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Occurrence of β -Aspartyl and γ -Glutamyl Oligopeptides in Human Urine*

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By means of column and paper chromatography 16 aspartyl di- and tripeptides and 3 glutamyl dipeptides have been isolated from pooled collections of human urine. All but one of the aspartyl peptides have the β linkage and the glutamyl peptides have the γ linkage. β -L-Aspartylglycine is the most abundant of these, and by isotopic means it was shown that it does not arise by isomerization of α -aspartylglycine during the isolation. The more abundant of these peptides, β -aspartylglycine and β -aspartylserine, were also found in 24-hour urine collections from fasted patients.

It has long been known that the amino acid content of urine increases considerably on acid hydrolysis (Henriques and Sørensen, 1909; Sauberlich and Baumann, 1946; Steele *et al.*, 1947; Hier, 1948; Woodson *et al.*, 1948; Stein, 1953). Hippuric acid and phenylacetylglutamine (Stein *et al.*, 1954) account for sizable fractions of the increase in glycine (Stein, 1953) and glutamic acid, but more of other amino acids, especially aspartic acid (Dent, 1947; Steele *et al.*, 1947; Woodson *et al.*, 1948; Hier, 1948; Uzman and Hood, 1952; Stein, 1953), are liberated on hydrolysis than exist free. That urinary peptides are involved has been demonstrated by several studies (Dent, 1947, 1948; Boulanger *et al.*, 1952; Carsten, 1952; Uzman and Hood, 1952; Bode *et al.*, 1953; Stein, 1953; Westall, 1955; Hanson and Fittkau, 1958; Ansorge *et al.*, 1961; Sarnicka-Keller, 1961) in which substances have been isolated that give free amino acids on total hydrolysis. Nearly all workers have found aspartic and glutamic acids to be among the more abundant of these.

Although many of the isolated peptides have been partially characterized, few have been completely identified. The present report describes the isolation and identification of several β -aspartyl oligopeptides and of three γ -glutamyl dipeptides. Preliminary experiments reported in abstract form (Haley *et al.*, 1961; Buchanan *et al.*, 1961) indicated that the urinary dipeptides,

β -aspartylglycine and β -aspartylserine, might have been artifacts, produced by isomerization of the corresponding α peptides (*cf.* John and Young, 1954; Swallow and Abraham, 1958; Bryant *et al.*, 1959). Data presented here demonstrate that L-aspartylglycine is excreted predominantly in the β form. With this finding, and the recent identification of β -L-aspartyl-L-histidine as a normal urinary constituent (Kakimoto and Armstrong, 1961), it is likely that the fourteen other β -aspartyl di- and tripeptides described here are actually excreted with the β structure and are not artifacts.

MATERIALS AND METHODS

Urine Collections.—Urine collections (24 hour) were from male hospital patients or laboratory employees, and all voidings were refrigerated immediately and desalted the day that the collection ended. Patients were selected who had no known metabolic or debilitating disease, but two of these had been subjected to gastrointestinal surgery and had received neither oral nor parenteral nitrogen-containing nutriment for 3 and 4 days prior to the start of the collection. In some instances α -L-aspartylglycine-1-C¹⁴ (10 mg, 50,000 dpm) was added to the urine before desalting or to the urine vessel prior to the collection.

Paper Chromatography.—The solvents used were: (I), *n*-butanol-acetic acid-water (4:1:1); (II), methylethylketone-propionic acid-water (15:5:6); (III), *n*-butanol-acetic acid-pyridine-water (15:3:10:12); (IV), methanol-water-pyridine (20:5:1); (V), methylethylketone-*t*-butanol-

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ammonium hydroxide-water (10:10:3:5); and (VI), *t*-butanol-methylethylketone-water (2:2:1). The filter papers used for spot tests and chromatography were: (A), Whatman No. 3; (B), Whatman No. 1; (C), Schleicher and Schull 470 A; (D), Whatman No. 17. All chromatograms were descending, and in preparative chromatography on heavy paper where solvent was allowed to drip from the lower edge this edge was serrated to prevent lateral flow. Material was spotted or streaked 7.5 cm from the edge.

Resins.—The resins used for desalting were Dowex 50 (X8), 200 to 400 mesh, graded by repeated sedimentation to retain the fraction settling 50 cm in 10 minutes and regenerated from the sodium form with 6 N HCl until a flame test of the effluent was negative for sodium; and Dowex 2 (X8), 100 mesh, graded by sedimentation to discard "fines" and regenerated from the chloride form with N NaOH until the effluent was negative for chloride (silver nitrate-nitric acid). For chromatography the resins were Dowex 50 W (X4), 200 to 400 mesh, graded and regenerated as was Dowex 50 above; and Dowex 2 (X8) 200 to 400 mesh, graded and regenerated as was Dowex 2 above. The Dowex 2-succinimide (Buchanan, 1957a) was prepared just prior to use.

Reagents and Reference Compounds.—Ninhydrin (Dougherty Chemicals, Richmond Hill, N.Y.) was recrystallized (Moore and Stein, 1948). Carbo-benzoxy-L-aspartic acid was either purchased (Nutritional Biochemicals Corporation, Cleveland, Ohio) or synthesized (Bergmann and Zervas, 1932). Glycine-1-C¹⁴ (Nuclear Chicago, Chicago, Ill.) was purified chromatographically (Buchanan, 1957a) before use.

Several samples of synthetic aspartyl and glutamyl dipeptides were received as gifts. Dr. G. T. Young (Oxford University) provided the α - and β -L-aspartyl-L-leucines (Bryant *et al.*, 1959) and the α - and β -L-aspartyl-L-valines (John and Young, 1954). Dr. Y. Liwischitz (The Hebrew University of Jerusalem) sent samples of β -aspartyl valine, -phenylalanine, - α -aminobutyric acid, and - β -alanine that had been prepared from racemic amino acids (Liwischitz and Zilkha, 1955a,b). The α - and γ -L-glutamyl-L-leucines were from Dr. Manfred Loefflender (1960) (Max-Planck-Gesellschaft, Göttingen, Germany).

Detection and Isolation Procedures.—Single 24-hour collections or larger samples of pooled urine were desalted by displacement (Carsten, 1952; Buchanan, 1957b), and the amphoteric fraction was subjected to carrier displacement chromatography (Buchanan, 1957a). Paper chromatography was carried out on all fractions (I,A). In a typical experiment 1160 ml of urine was first put through a 500-ml column of Dowex 50 (hydrogen), 30 cm in length, and this was washed with water until the effluent gave no color with a ninhydrin spot test on filter paper (B). The column was then eluted with 1500 ml of 1.5 M ammonium hydroxide, after which the effluent again became

ninhydrin negative. The eluted material was taken to dryness with a rotary evaporator and the residue was taken up in 60 ml of water. Even though some material remained undissolved the mixture was added to a 100-ml, 20-cm column of Dowex 2 (OH form). Water (670 ml) was added until the effluent became ninhydrin negative (270 ml) and until the precipitate dissolved (400 ml).

The material remaining on the column was then eluted with M acetic acid until the ninhydrin test became weak. This effluent (300 ml) was reduced to a small volume and subjected to chromatography with carriers (Buchanan, 1957a) on a 120-ml, 120-cm column of Dowex 50 W. Paper chromatograms of fractions showed, in addition to the common amino acids, many unidentified ninhydrin-reactive substances.

Because the more abundant of these ninhydrin-reactive substances, as judged by ninhydrin color intensity on paper, accompanied the acidic and hydroxy amino acids and proline (Group I, *cf.* Buchanan, 1957a), this group was rechromatographed on a 100-ml, 100-cm column of Dowex 2 in the succinimide form and displaced with 0.2 M acetic acid. Paper chromatograms of the fractions again revealed a number of unidentified substances, mostly in the fractions that contained or followed the acidic amino acids.

Rapid Isolation Procedure for Acidic Oligopeptides.—Because of their relative abundance, interest was centered mainly on the unidentified compounds with acidic properties, and the following more rapid method was developed for separating this fraction from urine. After filtration the urine is put through a large Dowex 50 column and this is then washed and eluted as above. The ninhydrin-positive eluate is evaporated to dryness, redissolved in water, and put on a 100-ml, 100-cm column of Dowex 2 in the acetate form. After washing with water until the effluent is ninhydrin negative the column is developed with 0.2 M acetic acid. In a typical separation glutamic acid was present in the eluate collected between 630 and 700 ml; aspartic acid between 740 and 1350 ml. Mixed with glutamic and aspartic acids and in the fractions following each were found the compounds with which this report is mainly concerned.

Purification and Identification Procedures.—Fractions containing unidentified material were pooled and reduced in volume, and the entire residue was chromatographed on heavy paper (I,C or D). Mobilities were always greater with preparative papers than with those used for assay (A,B), and substances with low R_f values could be made to move to near the end of a paper sheet with predetermined quantities of solvent. After the position of each zone had been determined by ninhydrin spraying of test strips cut from the lateral edges and the center of the paper, the zone containing a given substance was eluted with water. The eluate was put through a 1-ml column of Dowex 50 (H form), which was then displaced

with *m* pyridine. If sufficient material was present after evaporation of the displaced effluent to dryness, it was dissolved in a small amount of water and precipitated with alcohol. If too little was present the step was omitted.

The material was then dissolved in 0.1 to 0.5 ml of water, and suitable aliquots were subjected to acid hydrolysis, end-group determination, hydrazinolysis, and ninhydrin reactions. Hydrolysis was usually carried out with 0.1 ml of the solution in 1 or 2 ml of redistilled 6 *N* HCl in a sealed tube at 105° for 20 hours. Excess acid was removed by evaporation and identification of the products carried out by two-dimensional chromatography (B, IV, V).

Determination of the molar ratio of the amino acids in each peptide was performed either by separating the amino acids of a hydrolysate on a small column of Dowex 2 acetate and performing quantitative ninhydrin assays (Moore and Stein, 1948) or by separating the amino acids on paper, saturating the paper with ninhydrin solution, developing the color by heating at 100°, and then eluting the color into colorimeter tubes with 50% ethanol for comparison with standard amino acids chromatographed and treated in the same way. Tests on known amino acid mixtures showed this procedure (Greenstein and Winitz, 1961, p. 1428) to be precise enough to establish unequivocally the molar ratios in di- and tripeptides.

The 1-fluoro-2,4-dinitrobenzene (FDNB) method of Sanger and Thompson (1953) was used to identify the NH₂-terminal amino acid of each peptide isolated. For adequate detection on chromatograms the aliquots taken of the DNP-amino acid fraction were 5- to 10-fold greater than of the free amino acid fraction.

For hydrazinolysis 0.1 ml of the peptide solution was evaporated to dryness in a test tube over phosphorus pentoxide and the procedure of Pechère and Neurath (1957) was then carried out. In some of the procedures the benzaldehyde extraction was omitted and separation of free amino acids from the hydrazides was satisfactorily achieved by passing the reaction mixture through a small column of Dowex 50 (hydrogen) and then displacing the free amino acids with 0.2 *M* pyridine and the hydrazides with 1 *M* ammonia.

Ninhydrin Reactions.—It has been reported repeatedly that several β -aspartyl peptides give a blue color reaction on paper, whereas the α counterparts produce a purple color (LeQuesne and Young, 1952; John and Young, 1954; Liwschitz and Zilkha, 1955a,b; Bryant *et al.*, 1959). Although Kakimoto and Armstrong (1961) report that β -L-aspartyl-L-histidine gives a yellow color, this seems to be an exception, and each of six synthetic β -aspartyl peptides available to us gave a blue color. John and Young (1954) observed that with ninhydrin in aqueous solution β -aspartyl peptides and asparagine give a brown color.

Saidel (1957) found that ammonia was liberated from γ -glutamyl but not from α -glutamyl peptides when they were heated with aqueous ninhydrin at pH 2.5. In a number of trials with varying conditions we found that, when α - and β -aspartyl peptides were heated with aqueous ninhydrin, ammonia as well as CO₂ (John and Young, 1954) were liberated from both. Heating at 100° in citrate buffer at pH 2.5 for 10 minutes without ninhydrin caused no detectable α , β inter-conversion but produced 10% hydrolysis of α -aspartylglycine and 3% hydrolysis of β -aspartylglycine. The peptides also yielded 8% and 13%, respectively, of a compound believed to be the cyclic derivative, aminosuccinimido acetic acid (Swallow and Abraham, 1958).

Allowing the ninhydrin reaction to occur at 37°, which, in control experiments with buffer alone, produced no detectable change on paper chromatography, resulted in a clear distinction between α - and β -aspartyl peptides. At the lower temperature ammonia liberation and the characteristic brown color were not noted with known α -aspartyl peptides. For the determination of ammonia liberation 25 mg of ninhydrin and 0.5 ml of 5% citrate buffer were added to about 0.5 μ mole of peptide. After 16 hours at 37° the excess ninhydrin was destroyed with 1 ml of 4% hydrogen peroxide in the same buffer (Saidel, 1957) and the ammonia determined on a 1-ml aliquot of the supernatant solution by microdiffusion in Conway dishes and Nesslerization (0.2 cc) of the trapping acid (1 ml of 0.1 *N* sulfuric acid). With known β -aspartyl and γ -glutamyl peptides recovery of ammonia was nearly mole for mole when approximately 5 μ moles of peptide were used. With one tenth that quantity, recoveries were less complete (Table I) but adequate to distinguish clearly the α peptides from the β -aspartyl and γ -glutamyl compounds.

For the color reaction approximately 0.2 μ mole of each peptide in solution was taken to dryness in a micro test tube (0.7 ml) and 20 μ l of 1% ninhydrin in 2.5% citrate buffer was added. At 37° maximum color intensity is reached with asparagine and β -aspartyl peptides in 8 to 10 hours. Pure α -aspartyl peptide gives no color. The solutions were diluted to 0.7 ml and the optical density measured in micro silica cells in a Beckman DU spectrophotometer at 350 m μ . The absorbance of a ninhydrin blank was subtracted. For testing small quantities of material by this method it is necessary to develop the color in small volume because the color formation is much less efficient with dilute ninhydrin, and when the ninhydrin is in great excess in a larger volume the blank reading is excessive.

Synthesis of Aspartylglycines.—These dipeptides were prepared several times by methods modified from LeQuesne and Young (1952). Higher yields were obtained with the *p*-toluenesulfonate salt of the benzyl ester of glycine (Cipera and Nicholls, 1955) than with the ethyl ester or

TABLE I
NINHYDRIN REACTIONS OF SYNTHETIC AND URINARY ASPARTYL AND GLUTAMYL OLIGOPEPTIDES

Compound	Color Reaction on Paper	Color Reaction in Solution ^a		Ammonia Liberation ^b (moles/mole of peptide)
		Visual	Absorbance at 350 mμ	
Synthetic				
α-Aspartylglycine	purple	none	0.000	0.03
α-Aspartylleucine	purple	none	0.016	0.03
α-Aspartylvaline	purple	none	0.032	0.03
β-Aspartylglycine	blue	brown	0.200	0.56
β-Aspartylleucine	blue	brown	0.158	0.52
β-Aspartylvaline	blue	brown	0.266	0.86
β-Aspartyl-α-aminobutyric acid	blue	brown	0.216	0.72
β-Aspartyl-β-alanine	blue	brown	0.126	0.60
β-Aspartylphenylalanine	blue	brown	0.108	0.60
Asparagine	blue-grey or brown	brown	0.193	0.76
α-Glutamylleucine	purple	none	---	0.01
γ-Glutamylleucine	purple	none	---	0.56
Glutathione	purple	faint purple	---	0.50
Urinary				
α-Aspartylglycine	purple	none	0.076	0.21
β-Aspartylglycine	blue	brown	0.288	0.78
β-Aspartylserine	blue	brown	0.170	0.59
β-Aspartylthreonine	blue	brown	0.158	0.96
β-Aspartylalanine	blue	brown	0.166	0.66
β-Aspartylleucine	blue	---	---	0.84
β-Aspartylasparagine	blue	brown	0.210	0.62
β-Aspartylglutamine	blue	---	---	1.29
β-Aspartylvaline?	blue	---	---	---
β-Aspartylglycylvaline	blue	brown	0.255	1.02
β-Aspartylglycylalanine	blue	---	---	---
β-Aspartylglycylproline	blue	---	---	---
β-Aspartylglycylasparagine	blue	---	---	---
β-Aspartylglycylglycine	blue	---	---	0.91
β-Aspartylglycylglutamine	blue	---	---	---
γ-Glutamylleucine	blue	none	---	0.65
γ-Glutamylvaline	blue	none	---	0.44

^a Approximately 0.2 μ mole of peptide and 0.2 mg ninhydrin in 2.5% citrate buffer, pH 2.5. Read at 8-12 hours. ^b 0.5 μ mole peptide, 25 mg ninhydrin in 0.5 ml 5% citrate buffer, pH 2.5. Reaction time 16 hours. Ammonia determined by Conway procedure (see text).

the free benzyl ester. In a typical procedure, glycine-1-C¹⁴ benzyl ester *p*-toluenesulfonate of low radioactivity (1 mmole) was placed in a test tube in a small flask of crushed ice and 4 ml ethyl acetate-pyridine (1:1) was added. Carbobenzoxy aspartic anhydride (1 mmole), prepared according to Miller *et al.* (1941), was dissolved in 2 ml of ethyl acetate. This was added dropwise with shaking to the glycine ester salt. After the tube, protected from moisture, had stood overnight in the flask, to which no more ice had been added, the clear solution was taken to dryness *in vacuo*. The residue was dissolved in 5 ml of ethyl acetate and extracted three times with 5 ml of hydrochloric acid and then three times with 5 ml of 2% sodium carbonate. The sodium carbonate extract was acidified with N HCl and the oily precipitate extracted into 5 ml of ethyl acetate, which was dried with anhydrous sodium sulfate and then evaporated. The residue was dissolved in 5 ml of 75% ethanol, and 0.3 ml of

glacial acetic acid and 100 mg of 10% palladium on charcoal catalyst (Matheson Coleman Bell) were added. Hydrogen was bubbled through the solution until CO₂ evolution ceased. The solution was filtered, evaporated to dryness, taken up in a few ml of water, and put on a 100-ml, 100-cm column of Dowex 2 in the acetate form, and this was eluted with 0.2 M acetic acid. Radioactivity appeared in the fractions coming off between 530 and 850 ml and again between 1120 and 1620 ml. The first compound was free of contaminating ninhydrin-reactive material by paper chromatography with several solvents and on hydrolysis yielded theoretical quantities of aspartic acid and glycine.

Some of the fractions of the second compound were contaminated with aspartic acid, which was removed by chromatography on paper (I,C). Yields of the first and second compounds, respectively, were 47% and 21% based on the ester salt or 36% and 17% based on the original glycine.

The ninhydrin color of the second compound on paper was distinctly blue. This observation (see above) and the compound's higher apparent acidity as judged by its behavior on ion-exchange chromatography (*cf.* Davies, 1949; Buchanan, 1959) suggested that it was β -aspartylglycine. β -Aspartylglycine was prepared also from the α -benzyl ester of β -carbobenzoxy-L-aspartic acid chloride by a method not previously reported. Carbobenzoxy-L-aspartic acid α -benzyl ester, 0.3 g, m.p. 85°, was prepared and converted to the corresponding β acid chloride, both by the procedure of Bergmann *et al.* (1933). The crude acid chloride was then coupled with glycine benzyl ester to yield 100 mg of α -benzyl ester of β -carbobenzoxy-L-aspartylglycine benzyl ester, m.p. 126–7° (white needles). After catalytic hydrogenation the compound isolated was chromatographically identical to the compound first suspected of being the β peptide.

The melting points and optical rotations of the synthetic peptides were in agreement with those reported by LeQuessne and Young (1952). For the β peptide we found m.p. 154–7° and $[\alpha]_D^{25} + 14.2$ (c, 2.59 with one equivalent of HCl). For α -L-aspartylglycine we found m.p. 184–5° (decomposition) and $[\alpha]_D^{25} + 32.5$ (c, 2.16 with one equivalent of HCl).

RESULTS

Of numerous unidentified ninhydrin-reactive zones seen on paper chromatograms of fractions from resin columns, at least 26, when eluted and hydrolyzed in acid, released from two to five additional ninhydrin-reactive substances with chromatographic mobilities of known amino acids. In several cases the zones seemed to contain mixtures, because after hydrolysis the ninhydrin color intensities of the constituent amino acids were in ratios inconsistent with the small whole numbers expected with oligopeptides. Many of the presumed peptides were present in quantities too small for easy purification and identification. Consequently attention was centered on the more abundant substances that emerged from the Dowex 2 (acetate) column in the vicinity of aspartic acid.

Figure 1 is a composite diagram of paper chromatograms of these fractions from several separative procedures. With chromatograms from single 24-hour urine collections many of the zones shown were not seen. Some of the compounds when chromatographed preparatively, eluted, and hydrolyzed yielded the same spot when rechromatographed and were not studied further. One compound, β -L-aspartylglycine, was completely identified, and except for determinations of stereochemical configurations the materials in the zones with underlined labels were positively identified. The remainder were partially or presumptively identified with varying degrees of confidence.

β -L-Aspartylglycine. When chromatographed

as described, individual 24-hour urine collections from eighteen subjects and a pooled 24-hour collection from five subjects all showed ninhydrin reactivity in the area indicated as β -aspartylglycine on Figure 1. The fractions containing this substance were pooled, concentrated, and rechromatographed by streaking on heavy paper (I,C), and when 550 ml of solvent had run through the paper the substance was found in a 6-cm zone, the leading boundary of which was 5 cm from the lower edge of the paper.

When eluted from the paper and purified as described, 6.4, 8.8, 21.0 and 25.7 mg of material were recovered from 24 hour urines of four subjects where the material was weighed. A repeat isolation on the individual with the highest quantity yielded 32 mg. Hydrolysis of the material yielded aspartic acid and glycine in equimolar quantities, and end-group analysis showed only DNP aspartic acid. The material migrated with synthetic β -aspartylglycine on the resin columns and on paper in several solvents (I-V). At pH 2.5 it gave the characteristic brown color with ninhydrin in solution (Table I). Although determined with too small a quantity for accuracy, its specific rotation was positive and approximately that of the synthetic compound.

The urine specimens from two postoperative patients who had fasted 3 and 4 days contained β -aspartylglycine in about the same abundance, as judged by ninhydrin color intensity on the paper chromatogram, as specimens from subjects who had not fasted.

α -Aspartylglycine.—With synthetic α -L-aspartylglycine-1-C¹⁴ as a marker, the position of α -aspartylglycine on chromatograms (Fig. 1) was determined. The compound was obtained in small amount from the pooled urine collection but some of it had apparently converted to the β form during the isolation (Table I). On hydrolysis the isolated materials yielded equimolar quantities of aspartic acid and glycine, and the NH₂-terminal amino acid was aspartic acid. Urinary aspartylglycine migrated with the synthetic material on paper chromatograms with all solvents (I-V). It gave a purple ninhydrin color on paper and failed to give color in solution with ninhydrin at pH 2.5 (Table I).

Other β -Aspartyl Dipeptides.—On acid hydrolysis each of these yielded equimolar quantities of aspartic acid and the other constituent amino acid, and in each case the NH₂-terminal amino acid was aspartic acid. All gave a blue ninhydrin color on paper and a brown color with ninhydrin in solution at pH 2.5 and 37°. Each liberated a substantial quantity of ammonia when treated with ninhydrin for 16 hours at pH 2.5 and 37° (Table I).

In separation on heavy paper (I,C), β -aspartylserine migrated just behind β -L-aspartylglycine and was found in all urines. As judged by ninhydrin color intensity and the quantity of material precipitated with alcohol, this compound

On hydrolysis of β -aspartylasparagine aspartic

acid was the only amino acid found. Treated with 2,4-dinitrofluorobenzene a hydrolysate of the peptide yielded twice as much DNP-aspartic acid as did the unhydrolyzed peptide. After hydrazinolysis, no free amino acid was found and a single hydrazide resulted which had the same mobility (I,A) as that obtained with asparagine and which was different from that obtained with synthetic α -aspartylglycine. After hydrolysis one mole of ammonia was found by Conway diffusion and Nesslerization.

Hydrolysis of the material from the area designated β -aspartylglutamine (Fig. 1) gave approximately equimolar amounts of aspartic and glutamic acids with faint spots of glycine and serine, probably from a contaminating peptide. The fact that no free amino acid was seen after hydrazinolysis suggests that the second amino acid was glutamine. Insufficient material was present to identify the hydrazides.

Evidence for the existence of β -aspartylvaline was only suggestive. Faint blue spots appeared in the indicated position (Fig. 1) on the paper chromatogram from the pooled urine. On hydrolysis aspartic acid and valine were identified but other spots also appeared. There was insufficient material for further purification or study. Synthetic β -aspartylvaline had essentially the same R_F (I,A) as the unknown compound, and the relative positions on Dowex 2 (acetate) and paper (I,A) of the unknown and β -aspartylleucine, of γ -glutamylvaline and γ -glutamylleucine, and of valine and leucine were all similar.

α -Aspartyl Tripeptides.—In each of these tripeptides acid hydrolysis yielded the constituent amino acids in the quantities indicated and in each case the NH_2 -terminal amino acid was aspartic acid. Ninhydrin reactions characteristic of β -aspartyl peptides were obtained in each instance (Table I).

β -Aspartylglycylvaline was found in small amount only in the pooled urine sample and was not completely purified. After hydrolysis traces of leucine and alanine were present in addition to the constituent amino acids. Hydrazinolysis yielded only free valine. The quantity available was too small to permit identification of the hydrazides, which give less intense ninhydrin colors than free amino acids.

β -Aspartylglycylalanine and β -aspartylglycylproline were present as a mixture. Hydrolysis gave approximately equimolar quantities of aspartic acid glycine, with lesser quantities of alanine and proline. All aspartic acid was NH_2 -terminal. After hydrazinolysis of the mixture only free alanine and proline were detected. With electrophoresis on paper in 0.04 M citrate buffer, pH 3.5, two zones separated. Material from one of these zones when hydrolyzed yielded aspartic acid, glycine, and alanine in equimolar amounts. The other zone, which had a higher mobility toward the anode, gave equivalent quantities of aspartic acid and glycine and a proline spot that,

because of the amount available, was not quantitated.

β -Aspartylglycylasparagine was believed to be present because DNP-aspartic acid was found with spots of free aspartic acid and glycine approximately equal visually. The fact that no free amino acid remained after hydrazinolysis indicated that glycine was not COOH-terminal. Similar results indicated the existence of β -aspartylglycylglutamine.

On hydrolysis the material believed to be β -aspartylglycylglycine yielded approximately twice as much glycine as aspartic acid, but traces of serine were also present. Traces of free serine were also found mixed with glycine during the end-group analysis. The compound was possibly contaminated with a smaller quantity of β -aspartyl (serine, glycine).

γ -Glutamyl Dipeptides.—On hydrolysis material from the zone designated γ -glutamylleucine and γ -glutamylisoleucine (Fig. 1) yielded equimolar quantities of glutamic acid and "leucine." The NH_2 -terminal amino acid was glutamic acid. Chromatography of the hydrolysate in solvent VI showed both leucine and isoleucine to be present, the latter being more abundant. Because these peptides followed glutamic acid on the Dowex 2 column, it was suspected from analogy with aspartic acid and the aspartyl dipeptides that they might have the γ structure. Chromatographic comparison (I,A) with synthetic α - and γ -glutamylleucine showed the urinary compounds to be γ peptides. This was confirmed by ammonia liberation on treatment with ninhydrin at 37° (Table I).

On hydrolysis γ -glutamylvaline yielded equimolar quantities of glutamic acid and valine. The DNP-amino acid was glutamic acid, and the liberation of ammonia with ninhydrin at 37° as well as the chromatographic relationship to γ -glutamylleucine indicated that the peptide had the γ -glutamyl linkage.

Natural Occurrence of β -L-Aspartylglycine.—It was first suspected that the β -aspartylglycine was an artifact (Haley *et al.*, 1961; Buchanan *et al.*, 1961) and that the α form had converted to the β structure on the acidic Dowex 50 column during the desalting procedure. This type of isomerization has been reported during heating in aqueous solution with α -L-aspartyl-L-tyrosine, -L-glutamic acid, and -L-valine (*cf.* John and Young, 1954); and with the α -(α -L-aspartyl)-L-lysines (Swallow and Abraham, 1958), α -L-aspartyl-L-leucine, -L-glutamic acid, and -L-aspartic acid (Bryant *et al.*, 1959) under strongly acid conditions. The suspicion was strengthened when much smaller quantities of blue ninhydrin-reactive material appeared on paper chromatograms when the initial desalting on the Dowex 50 (H form) column was omitted from the isolation procedure. However it later became evident that the low recovery of β -aspartylglycine when the desalting step was omitted had resulted

from its elution and loss from the Dowex 2 column and that it was actually present in considerable amount.

When labeled α -L-aspartylglycine (10 mg, 50,000 dpm) was added to the urine samples prior to the isolation procedure or to the vessels used for urine collection, the specific radioactivity of the isolated β peptide was only 5.4, 0.7, 0.7, and 0.3%, respectively, of that of the isolated α peptide. The last three determinations are considered more reliable because the chromatography was performed more promptly. Experiments to be published demonstrate a slow conversion of α -aspartylglycine to the β form even under mild conditions.

In the last two experiments the recovered α peptide when purified had a specific radioactivity essentially equal to the added material, and it became evident that its concentration was too low to measure accurately by isotope dilution with the addition of 10 mg of labeled peptide. Addition of much less of the material at hand would not have allowed the isolation of enough material for the accurate determination of specific radioactivity.

DISCUSSION

The presence in urine of a considerable number of aspartyl oligopeptides explains, at least in part, the appearance of this amino acid on acid hydrolysis. Woodson *et al.* (1948), in a study of eighteen normal subjects, found that on total hydrolysis a mean of 163 mg of aspartic acid was liberated from each 24-hour urine sample. Stein (1953) found an average of 212 mg in three subjects, 31% coming from asparagine. From one of our subjects the equivalent of about 10% of this amount was isolated in pure form as β -aspartylglycine. When it is considered that the procedures employed here were not quantitatively efficient this percentage must be regarded as a minimal value. β -L-Aspartylglycine, though the most abundant of the peptides of this type, is but one of many. The reported quantities of β -aspartylhistidine found by Kakimoto and Armstrong (1961) would account for 2-3% of the bound aspartic acid in urine, and it is possible that β -aspartyl oligopeptides may be found to account for most of the bound form of this amino acid that does not arise from asparagine.

The aspartyl peptides reported here and the one reported by Kakimoto and Armstrong (1961) are certainly not the only ones present in urine. During the procedures of isolation and purification there were indications in several instances that smaller quantities of similar compounds were contaminating the isolated material. From the behavior of these contaminating substances on resin columns they would, of necessity, be acidic, and by analogy with the compounds identified in this group they should be mostly of the β -aspartyl structure.

Aspartyl and glutamyl dipeptides of the basic,

acidic, aromatic, and sulfur-containing amino acids were not found. Those with basic amino acids would not be retained on Dowex 2 (acetate), while those with aromatic and acidic amino acids would be expected to be eluted much later (Buchanan, 1959). The number of aspartyl dipeptides and tripeptides that were found suggests that these compounds do not play a specific metabolic role. Their presence in the urine of fasting patients indicates that they are at least partially of metabolic and not entirely of dietary origin.

Although the γ -glutamyl linkage is present in glutathione and in several other naturally occurring peptides (Greenstein and Winitz, 1961, p. 772 ff.), the β -aspartyl structure has been reported to occur naturally only in Bacitracin A (Swallow and Abraham, 1959), in which both ends of one aspartic acid residue appear to be in peptide linkage. The results presented here show that at the time of voiding β -aspartylglycine is far more abundant in urine than α -aspartylglycine, and it is possible that the small amount of the α form found arose from the β form during the isolation. It seems likely that the other aspartyl peptides are actually excreted with the β linkage.

A possible explanation for the presence of β -aspartyl peptides in urine is the spontaneous or even catalyzed isomerization of α linkages during some phase of *in vivo* metabolism. The β peptides formed may then be less susceptible to the action of tissue peptidases and more susceptible to renal excretion. This hypothesis is supported by the failure to find appreciable quantities of α -aspartyl peptides or of oligopeptides of aspartic acid other than those with this amino acid in the NH_2 -terminal position. It is also supported by *in vivo* and *in vitro* studies in progress. Whether a similar mechanism can account for the presence in urine of γ -glutamyl peptides is conjectural.

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Studies on the *in vivo* Metabolism of α - and β -Aspartylglycine-1-C¹⁴*

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Human subjects were given 4-mg intravenous doses of α -L-aspartylglycine-1-C¹⁴, β -L-aspartylglycine-1-C¹⁴, or an equivalent molar amount of free glycine-1-C¹⁴ and unlabeled aspartic acid. Much more radioactivity appeared in the expired carbon dioxide, in the urinary hippuric acid, and in the glycine and serine of plasma protein when the α rather than the β peptide was given. The results with free glycine and the α peptide were similar. When the β peptide was administered most of the radioactivity promptly appeared in the urine in the same chemical form but mixed with a larger quantity of endogenous β -aspartylglycine.

The accompanying report from this laboratory (Buchanan *et al.*, 1962) describes the isolation and identification of a number of β -aspartyl and γ -glutamyl oligopeptides from human urine. The most abundant of these, β -aspartylglycine, was shown not to arise by isomerization of α -aspartylglycine (John and Young, 1954; Swallow and Abraham, 1958; Byrant *et al.*, 1959) during the isolation procedure, and it is likely that the other β -aspartyl oligopeptides found are actually excreted in the β form. The present paper reports

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studies on the metabolic fates of synthetic α - and β -aspartylglycines given intravenously to human subjects. The results support a hypothesis (Buchanan *et al.*, 1962) that may explain the presence of β -aspartyl peptides in urine.

METHODS

The α - and β -L-aspartylglycines were prepared from carbobenzoxy-L-aspartic acid and glycine-1-C¹⁴ by a slight modification of the procedure of LeQuesne and Young (1952), separated chromatographically, and recrystallized from alcohol (Buchanan *et al.*, 1962). The specific radioactivity of crystalline peptides was 2.5 μ c per mg.