remarkable influence for the separation; the order of elution of the diastereomers of the dipeptide was found to be inverted at near pH 5.0. On a preparative scale L-leucyl-DL-valine was successfully separated into the two diastereomers by using a fairly large column. Attempt to separate benzyloxycarbonyl-L-leucyl-DL-valine benzyl ester with a column of Sephadex LH-20 was described, also.

Previous papers from this laboratory have dealt with the analytical and preparative separation of hydroxyproline diastereomers on columns of the ion-exchange resin.[1] In the present paper, we describe the manipulation of the variables in the ion-exchange technique and the preparative separation of leucyl dipeptide diastereomers.

Several investigators have reported the separation of the diastereomers of dipeptide on paper[2] and thin-layer chromatography.[3] On an ion-exchange column chromatography of Dowex 50, Blackburn and Tetley reported the separation of L-leucyl-L-tyrosine and L-leucyl-D-tyrosine with citrate buffer of pH 4.8,[4] and Neuhaus separated alanylalanine diastereomers.[5] Yanari *et al.* separated a mixture of leucyltyrosine diastereomers by the use of DEAE-cellulose column.[6] Although these investigators cited above carried out the chromatographies in analytical manner using several mg or less of dipeptide diastereomers, Wieland and Bende reported recently the separation of several dipeptide diastereomers on a preparative scale; for example, they separated 200 mg of DL-alanyl-L-tyrosine into each pure diastereomer with a Sephadex G-50 column (1.5 × 100 cm) using a solvent of aqueous pyridine.[3]

In this investigation, each diastereomer of leucylalanine, leucylvaline, leucylleucine and leucyltyrosine were prepared in the conventional manner as the first step of the experiment. For

1) H. Aoyagi, H. Okai, M. Ohno and N. Izumiya, *Nippon Kagaku Zasshi (J. Chem. Soc. Japan, Pure Chem. Sect.)*, **85**, 656 (1964); H. Aoyagi, M. Ohno, N. Izumiya and B. Witkop, *J. Org. Chem.*, **29**, 1382 (1964).

2) For example, T. Sokolowska and J. F. Biernat, *J. Chromatogr.*, **13**, 269 (1964).

3) T. Wieland and H. Bende, *Chem. Ber.*, **98**, 504 (1965).

4) S. Blackburn and P. Tetley, *Biochim. Biophys. Acta*, **20**, 423 (1956).

5) F. C. Neuhaus, *J. Biol. Chem.*, **237**, 778 (1962).

6) S. Yanari, M. Volini and M. A. Mitz, *Biochim. Biophys. Acta*, **45**, 595 (1960).

example, L-leucyl-L-alanine was synthesized as follows:

$$\text{Z-Leu-OH} + \text{H-Ala-OBzl} \xrightarrow{\text{mixed anhydride method}}$$

$$\text{Z-Leu-Ala-OBzl} \xrightarrow{H_2/Pd} \text{H-Leu-Ala-OH}$$

(Z, benzyloxycarbonyl; OBzl, benzyl ester)

It was observed that a diastereomeric mixture of any peptide was separated appreciably enough by paper chromatography (Table 1).

As a preliminary experiment of a column chromatography, ion-exchange (strong acid type) paper chromatography was carried out with leucylleucine and leucyltyrosine in order to find the effect of pH and alcohols in an ammonium acetate solvent system (Tables 2 and 3). However, it was observed that anticipation to find the optimal condition for a separation of the diastereomers on a column through the study of ion-exchange paper chromatography appeared to be difficult. Then, the effects of variations of pH, alcohol, and concentration of ammonium acetate in the solvent system were studied by using a small column (0.9 $\times$ 50 cm) with Dowex 50X8.

In the course of the column chromatographic experiment, we observed the interesting phenomena; each L-L diastereomer of leucylalanine, leucylvaline or leucylleucine with a solvent of higher pH than 5.0 was eluted more rapidly than the corresponding L-D isomer, whereas each L-L isomer was eluted slower at pH 4.5 (Figs. 4—6). To elucidate these inversions, the titration experiments of the L-L and L-D isomer of each peptide were carried out by using a pH-stat, and it was found that the titration curves for the diastereomers of each peptide crossed each other around pH 5 (Figs. 10—12). Then, the inversion in the chromatographic pattern may be explained by the interpretation that the main interaction between the resin and the dipeptide is due to ionic forces and the ionic conditions of the diastereomers of the dipeptide are inverted each other at a definite pH near 5.

As an experiment on a preparative scale, a L-leucyl-DL-valine preparation was tried to separate with a large column (1.8 $\times$ 110 cm) of Dowex 50 under the good condition which was found previously by using smaller column. For the synthesis of L-leucyl-DL-valine, the mixed anhydride of benzyloxycar-L-leucinebonyl was coupled with DL-valine benzyl ester and the benzyloxycarbonyl-dipeptide ester obtained as syrup was hydrogenated to crude dipeptide, L-Leu-DL-Val, which was subjected to the separation experiment without further purification.[7] With the large column using 0.2 M ammonium acetate of pH 7.0, we could separate 1 g of the crude peptide into 0.40 g of pure L-leucyl-L-valine and 0.48 g of L-leucyl-D-valine.

Separation of the oily Z-L-Leu-DL-Val-OBzl preparation with a column (0.9 $\times$ 50 cm) of Sephadex LH-20 using dioxane or methanol as solvent was attempted; it was found that the separation was occurred partially (Fig. 13). Reviewing the literature on the separation of acyldipeptide ester, Weygand *et al.* reported the separation of the diastereomeric mixture of trifluoroacetyl-phenylalanylvalyl methyl ester by gas chromatographic technique.[8] As the studies using a Sephadex LH-20 or methylated Sephadex column, Waki and Izumiya applied it successfully for the separation of benzyloxycarbonyl-substituted gramicidin S and cyclosemigramicidin S,[9] and Mutt *et al.* reported the separation of Z-L-Leu-L-Leu-OMe and *p*-nitrophenol during a peptide synthesis by the active ester method.[10]

Experimental

The melting points were uncorrected. Optical rotations were determined with a Yanagimoto Photometric Polarimeter, OR-20 type.

D-Valine Benzyl Ester *p*-Toluenesulfonate (I). A mixture of D-valine, benzyl alcohol, *p*-toluenesulfonic acid monohydrate, and benzene was heated as has been described for the preparation of the corresponding L-isomer.[11] Yield, 78%; mp 157—158°C; $[\alpha]_D^{25}$ +4.8° (*c* 2, ethanol) (Found: C, 60.05; H, 6.74; N, 3.68%).

DL-Valine Benzyl Ester *p*-Toluenesulfonate (II). The crude crystals obtained by the usual way were recrystallized from ethanol-ether; yield, 94%, mp 138—140°C (Found: C, 59.88; H, 6.88; N, 3.75%).

D-Leucine Benzyl Ester *p*-Toluenesulfonate (III). This compound was obtained according to the procedure for the L-isomer.[11] Yield, 75%; mp 155—156°C; $[\alpha]_D^{25}$ −0.5° (*c* 2, ethanol) (Found: C, 60.96; H, 7.05; N, 3.67%).

Benzyloxycarbonyl-L-leucyl-L-alanine Benzyl Ester (IV)[12] To a chilled solution of benzyloxycarbonyl-L-leucine[13] (2.65 g) and triethylamine (1.4 m*l*) in tetrahydrofuran (20 m*l*), isobutyl chloroformate (1.32 m*l*) was added at −5°C. After 15 min, a mixture of L-alanine benzyl ester *p*-toluenesulfonate[11] (3.51 g) and triethylamine (1.4 m*l*) in chloroform (20 m*l*) was added. The mixture was left to stand overnight at room temperature, and then evaporated *in vacuo*. The residue was dissolved in ethyl acetate (30 m*l*), then the solution was successively washed with 4% sodium bicarbonate, 2% hydrochloric acid and water, and dried

7) It was found that the ratio of the L-L and L-D isomer in the crude dipeptide was 82 : 100 by an amino acid analyzer.

8) F. Weygand, A. Prox and W. König, *Chem. Ber.*, **99**, 1451 (1966).

9) M. Waki and N. Izumiya, *J. Am. Chem. Soc.*, **89**, 1278 (1967); This Bulletin, **40**, 1687 (1967).

10) V. Mutt, E. Nyström and J. Sjövall, *J. Chromatogr.*, **24**, 205 (1966).

11) N. Izumiya and S. Makisumi, *Nippon Kagaku Zassi (J. Chem. Soc. Japan, Pure Chem. Sect.)*, **78**, 662, 1768 (1957).

12) We are indebted to Mr. M. Waki in this laboratory who performed the syntheses of the L-L and L-D isomer of leucylalanine.

13) M. Bergmann, L. Zervas and W. F. Ross, *J. Biol. Chem.*, **111**, 245 (1935).

over sodium sulfate. The filtrate was evaporated *in vacuo*, and the oily residue was solidified by the addition of petroleum ether. The product was recrystallized from ethyl acetate - ether - petroleum ether; yield, 2.78 g (65%), mp 111°C; $[\alpha]_D^{25}$ −45.5° (*c* 2, ethanol).

Found: C, 67.55; H, 7.25; N, 6.69%. Calcd for $C_{24}H_{30}O_5N_2$: C, 67.58; H, 7.09; N, 6.57%.

Benzyloxycarbonyl-L-leucyl-D-alanine Benzyl Ester (V). The mixed anhydride of benzyloxycarbonyl-L-leucine was coupled with a mixture of D-alanine benzyl ester *p*-toluenesulfonate[14] and triethylamine in chloroform as described above; yield, 67%; mp 120—121°C; $[\alpha]_D^{25}$ +1.2 °(*c* 2, ethanol).

Found: C, 67.80; H, 7.14; N, 6.54%.

Benzyloxycarbonyl-L-leucyl-DL-valine Benzyl Ester (VI). Benzyloxycarbonyl-L-leucine (2.65 g, 10 mmol) was coupled with II (10 mmol) by the mixed anhydride, and the compound (VI) was obtained as syrup; yield, 4.01 g (88%).

Benzyloxycarbonyl-L-leucyl-D-leucine Benzyl Ester (VII). This compound was obtained from benzyloxycarbonyl-leucine and III; yield, 68%; mp 110—112°C; $[\alpha]_D^{25}$ +2.3° (*c* 2, ethanol).

Found: C, 68.96; H, 7.68; N, 5.99. Calcd for $C_{27}H_{36}O_5N_2$: C, 69.20; H, 7.74; N, 5.98%.

L-Leucyl-L-alanine. A solution of IV (2.13 g) in a mixture of acetic acid (30 m*l*), methanol (15 m*l*), and water (5 m*l*) was treated with hydrogen in the presence of palladium black. The filtrate from the catalyst was evaporated *in vacuo* to dryness. The residual crystals were recrystallized from methanol-acetone-ether; yield of L-Leu-L-Ala monohydrate, 0.96 g (87%); $[\alpha]_D^{25}$ +27.6° (*c* 1, H_2O), +21.8°C (*c* 1, methanol) (Found: C, 49.52; H, 9.15; N, 12.96%). Polglase and Smith reported the synthesis of L-Leu-L-Ala by the hydrogenolysis of Z-Leu-Ala-OH which was obtained by saponification of Z-Leu-Ala-OMe; $[\alpha]_D^{24}$ +22.9° (methanol).[15]

L-Leucyl-D-alanine. V (2.13 g) was hydrogenolyzed as described above; yield of L-Leu-D-Ala monohydrate, 0.93 g (85%); $[\alpha]_D^{25}$ +77.8° (*c* 1, H_2O) (Found: C, 45.67; H, 9.21; N, 12.02%). Reported value, $[\alpha]_D^{24}$ +76° (H_2O).[15]

L-Leucyl-L-valine. Benzyloxycarbonyl-leucine (1.32 g, 5 mmol) was coupled with L-valine benzyl ester as described for the preparation of IV. The oily Z-Leu-Val-OBzl thus obtained was hydrogenolyzed, and the residue was recrystallized from methanol-ether. Yield of L-Leu-L-Val monohydrate, 1.05 g (85%); $[\alpha]_D^{20}$ +18.0° (*c* 1, N HCl) (Found: C, 53.25; H, 9.46; N, 11.17%). Reported value, $[\alpha]_D^{23}$ +18.2° (N HCl).[16]

L-Leucyl-D-valine. Benzyloxycarbonyl-leucine (1.23 g) was coupled with I, and the oily Z-Leu-Val-OBzl obtained was hydrogenolyzed; yield of L-Leu-D-Val monohydrate, 90%; $[\alpha]_D^{20}$ +55.0° (*c* 1, H_2O) (Found: C, 53.41; H, 9.71; N, 11.32%). Reported value, $[\alpha]_D^{24}$ +55.3° (H_2O).[16]

L-Leucyl-DL-valine (VIII). The oily VII (2.27 g, 15 mmol) was hydrogenated in the usual manner, and the filtrate from the catalyst was evaporated to dryness; the yield of the residual powder was 1.18 g (95%, calcd as monohydrate). This powder was used for the experiment of separation without recrystallization. The weight ratio between the L-L and L-D diastereomer in the powder was determined as 82 : 100 by the use of an amino acid analyzer.

L-Leucyl-L-leucine. Benzyloxycarbonyl-leucine (1.32 g) was coupled with leucine benzyl ester, and the oily Z-Leu-Leu-OBzl obtained was hydrogenolyzed. Recrystallization from hot water yielded 0.79 g (63%) of L-Leu-L-Leu hemihydrate; $[\alpha]_D^{20}$ −13.4° (*c* 1, N NaOH) (Found: C, 56.67; H, 9.84; N, 10.89%). Reported value, $[\alpha]_D^{23}$ −13.4° (N NaOH).[16]

L-Leucyl-D-leucine. This compound was obtained from VII by the hydrogenolysis; yield of L-Leu-D-Leu dihydrate, 72%; $[\alpha]_D^{20}$ +68.0° (*c* 1, N HCl) (Found: C, 51.47; H, 9.63; N, 9.80). Reported value, $[\alpha]_D^{24}$ +68.5° (N HCl).[16]

L-Leucyl-L-tyrosine and L-Leucyl-D-tyrosine. These dipeptides were prepared as has been described in the literatures.[17]

Separation Studies on Paper Chromatographies. *a) Paper Chromatography.* A paper chromatography was carried out with ascending technique using Toyo Roshi No. 52 paper, and a peptide on the paper was revealed with ninhydrin. As shown in Table 1, it was observed that the diastereomers of each peptide were clearly separated on a chromatogram.

Table 1. R_f values of dipeptides on paper chromatography

Dipeptide	R_f	
	L-L	L-D
Leu-Ala[a]	0.73	0.52
Leu-Ala[b]	0.66	0.64
Leu-Val[b]	0.83	0.75
Leu-Leu[b]	0.90	0.81
Leu-Tyr[b]	0.78	0.66

a) Solvent system: pyridine-water (4 : 1, v/v)
b) Solvent system: *n*-butanol - acetic acid - pyridine - water (4 : 1 : 1 : 2, v/v).

b) Ion-exchange Paper Chromatography. As the preliminary experiment for a column chromatography, a paper of Amberlite SA-II, NH_4^+ form, was applied for a dipeptide or a mixture of the two diastereomers using 0.2 M ammonium acetate solvent at a chosen pH. As shown in Tables 2 and 3, a mixture of the diastereomers was successfully separated at the different pH. Since a diastereomeric mixture of 4-hydroxyproline or 3-hydroxy-2-aminobutyric acid had been separated more effectively by the addition of alcohol such as methanol in the ammonium acetate solvent system,[1] the effect of alcohol to the solvent was studied; the addition of various alcohol afforded little effect on the separation pattern (Table 3).

14) M. Winitz, L. Bloch-Frankenthal, N. Izumiya, S. M. Birnbaum, C. G. Baker and J. P. Greenstein, *J. Am. Chem. Soc.*, **78**, 2423 (1956).
15) W. J. Polglase and E. L. Smith, *ibid.*, **71**, 3081 (1949).
16) E. L. Smith, D. H. Spackman and W. J. Polglase, *J. Biol. Chem.*, **199**, 801 (1952).
17) N. Izumiya, H. Uchio and T. Yamashita, *Nippon Kagaku Zassi (J. Chem. Soc. Japan, Pure Chem. Sect.)*, **79**, 420 (1958); B. F. Erlanger, W. V. Curran and N. Kokowsky, *J. Am. Chem. Soc.*, **80**, 1128 (1958).

TABLE 2. R_f VALUES OF DIPEPTIDES ON ION-EXCHANGE PAPER CHROMATOGRAPHY (EFFECT OF pH)

pH	R_f			
	Leu-Leu		Leu-Tyr	
	L-L	L-D	L-L	L-D
6.0	0.44	0.52	0.28	0.39
4.0	0.14	0.20	0.08	0.14

TABLE 3. R_f VALUES OF DIPEPTIDES ON ION-EXCHANGE PAPER CHROMATOGRAPHY (EFFECT OF ALCOHOL)

Alcohol (20%)	R_f			
	Leu-Val[a]		Leu-Leu[b]	
	L-L	L-D	L-L	L-D
—	0.73	0.68	0.34	0.28
Methanol	0.74	0.70	0.31	0.27
Ethanol	0.76	0.71	0.30	0.26
n-Propanol	0.73	0.67	0.32	0.28
Isopropanol	0.70	0.64	0.30	0.25
t-Butanol	0.75	0.70	0.31	0.26

a) pH of buffer: 7.0
b) pH of buffer: 4.5

Separation Studies on Column Chromatography.
a) Column and Solvent System. A column (0.9×50 cm) was filled with Dowex 50WX8 (200—400 mesh), NH_4^+ form, and equilibrated by washing with an appropriate solvent system. A solvent of 0.2 M ammonium acetate, which was used in most cases, was prepared in the following manner. Ammonium acetate (15.4 g, 0.2 mol) was dissolved in water (500 m*l*), and acetic acid was added until the solution reached the desired pH. The solution was made up to 1 *l* with water, and adjusted again to the desired pH by the addition of acetic acid or ammonia.

b) Chromatography of Model Mixture. A mixture of each 0.05 mmol of a L-L form and L-D form of each dipeptide was dissolved in water (1 m*l*), and the solution was applied to the column and eluted with the appropriate solvent system at room temperature and a flow rate of about 10 m*l*/hr. Two-milliliter fractions were collected, and 0.01 m*l* of the eluate in each test tube was spotted on a strip of filter paper. The strip was dried up, immersed in a 0.2% ninhydrin-acetone solution, and heated at 100°C to find the presence of the peptide (Fig. 1A). Then, 0.01 m*l* of the eluate in the tubes which contain the peptide was applied on the paper chromatography (Fig. 1B). The amounts of color developed were determined by Atago AG-4 densitometer with slit 1×8 mm and 610 mμ (Fig. 1C) and

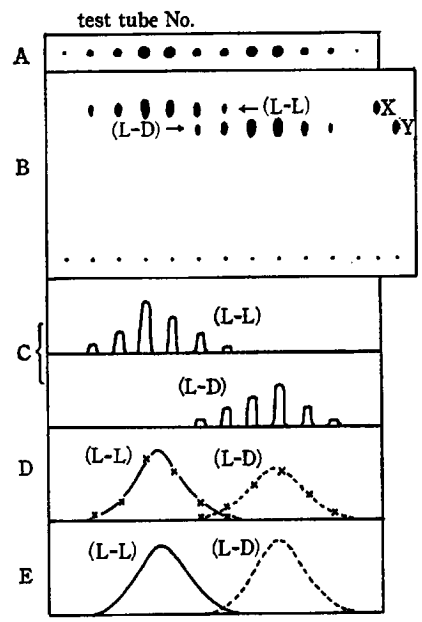

Fig. 1. Separation and assay of a mixture of dipeptide diastereomers on Dowex 50WX8.
Markers: X, spot by a L-L diastereomer of dipeptide; Y, spot by a L-D diastereomer

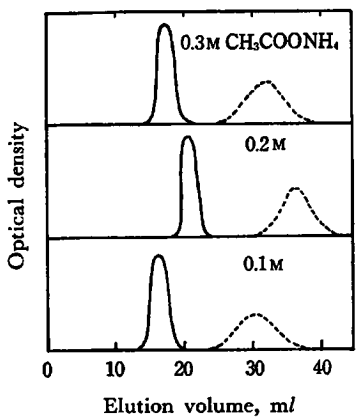

Fig. 2. Effect of concentration of ammonium acetate in buffer system (pH 7.0) on separation of Leu-Val.
Solid line, L-Leu-L-Val; dotted line, L-Leu-D-Val

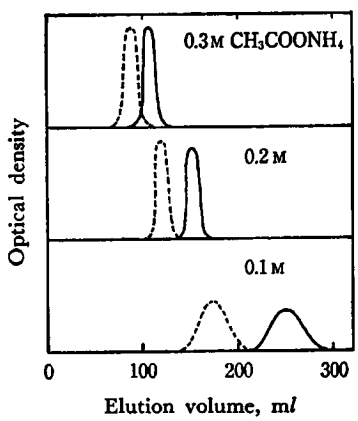

Fig. 3. Effect of concentration of ammonium acetate in buffer system (pH 4.5) on separation of Leu-Leu.
Solid line, L-Leu-L-Leu; dotted line, L-Leu-D-Leu

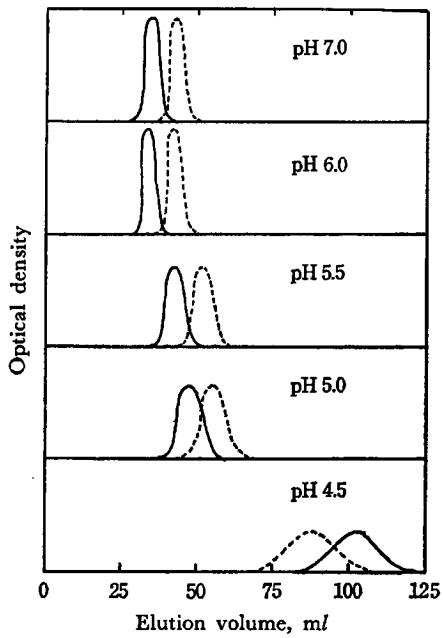

Fig. 4. Effect of pH in buffer system on separation of Leu-Ala.
Solid line, L-Leu-L-Ala; dotted line, L-Leu-D-Ala

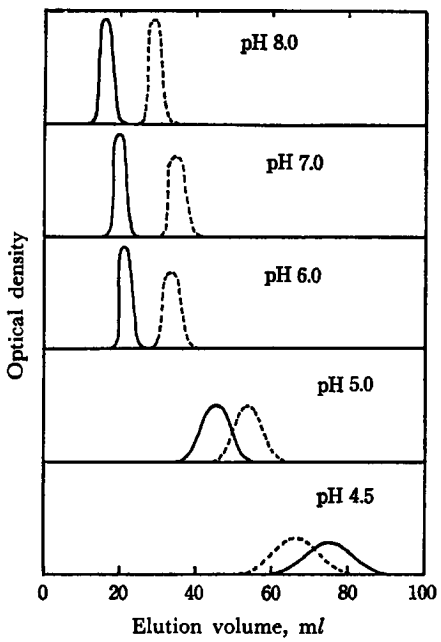

Fig. 5. Effect of pH in buffer system on separation of Leu-Val.
Solid line, L-Leu-L-Val; dotted line, L-Leu-D-Val

the integrated areas were plotted on a graph (Fig. 1D). It was observed that the color intensity of a L-D isomer was about 70% compared with that of the corresponding L-L isomer. In many figures (*e. g.*, Figs. 2—8) the area of the L-D isomer was corrected as of the value of the L-L isomer (Fig. 1E).

c) Effect of Concentration of Salt in Solvent System. Effect of variation in concentration (0.1—0.3 M) of ammonium acetate in a solvent was examined, and the patterns obtained were shown in Fig. 2 for Leu-Val and Fig. 3 for Leu-Leu. Since the 0.2 M ammonium acetate solvent gave best results for the separation, this solvent system was used in most cases in a series of experiments.

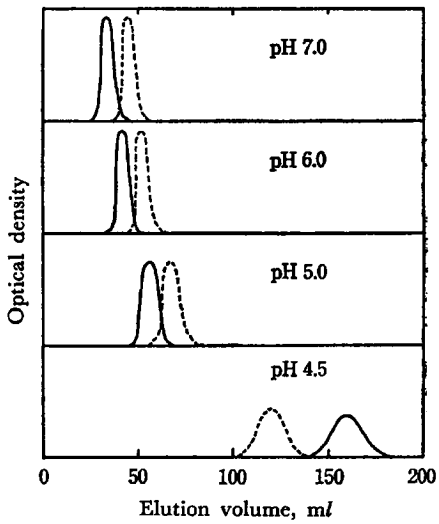

Fig. 6. Effect of pH in buffer system on separation of Leu-Leu.
Solid line, L-Leu-L-Leu; dotted line, L-Leu-D-Leu

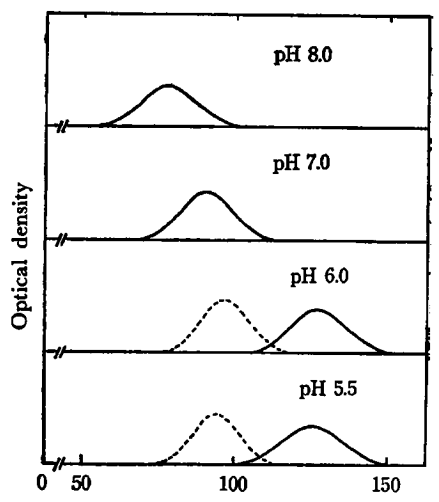

Fig. 7. Effect of pH in buffer system on separation of Leu-Tyr.
Solid line, L-Leu-L-Tyr; dotted line, L-Leu-D-Tyr
In the case of pH 7.0 or 8.0 buffer, the curve by the L-L isomer coincided with that by the L-D isomer.

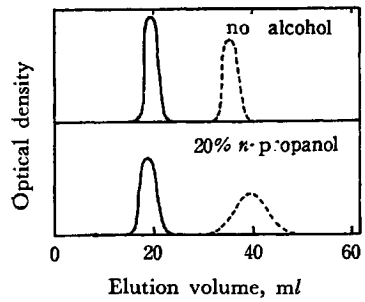

Fig. 8. Effect of alcohol in buffer system (pH 7.0) on separation of Leu-Val.
Solid line, L-Leu-L-Val; dotted line, L-Leu-D-Val

d) Effects of pH and Alcohol in Solvent System. Change of pH in the solvent was examined. As noted in Figs. 4—6, each L-L diastereomer of Leu-Ala, Leu-Val or Leu-Leu dipeptide with a solvent at higher pH than 5.0 was eluted more rapidly than the corresponding L-D isomer, on the contrary each L-L isomer was eluted slower at pH 4.5. In the case of Leu-Tyr, the observation of such an inversion of the order of elution was of difficult since the elution of this dipeptide was retarded and broadend remarkably at lower pH than 5. An optimum pH for best separation appeared to differ toward a diasteremeric mixture of each peptide; that pH are about 7.0 for Leu-Ala and Leu-Val, and 4.5 for Leu-Leu. Although it had been observed in the experiment with the ion-exchange paper that the addition of an alcohol in a solvent system hardly affected on separation, the effect of *n*-propanol in a column chromatography was examined (Fig. 8). The result showed again that the addition of the alcohol did not improve the separation.

e) Separation with Amino Acid Analyzer. A mixture of each 0.2 μmol of the L-L form and L-D form of Leu-Val was analyzed with Hitachi amino acid analyzer, model KLA-3, under these conditions: length of the column with spherical resin, 0.9×50 cm; solvent, citrate buffer at pH 4.25; flow rate, 60 ml/hr; jacket temperature, 55°C. The chromatogram obtained was shown in Fig. 9. The ratio of color intensities between L-Leu-L-Val and L-Leu-D-Val, which was calculated from the integrated areas on the chromatogram, was found to be 100 : 63.[18]

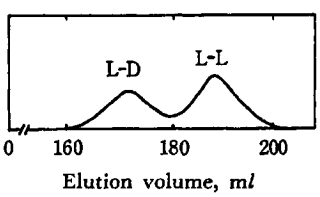

Fig. 9. Chromatogram of a diastereomeric mixture of Leu-Val by an amino acid analyzer.

18) In this connection, it would be noteworthy that the ratio of color intensities between L-Alloile-L-Tyr and D-Alloile-L-Tyr was 100 : 45; see, T. Yamashita, H. Uchio and N. Izumiya, *Nippon Kagaku Zasshi (J. Chem. Soc. Japan, Pure Chem. Sect.)*, **80**, 767 (1959).

pH Titration Curve. Each diastereomer (0.05 mmol) of Leu-Ala, Leu-Val and Leu-Leu was dissolved in 1/30 N sodium hydroxide (3 ml), and automatic titration was carried out with 0.5 N hydrochloric acid with Radiometer pH-Stat, model TTT-1, at room temperature (Figs. 10—12). As noted from the figures, it was

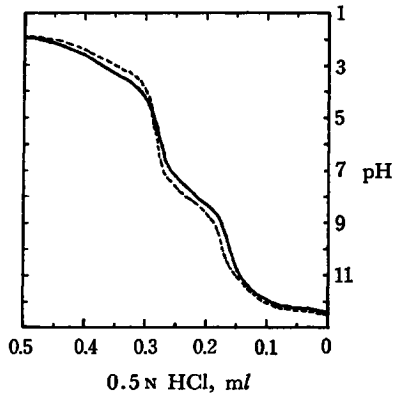

Fig. 10. pH titration curves of Leu-Ala. Solid line, L-Leu-L-Ala; dotted line, L-Leu-D-Ala

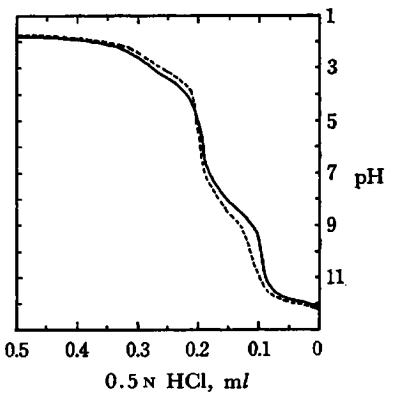

Fig. 11. pH titration curves of Leu-Val. Solid line, L-Leu-L-Val; dotted line, L-Leu-D-Val

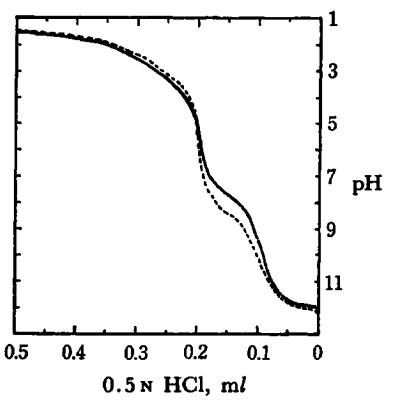

Fig. 12. pH titration curves of Leu-Leu. Solid line, L-Leu-L-Leu; dotted line, L-Leu-D-Leu

observed that the titration curves of the diastereomers crossed each other around pH 5 in which the inversions of the elution patterns of the diastereomers were observed as shown in Figs. 6—8.

Preparative Separation of L-Leu-DL-Val (VIII). A solution of VIII (1 g, 5 mmol) in a 0.2 M ammonium acetate solvent (40 m*l*) of pH 7.0 was put on a column (1.8×110 cm) of the Dowex 50, and eluted with the same solvent at a flow rate of 20 m*l*/hr. Each fraction (5 m*l*) was tested on a paper strip with ninhydrin, and the strip demonstrated that a mixture of the diastereomers was separated into two portions with the column. The faster eluting fractions from tube number 22 to 33 were evaporated *in vacuo* to dryness. Most of the ammonium acetate was removed by sublimation *in vacuo* for about 1 hr at 100°C. To remove completely the ammonium acetate still contaminated, a solution of the residue in water was put on a column of Dowex 50 (0.9×10 cm), H^+ form, and the column was eluted with 2 N ammonium hydroxide. The eluate was evaporated, and the residual crystals were recrystallized from methanol-ether; yield of L-Leu-L-Val monohydrate, 0.396 g (40%); $[\alpha]_D^{20}$ +17.5° (*c* 1, N HCl).

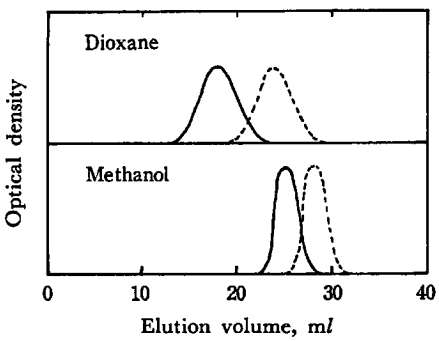

Fig. 13. Chromatogram of Z-L-Leu-DL-Val-OBzl on Sephadex LH-20 column.
Solid line, Z-L-Leu-L-Val-OBzl; dotted line, Z-L-Leu-D-Val-OBzl

Found: C, 53.13; H, 9.49; N, 11.15%. Calcd for $C_{11}H_{22}O_3N_2 \cdot H_2O$: C, 53.20; H, 9.74; N, 11.28%. The slower eluting fractions from tube number 34 to 47 were treated in the same manner as described above. Yield of L-Leu-D-Val monohydrate, 0.476 g (48%); $[\alpha]_D^{20}$ +55.0° (*c* 1, H_2O).

Found: C, 53.56; H, 9.53; N, 11.24%.

The weight ratio between the L-L and L-D isomer was calculated as 84 : 100 which agreed with the ratio of 82 : 100 observed for the crude L-Leu-DL-Val (VIII) by the amino acid analyzer.

Separation Studies of Z-L-Leu-DL-Val-OBzl (VII). Sample of the syrup (VII) (23 mg, 0.05 mmol) was applied to a column (0.9×50 cm) of Sephadex LH-20, and the column was eluted with dioxane or methanol at room temperature and a flow rate of about 5 m*l*/hr. One-m*l* fractions were collected, and small amount of each test tube was spotted on a thin-layer plate. The compound on the plate was detected by spraying 47% hydrobromic acid and then ninhydrin. Then, 0.05 m*l* of the elute in the test tubes which contain benzyloxycarbonyl-dipeptide ester was hydrogenated, and the filtrate from the catalyst was applied on paper chromatography, and Fig. 13 was prepared in the same manner as described for the preparation of Fig. 1B-1E.

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