Quantitative Studies of Urinary β-Aspartyl Oligopeptides*

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ABSTRACT: The daily excretions of four individual β-aspartyl peptides, four other β-aspartyl peptides as a group, and α-aspartylglycine were measured by analytical chromatography. The method involved fractionation of a 5% aliquot of 24-hr urine on cation- and anion-exchange resins followed by analysis using an automatic amino acid analyzer. Individuals on unrestricted diets, on a complete diet, identical for each of 6 consecutive days, on a very low protein diet, on intravenous glucose, and a fasting person were studied.

In the absence of dietary protein the daily excretion of each peptide assumes a minimal and narrow range of values among individuals and a narrower range for a single subject. When diets are not restricted, daily excretions of β-aspartyl peptides are greater, vary widely, and do not approximate a constant ratio to each other. α-Aspartylglycine excretion is unrelated to protein intake. The results suggest a reasonably constant release of β-aspartyl peptides during endogenous metabolism with additional contributions from dietary protein.

With varying degrees of confidence, 15 β-aspartyl di- and tripeptides were identified in normal human urine in this laboratory (Buchanan *et al.*, 1962a). The paper chromatographic methods used for the identifications were not quantitative, and except in the case of Asp(Gly)[1] and Asp(Ser), the peptides were found only in pooled urine samples. Because Asp(Gly) and Asp(Ser) were present in the urines of two patients receiving no nutriment but intravenous glucose, it was believed that at least a fraction of these compounds arose endogenously.

Asp(His) has been identified in human urine (Kakimoto and Armstrong, 1961). Pisano *et al.* (1963) reported Asp(His) in the urine of an individual receiving nothing but carbohydrate. High collagen diets tended to elevate the urinary level of this peptide and possibly the levels of Asp(Gly) and Asp(Ser) as well. Using paper electrophoresis and paper chromatography, Wright and Nicholson (1965) reported the presence of Asp(Gly), Asp(Val), and Asp-Gly in dog urine.

An automatic ion-exchange method has permitted a quantitative study of the excretion pattern of the more abundant peptides using aliquots of 24-hr urine collections. Evidence for the identity of many of the peptides has been strengthened by comparison with the synthetic compounds (Buchanan *et al.*, 1966). The results indicate that at least for the more abundant of the urinary β-aspartyl peptides there is a substantial level of endogenous excretion, quite constant for a given individual, and reasonably so among different individuals. The endogenous level of Asp(Gly) excretion was found to average approximately 60 μmoles/day, but amounts up to three times this were measured when protein-containing foods were included in the diet.

Experimental Section

Materials. Synthetic α- and β-L-aspartyl peptides were prepared in this laboratory (Buchanan *et al.*, 1966). The components of the amino acid analyzer were purchased from Technicon Chromatography Corp.

Collection and Fractionation of Urine. Urine samples (24 hr), collected from laboratory personnel and hospital patients, were kept in a refrigerator and were desalted within 8 hr after the end of the collection period. Collection periods began in the morning after voiding and before breakfast. Repeat analyses were sometimes performed on aliquots of urine stored in a freezer for weeks or months. Freezing for 6 months or storage at room temperature for 1 week did not affect the recovery of the peptides.

Routinely, 5% of a 24-hr urine sample was desalted on a 2.2 × 13 cm column of Dowex 50W-X4, 100–200 mesh (hydrogen). After the sample was applied, the column was washed with water only until the pH of the effluent solution became neutral. We have noted that excess water will elute the more acidic β-aspartyl peptides. The ampholytes were displaced with 500 ml of 1 M aqueous pyridine, taken to dryness on a rotary evaporator, dissolved in 20 ml of water, and put on a 0.8 × 30 cm column of Dowex 2-X8, 100–200 mesh (acetate). The column was washed with 75 ml of water and then eluted by gravity with an acetic acid gradient (350 ml of 0.1 M in the mixing chamber and

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[1] To conserve space, the names of peptides will be abbreviated as follows: Asp-Ser, α-L-aspartyl-L-serine; Asp(Ser), β-L-aspartyl-L-serine; Asp(Gly-Ala), β-L-aspartylglycyl-L-alanine. The naturally occurring peptides are assumed to be of the L configuration.

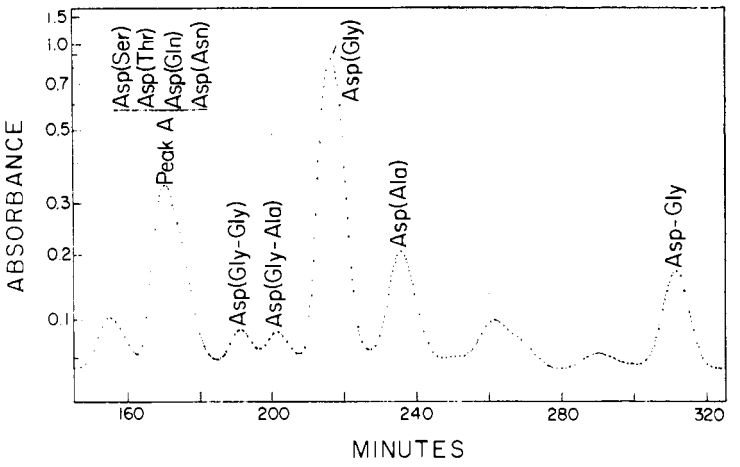

FIGURE 1: The elution pattern of the Dowex 2 fraction (representing 1% of a 24-hr collection) of urine from a subject on an unmeasured diet. The 0.624 × 128 cm analytical column, operated at 60°, was eluted with pH 1.82 sodium phosphate buffer, 0.5 M (Na^+), at a flow rate of 0.50 ml/min. One-fifth of the column effluent went to the analytical system (ninhydrin color measured at 570 mμ), the rest being diverted to a fraction collector.

1000 ml of 0.2 M in the reservoir chamber) at a flow rate of about 0.9 ml/min. The first 120 ml were discarded, and the subsequent 800 ml were collected, taken to dryness on a rotary evaporator, and stored in a freezer until subjected to analytical column chromatography. Preliminary experiments showed that this fraction, hereafter designated the Dowex 2 fraction, contains all of the β-aspartyl peptides previously reported (Buchanan *et al.*, 1962a). Experiments in which known amounts of synthetic peptides were added to urine and carried through the fractionation and analytical procedure showed a satisfactory recovery of the aspartyl peptides.

Analytical Chromatography and Identification of Aspartyl Peptides. The Dowex 2 fraction was dissolved in 2.5 ml of water, and 0.5 or 1.0 ml (corresponding to 1 or 2% of a 24-hr excretion) was applied to the Technicon column (Chromobeads Type A or Dowex 50) and eluted with sodium phosphate buffer. The elution stream was divided so that 80% was collected in 5- or 10-min fractions. The elution pattern of the Dowex 2 fraction of a typical urine is shown in Figure 1. The content and purity of each of these peaks were determined by comparison with synthetic peptides in elution time on the analytical column and by thin layer and paper chromatography (Haley *et al.*, 1966) of the desalted fractions. The identities of the peaks were further substantiated by analytical column chromatography of acid hydrolysates of the desalted fractions (Technicon Chromatography Corp., Instruction Manual, 1964). These analyses showed that of the six peaks measured, four, Asp(Gly-Ala), Asp(Gly), Asp(Ala), and Asp-Gly, were homogeneous. The peak identified as Asp(Gly-Gly) appeared to be contaminated with ninhydrin-positive material, and therefore the values given for this peptide should be considered as maximal values. Peak A contains four peptides: Asp(Ser), Asp(Thr), Asp(Gln), and Asp(Asn). This peak has been measured as the sum of all four components, using as a color value the average of the four peptides. In all cases where the fractions corresponding to this peak were examined by thin layer or paper chromatography only these four peptides were present. The relative amounts of each of them were determined by rechromatography of the constituent amino acids on the analytical column after acid hydrolysis of peak A (Table I).

TABLE I: Partition of Peak A.

Peptide	Subject (μmoles/24 hr) 4[a]	13[b]
Peak A[c]	139	18.9
Asp(Ser)[d]	72	8.0
Asp(Thr)[d]	27	5.6
Asp(Gln)[d]	13	2.8
Asp(Asn)[d]	26	2.9

[a] Unmeasured diet. [b] Fasting. [c] Measured before hydrolysis. [d] Calculated from values for free amino acids after acid hydrolysis.

To estimate the reproducibility of the entire method, duplicate aliquots were taken from each of 23 urine collections, fractionated, and analyzed separately. The coefficient of variation (standard deviation × 100/mean value) was computed for each pair of values and the mean of these coefficients calculated for each

TABLE II: Excretion of Aspartyl Peptides by Subjects on Unmeasured Diets on Each of 2 Days.[a]

Peptide	Day	Subject (μmoles/24 hr) 1	2	3	4	5	6
Peak A	1	47	113	45	139	123	60
	2	44	35	—	58	53	31
Asp(Gly-Gly)	1	3.2	8.9	5.0	16	4.1	12
	2	2.2	6.0	—	9.0	6.0	6.6
Asp(Gly-Ala)	1	2.7	2.5	1.5	5.1	1.8	1.3
	2	1.1	1.2	—	2.7	3.1	0.7
Asp(Gly)	1	85	144	118	200	134	102
	2	66	74	—	114	97	62
Asp(Ala)	1	22	77	18	99	54	20
	2	16	13	—	22	19	12
Asp-Gly	1	7.2	10	13	11	11	9.8
	2	6.8	8.2	—	7.7	9.5	9.0

[a] For a given subject the two daily collections were separated by at least 2 weeks.

TABLE III: Excretion of Aspartyl Peptides by Subject 2 Eating Identical Foods[a] on each of 6 Consecutive Days.

Peptide	Day (μmoles/24 hr) 1	2	3	4	5	6
Peak A	43	45	53	58	50	47
Asp(Gly-Gly)	7.4	8.9	9.6	10	12	10
Asp(Gly-Ala)	1.0	1.4	1.0	1.3	1.7	1.5
Asp(Gly)	80	76	92	82	85	80
Asp(Ala)	17	19	23	26	19	19
Asp-Gly	8.0	7.6	9.4	7.7	9.0	7.8

[a] Diet contained protein, 119 g; carbohydrate, 302 g; and fat, 147 g.

peptide. The values obtained for the peptides were: peak A, 2.4%; Asp(Ala), 2.7%; Asp(Gly), 3.3%; Asp-Gly, 5.2%; Asp(Gly-Gly), 8.0%; and Asp(Gly-Ala), 16.6%.

Diets. The diet of subject 2 was identical for each of 6 days, and with ground beef as the main protein food, had 119 g of protein, 302 g of carbohydrate, and 147 g of fat/day. For 3 days subject 3 and for 1 day subject 4 consumed nothing but canned fruit and a shortbread of purified potato starch, sucrose, and butter. This diet contained less than 1 g of protein/day. Subjects 11 and 12 were postsurgical patients getting only parenteral glucose, and subject 13 was an obese female patient who fasted completely for 1 week.

Results

Of the 16 aspartyl peptides reported as normal constituents of urine (Buchanan *et al.*, 1962a), 10 were present in sufficient amount to be assayed by the present method, but the peak of one of these, Asp-(Leu), contained a considerable quantity of unidentified ninhydrin-reactive material, and the measurement of this peak is not included in the results. The analytical chromatograms showed peaks corresponding in position to the synthetic standards of five more of the previously reported compounds. Material in these peaks matched the synthetic compounds by paper and thin layer chromatography but was present in very small amount and usually mixed with unknown ninhydrin-reactive compounds. In the peak preceding peak A (Figure 1) Asp(Gly-Asn) was mixed with three unidentified compounds that gave the characteristic blue reaction of β-aspartyl peptides. By chromatographic analogy one of these may be Asp(Gly-Gln), but the synthetic standard was not available.

Of the nine peptides that were assayed, the four that emerge in the mixed peak A were individually measured only in two urine samples, one that gave a high and the other a low level for the peak (Table I). Despite a sevenfold difference between the values determined for this mixture in the two samples, the relative quantities of the four compounds were in the

same order. Asp(Ser), previously noted on the basis of paper chromatograms to be the second most abundant β-aspartyl peptide (Buchanan *et al.*, 1962a), accounted for about one-half of peak A and in these two samples gave values 35 and 36% of those obtained for Asp(Gly) in the same urine. In only two cases of all the urines analyzed did Asp(Ala) appear to be the second most abundant peptide.

The peptide excretions of six healthy male subjects on unmeasured diets are shown in Table II. Except for subject 3, analyses were performed for each of 2 days. There was more than a threefold spread in the daily excretion of Asp(Gly), always the most abundant peptide. Even larger ranges were noted with some of the other β-aspartyl peptides, and all varied more than Asp-Gly. Variations with a single individual were no less striking than those between individuals. No obvious correlation was evident between peptide excretion and the volume or pH of the urine or the age or body weight of the subject.

When subject 2 consumed identical foods on each of 6 consecutive days (Table III) the daily excretion of each peptide was reasonably constant. When subject 3 consumed food essentially devoid of protein for 3 consecutive days, his daily excretion of each of the β-aspartyl peptides fell and became remarkably constant (Table IV). These findings and the analysis obtained with subject 4 on the same diet for 1 day indicate that dietary protein augments the daily output of β-aspartyl peptides and that the peptides of dietary origin are rapidly excreted. Further confirmation of these findings (Table V) was obtained from urine collections from an obese female in the fasting state for 7 days and from two male postsurgical patients receiving only parenteral glucose.

Diet had no noticeable influence on the daily output of Asp-Gly. The range of values found for this peptide was as great for the subjects deprived of protein as it was for those on *ad libitum* diets.

TABLE IV: Excretion of Aspartyl Peptides by Subjects on a Diet Containing Less Than 1 g of Protein/Day.

	μmoles/24 Hr			
	Subject 3			Subject 4
Peptide	Day 1	Day 2	Day 3	Day 1
Peak A	33	28	28	24
Asp(Gly-Gly)	3.2	2.2	2.3	4.8
Asp(Gly-Ala)	1.5	1.2	1.3	0.8
Asp(Gly)	70	67	66	55
Asp(Ala)	12	10	11	8.6
Asp-Gly	9.6	9.2	9.4	6.4

Discussion

Except for an isolated measurement of Asp(Gly) by isotope dilution (Buchanan *et al.*, 1962b) and semiquantitative determinations of β-aspartylhistidine by paper chromatography (Pisano *et al.*, 1963), the daily output of β-aspartyl peptides in human urine has not been reported. As contrasted with our previous work, where entire 24-hr urine collections or even pooled samples of several such collections were fractionated to obtain qualitative results, the present method requires only 5% or less of the daily urine output for the determination of several compounds of this group.

The data indicate that for each β-aspartyl peptide there is a base level of excretion which arises from endogenous metabolism. These levels seem to be quite constant for a given individual and reasonably so among individuals. To these levels are added increments from the diet, presumably from protein. Other research from this laboratory (Haley *et al.*, 1966) demonstrates the presence of β-aspartyl peptides in the enzymatic

TABLE V: Excretion of Aspartyl Peptides by Subjects on Protein-Free Diets.

	μmoles/24 Hr										
	Subject 13[a]							Subject 11[b]			Subject 12[b]
Peptide	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 1	Day 2	Day 3	Day 1
Peak A	16	19	17	20	14	18	15	38	23	20	20
Asp(Gly-Gly)	2.2	3.1	2.4	4.2	3.8	7.6	7.6	7.1	—	—	2.3
Asp(Gly-Ala)	0.4	0.6	0.3	0.2	0.0	0.0	0.0	1.5	—	—	1.2
Asp(Gly)	41	54	46	55	40	50	41	84	71	65	54
Asp(Ala)	4.5	5.5	4.1	5.3	3.9	5.5	5.0	13	13	12	5.8
Asp-Gly	5.4	8.2	6.5	7.9	6.2	8.0	6.8	12	11	11	10

[a] From an obese, hospitalized, fasting, female patient. Urine collection for day 1 was begun on first morning of fasting period. [b] From postgastrectomy patients receiving only parenteral glucose. Urine collections were begun 24 and 48 hr after surgery for subjects 11 and 12, respectively.

digests of several purified proteins, but it has not been determined whether β-aspartyl linkages exist in protein or are formed during its hydrolytic breakdown. Such β-aspartyl peptides would be released during the digestion of dietary protein, would resist further hydrolysis, and would be rapidly excreted (Buchanan *et al.*, 1962b). In contrast, the level of Asp-Gly excretion seems to be independent of the amount of dietary protein.

The consumption of protein foods does not cause the daily excretions of various β-aspartyl peptides to increase in the same proportions. This leads to the belief that certain proteins may be major sources of specific urinary β-aspartyl peptides, an hypothesis currently being investigated further.

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Preparation of Aspartyl Peptides*

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ABSTRACT: Mixtures of α- and β-aspartyl di- and tripeptides can be prepared in high yield by adding carbobenzoxyaspartic anhydride in dimethylformamide to aqueous solutions of free amino acids or dipeptides. After removal of the protecting group the isomers are easily separated by preparative chromatography.

Several workers (Bergmann and Zervas, 1932; Grassman and Schneider, 1934; Bergmann *et al.*, 1935; Le Quesne and Young, 1952; John and Young, 1954; Buchanan *et al.*, 1962) have prepared mixtures of α- and β-aspartyl dipeptides by coupling carbobenzoxyaspartic anhydride (Cbz-Asp-Anh)[1] to an ester of the desired carboxy-terminal amino acid, both reactants being in organic solvents. After removal of protecting groups, separation of the isomers by partial extraction with sodium bicarbonate solution has often been inefficient and tedious (Le Quesne and Young, 1952; John and Young, 1954) or unsuccessful (Bryant *et al.*, 1959).

We have found that the coupling step proceeds better when amino acids or dipeptides are in aqueous solution and not esterified, a modification especially useful with radioactive amino acids. Furthermore, with syntheses at a 10-mmole level, a 1.9 × 140 cm column of Dowex 1 allows nearly quantitative isolation of each peptide in pure form. These modifications have enabled the preparation in high yield of approximately equal quantities of the α and β isomers of 15 aspartyl di- and tripeptides, all derived from L-amino acids. The peptides reported here include all but one of the β-aspartyl peptides previously found in human urine (Buchanan *et al.*, 1962), the α-aspartyl isomer of each, and the aspartylphenylalanines. Of the 30 peptides, five have been prepared by other methods (Grassman and Schneider, 1934; Bergmann *et al.*, 1935; Le Quesne and Young, 1952; John and Young, 1954; Liwschitz and Zilka, 1954, 1955).

Results and Discussion

Yields. The combined yields of α- and β-aspartyl peptides (Table I) were from 65 to 95%, higher than heretofore reported. The lowest was obtained in the preparation of the aspartylglutamines where the coupling step was carried out near 0°. A reaction at room temperature had resulted in extensive decomposi-

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[1] Abbreviations used: Cbz-Asp-Anh, carbobenzoxy-L-aspartic anhydride; Asp-Gly, α-aspartylglycine; Asp(Gly), β-aspartylglycine; Asp(Gly-Ala), β-aspartylglycylalanine.