Previous studies of the specificity of leucine aminopeptidase have shown that this enzyme can hydrolyze a variety of aliphatic amino acid amides and peptides (1-4). The most rapid action is on compounds which possess the free amino group of leucine. For dipeptides of the structure shown in I, the dash line indicates the hydrolytic action.

\[
\text{NH}_2 \quad \text{O} \\
\text{R'}\text{--CH--C--}\text{NH--CHCOOH} \\
\text{R''}
\]

(1)

The nature of the amino acid residue bearing R' has a profound influence on the sensitivity of the peptide, whereas the residue with R'' has much less effect on the rate of hydrolysis by the enzyme. Such peptides as L-leucylglycine, L-leucyl-β-alanine, and L-leucyl-L-alanine are split at rates which vary only by a factor of about 2. The residue possessing R' must be of the L configuration; D compounds are completely resistant to hydrolysis. It has been found, however, that L-leucyl-D-alanine (4) is hydrolyzed at about 5 per cent of the rate of the L-L diastereoisomeric peptide. It is apparent that the relationship between the configuration of the R'' residue and the activity of the enzyme is of a different character from that of the R' residue inasmuch as the action of the enzyme is, in one case, completely blocked, and in the other only partially hindered.

This phenomenon has been studied further by investigating the action of the enzyme on a number of diastereoisomeric dipeptides containing L-leucyl as the R' residue combined with the leucines, isoleucines, valines, and phenylalanines of both the D and L configurations. In addition, the action of leucine aminopeptidase on the amides of valine, isoleucine, and

* This investigation was aided by research grants from the National Institutes of Health, United States Public Health Service.
α-aminocaprylic acid has been studied for comparison with the rates of hydrolysis of other aliphatic amino acid amides previously reported (4).

The synthesis of several new amino acid ester and amide hydrochlorides and of a number of diastereoisomeric peptides containing L-leucine is also described.

EXPERIMENTAL

Enzyme Preparations—The source of enzyme was swine intestinal mucosa. Two types of preparations at different levels of purification were used. Preparation I of this study was obtained from an extract dried from the frozen state of intestine¹ by the procedure described for Preparation B of an earlier investigation (4). Preparation II was isolated from fresh mucosa by the method of Smith and Bergmann (2) with some further purification by fractionation with acetone (3).

Procedure—Enzyme preparations were incubated at pH 7.8 to 8.0 with 0.02 M MnCl₂ for 2 to 4 hours at 40° in order to achieve maximal activities (2). The mixtures were buffered with 0.035 M Veronal or 0.1 M tris(trihydroxymethyl)aminomethane (Tris); identical results were obtained with either buffer. Aliquots of the incubated solution were added to the buffered substrate solutions for measurement of the hydrolytic reactions. The concentration of Mn²⁺ in the test solutions was 0.002 to 0.008 M. Control measurements with L-leucinamide showed that optimal and identical rates were achieved with final concentrations of Mn²⁺ between 0.001 and 0.03 M. Enzyme concentrations are given for the final reaction mixtures. Substrate concentrations were 0.05 M unless otherwise indicated.

Hydrolysis was measured by the titration of liberated carboxyl groups (5). Proteolytic coefficients (C) were estimated in the usual manner from $C = K/E$, where $K$ is the first order velocity constant calculated in decimal logarithms and $E$ is the enzyme concentration given in mg. of protein N per ml. of test solution. The complete hydrolysis of one peptide or amide bond is given as 100 per cent.

Substrates—The following compounds were prepared as previously described: L-Leucinamide hydrochloride (3), L-leucyl-L-alanine (6), and L-leucyl-D-alanine (6). L-Leucyl-L-tyrosine was a commercial preparation obtained from Hoffmann-La Roche. We are indebted to Dr. A. Meister of the National Cancer Institute for the sample of DL-α-aminocaprylic acid amide (7).

It is much more difficult to prepare the ester and amide hydrochlorides of valine and isoleucine than the similar derivatives of other aliphatic amino acids. Esterification of valine or isoleucine requires boiling with

¹ We are indebted to Dr. E. E. Hays of the Armour Laboratories for supplying us with samples of dried intestinal extract.
alcoholic HCl in contrast to other amino acids which are easily esterified in the cold or at room temperature. Amidation of valine and isoleucine ester hydrochlorides is incomplete after 10 days at room temperatures (20–25°), whereas amides of other amino acids are obtained in high yield after 1 or 2 days under identical conditions (2–4). It is apparent that the branching at the β-carbon atom greatly retards the reactions of the carboxyl group of valine or isoleucine.

It was observed that melting points previously reported for some of the amino acid ester hydrochlorides are much too low, despite satisfactory analytical figures for nitrogen. Our experience has been that the determination of the nitrogen content of amino acid methyl ester hydrochlorides as a criterion of purity is of little value since, with incomplete esterification, the main impurity is the amino acid hydrochloride whose N content differs little from that of the methyl ester hydrochloride. More reliance can be placed, as in the present investigation, on equivalence of the melting points and of the optical rotations of L and D isomers, or on methoxyl determinations.

The methoxyl determinations reported below were carried out by the procedure of Hoffman and Wolfrom (8). Elementary analyses were performed by Weiler and Strauss of Oxford.

L-Isoleucine Methyl Ester Hydrochloride—10 gm. of L-isoleucine were suspended in 125 ml. of methanol, which was saturated with dry HCl gas and refluxed for 3 hours. Repeated concentration in vacuo with methanol yielded a syrup which was reesterified twice more by the same procedure. Repeated evaporation in vacuo with acetone yielded a partially crystalline product which was recrystallized from acetone-ether and dried in vacuo over KOH. Yield, 7.6 gm.; m.p., 98–100°.

C7H15O2NCl (181.7). Calculated, CH1O 17.1; found, CH1O 16.5
\[\alpha\]D = +26.6° (2% in water)

L-Isoleucinamide Hydrochloride—L-Isoleucine methyl ester hydrochloride (4.3 gm.) was dissolved in 100 ml. of methanol which was saturated with dry ammonia gas at 0°. The solution was allowed to remain in a pressure bottle at room temperature for 10 days. The solution was concentrated in vacuo repeatedly with methanol and the product recrystallized from methanol-ether; yield, 1.6 gm. A second amidation of the substance in the mother liquor as described above yielded another 0.7 gm. The melting point was 246° with slight decomposition. For analysis, the compound was recrystallized once more from methanol-ethyl acetate.

C9H19ON3Cl. Calculated. C 43.2, H 9.1, N 16.8
\[\alpha\]D = +21.2° (1% in water)
d-Isoleucine Methyl Ester Hydrochloride—9.8 gm. of d-isoleucine were esterified as described above. After recrystallization from methanol-ether, 4.25 gm. of product were obtained. After a second recrystallization, the substance melted at 98–100°, identical with that of the L compound.

\[ [\alpha]_b^0 = -26.6^\circ \text{ (2\% in water)} \]

d-Isoleucinamide Hydrochloride—This was prepared as already described from 1.7 gm. of the ester hydrochloride. After two amidations, a total yield of 1.1 gm. was obtained. Recrystallization from methanol-ether gave a product which melted at 246–247° with decomposition.

C₆H₁₃ON₂Cl. Calculated. C 43.2, H 9.1, N 16.8
166.7 Found. ' 43.3, ' 9.4, ' 16.7

\[ [\alpha]_b^0 = -21.0^\circ \text{ (1\% in water)} \]

L-Valine Methyl Ester Hydrochloride—9.8 gm. of L-valine were esterified as described above, yielding 8.6 gm. of rectangular plates which, after recrystallization from methanol ether, melted at 167.5–168°.

C₆H₁₃O₂N₂Cl. Calculated. N 8.4, CH₃O 18.5
167.6 Found. ' 8.3, ' 18.2

\[ [\alpha]_b^0 = +15.5^\circ \text{ (2\% in water)} \]

Synge (9) reported m.p., 146–149°, and \([\alpha]_b^{20} = +14^\circ \text{ (3 per cent in water)}\) for this compound prepared by a different method.

L-Valinamide Hydrochloride—The ester hydrochloride (3.0 gm.) was amidated as described above. After recrystallization from methanol-ethyl acetate, the product melted at 208° with decomposition; yield, 1.85 gm.

C₆H₁₃O₂N₂Cl. Calculated. C 39.3, H 8.6, N 18.4
152.6 Found. ' 39.2, ' 8.6, ' 18.5

\[ [\alpha]_b^0 = +28.3^\circ \text{ (1\% in water)} \]

d-Valine Methyl Ester Hydrochloride—Esterification of 5.0 gm. of d-valine yielded 6.5 gm. of hexagonal plates melting at 167.5–168° after recrystallization from methanol-ether.

C₆H₁₃O₂N₂Cl. Calculated. N 8.3, CH₃O 18.5
167.6 Found. ' 8.3, ' 17.2

\[ [\alpha]_b^0 = -15.6^\circ \text{ (2\% in water)} \]

d-Valinamide Hydrochloride—2.1 gm. of the ester hydrochloride were amidated to yield 1.4 gm. of crystalline product. After recrystallization from methanol-ether, the product separated in square plates; m.p., 268–269° with decomposition.

C₆H₁₃O₂N₂Cl (152.6). Calculated, N 18.4; found, N 18.4

\[ [\alpha]_b^0 = -28.3^\circ \text{ (1\% in water)} \]
DL-Valine Methyl Ester Hydrochloride—Esterification of 20 gm. of DL-valine yielded 27.4 gm. of product which, after recrystallization from methanol-ether, gave 16.8 gm. of large plates. A sample was recrystallized from the same solvents for analysis; m.p., 120-122°.

C_{4}H_{10}O_{2}NCl. Calculated. N 8.3, CH_{2}O 18.5
107.6  Found. " 8.3, " 17.8

Fox and Minard (10) report a melting point of 90-97° for this compound.

DL-Valinamide Hydrochloride—The ester hydrochloride (4.05 gm.) gave, after recrystallization from methanol-ether, 2.9 gm. of product; m.p., 245° with decomposition.

C_{6}H_{14}ONCl. Calculated. C 39.3, H 8.6, N 18.4
152.6  Found. " 39.2, " 8.6, " 18.3

Errera and Greenstein (11) prepared DL-valinamide as the free base by amination of the free ester. Yields and properties were not given.

Carbobenxoxy-L-leucyl-L-leucine Methyl Ester—Carbobenzoxy-L-leucine azide from 7.6 gm. of carbobenzoxy-L-leucine hydrazide (12) was added to an ethereal solution of L-leucine methyl ester prepared from 5.0 gm. of the hydrochloride. The reaction mixture was allowed to stand overnight at room temperature and was then washed consecutively with N HCl, water, 2 per cent sodium bicarbonate, and finally water; it was then dried over sodium sulfate. Upon addition of petroleum ether (b.p. 30-60°), 3.5 gm. of needles were obtained. The melting point was 97.5-98.5° after recrystallization from methanol-water.

C_{21}H_{32}O_{5}N_{2} (392.5). Calculated, N 7.1; found, N 7.0
[α]_{D}^{25} = -33.8° (1% in ethanol)

Carbobenzoxy-L-leucyl-L-leucine—3.5 gm. of the above ester were saponified in acetone at room temperature for 45 minutes by addition of 9.8 ml. of N NaOH. The solution was acidified to Congo red, concentrated in vacuo, and the product extracted into ethyl acetate and dried over sodium sulfate. Upon addition of petroleum ether (b.p. 65-110°), 3.2 gm. of the substance were obtained; m.p., 98-101°, after recrystallization from the same solvents.

C_{20}H_{30}O_{6}N_{2} (378.2). Calculated, N 7.4; found, N 7.5
[α]_{D}^{25} = -24.7° (1% in ethanol)

L-Leucyl-L-leucine—2.74 gm. of the carbobenzoxydipeptide were hydrogenated in 30 ml. of methanol containing 1.5 ml. of water and 1.5 ml. of glacial acetic acid in the presence of palladium black. The catalyst was removed and the filtrate concentrated repeatedly in vacuo with methanol;
ether was added to complete the crystallization. After recrystallization from ethanol-ether, 1.56 gm. of product were obtained as the monohydrate.

\[
\text{C}_{13}\text{H}_{24}\text{O}_{3}\text{N}_{2} \cdot \text{H}_{2}\text{O}. \quad \text{Calculated. C 54.9, H 10.0, N 10.7} \\
262.3 \quad \text{Found. “ 54.7, “ 9.6, “ 10.6} \\
[\alpha]_{D}^{H} = -13.6^\circ (5\% \text{ in } \mathrm{NaOH})
\]

Fischer (13) reported \([\alpha]_{D}^{90} = -13.43^\circ (5 \text{ per cent in } \mathrm{NaOH}), -13.36^\circ (10 \text{ per cent in } \mathrm{NaOH})\) for the same peptide prepared by amination of \(d\alpha\)-bromoisocapronyl-\(L\)-leucine.

Carbobenzyoxy\(L\)-leucyl-\(D\)-leucine Methyl Ester—The azide from 5.76 gm. of carboxbenzyoxy-\(L\)-leucine hydrazide and the ester from 4.5 gm. of \(L\)-leucine methyl ester hydrochloride were coupled and washed as described above. The product crystallized from ether-petroleum ether (b.p. 30-60°). Yield, 4.53 gm.; m.p., 81.5-82.5°, after recrystallization from the same solvents.

\[
\text{C}_{17}\text{H}_{22}\text{O}_{4}\text{N}_{2}. \quad \text{Calculated. C 64.3, H 8.2, N 7.1} \\
392.5 \quad \text{Found. “ 64.3, “ 8.3, “ 7.4} \\
[\alpha]_{D}^{H} = +2.30^\circ (1\% \text{ in ethanol})
\]

\(L\)-Leucyl-\(D\)-leucine—4 gm. of the above ester were saponified with 10 per cent excess \(\mathrm{NaOH}\) for 2 hours at room temperature. Plates melting at 96-102° were obtained from ethyl acetate-ether; these were not obtained analytically pure; yield, 2.0 gm. 1.1 gm. of the carboxbenzyoxydipeptide were reduced in the usual manner. The product was obtained as microcrystals from methanol-ether; yield, 0.63 gm. It was recrystallized from methanol-ethyl acetate and obtained as the monohydrate on being dried in air.

\[
\text{C}_{15}\text{H}_{24}\text{O}_{3}\text{N}_{2} \cdot \text{H}_{2}\text{O}. \quad \text{Calculated. C 54.9, H 10.0, N 10.7} \\
262.3 \quad \text{Found. “ 54.6, “ 10.0, “ 10.7} \\
[\alpha]_{D}^{H} = +74.4^\circ (1\% \text{ in } \mathrm{HCl})
\]

Fischer and Koelker (14) reported \([\alpha]_{D}^{90} = +68.95^\circ (10 \text{ per cent in } \mathrm{HCl})\) for the same compound prepared from \(d\alpha\)-bromoisocapronyl-\(D\)-leucine. The different temperatures and concentrations probably account for the discrepancy in the rotations.

Carbobenzyoxy\(L\)-leucyl-\(L\)-isoleucine—Carboxbenzyoxy-\(L\)-leucine azide from 4.0 gm. of the hydrazide was coupled to \(L\)-isoleucine methyl ester prepared from 2.4 gm. of the hydrochloride and worked up as described. The resulting syrupy compound was saponified in acetone with 13.0 ml. of \(\mathrm{NaOH}\) at room temperature for 45 minutes. The product was crystallized from ethyl acetate by addition of ether-petroleum ether (b.p. 65-110°). Yield, 2.9 gm.; m.p., 101-101.5°, after recrystallization from the same solvents.

\[
\text{C}_{25}\text{H}_{30}\text{O}_{4}\text{N}_{2}. \quad \text{Calculated. C 63.5, H 8.0, N 7.4} \\
378.2 \quad \text{Found. “ 63.6, “ 8.2, “ 7.5} \\
[\alpha]_{D}^{H} = -12.0^\circ (1\% \text{ in ethanol})
\]
L-Leucyl-L-isoleucine—2.0 gm. of the carbobenzoxydipeptide were reduced in the usual manner and isolated from methanol-ether; yield, 1.2 gm. The dipeptide was dissolved in water and a trace of insoluble material was removed by filtration; the compound was isolated by concentration in vacuo.

\[
\text{C}_{12}\text{H}_{24}\text{O}_{2}\text{N}_2. \quad \text{Calculated.} \quad \text{C 59.0, H 9.9, N 11.5} \\
244.3 \quad \text{Found.} \quad " 58.5, " 10.3, " 11.5 \\
\text{[\(\alpha\)\text{D}]} = \ +20.9^\circ (1\% \text{ in water}); \quad \text{[\(\alpha\)\text{D}]} = \ +26.1^\circ (1\% \text{ in } \text{HCl})
\]

Abderhalden and Hirsch (15) reported \([\alpha]\text{D} = \ +25.7^\circ (3 \text{ per cent in } \text{HCl})\) for the same dipeptide prepared from \(d\)-\(\alpha\)-bromoisocapronyl-L-isoleucine.

Carbobenzoxy-L-leucyl-D-isoleucine—Carbobenzoxy-L-leucine azide from 5.0 gm. of the hydrazide was coupled to D-isoleucine methyl ester prepared from 3.25 gm. of the hydrochloride. The syrup was saponified as previously described, and crystallized twice from ethyl acetate-petroleum ether (b.p. 30–60º); m.p., 70–71º.

\[
\text{C}_{20}\text{H}_{30}\text{O}_{6}\text{N}_{2}. \quad \text{Calculated.} \quad \text{C 63.5, H 8.0, N 7.4} \\
378.2 \quad \text{Found.} \quad " 63.1, " 8.4, " 7.5 \\
\text{[\(\alpha\)\text{D}]} = \ -29.6^\circ (1\% \text{ in ethanol})
\]

L-Leucyl-D-isoleucine—2.0 gm. of carbobenzoxy-L-leucyl-D-isoleucine were reduced in the usual manner. After repeated concentration in vacuo with methanol, a gel-like product was obtained from methanol-ether, which was recrystallized from ethyl acetate-petroleum ether (b.p. 65–110º); yield, 1.32 gm. of microneedles. The substance was recrystallized from methanol-ethyl acetate-petroleum ether (b.p. 65–110º) and obtained as the monohydrate after drying in air. A sample was dried to constant weight in vacuo at 94º.

\[
\text{C}_{12}\text{H}_{21}\text{O}_{1}\text{N}_{2}\cdot\text{H}_{2}\text{O}. \quad \text{Calculated.} \quad \text{C 54.9, H 10.0, H}_{2}\text{O} 6.9 \\
262.3 \quad \text{Found.} \quad " 54.8, " 10.2, " 7.2 \\
\text{[\(\alpha\)\text{D}]} = \ +58.4^\circ (1\% \text{ in water})
\]

Abderhalden and Schuler (16) reported \([\alpha]\text{D} = \ +53.1^\circ (2.1 \text{ per cent in water})\) for the same dipeptide prepared from \(d\)-\(\alpha\)-bromoisocapronyl-D-isoleucine.

Carbobenzoxy-L-leucyl-L-valine—The coupling was performed with carbobenzoxy-L-leucine azide from 4.6 gm. of the hydrazide and L-valine methyl ester prepared from 2.5 gm. of the hydrochloride. The syrupy product was saponified and the free acid was crystallized twice from ethyl-petroleum ether (b.p. 65–110º); yield, 3.8 gm. of rectangular plates which melted at 108–109º.

\[
\text{C}_{14}\text{H}_{26}\text{O}_{5}\text{N}_{2} (364.4). \quad \text{Calculated, N 7.7; found, N 7.3} \\
[\(\alpha\)\text{D} ] = \ -15.0^\circ (1\% \text{ in ethanol})
\]
**Leucine Aminopeptidase. III**

**L-Leucyl-L-valine**—The carbobenzyloxydipeptide (2.0 gm.) was hydrogenated and the peptide isolated from methanol-ether. The substance was obtained as the monohydrate after recrystallization from water-methanol-ethyl acetate; yield, 1.35 gm.

C₃₆H₅₀O₅N₂·H₂O. Calculated. C 53.2, H 9.8, N 11.3
248.3 Found. " 53.5, " 9.7, " 11.1
[α]ᵣ₀ = +18.2° (1% in HCl); +17.7° (1% in water)

Fischer and Scheibler (17) reported [α]ᵣ₀ = +18.0° (10 per cent in water) for the same peptide prepared by amination of d-α-bromoisocaproyl-L-valine.

**Carbobenzyo-L-leucyl-D-valine Methyl Ester**—Carbobenzyo-L-leucine azide prepared from 11 gm. of the hydrazide was coupled to D-valine methyl ester from 6 gm. of the hydrochloride. Addition of petroleum ether (b.p. 30-60°) to the washed and dried ethereal solution yielded 10.4 gm. of product. After recrystallization from ether, the melting point was 102-102.5°.

C₂₃H₃₈O₁₃N₂ (378.4). Calculated, N 7.4; found, N 7.5
[α]ᵣ₀ = -7.92° (1% in ethanol)

**L-Leucyl-L-valine**—2.5 gm. of the above ester were saponified to give a product which was crystallized from ethyl acetate-ether-petroleum ether (b.p. 65-110°), but was not obtained analytically pure; yield, 2.0 gm. 2.5 gm. of the carbobenzyloxydipeptide were reduced to give 1.6 gm. of the peptide from methanol-ether; m.p., 276-278°, with decomposition. After recrystallization, the compound gave analytical figures for the monohydrate.

C₃₆H₅₀O₅N₂·H₂O. Calculated. C 53.2, H 9.7, N 11.3
248.3 Found. " 53.0, " 9.8, " 11.4
[α]ᵣ₀ = +55.3° (1% in water)

**Carbobenzyo-L-leucyl-L-phenylalanine Ethyl Ester**—The coupling was performed with carbobenzyo-L-leucine azide from 5 gm. of the hydrazide and L-phenylalanine ethyl ester prepared from 4.1 gm. of the hydrochloride. The product was obtained from ether-petroleum ether (b.p. 30-60°); yield, 6.2 gm. The melting point, after recrystallization from ethyl acetate-petroleum ether (b.p. 65-110°), was 95-96°.

C₃₈H₄₂O₄N₂ (440.5). Calculated, N 6.4; found, N 6.3
[α]ᵣ₀ = -21.6° (1% in ethanol)

**Carbobenzyo L-leucyl-L-phenylalanine**—4.0 gm. of the ester were saponified as already described. After extraction into ethyl acetate and concen-
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Treatment to dryness in vacuo, the product was crystallized from ethanol-water. Yield, 2.3 gm.; m.p., 119–121°.

\[ \text{C}_{29}\text{H}_{28}\text{O}_{6}\text{N}_{2} \] (412.5). Calculated, N 6.8; found, N 6.8

**L-Leucyl-L-phenylalanine**—2.0 gm. of the carbobenzyloxydipeptide were hydrogenated in the manner described above. After repeated concentration in vacuo with methanol, 1.2 gm. of the product were isolated as the monohydrate. A sample was dried in vacuo at 106°.

\[ \text{C}_{16}\text{H}_{17}\text{O}_{5}\text{N}_{2}, \text{H}_{2}\text{O}. \] Calculated, N 9.4, H_{2}O 6.1

296.4 Found, " 9.4, " 6.4

\[ [\alpha]_{D}^{29} = +9.7° \text{ (1% in water)} \]

**Carbobenzyloxy-L-leucyl-D-phenylalanine Ethyl Ester**—Carbobenzyloxy-L-leucine azide from 4 gm. of the hydrazide and the free ester from 3 gm. of D-phenylalanine ethyl ester hydrochloride were coupled in ether as described above. 3.5 gm. of the product (m.p., 107.5–108.5°) crystallized from the reaction mixture. A second crop (0.7 gm.) was obtained upon addition of petroleum ether (b.p. 65–110°) to the mother liquor.

\[ \text{C}_{29}\text{H}_{29}\text{O}_{6}\text{N}_{2}. \] Calculated. C 68.1, H 7.8, N 0.4

440.5 Found, " 68.4, " 7.1, " 6.2

\[ [\alpha]_{D}^{29} = -16.9° \text{ (1% in ethanol)} \]

Synge (9) prepared this compound by coupling carbobenzyloxy-L-leucyl chloride to D-phenylalanine ethyl ester and reported a melting point of 103–105°; \([\alpha]_{D}^{29} = -19° \text{ (2.7 per cent in ethanol)}\).

**Carbobenzyloxy-L-leucyl-D-phenylalanine**—3.4 gm. of the above ester were saponified as described. 2.9 gm. of fine needles were obtained from ethyl acetate-petroleum ether (b.p. 65–110°); m.p., 114–115°.

\[ \text{C}_{29}\text{H}_{28}\text{O}_{6}\text{N}_{2}. \] Calculated. C 66.9, H 6.8, N 6.8

412.5 Found, " 67.0, " 7.2, " 6.8

\[ [\alpha]_{D}^{29} = -39.5° \text{ (1% in ethanol)} \]

This compound was obtained by Synge (9) as an oil.

**L-Leucyl-D-phenylalanine**—2.0 gm. of the carbobenzyloxydipeptide were hydrogenated to yield 1.28 gm. of product from methanol-ether. The dihydrate melted at 253–255° with decomposition.

\[ \text{C}_{16}\text{H}_{21}\text{O}_{5}\text{N}_{2}, \text{2H}_{2}\text{O}. \] Calculated. C 57.3, H 8.3, N 8.9

314.4 Found, " 57.7, " 8.6, " 8.4

\[ [\alpha]_{D}^{29} = +29.7° \text{ (1% in glacial acetic acid)} \]

Synge (9) reported \([\alpha]_{D}^{29} = +25° \text{ (2.4 per cent in glacial acetic acid)}\) for the hydrated material; this gives \([\alpha]_{D}^{29} = +28.2° \text{ (1 per cent in glacial acetic acid)}\) for the anhydrous compound.
Enzymatic Studies

Effect of pH—In earlier studies, Johnson and coworkers (18) investigated the effect of pH on the activity of the Mg++-activated enzyme from swine intestinal mucosa. They found an optimum at pH 8.0 with a slight decline up to pH 9.2, where a sharp decrease was encountered because of the precipitation of Mg(OH)₂. In the present investigation, the activity of the Mn++-activated enzyme was found to increase rapidly from pH 6.0 to 7.8 and then remained constant to the highest value tested, pH 9.3. For the determinations given in Fig. 1, the buffer mixture contained both Tris and cacodylate (19). Neither substance had any inhibitory effect on the enzyme, as determined in comparable tests with Tris alone or with Veronal alone.

Although the form of the S-shaped curve in Fig. 1 suggests that of a simple titration curve the data cannot be described by the equation for the titration of a single ionic group. However, the points are readily, although arbitrarily, fitted by the titration curves of two ionic groups whose pK' values fall at 6.8 and 7.6. If it is assumed that the formation of the metal-protein complex involves coordination of Mn++ with at least two groups (1, 4), these pK values are consistent with an assumed binding with an unionized imidazole group and an unionized terminal α-amino group in peptide linkage or with two imidazole groups of different pK values. Moreover, it is well known that metal-enzymes of the type of leucine

![Graph](http://www.jbc.org/)

**Fig. 1.** Effect of pH on the hydrolysis of L-leucinamide by leucine aminopeptidase. Enzyme preparation was activated by preincubation at 40° with Mn++ at pH 8.0 and aliquots were added to substrate buffered with a mixture of Tris and cacodylate. For the points at the two most acid pH values, the concentration of each buffer ion was 0.04 M. In all other determinations, each substance was present at 0.02 M.
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Aminopeptidase lose activity at slightly acid pH values because of loss of the metal ion; this is readily effected by dialysis, whereas at alkaline pH values it is difficult to remove the metal ion by dialysis. The increased enzymatic activity with increasing pH may then be explained as due to an increased coordination of the metal ion with the protein.

Hydrolysis of Amides—In Table I are shown the results for the action of leucine aminopeptidase on several amino acid amides for the two enzyme preparations at different levels of purity, as indicated by the C values. The action on L-leucinamide is given as 100 per cent to facilitate comparison with earlier studies in which this substrate was used as a standard. No hydrolysis of the D-amino acid amides was detected after 24 hours incubation with the enzyme. The resistance of these compounds to the action of the enzyme is to be expected from earlier investigations of optical specificity (4).

L-Isoleucinamide and L-valinamide are hydrolyzed at comparable relative rates by both enzyme preparations, but these rates are extremely slow when compared with the corresponding straight chain amino acid amides. It was found earlier that norleucinamide is hydrolyzed at the same rate as DL-leucinamide.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Preparation I</th>
<th>Preparation II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E</td>
<td>C</td>
</tr>
<tr>
<td>L-Leucinamide</td>
<td>25.6</td>
<td>0.14</td>
</tr>
<tr>
<td>L-Isoleucinamide</td>
<td>205</td>
<td>0.010</td>
</tr>
<tr>
<td>D-Isoleucinamide</td>
<td>128</td>
<td>0</td>
</tr>
<tr>
<td>L-Valinamide</td>
<td>205</td>
<td>0.012</td>
</tr>
<tr>
<td>D-Valinamide</td>
<td>128</td>
<td>0</td>
</tr>
<tr>
<td>DL-Valinamide*</td>
<td>205</td>
<td>0.011</td>
</tr>
<tr>
<td>DL-x-Aminocaprylic amide*†</td>
<td>25.6</td>
<td>0.086</td>
</tr>
</tbody>
</table>

* Present at 0.1 m. Rate of hydrolysis is computed only for the susceptible L form.
† The L-aminocaprylic acid begins to crystallize after about 25 to 30 per cent hydrolysis of the L-amide.
leucinamide, whereas norvalinamide is cleaved at about 80 per cent of the rate of leucinamide (4).

The data obtained with the amide of \( \alpha \)-aminocaprylic acid are perhaps less reliable because of the crystallization of the liberated acid during the assay. However, the long chain compound is somewhat less sensitive than leucinamide to the action of the enzyme.

**Table II**

*Hydrolysis of Peptides*

Each \( C \) value is the average of two or more independent kinetic runs. Mn\(^{++} \) = 0.002 to 0.008 m; pH 8.0; \( t 40^\circ \). Enzyme concentration \( (E) \) is in mg. of protein N per ml. \( \times 10^3 \).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Preparation I</th>
<th></th>
<th></th>
<th>Preparation II</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( E )</td>
<td>( C )</td>
<td>Relative activity</td>
<td>( E )</td>
<td>( C )</td>
</tr>
<tr>
<td>L-Leucyl-L-leucine</td>
<td>25.6</td>
<td>0.14</td>
<td>100</td>
<td>1.92</td>
<td>2.0</td>
</tr>
<tr>
<td>L-Leucyl-D-leucine</td>
<td>256</td>
<td>0.0013</td>
<td>0.9</td>
<td>96</td>
<td>0.014</td>
</tr>
<tr>
<td>L-Leucyl-L-isoleucine</td>
<td>25.6</td>
<td>0.12</td>
<td>86</td>
<td>1.92</td>
<td>1.6</td>
</tr>
<tr>
<td>L-Leucyl-D-isoleucine</td>
<td>256</td>
<td>0</td>
<td>96</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>L-Leucyl-L-valine</td>
<td>25.6</td>
<td>0.085</td>
<td>61</td>
<td>1.92</td>
<td>1.3</td>
</tr>
<tr>
<td>L-Leucyl-D-valine*</td>
<td>256</td>
<td>0</td>
<td>96</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>L-Leucyl-L-alanine</td>
<td>38.4</td>
<td>0.058</td>
<td>3</td>
<td>1.92</td>
<td>0.44</td>
</tr>
<tr>
<td>L-Leucyl-D-alanine</td>
<td>96</td>
<td>0.009</td>
<td>0.45</td>
<td>4.80</td>
<td>0.30</td>
</tr>
<tr>
<td>L-Leucyl-L-phenylalanine</td>
<td></td>
<td></td>
<td></td>
<td>1.92</td>
<td>2.1</td>
</tr>
</tbody>
</table>

\* No detectable hydrolysis of these compounds was observed after 24 hours incubation.

\( \dagger \) Crystallization of L-tyrosine during the course of the reaction.

**Action on Diastereoisomeric Peptides**—The data in Table II show that all of the dipeptides of the L-L type are rapidly hydrolyzed by leucine aminopeptidase. The compounds which contain L-phenylalanine and L-tyrosine are, however, much less sensitive than the peptides which contain only aliphatic amino acids. This is particularly striking since the peptides which contain L-leucyl-L-amino acid are influenced only to a small extent by the nature of the terminal aliphatic residue. The larger L-leucyl derivatives are hydrolyzed in the order L-leucine > L-isoleucine > L-valine > L-alanine; however, L-leucylglycine is hydrolyzed at about the same rate as L-leucyl-L-leucine.

It has been reported earlier that L-leucyl-D-alanine is hydrolyzed by this
enzyme (4). It has now been found that L-leucyl-d-leucine and L-leucyl-
d-phenylalanine are hydrolyzed by leucine aminopeptidase, although these
are cleaved more slowly than the compound containing d-alanine. It will
be noted that the diastereoisomeric leucylleucines show the same relative
rates of hydrolysis for Preparations I and II.

In order to test further whether leucine aminopeptidase is responsible for
the hydrolysis of the susceptible dipeptides containing d residues as well as
other substrates, experiments were performed in the absence of added

\[\text{Table III}\]

**Hydrolysis of Tested Substrates without Added Mn\textsuperscript{++}\]**

The amount of activity has been computed in comparison with the fully activated
preparations given in Tables I and II.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Preparation I</th>
<th></th>
<th>Preparation II</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>Activity</td>
<td>C</td>
<td>Activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>compared to</td>
<td></td>
<td>compared to</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mn\textsuperscript{++}-activated</td>
<td></td>
<td>Mn\textsuperscript{++}-activated</td>
</tr>
<tr>
<td>L-Isoleucinamide</td>
<td>0.0015</td>
<td>15</td>
<td>0.010</td>
<td>6</td>
</tr>
<tr>
<td>L-Valinamide</td>
<td>0.0015</td>
<td>13</td>
<td>0.008</td>
<td>4</td>
</tr>
<tr>
<td>DL-(\alpha)-Aminooctanoic amide*</td>
<td>0.021</td>
<td>24</td>
<td>0.18</td>
<td>15</td>
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<tr>
<td>L-Leucyl-L-leucine</td>
<td>0.028</td>
<td>20</td>
<td>0.23</td>
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<tr>
<td>L-Leucyl-D-leucine</td>
<td>0.0003</td>
<td>23</td>
<td>0.0013</td>
<td>9</td>
</tr>
<tr>
<td>L-Leucyl-L-isoleucine</td>
<td>0.016</td>
<td>13</td>
<td>0.11</td>
<td>7</td>
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<tr>
<td>L-Leucyl-L-valine</td>
<td>0.012</td>
<td>14</td>
<td>0.080</td>
<td>6</td>
</tr>
<tr>
<td>L-Leucyl-L-alanine</td>
<td></td>
<td></td>
<td>0.15</td>
<td>12</td>
</tr>
<tr>
<td>L-Leucyl-D-alanine</td>
<td></td>
<td></td>
<td>0.0069</td>
<td>12</td>
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<tr>
<td>L-Leucyl-L-phenylalanine</td>
<td></td>
<td></td>
<td>0.089</td>
<td>21</td>
</tr>
<tr>
<td>L-Leucyl-D-phenylalanine</td>
<td></td>
<td></td>
<td>0.0021</td>
<td>23</td>
</tr>
<tr>
<td>L-Leucyl-L-tyrosine</td>
<td>0.012</td>
<td>28</td>
<td>0.092</td>
<td>31</td>
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</tbody>
</table>

* Present at 0.1 M.

The data shown in Table III indicate that the rate of hydrolysis of all of the
tested compounds is markedly accelerated by the presence of added Mn\textsuperscript{++},
although there is some variation in the amount of activation. Despite
these variations, it is noteworthy that the susceptible pairs of diastereoisomeric
dipeptides each showed similar amounts of activation; that is, in the
absence of added Mn\textsuperscript{++}, the leucylleucines had about 11 per cent of the
activity of the fully activated enzyme, the leucylalanines 12 per cent, and
the leucylphenylalanines about 22 per cent for Preparation II. It will be
noted that greater activation is obtained with the more highly purified
Preparation II, although no particular efforts were made to remove the
natural activator.
Kinetics—Under the conditions of the tests, all of the sensitive substrates gave satisfactory first order kinetics for the hydrolytic reaction. In each instance, even with less sensitive compounds, the reaction was followed until at least 50 per cent hydrolysis had occurred, and, in many cases, was allowed to proceed to completion. Representative data for several com-

TABLE IV

Kinetics of Hydrolysis for Several Substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Preparation No.</th>
<th>Mn** added</th>
<th>E</th>
<th>Time (min)</th>
<th>Hydrolysis (per cent)</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Isoleucinamide</td>
<td>I</td>
<td>0.006</td>
<td>205</td>
<td>35</td>
<td>14</td>
<td>0.009</td>
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<tr>
<td></td>
<td></td>
<td></td>
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<td>63</td>
<td>27</td>
<td>0.011</td>
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<tr>
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<td></td>
<td></td>
<td>88</td>
<td>36</td>
<td>0.011</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>141</td>
<td>53</td>
<td>0.011</td>
</tr>
<tr>
<td>L-Leucyl-L-leucine</td>
<td>II</td>
<td>0.002</td>
<td>1.92</td>
<td>24</td>
<td>20</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
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<td>60</td>
<td>41</td>
<td>2.0</td>
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<td></td>
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<td>80</td>
<td>54</td>
<td>2.2</td>
</tr>
<tr>
<td>L-Leucyl-D-leucine</td>
<td>I</td>
<td>0.002</td>
<td>25.6</td>
<td>21</td>
<td>15</td>
<td>0.13</td>
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<tr>
<td></td>
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<td></td>
<td></td>
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<td>27</td>
<td>0.15</td>
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<td>66</td>
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<td></td>
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<td></td>
<td>94</td>
<td>51</td>
<td>0.13</td>
</tr>
<tr>
<td>L-Leucyl-L-phenylalanine</td>
<td>II</td>
<td>0.003</td>
<td>96</td>
<td>46</td>
<td>14</td>
<td>0.015</td>
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<td>33</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>268</td>
<td>54</td>
<td>0.014</td>
</tr>
<tr>
<td>L-Leucyl-D-phenylalanine</td>
<td>II</td>
<td>none</td>
<td>15.4</td>
<td>90</td>
<td>24</td>
<td>0.084</td>
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<td>123</td>
<td>32</td>
<td>0.091</td>
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<tr>
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<td></td>
<td>180</td>
<td>44</td>
<td>0.091</td>
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<td>241</td>
<td>55</td>
<td>0.091</td>
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<td>18</td>
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<td>0.0099</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>379</td>
<td>52</td>
<td>0.0088</td>
</tr>
</tbody>
</table>

pounds with both enzyme preparations are shown in Table IV. The falling constants obtained with L-leucyl-D-phenylalanine should probably be ascribed to enzyme inactivation during the long incubation required for the hydrolysis of this relatively resistant compound.

DISCUSSION

Several years ago, in an attempt to explain the specificity of the metal-peptidases, it was suggested that the metal ion acts as a ligand in forming a coordination complex with both the substrate and the enzyme (20, 4). For the hydrolysis of simple peptides or amides by leucine aminopeptidase,
it was assumed that the metal ion, Mn^{++} in this instance, binds with the free amino group and the peptide nitrogen whereas the aliphatic side chain (R' in I) interacts through van der Waals' forces directly with the protein moiety of the enzyme (4). No major effect on the enzyme interaction was assigned to R'' in view of the essentially similar sensitivity of L-leucinamide, L-leucylglycine, and L-leucyl-β-alanine (4, 21). The slower rate of hydrolysis of L-leucyl-D-alanine was ascribed to a steric interference by the terminal methyl group of the D-alanine residue, sterically hindering the binding of the metal to the peptide nitrogen. In order to test this idea further, the present experiments with several diastereoisomeric L-leucyl peptides were designed.

If the above hypothesis is correct, it would be anticipated that the nature of the terminal residue R'' in dipeptides of the L-D type would have different effects, depending upon the size and configuration of its structure. This is, in general, borne out by the data reported in Table II. The peptides containing the larger residues D-leucine and D-phenylalanine are hydrolyzed at rates corresponding to about 0.8 and 2 per cent of those of the corresponding L-leucyl-L-leucine and L-leucyl-L-phenylalanine, although the compound containing D-phenylalanine is the most slowly hydrolyzed in absolute terms. Moreover, the peptides containing D-isoleucine and D-valine are, within present experimental limits, completely resistant to the action of the enzyme. It must be assumed that the branched aliphatic side chains of these residues offer even greater steric hindrance near the peptide bond than do other residues. The resistance of L-leucyl-D-valine and L leucyl D isoleucine is probably not due to a bond strength influence since both of the corresponding diastereoisomeric peptides of the L-L configuration are hydrolyzed slightly more rapidly than L-leucyl-L-alanine. Moreover, the N-linked derivatives of valine and isoleucine do not appear to possess any abnormal properties.

The relative resistance to enzymatic hydrolysis of L-isoleucinamide and L-valinamide should probably be ascribed to two different factors. The sluggishness of these β-branched amino acids in forming ester and amide derivatives has already been mentioned. The unique properties of these amino acids are also shown by the observation that in the amination of the optically active α-bromo compounds to form isoleucine and valine Walden inversion does not occur; this is in contrast to results with other α-bromo acids which yield amino acids of the inverted form (22-24). Carboxyl-linked derivatives of α-bromoisovaleric acid and α-bromo-β-methylvaleric acid do show normal inversion on amination (24). Studies with valylglycine show that this peptide is hydrolyzed very slowly by alkali (25) or by acid (26) when compared with other dipeptides which are also aliphatic amino acid derivatives of glycine such as leucylglycine.
Regardless of the mechanism by which the aminopeptidase operates, the resistance to both acidic and alkaline hydrolysis of valyl and isoleucyl derivatives must be reflected in the rates of enzyme-catalyzed hydrolysis of these amides. This cannot be the only factor involved, however, since neither L-isoleucinamide nor L-valinamide inhibits the hydrolysis of L-leucinamide when these are present in equal concentrations (0.05 M). The absence of inhibition indicates that the \( \beta \)-branched compounds do not interact with the enzyme as strongly as does leucinamide. Thus, weakness of interaction and strength of bond are both reflected in the action of the enzyme. The quantitative evaluation of these two effects must await the availability of more highly purified enzyme preparations.

In contrast to the inertness of valylglycine, glycylvaline is hydrolyzed by acid or by alkali at rates comparable with those of glycylleucine (25, 26). Correspondingly, the aminopeptidase hydrolyzes the L-leucyl derivatives of L-leucine, L-isoleucine, and L-valine at rates which differ only slightly from one another. Thus, the "normal" character of \( N \)-substituted derivatives of valine and isoleucine is also reflected in their similar sensitivity to enzymatic hydrolysis.

**SUMMARY**

1. The synthesis by the carbobenzyloxyl method of a number of diastereoisomeric peptides is described. These include L-leucyl-L-leucine, L-leucyl-D-leucine, L-leucyl-L-isoleucine, L-leucyl-D-isoleucine, L-leucyl-L-valine, L-leucyl-D-valine, L-leucyl-L-phenylalanine, and L-leucyl-D-phenylalanine. In addition, many of the intermediate carbobenzyldipeptide esters and carbobenzyldipeptides have been isolated and characterized.

2. The synthesis and characterization of the hydrochlorides of L-valine methyl ester, D-valine methyl ester, DL-valine methyl ester, L-valinamide, D-valinamide, DL-valinamide, L-isoleucine methyl ester, D-isoleucine methyl ester, L-isoleucinamide, and D-isoleucinamide are described.

3. Leucine aminopeptidase hydrolyzes L-valinamide and L-isoleucinamide at about 10 per cent of the rate at which L-leucinamide is split. The lesser sensitivity of \( \beta \)-branched amino acid amides is ascribed to the chemical inertness and unusual character of the carboxyl derivatives of these amino acids rather than to any specific enzymatic factor. The amide of \( \alpha \)-aminocaprylic acid is hydrolyzed at about 70 per cent of the rate of leucinamide. None of the D-amino acid amides is split.

4. All of the leucyl dipeptides in which both residues are of the L configuration are hydrolyzed; those with terminal aliphatic amino acids are split more rapidly than those with terminal L-phenylalanine or L-tyrosine.

5. Leucine aminopeptidase has no detectable action on L-leucyl-D-valine or L-leucyl-D-isoleucine. However, L-leucyl-L-leucine, L-leucyl-D-phenyl-
alanine, and L-leucyl-d-alanine are slowly hydrolyzed by the enzyme. The nature of this type of optical specificity is discussed.

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Emil L. Smith, Darrel H. Spackman and W. J. Polglase


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