Isolation and Identification of L-β-Aspartyl-L-lysine and L-γ-Glutamyl-L-ornithine from Normal Human Urine†

Majorie F. Lou

ABSTRACT: L-β-Aspartyl-L-lysine and L-γ-glutamyl-L-ornithine were isolated from pooled normal human urine and each peptide was shown to be identical with the authentic peptides. The concentrations of these dipeptides in the urine of individual subjects were determined directly by using a new buffer sequence on a standard ion exchange chromatographic amino acid analyzer with a sensitivity of 10^{-10} mol. In urine from normal subjects ranging in age from 12 to 64 years, mean values of 1.47 μmol/g of creatinine of L-γ-glutamyl-L-ornithine and 8.24 μmol/g of creatinine of L-β-aspartyl-L-lysine were found. The urine of children under 10 years of age contained, relative to creatinine excretion, more L-β-aspartyl-L-lysine and L-γ-glutamyl-L-ornithine than that of older children and adults. All urines contained substantially larger concentrations of L-β-aspartyl-L-lysine than of L-γ-glutamyl-L-ornithine. Both peptides were found in urine collected after 21 hr of fasting in lower concentrations than found in urine from nonfasting subjects. The urinary concentrations of both peptides did not appear to be influenced by race or sex.

Normal human urine contains a large number of ninhydrin-positive constituents (Stein, 1953; Hamilton, 1962; King, 1964) both in free and bound form. Most of these have not yet been identified.

Several laboratories have reported the presence of L-β-aspartyl peptides in normal human urine (Kakimoto and Armstrong, 1961; Buchanan et al., 1962; Pisano et al., 1966). Only three γ-glutamyl dipeptides have thus far been isolated from this source (Buchanan et al., 1962). They include γ-glutamylvaline, -leucine, and -isoleucine.

Using a gel column chromatographic technique developed in this laboratory for the separation of amino acids, peptides, and proteins in urine (Hamilton and Lou, 1972), two dipeptides have been isolated and purified from normal human urine. One has been identified as L-β-aspartyl-L-lysine, referred to subsequently as peak 796, the retention time in minutes on the amino acid chromatogram; and the other as L-γ-glutamyl-L-ornithine (peak 735). The concentrations of these two peptides in urine from 44 normal individuals have been determined. Although the concentrations varied considerably from subject to subject, the average excretion of both peptides relative to creatinine excretion appeared to be greater in urine of subjects 10 years of age and under. As noted below, the data were obtained from the analysis of single urine specimens and not 24-hr collections.

Materials and Methods

Collection of Urine. Fresh morning specimens from three normal adult subjects were collected, pooled, and, pending analysis, stored without chemical preservatives at −85°. Single specimens from 44 subjects who varied in sex, race (including Caucasian, Negroid, and Mongoloid), and age (from 3 to 64 years) were collected in the early morning. From the fasting group, eight adult subjects were allowed a light meal (crackers, tomato soup, skim milk, gelatin) before fasting began. Urine specimens were collected individually 21 hr later and stored in the same manner as the pooled urine.

Urinary creatinine was determined manually by a revised method for the Jaffe reaction (DiGiorgio, 1974).

Gel Column Chromatography. Pooled untreated urine (130 ml) was applied to the top of a 5 × 264 cm column of Sephadex G-10 and eluted with 0.1 *N* acetic acid (pH 3.0) saturated with chloroform, at a flow rate of 66 ml/hr, as described previously (Hamilton and Lou, 1972). In accordance with their procedure, the effluent was collected at 10-min intervals and the absorbance of each fraction was measured at 280, 260, and 220 mμ on a Zeiss PMQII spectrophotometer. Fractions were divided according to the absorbance pattern at 280 mμ. Each fraction was pooled, concentrated to dryness in a flash evaporator, and dissolved in 1 ml of water. The first fraction was found to contain proteins and large peptides. Fractions II–V contained peptides and basic amino acids and fraction VI contained most of the acidic and neutral amino acids. Aromatic amino acids and urea emerged in the later fractions.

Isolation and Purification Procedure. Samples of fraction III (or fraction IV) were applied to an amino acid analyzer using a buffer sequence previously described and operated in a split stream mode (Lou, 1973). The buffer contained neither thiodiglycol nor Brij 35. Ninety percent of the effluent was directed to a fraction collector, while the remaining 10% was reacted with ninhydrin to provide an analog display on a strip chart recorder. The peptides which were isolated by this technique were subsequently desalted on a Sephadex G-10 column (0.9 × 184 cm). Each peptide when eluted with water emerged ahead of NaCl and citrate. The peptides were concentrated on a flash evaporator and stored at −85° until further analysis.

High Voltage Electrophoresis and Paper Chromatography. The details of the high voltage electrophoresis–paper chromatography method have been reported previously (Lou and Hamilton, 1971). Whatman No. 3 mm paper was used for both electrophoresis and chromatography. A Model D high voltage electrophoresis apparatus (Gilson Medical Electronics, Middleton, Wis.) was employed. Pyridine–acetic acid–H_2O buffers at pH 5.3 and 3.5 were used for electrophoresis. Butanol–acetic acid–H_2O (4:1:5) and

† From the Thomas R. Brown Memorial Laboratories, Department of Biochemistry, The Alfred I. duPont Institute, Wilmington, Delaware 19899. *Received March 6, 1975.* Preliminary reports of this work were presented at the American Society of Biological Chemists Meeting, 1971, and the 9th International Congress of Biochemistry, 1973.

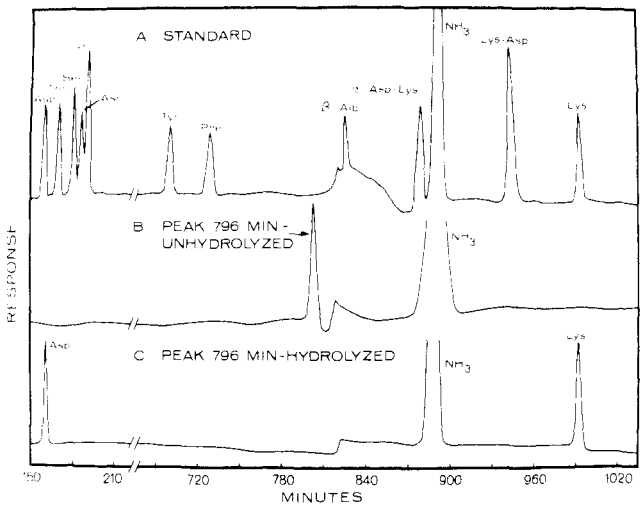

FIGURE 1: Ion-exchange chromatography of peak 796. (A) The chromatographic pattern of standard amino acids and dipeptides (0.01 μmol each). (B) Peak 796 isolated from Sephadex fractions III and IV (see Materials and Methods). (C) Complete acid hydrolysate of peak 796.

2-propanol–formic acid–H_2O (8:1:1) were the solvent systems for descending paper chromatography. Compounds were visually detected by dipping the paper into a 0.1% ninhydrin solution (in acetone–H_2O 19:1, v/v).

Ion Exchange Chromatography. A single column ion exchange chromatographic system was used according to Hamilton (1963), with the ninhydrin reagent of Moore (1968). Two buffer sequences were used for routine analysis: a modified procedure of Hamilton (1963) was designated as buffer sequence I; a second buffer sequence (sequence II) was described by Lou and Hamilton (1971). To determine the concentrations of peak 735 and peak 796 in the urine of individual subjects, sequence II was modified by changing the eluting buffer from pH 3.55 to pH 3.465; this was designated as sequence IIB.

Preparation of Samples for Amino Acid Analysis. All urinary specimens were deproteinized by the addition of sulfosalicylic acid followed by centrifugation (Hamilton, 1968). For quantitative determination of peak 735 and peak 796, 0.5 ml of supernatant was applied to the column of the amino acid analyzer. The purified urinary peptides were hydrolyzed in 2 ml of constant boiling 6 *N* HCl in a 5-ml glass ampoule (Bellco Glass Co.), which was sealed and placed in 110° dry bath (Hallikainen Instruments, Slaco Division, Richmond, Calif.) for 21 hr. The hydrolysate was evaporated to dryness by a flash evaporator, dissolved in 0.5 ml of water, and applied to the column of the analyzer.

N-Terminal Group Determination. The dansylation procedure of Gray (1967) was followed for N-terminal amino acid determination. The dansylated product was hydrolyzed and applied to polyamide-6 thin-layer sheet (Baker Co.) followed by development first in 1.5% formic acid (solvent A), and then in a perpendicular direction, in benzene–acetic acid (9:1, v/v) (solvent B). The sheet was developed a third time, in the same direction as solvent B, in ethyl acetate–chloroform–acetic acid (20:1:1, v/v) (solvent C). A rectangular glass tank (SJ-1003, Analtech, Wilmington, Del.) was used as a developing chamber. Standard dansylated amino acids were obtained from Seikagaku Fine Biochemicals, Tokyo, Japan. A mixture of N^{ϵ}-dansyllysine and N^{α}-dansyllysine was obtained by hydrolyzing authentic dansyldilysine in 6 *N* HCl at 105° for 16 hr. To distinguish N^{ϵ}-dansyllysine from its α derivative, the technique of Cole et al. (1965) was followed. The hydrolysate was applied to silica gel G, 250 μm thickness (Analtech, Inc.) and developed in 1-butanol saturated with 0.2 *N* NaOH (solvent D) for 4.5 hr. Authentic N^{δ}-dansylornithine and N^{α}-dansylornithine were prepared by dansylation followed by hydrolysis of N^{α}-acetylornithine and N^{δ}-acetylornithine, respectively. The derivatives were separated on a thin-layer polyamide-6 sheet after development in solvent B.

C-Terminal Group Determination. Hydrazinolysis was used for C-terminal amino acid determination following the procedure of Fraenkel-Conrat and Tsung (1967), without catalyst. An aliquot of hydrazine (Eastman Organic Chemicals, 95%+) was added to 0.1 μmol of dried sample in a 2-ml glass ampoule which was then sealed and heated at 80° for 24 hr. Excess hydrazine in the sample was removed by flash evaporation and by placing it over concentrated H_2SO_4 in a desiccator overnight under vacuum. The hydrazinolysates were analyzed by both high voltage electrophoresis and ion-exchange chromatography without further treatment. Authentic α-aspartylhydrazide and β-aspartylhydrazide were synthesized in solution from isoasparagine and asparagine, respectively. γ-Glutamylhydrazide and α-glutamylhydrazide, however, were synthesized in solution from glutamine and isoglutamine, respectively.

Ninhydrin Reaction of β-Aspartyl Peptides. Buchanan et al. (1962) have reported that β-aspartyl peptides give a blue ninhydrin color on filter paper, whereas α-aspartyl peptides give a purple color. They have also found that upon incubation with 1% ninhydrin (in 2.5% citrate buffer, pH 2.5) at 37° for 8–10 hr in solution, the β-aspartyl peptides produced a brown color while α-aspartyl peptides produced none. We used 5 nmol each of peak 796, standard α-aspartyllysine, α-aspartylglycine, and β-aspartylglycine (Cyclo Chemical Co.) for this test.

Enzymatic Determination of Ornithine. Ornithine was determined as citrulline following enzymatic carbamylation by ornithine transcarbamylase in the presence of carbamyl phosphate.

Ornithine transcarbamylase was partially purified from fresh calf liver according to the procedure of Burnett and Cohen (1957). The enzyme was purified up to the ammonium sulfate fractionation step described by these authors. The protein precipitate was dissolved in 0.005 *M* sodium phosphate buffer (pH 7.0), and dialyzed for 18 hr against the same buffer. This enzyme preparation contained 1.25 mg of protein/ml and had a specific activity of 2 nmol of citrulline per min per mg of protein assayed by the method of Koritz and Cohen (1957).

No free amino acids were detected in this enzyme preparation following deproteinization by sulfosalicylic acid.

The reaction mixture for the carbamylation reaction contained in a total volume of 0.35 ml, 0.02 μmol of peak 735 hydrolysate, 5 μmol of carbamyl phosphate, 0.125 mg of enzyme protein, and 30 μmol of borate buffer (pH 9.0). After incubation at 37° for 30 min, the reaction was stopped by the addition of 0.2 ml of 15% sulfosalicylic acid. After centrifugation, the supernatant was assayed for citrulline by ion-exchange chromatography.

Colorimetric Determination of Ornithine. Ornithine was determined as described by Chinard (1952). Under the conditions of this assay, ninhydrin reacts with ornithine to produce a distinct red color which can be measured spectrophotometrically.

Table I: Chromatographic and Electrophoretic Properties of Peak 796 and Authentic Peptide L-β-Aspartyl-L-lysine.

	High Voltage Electrophoresis		Paper Chromatography (R_f)		Ion-Exchange Chromatography Retention Time (min)		
	Pyridine–Acetic Acid–H_2O (5:50: 945, v/v) pH 3.5	Pyridine–Acetic Acid–H_2O (2:1: 97, v/v) pH 5.3	Butanol–Acetic Acid–H_2O (4:1:5, v/v)	Propanol–Formic Acid–H_2O (8:1:1, v/v)	Buffer Sequence		
	Migration Distance toward Anode at 35.8 V/cm, 1 hr (cm)				I[a]	II[b]	IIB[c]
Peak 796	6.3	3.7	0.064	0.14	830	780	890
L-β-Aspartyl-L-lysine	6.5	3.8	0.064	0.15	830	780	890

[a] Hamilton (1963). [b] Lou and Hamilton (1972). [c] See Materials and Methods.

Ornithine, citrulline, and carbamyl phosphate were obtained from Sigma Co.

Results

L-β-Aspartyl-L-lysine (Peak 796)

Isolation and Purification of Peak 796. Upon analysis by the split stream ion exchange chromatographic procedure (see Materials and Methods), an unknown peak at 796 min was found in both Sephadex fractions III and IV and was designated peak 796. It was isolated from the effluent of the split stream column and desalted. A portion on two-dimensional high voltage paper electrophoresis–paper chromatography showed one ninhydrin-positive spot. Another portion on an ion-exchange chromatogram showed a single symmetrical peak which was resolved immediately ahead of β-aminoisobutyric acid and after phenylalanine (Figure 1B). The isolated material was therefore considered to be homogeneous.

Identification of Peak 796. After hydrolysis of peak 796 (in Materials and Methods), equimolar amounts of aspartic acid and lysine were found (Figure 1C). α-Aspartyllysine or lysylaspartic acid were eliminated as possible structures by comparison of peak 796 with authentic samples of these peptides (Cyclo Chemical Co.). Both occupied positions on the ion-exchange chromatogram that were later than peak 796 as shown in Figure 1A. The presence of an amide group in the peptide structure was unlikely, since peak 796 was not susceptible to hydrolysis by amidopeptidase M (Henley Co., New York). In addition, there was no liberation of ammonia when peak 796 was hydrolyzed in 2 *N* HCl, 110°, at time intervals from 30 min to 6 hr (Wilcox, 1967). With even milder hydrolysis condition, i.e., 0.03 *N* HCl, 100° for 2 hr, no other ninhydrin-positive peaks appeared on the chromatogram other than aspartic acid, lysine, and the unhydrolyzed peptide. This result suggested that peak 796 was a dipeptide of simple structure.

N-Terminal Group Studies of Peak 796. After dansylation of peak 796, the reaction mixture was hydrolyzed in acid. The hydrolysate was evaporated to dryness in a flash evaporator, and suspended in 10 μl of 50% pyridine. It was applied on the lower left corner of the polyamide-6 sheet and developed first with solvent A and then in perpendicular direction, subsequently by solvents B and C (see Materials and Methods). Two fluorescent spots were found. One moved to the center of the sheet in the same position as standard dansylaspartic acid. The other spot moved faster than dansylaspartic acid in solvent A but remained stationary when developed by both solvents B and C. The dansylation patterns of peak 796 and standard α-aspartyllysine

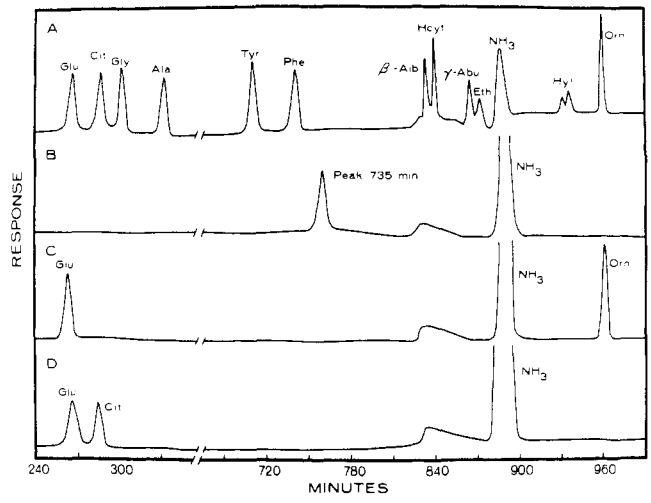

FIGURE 2: Ion-exchange chromatography of peak 735. (A) Standard mixtures of amino acids (0.01 μmol each). (B) Peak 735 isolated from Sephadex fractions III and IV (see Materials and Methods). (C) Complete acid hydrolysate of peak 735. (D) Acid hydrolysate of peak 735 after incubation with partially purified ornithine transcarbamylase and carbamyl phosphate (see Materials and Methods).

were identical. To clarify the nature of the second dansylated spot which is present in both peak 796 and α-aspartyllysine, the spot was scraped off the thin-layer sheet and the scrapings were extracted by repeated suspension and centrifugation of the polyamide gel in 50% pyridine. The extract was pooled, concentrated, and reapplied onto a silica gel G plate (250 μm thickness), parallel with the spots of standard N^{ϵ}-dansyllysine and N^{α}-dansyllysine. The gel plate was developed in solvent D and showed that both the isolated dansyl spots of peak 796 and α-aspartyllysine had the same R_f values as N^{ϵ}-dansyllysine, moving faster than the N^{α} derivative. These results clearly indicated that aspartic acid was the N-terminal amino acid of peak 796 and that the ε-amino group of lysine was free while the α-amino group was involved in the peptide bond.

C-Terminal Group Studies of Peak 796. The hydrazinolysate of peak 796 was spotted on Whatman No. 3 mm paper for high voltage electrophoresis (pyridine–acetic acid buffer (pH 5.3)). A ninhydrin-positive spot moved to the same location as lysine. The hydrazinolysate was also analyzed by ion-exchange chromatography. Only a lysine peak appeared. After hydrolyzing the hydrazinolysate of peak 796, both aspartic acid and lysine were recovered in equal amounts. This result indicated that lysine was the C-terminal group of the peak 796 peptide.

Examination of the Configuration of Peak 796 Peptide

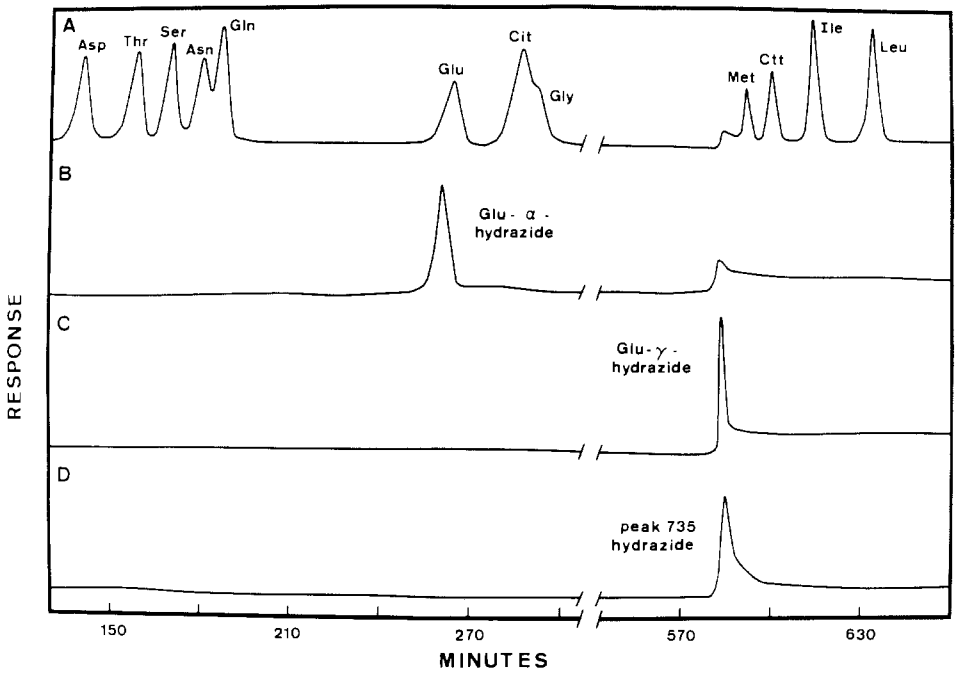

FIGURE 3: Ion-exchange chromatographic pattern of glutamylhydrazides. (A) Standard amino acid mixture (0.01 μmol each); (B) standard glutamyl-α-hydrazide synthesized from isoglutamine; (C) standard glutamyl-γ-hydrazide synthesized from glutamine; (D) hydrazinolysate of peak 735.

Table II: Chromatographic and Electrophoretic Properties of Peak 735 and Authentic Dipeptide L-γ-Glutamyl-L-ornithine.

	High Voltage Electrophoresis		Paper Chromatography (R_f)		Ion-Exchange Chromatography		
	Pyridine–Acetic Acid–H_2O (5:50: 945, v/v) pH 3.5	Pyridine–Acetic Acid–H_2O (2:1: 97, v/v) pH 5.3			Retention Time (min) Buffer Sequence		
	Migration Distance toward Anode at 35.8 V/cm, 1 hr (cm)		Butanol–Acetic Acid–H_2O (4:1:5, v/v)	Propanol–Formic Acid–H_2O (8:1:1, v/v)	I[a]	II[b]	IIB[c]
Peak 735	5.0	3.05	0.0925	0.223	775	600	765
L-γ-Glutamyl-L-ornithine	5.1	3.25	0.0950	0.221	775	600	765

[a] Hamilton (1963). [b] Lou and Hamilton (1972). [c] See Materials and Methods.

Bond. After incubation with acidic ninhydrin (see Materials and Methods), peak 796 turned brown in color similar to standard β-aspartylglycine. Both α-aspartylglycine and α-aspartyllysine were colorless under the same conditions. When comparing the ninhydrin reaction of each peptide on filter paper, both peak 796 and β-aspartylglycine turned greenish-blue, whereas both α-aspartyl peptides were purple. These results suggested that peak 796 was a β-aspartyl peptide.

Comparison of the Characteristics of Authentic L-β-Aspartyl-L-lysine with Unknown Peak 796. Peak 796 and authentic L- β-aspartyl-L-lysine (Bachem Fine Chemicals, Inc., Marina Del Rey, Calif. 90291) were identical when analyzed by paper electrophoresis chromatography, as well as by ion exchange chromatographic techniques. The results are summarized in Table I.

L-γ-Glutamyl-L-ornithine (Peak 735)

Isolation and Purification of Peak 735. Examination of Sephadex fractions III and IV by the split stream ion-exchange column procedure showed a well-resolved peak at 735 min and was designated peak 735. It was isolated by the split stream technique (Lou, 1973). A single symmetrical peak, resolved immediately after phenylalanine and considerably before β-aminoisobutyric acid, was observed by ion-exchange chromatography (Figure 2B). Two-dimensional high voltage electrophoresis–paper chromatography confirmed the homogeneity of peak 735.

Identification. Ion-exchange chromatographic studies of a 21-hr acid hydrolysate of peak 735 showed two peaks with equimolar ratio, one in the position of glutamic acid and the other in the position of ornithine (Figure 2C). Authentic glutamic acid and ornithine each overlapped completely with these two peaks. These two components also showed the same mobility with authentic glutamic acid and ornithine on high voltage electrophoresis.

To further identify the presence of ornithine in this peptide, two tests were employed. Using the Chinard reaction (Chinard, 1952), the hydrolysate of peak 735 produced a distinct red color and was calculated to contain 1.05 μmol of ornithine/μmol of peptide. In addition, the hydrolyzed peptide was treated with ornithine transcarbamylase. Citrulline was found in the reaction mixture at the end of incubation at a concentration of 0.8 μmol/μmol of peptide (Figure 2D).

N-Terminal Determination of Peak 735. The hydroly-

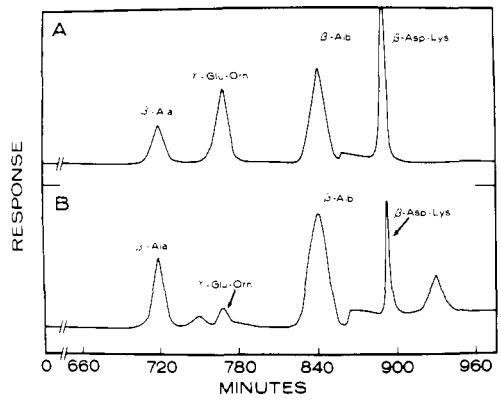

FIGURE 4: Ion-exchange chromatography with elution sequence IIB (see Materials and Methods). β-Alanine (5 nmol) and β-aminoisobutyric acid (10 nmol) were added to every run as markers. (A) Standard peptides: L-γ-glutamyl-L-ornithine (11.3 nmol); L-β-aspartyl-L-lysine (10.0 nmol). (B) Normal human urine 500-μl supernatant of sulfosalicylic acid treated (see Materials and Methods) equivalent to 416.5 μl of urine. L-γ-Glutamyl-L-ornithine, 2.1 nmol; L-β-aspartyl-L-lysine, 6.6 nmol.

sate of dansylated peak 735 was applied to a polyamide-6 sheet and developed in solvent A. One fluorescent spot moved to the same position as standard dansylglutamic acid. Another faster moving spot was scraped off the thin-layer sheet and was isolated by the same technique as described for the isolation of N^{ϵ}-dansyllysine of peak 796. For further identification, it was applied to another polyamide-6 sheet parallel to standard N^{α}-dansylornithine and N^{δ}-dansylornithine and developed with solvent B. It had the same mobility as N^{δ}-dansylornithine. This result indicated that glutamic acid was the N-terminal group of peak 735 while the α-amino group of ornithine was bound and the δ-amino group was free.

Determination of C-Terminal Group and Peptide Bond Configuration. The hydrazinolysis data showed that ornithine is the C-terminal end of peak 735. This conclusion was based on the following findings. An ornithine peak was observed when the hydrazinolysate of peak 735 was analyzed by ion-exchange chromatography. In addition, a low ninhydrin color intensity peak emerged at 580 min which coincided with standard γ-glutamylhydrazide. Standard α-glutamylhydrazide emerged at 360 min under the same conditions (Figure 3). High voltage electrophoresis of the hydrazinolysate also confirmed the presence of ornithine and γ-glutamylhydrazide, having the same mobilities as the respective authentic compounds. Furthermore, the γ-glutamyl bond test of Ramakrishna and Krishnaswamy (1967) on peak 735 was also positive. Under very mild acid hydrolysis, i.e., 0.1 *N* HCl 100° for 30 min to 2 hr, only ornithine was found to increase at a rate directly proportional to the rate of degradation of the peptide. A glutamic acid peak appeared after 2 hr, but the peak was considerably smaller than ornithine. An equimolar ratio was reached faster only when the peptide was subjected to stronger acid hydrolysis. This phenomenon suggested that peak 735 was a γ-glutamyl peptide which, under mild acid hydrolysis conditions, had undergone cyclization to form a ninhydrin-negative product, pyrrolidonecarboxylic acid (Greenstein and Winitz, 1961; Levintow et al., 1955; Wilson and Cannon, 1937).

Comparison of the Characteristics of Synthetic Dipeptide L-γ-Glutamyl-L-ornithine with Unknown Peak 735. Peak 735 and L-γ-glutamyl-L-ornithine (Bachem Fine Chemicals, Inc.) showed identical behavior on paper electrophoresis and chromatography. Only one peak appeared on ion-exchange chromatography using three different buffer sequences, I, II, and IIB. The results are summarized in Table II.

Table III: The Influence of Sex, Race, and Age on the Concentrations of L-γ-Glutamyl-L-ornithine and L-β-Aspartyl-L-lysine in Normal Human Urine.

	Factor	L-γ-Glutamyl-L-ornithine Mean Value (μmol/g of Creatinine)	L-β-Aspartyl-L-lysine Mean Value (μmol/g of Creatinine)
Age	3–10 years (17)[a]	4.2	14.8
	12–64 years (27)	1.5	8.2
Race	Caucasian (22)	2.2	12.2
	Negroid (6)	2.6	10.8
	Mongoloid (16)	3.2	8.7
Sex	Male (20)	2.7	12.1
	Female (24)	2.6	9.6

[a] Values in parenthesis indicate the number of subjects in each group.

Determination of L-β-Aspartyl-L-lysine and L-γ-Glutamyl-L-ornithine in Normal Human Urine from Fasted and Nonfasted Subjects. When the urine filtrate of each subject was analyzed using buffer sequence IIB (Figure 4), L-γ-glutamyl-L-ornithine emerged at 765 min as a single peak after β-alanine and before β-aminoisobutyrate. It was well resolved from other ninhydrin-positive peaks in urine. L-β-Aspartyl-L-lysine appeared at 890 min, well resolved after β-aminoisobutyrate.

The urinary concentrations of L-β-aspartyl-L-lysine and L-γ-glutamyl-L-ornithine in normal individuals were determined in both sexes, various races (Caucasian, Negroid, Mongoloid), and a range of age groups from 3 to 64 years. As shown in Table III, race and sex appear to have less effect on the excretion of these two peptides as compared with age. Subjects below 10 years of age excreted L-γ-glutamyl-L-ornithine at a mean concentration of 4.25 μmol/g of creatinine, whereas subjects of 12–64 years of age excreted approximately one-third that amount. However, in both age groups, the excretion level varied considerably from individual to individual. In fact, among the 27 subjects of the older age group, three excreted less than the detection capability of our system, i.e., 0.1 μmol/g of creatinine.

The excretion level of L-β-aspartyl-L-lysine of the older group showed a much smaller variation from subject to subject than that found in the younger age group. However, as was the case for L-γ-glutamyl-L-ornithine, the mean level of excretion for the younger group was higher than that for the older group. The results indicated that normal urine contained more L-β-aspartyl-L-lysine than L-γ-glutamyl-L-ornithine.

After 21 hr of fasting, the eight adult subjects each excreted both L-β-aspartyl-L-lysine and L-γ-glutamyl-L-ornithine, but the average concentration of both peptides was half that found in urine from nonfasting subjects (Table IV). This result suggests that both peptides may be of an endogenous origin.

Discussion

Kakimoto and Armstrong (1961) first reported β-aspartylhistidine as a normal constituent in human urine. Bu-

Table IV: The Concentration of L-γ-Glutamyl-L-ornithine and L-β-Aspartyl-L-lysine in Urine from Fasting and Nonfasting Adults.

Diet	L-γ-Glutamyl-L-ornithine (μmol/g of Creatinine) Range	Mean	L-β-Aspartyl-L-lysine (μmol/g of Creatinine) Range	Mean
Nonfasting[a]	<0.1–4.6	1.4	5.5–12.7	7.5
Fasting[b]	<0.1–1.1	0.6	1.7–4.0	3.2

[a] 17 subjects. [b] 8 subjects.

chanan et al. (1962) later found a series of di- and tripeptides containing neutral amino acids bound to the β-carboxyl group of aspartic acid. These peptides were suggested to be the end products of normal endogenous metabolism as well as dietary protein (Dorer et al., 1966). Pisano et al. (1966) also reported the existence of β-aspartylglycine in normal human urine and in enzymic hydrolysate of collagen. The significance of these β-aspartyl peptides in metabolic systems is still unknown. The L-β-aspartyl-L-lysine reported here may be derived through similar mechanisms. The fact that this peptide was excreted by the 21-hr fasting subjects, although in lower concentration as compared to nonfasting individuals, suggests its presence in the metabolic pool. Whether it is the end product of protein degradation or it is actually involved as a mechanism for the excretion of free amino acids after β-peptide bond formation, needs to be further clarified. Other explanations are also conceivably possible.

Only three γ-glutamyl dipeptides have been isolated from normal human urine (Buchanan et al., 1962). To our knowledge, the presently reported L-γ-glutamyl-L-ornithine is the only basic γ-glutamyl peptide which has been isolated from urine. The origin and function of this dipeptide are unknown. Although free ornithine is known to be involved in the urea cycle in mammalian system, its existence as a structural unit in proteins has been reported only recently in urate-binding α_1–α_2 globulin (Sletten et al., 1971). However, to date confirmation of this finding has not been found in the literature by this laboratory.

The amino acid transport system in kidney and brain tissues recently proposed by Meister and his associates (Orlowski and Meister, 1970; Tate et al., 1973; Meister, 1973) seems to offer some logical explanation for the existence of γ-glutamyl peptides in both brain (Kanazawa et al., 1965; Kakimoto et al., 1964; Reichelt, 1970) and urine. Since ornithine is an even better substrate than lysine for the kidney γ-glutamyl transpeptidase (A. Meister and S. S. Tate, personal communication), it is probable that L-γ-glutamyl-L-ornithine could be formed in the kidney through the transfer of the γ-glutamyl group from glutathione to ornithine. The fact that children seem to excrete more of this dipeptide than adults may indicate that children may have a relative deficiency of the enzyme in the cycle that acts on L-γ-glutamyl-L-ornithine (γ-glutamyl cyclotransferase).

The observations of significant amounts of this peptide in fasting urine provided further evidence that the L-γ-glutamyl-L-ornithine may be of endogenous origin. Further work is planned to attempt to clarify the metabolic role and the origin of this dipeptide.

Acknowledgments

The author expresses her gratitude to Dr. Paul B. Hamilton and Dr. Priscilla Ortiz for their guidance both in the research and in preparation of the manuscript. The author also thanks Mrs. Linda St. Onge and Mrs. Roberta deFiore for skilled technical assistance, Mr. Robert M. Clemick for his expert photographic art work, and Miss Joyce McGinnis for the typing of the manuscript.

References

Buchanan, D. L., Haley, E. E., and Markiw, R. T. (1962), *Biochemistry 1,* 612.
Burnett, G. H., and Cohen, P. P. (1957), *J. Biol. Chem. 229,* 337.
Chinard, F. P. (1952), *J. Biol. Chem. 199,* 91.
Cole, M., Fletcher, J. C., and Robson, A. (1965), *J. Chromatogr. 20,* 616.
DiGiorgio, J. (1974), in Clinical Chemistry, Henry, R. J., Cannon, D. C., and Winkelman, J. W., Ed., Hagerstown, Md., Harper and Row, p 543.
Dorer, F. E., Haley, E. E., and Buchanan, D. L. (1966), *Biochemistry 5,* 3236.
Fraenkel-Conrat, H., and Tsung, C. M. (1967), *Methods Enzymol. 11,* 151.
Gray, W. R. (1967), *Methods Enzymol. 11,* 139.
Greenstein, J. P., and Winitz, M. (1961), in Chemistry of the Amino Acids, New York, N.Y., Wiley, p 1934.
Hamilton, P. B. (1962), *Ann. N.Y. Acad. Sci. 102,* 55.
Hamilton, P. B. (1963), *Anal. Chem. 35,* 2055.
Hamilton, P. B. (1968), in Automation in Analytical Chemistry, Technicon Symposia, 1967, Vol. 1, Scova, N. B. et al., Ed., White Plains, N.Y., Mediad, Inc., p 317.
Hamilton, P. B., and Lou, M. F. (1972), *Biochem. Med. 6,* 193.
Kakimoto, Y., and Armstrong, M. D. (1961), *J. Biol. Chem. 236,* 3280.
Kakimoto, Y., Nakajima, T., Kanazawa, A., Takesada, M., and Sano, I. (1964), *Biochim. Biophys. Acta 93,* 333.
Kanazawa, A., Kakimoto, Y., Nakajima, T., and Sano, I. (1965), *Biochim. Biophys. Acta 111,* 90.
King, J. S., Jr. (1964), *Clin. Chim. Acta 9,* 441.
Koritz, S. B., and Cohen, P. P. (1957), *J. Biol. Chem. 209,* 145.
Levintow, L., Meister, A., Hogeboom, G. H., and Kuff, E. L. (1955), *J. Am. Chem. Soc. 77,* 5304.
Lou, M. F. (1973), *Anal. Biochem. 55,* 51.
Lou, M. F., and Hamilton, P. B. (1971), *Clin. Chem. 17,* 782.
Meister, A. (1973), *Science 180,* 33.
Moore, S. J. (1968), *J. Biol. Chem. 243,* 6281.
Orlowski, M., and Meister, A. (1970), *Proc. Natl. Acad. Sci. U.S.A. 67,* 1248.
Pisano, J. J., Prado, E., and Freedman, J. (1966), *Arch. Biochem. Biophys. 117,* 394.
Ramakrishna, M., and Krishnaswamy, P. R. (1967), *Anal. Biochem. 19,* 338.
Reichelt, K. L. (1970), *J. Neurochem. 17,* 19.
Sletten, K., Aakesson, I., and Alvsaker, J. O. (1971), *Nature (London), New Biol. 231,* 118.
Stein, W. H. (1953), *J. Biol. Chem. 201,* 45.
Tate, S. S., Ross, L. L., and Meister, A. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1447.
Wilcox, P. E. (1967), *Methods Enzymol. 11,* 63.
Wilson, H., and Cannon, R. K. (1937), *J. Biol. Chem. 119,* 309.