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.

tion of glutamine. A reaction with glycine at 100° gave a total yield 12% lower than at room temperature. Yields were related to the solubility of the free amino acid; with less water they were higher. In only two of the preparations did the yield of β peptide exceed that of the α peptide, but in no instance was the yield of the α peptide twice that of the β isomer as has been the case when amino acid esters are used (Le Quesne and Young, 1952; John and Young, 1954; Buchanan *et al.*, 1962).

Removal of the Carbobenzoxy Group. Although slower than with flowing hydrogen, removal of the carbobenzoxy group in a closed flask requires little hydrogen and several preparations can be treated simultaneously. In five preparations the reaction was followed by manometric measurement of carbon dioxide evolution. During the first 24 hr 70–94% of the expected carbon dioxide was recovered and after 3 days from 89 to 96% had been collected. Because the recovery of free and coupled aspartic acid was always considerably less than the recovery of the free and coupled carboxy-terminal amino acid or dipeptide, it is believed that the uncleaved covering group was mainly on aspartic acid and not on peptides.

Identification of Reaction Products. Free aspartic acid and the unreacted free amino acid or dipeptide were identified by thin layer chromatography. The α- and β-aspartyl peptides were differentiated by analogy in their elution pattern and color reactions, by the chromatographic identity of all but one β-aspartyl peptide with metabolites that had been previously identified (Buchanan *et al.*, 1962), and finally by identification of the products of hydrazinolysis.

Because β-aspartyl peptides have two α-COOH groups their pK_2 values are lower than those of α-aspartyl peptides (Cohn and Edsall, 1943) and, without exception, they were eluted later from anion-exchange columns (Davies, 1949; Buchanan, 1959). Aspartic acid came off between the α and β peptides in all separations except with the aspartylphenylalanines. Here the aromatic nature of the peptides would be expected to retard the elution (Buchanan, 1959). Several investigators (Le Quesne and Young, 1952; John and Young, 1954; Buchanan *et al.*, 1962; Bryant *et al.*, 1959; Liwschitz and Zilka, 1954, 1955) have noted that β-aspartyl peptides give a bright blue color when paper spots are sprayed with ninhydrin and heated to 120°, whereas the α isomers turn purple; there were again no exceptions.

Thin layer chromatography of the products of hydrazinolysis with known hydrazides and amino acids as standards revealed a predominance of either the appropriate α- or the β-aspartylhydrazide. The tripeptides all gave glycylhydrazide and all peptides except the six with carboxy-terminal asparagine or glutamine showed the appropriate free amino acid. With the pyridine–water solvent these hydrazides have characteristic R_F values and ninhydrin colors. α-Aspartylhydrazide becomes gray-purple, its β isomer, pink, and glycylhydrazide, yellow–brown. Asp-Asn and Asp-Gly-Asn showed both aspartylhydrazides while their β isomers gave β-aspartylhydrazide with double intensity. Although the reaction with hydrazine is useful in confirming the structure of pure compounds it would be of little value in the detection of β-aspartyl linkages in mixtures because traces of β-aspartylhydrazide were noted with all α-aspartyl peptides.

Crystallization and Purity. All of the peptides were obtained in solid form, but Asp-Val, Asp-Ile, and Asp-Gly-Asn were amorphous. Difficulty was experienced in getting crystals with Asp-Asn, Asp(Gly-Val), Asp(Gly-Asn), and Asp-Gly-Ala, all of which finally formed silky needles containing water, ethanol, methanol, or water and ethanol (Table II). Although crystals and amorphous powders that are listed as containing water or alcohol all released gas bubbles when heated in mineral oil (Jelley, 1949), the only quantitative evidence for the solvent content was the elemental analysis after prolonged drying *in vacuo* over P_2O_5. Crystals of aspartyl peptides cannot be analyzed for solvent content by heating *in vacuo* because even the anhydrous ones slowly lose water as they cyclize to aminosuccinimido derivatives of the carboxy-terminal amino acids (Swallow and Abraham, 1958; E. E. Haley and D. L. Buchanan, unpublished data).

Each peptide gave a single spot on thin layer chromatography with at least two solvent systems and each gave a symmetrical peak with only trace impurities on automatic ion-exchange chromatography (Dorer *et al.*, 1966). The specific rotations of the five peptides that had been described in the literature were in satisfactory agreement with literature values.

Experimental Section

Materials. Cbz-Asp-Anh (mp 110°) was prepared according to Miller *et al.* (1941). Its optical purity was confirmed by hydrolysis to aspartic acid which was quantitatively recovered as a dry residue, and the rotation was then measured without recrystallization. To eliminate possible traces of reactive material dimethylformamide was distilled *in vacuo* several hours after the addition of 0.5% Cbz-Asp-Anh. The α- and β-hydrazides of aspartic acid and glycylhydrazide were prepared from the corresponding ethyl esters (Narita and Ohta, 1959). Amino acids and dipeptides,[2] ninhydrin,[3] and 10% palladium on charcoal,[4] were used as received. Anhydrous hydrazine was redistilled *in vacuo*.

Methods. Ninhydrin color tests were performed by spotting aqueous solutions on Whatman No. 1 filter paper, spraying with 0.3% ninhydrin in 95% ethanol, and heating to 120° for 3 min. Paper chromatograms were descending with Whatman No. 1 paper and 1 butanol–acetic acid–water (4:1:1). Thin layer chroma-

[2] Mann Research Laboratories, New York, N. Y., and Nutritional Biochemicals Corp., Cleveland, Ohio.
[3] Pierce Chemical Co., Rockford, Ill.
[4] Matheson Coleman and Bell, Norwood, Ohio.

TABLE I: Preparation, Chromatography, and Crystallization of Aspartyl Peptides.[a]

	Reagents				Preparative Chromatography[b]				Recrystallization			
						Eluent Vol (ml)		Recov[c] (%				
Prepn	Cbz-Asp-Anh (mmoles)	Amino Acid or Dipeptide Compd	(mmoles)	Water (ml)	Compd	from	to	theory)	Solvent I[d] (ml/g)		EtOH (ml/g)	Recov[e] (%)
1	10	Gly	20	6	Gly	176	242	51[f]				
					Asp-Gly	726	1254	54	0.5 M p	18	60	94
					Asp	1276	1320	1				
					Asp(Gly)	1540	2130	41	1.0 M p	13	50	98
2	11	Ala	10	5	Ala	176	264	4				
					Asp-Ala	860	1230	47	Water	5	54	93
					Asp	1252	1538	12				
					Asp(Ala)	1562	2090	48	0.5 M p	15	60	85
3	10	Val	10	13	Val	176	268	18				
					Asp-Val	652	1300	53	Water	7	115	85
					Asp	1300	1510	9				
					Asp(Val)	1532	2012	34	Water	8	52	84
4	11	Leu	10	50	Leu	205	520	5				
					Asp-Leu	850	1390	48	0.5 M p	20	125	84
					Asp	1390	1658	4				
					Asp(Leu)	1800	2420	37	1.0 M p	6	55	93
5	10	Ile	10	30	Ile	170	282	—				
					Asp–Ile	820	1430	38	Water	8	50	80
					Asp	1430	1650	12				
					Asp(Ile)	1650	2220	38	Water	10	50	87
6	10	Phe	10	50	Phe	344	486	22				
					Asp	1460	1890	19				
					Asp-Phe	2040	3230	40	0.5 M p	22	100	89
					Asp(Phe)[g]	3980	4250	33	0.6 M p	16	100	83
7	11	Asn	10	12	Asn	200	275	16				
					Asp-Asn	550	1220	41	Water	11	75	93
					Asp	1220	1395	8				
					Asp(Asn)	1670	2470	49	0.5 M p	11	63	94
8	6	Gln	5	10	Gln	175	275	26				
					Asp-Gln	600	1075	40	Water	16	50	91
					Asp	1100	1325	12				
					Asp(Gln)	1650	2025	25	Water	14	50	89
9	11	Ser	10	3	Ser	198	242	2				
					Asp-Ser	508	1150	51	Water	24	50	93
					Asp	1150	1260	6				
					Asp(Ser)	1560	2270	43	Water	11	69	90
10	11	Thr	9.1	3	Thr	220	310	4				
					Asp-Thr	570	1040	51	Water	15	110	84
					Asp	1100	1360	17				
					Asp(Thr)	1450	1920	41	Water	6.2	60	88
11	11	Gly-Gly	10	6	Gly-Gly	130	395	9				
					Asp-Gly-Gly	1500[h]	1870	48	0.6 M p	18	55	95
					Asp	1870	2055	13				
					Asp(Gly-Gly)	2100	2610	43	0.5 M p	15	45	91
12	4	Gly-Ala	2.9	1	Gly-Ala	220	270	—				
					Asp-Gly-Ala	615	915	51	Water	12	150	85
					Asp	1040	1365	29				
					Asp(Gly-Ala)	1365	1615	43	0.5 M p	11	55	87
13	6	Gly-Val	5	1.5	Gly-Val	72	150	—				
					Asp-(Gly-Val)	604	999	53	Water	6.7	100	80
					Asp	1053	1473	26				
					Asp(Gly-Val)	1473	1868	30[i]	MeOH	10	—	80

TABLE I (*Continued*)

Reagents					Preparative Chromatography[b]				Recrystallization			
						Eluent Vol (ml)		Recov[c] (%				
Prepn	Cbz-Asp-Anh (mmoles)	Amino Acid or Dipeptide Compd	(mmoles)	Water (ml)	Compd	from	to	theory)	Solvent I[d]	(ml/g)	EtOH (ml/g)	Recov[e] (%)
14	6	Gly-Pro	5	1.5	Gly-Pro	220	240	Trace				
					Asp-Gly-Pro	620	970	41	Water	5	50	78
					Asp	1010	1300	—				
					Asp(Gly-Pro)	1400	1840	48	Water	5	50	95
15	11	Gly-Asn	10	6	Gly-Asn	177	249	—				
					Asp-Gly-Asn	963	1613	43	Water	5	150	88
					Asp	1613	1928	11				
					Asp(Gly-Asn)	2091	2766	41	Water	8	100	86

[a] All amino acids except glycine are L. [b] On 400-ml column of Dowex 1-X4 eluted with 0.1 M formic acid (see text). [c] Yield of dry material based on limiting reagent added and corrected for water or alcohol of crystallization of final product. [d] Pyridine (p) solutions are aqueous. [e] Based on a single recrystallization. [f] Includes 5.6 mmoles of crystals filtered from reaction mixture. [g] Eluted with 1.0 M formic acid after 3250 ml. [h] Eluted with water for first 1000 ml, then with 0.1 M formic acid. [i] Accidental loss.

tography of peptides and amino acids was performed ascending on 20-cm plate-glass squares coated with Whatman Chromedia, CC 41, and developed with 1-butanol–acetic acid–water (3:1:1). Hydrazides were chromatographed identically in pyridine–water (65:35). Decomposition points are uncorrected and were performed in capillary tubes heated 3°/min. Microanalyses were performed by S. M. Nagy.[5]

Qualitative tests for solvent of crystallization were performed by heating crystals in mineral oil (Jelley, 1949). Hydrazinolyses were done by heating a 1% solution of peptide in anhydrous hydrazine at 100° for 15 min and then removing hydrazine *in vacuo* over sulfuric acid. Residues were diluted with water to 0.05 M before thin layer chromatography.

Optical rotation was measured with a Rudolph, Model 80, visual polarimeter in a 3.0 mm × 10 cm cell. Automatic column chromatography was performed on the Technicon amino acid analyzer with 0.65 × 130 cm columns of sulfonated polystyrene resin pumped at 0.50 ml/min with phosphate buffer, pH 1.82, and 0.5 M with respect to sodium (Dorer *et al.*, 1966).

Preparation of Peptide Mixtures. To a nearly saturated aqueous solution of the desired carboxy-terminal amino acid or glycyl dipeptide (Table I) was added 3 molar equiv of triethylamine. The solution was made up in a 1-l., round-bottom, standard taper flask unless the volume was 10 ml or less in which case a test tube was used. After the Cbz-Asp-Anh was dissolved in twice its weight of dimethylformamide it was rapidly added to the vigorously swirled amino acid or peptide solution using a magnetic stirrer with the flasks and a vortex mixer with the tubes. Reactions were carried out at room temperature except with glutamine where the aqueous solution was cooled on an ice bath just prior to the addition of Cbz-Asp-Anh; solutions were invariably warmed. After 15 min each mixture was reduced to a heavy syrup by rotary evaporation and then diluted with 50% ethanol (5 ml/mmole of Cbz-Asp-Anh. Mixtures in test tubes were washed into flasks with 50% ethanol at this point. After the addition of 80 mg of palladium–charcoal catalyst/mmole of Cbz-Asp-Anh, the flask was sealed to a vacuum stopcock with a greased, ground-glass fitting and evacuated until boiling occurred. The stopcock was closed and the flask charged to 1 atm with hydrogen and swirled at 120 rotations/min for 3 days. After the first and second days, flasks were evacuated to boiling and recharged.

Separation of Peptides. The palladium–charcoal was removed by filtration and washed with water and alcohol alternatively until the washings gave no color with ninhydrin. The filtrate and washings were taken to a syrup, diluted with water, and put on a 2.2 × 10 cm column of Dowex 50-X8 (hydrogen). After the column was washed with water the amino acids and peptides were displaced with 1 M aqueous pyridine and taken to dryness. The residue was dissolved in a minimum of water and put on a 1.9 × 140 cm column of Dowex 1-X4 (formate). The column was pumped usually with 0.1 M formic acid (Table I) at 2 ml/min and 22-ml fractions were collected overnight. A drop of each was tested on paper with ninhydrin. When compounds were confluent thin layer chromatography was used to detect boundaries. Mixed fractions

[5] Microanalytical Laboratory, Massachusetts Institute of Technology, Cambridge, Mass.

TABLE II: Analytical Data of Aspartyl Peptides.

Peptide	Dec Pt (°C)	$[\alpha]_D^{25}$, deg	Formula	Calcd (%) C	Calcd (%) H	Calcd (%) N	Found (%) C	Found (%) H	Found (%) N
α-L-Aspartylglycine·H_2O	175	+31.0 (*c* 2.0, water with 1 equiv of HCl)	$C_6H_{12}N_2O_6$	34.62	5.81	13.45	34.39	5.98	13.39
β-L-Aspartylglycine·H_2O	163	+12.7 (*c* 2.0, water with 1 equiv of HCl)	$C_6H_{12}N_2O_6$	34.62	5.81	13.45	34.66	5.93	13.40
α-L-Aspartyl-L-alanine	197	−6.5 (*c* 2.0, water)	$C_7H_{12}N_2O_5$	41.18	5.92	13.72	41.19	6.05	13.68
β-L-Aspartyl-L-alanine	220	−39.7 (*c* 2.0, water)	$C_7H_{12}N_2O_5$	41.18	5.92	13.72	41.44	5.96	13.66
α-L-Aspartyl-L-valine·$^1/_2$ H_2O	172	+13.5 (*c* 1.2, glacial acetic acid)	$C_9H_{17}N_2O_{5.5}$	44.80	7.10	11.61	45.13	7.03	11.80
β-L-Aspartyl-L-valine	208	−10.7 (*c* 2.0, water)	$C_9H_{16}N_2O_5$	46.55	6.94	12.06	46.17	6.93	11.96
α-L-Aspartyl-L-leucine	204	−11.1 (*c* 1.0, water)	$C_{10}H_{18}N_2O_5$	48.77	7.37	11.37	48.33	7.42	11.18
β-L-Aspartyl-L-leucine	215	−29.6 (*c* 1.0, water)	$C_{10}H_{18}N_2O_5$	48.77	7.37	11.37	48.64	7.49	11.40
α-L-Aspartyl-L-isoleucine·$^1/_2$ H_2O	190	+9.1 (*c* 2.0, water)	$C_{10}H_{19}N_2O_{5.5}$	47.05	7.50	10.97	47.13	7.53	10.83
β-L-Aspartyl-L-isoleucine	205	−4.7 (*c* 2.0, water)	$C_{10}H_{18}N_2O_5$	48.77	7.37	11.37	49.14	7.28	11.31
α-L-Aspartyl-L-phenylalanine	210	+13.0 (*c* 2.0, water with 1 equiv of HCl)	$C_{13}H_{16}N_2O_5$	55.71	5.75	9.99	55.94	5.79	9.70
β-L-Aspartyl-L-phenylalanine	204	+46.6 (*c* 2.0, water with 1 equiv of HCl)	$C_{13}H_{16}N_2O_5$	55.71	5.75	9.99	55.30	5.64	9.66
α-L-Aspartyl-L-asparagine·H_2O·C_2H_5OH	129	+4.8 (*c* 2.1, water)	$C_{10}H_{21}N_3O_8$	38.58	6.80	13.50	38.70	6.70	13.57
β-L-Aspartyl-L-asparagine	242	−4.8 (*c* 2.1, water)	$C_8H_{13}N_3O_6$	38.87	5.30	17.00	38.98	5.35	16.66
α-L-Aspartyl-L-glutamine	191	+11.3 (*c* 2.0, water)	$C_9H_{15}N_3O_6$	41.38	5.79	16.08	41.48	5.90	15.83
β-L-Aspartyl-L-glutamine	219	−7.8 (*c* 2.0, water)	$C_9H_{15}N_3O_6$	41.38	5.79	16.08	41.36	5.98	15.96
α-L-Aspartyl-L-serine	178	+17.8 (*c* 2.0, water)	$C_7H_{12}N_2O_6$	38.19	5.49	12.73	38.30	5.61	12.51
β-L-Aspartyl-L-serine	181	−0.9 (*c* 2.0, water)	$C_7H_{12}N_2O_6$	38.19	5.49	12.73	37.96	5.72	12.66
α-L-Aspartyl-L-threonine	175	+6.0 (*c* 2.0, water)	$C_8H_{14}N_2O_6$	41.03	6.03	11.96	40.75	6.06	11.83
β-L-Aspartyl-L-threonine	201	+1.3 (*c* 2.0, water)	$C_8H_{14}N_2O_6$	41.03	6.03	11.96	41.30	5.75	12.03
α-L-Aspartylglycylglycine	221	+32.3 (*c* 2.0, water with 1 equiv of HCl)	$C_8H_{13}N_3O_6$	38.87	5.30	17.00	39.21	5.06	17.10
β-L-Aspartylglycylglycine	234	+6.0 (*c* 2.0, water with 1 equiv of HCl)	$C_8H_{13}N_3O_6$	38.87	5.30	17.00	39.22	5.43	16.96
α-L-Aspartylglycyl-L-alanine·$^1/_2C_2H_5OH$	170	−1.8 (*c* 1.3, water)	$C_{10}H_{18}N_3O_{6.5}$	42.25	6.38	14.78	41.95	6.32	14.92
β-L-Aspartylglycyl-L-alanine	223	−39.2 (*c* 2.1, water)	$C_9H_{15}N_3O_6$	41.38	5.79	16.09	41.57	6.02	16.04
α-L-Aspartylglycyl-L-proline	200	−53.0 (*c* 2.0, water)	$C_{11}H_{17}N_3O_6$	45.99	5.96	14.63	45.68	6.00	14.24
β-L-Aspartylglycyl-L-proline	214	−93.7 (*c* 2.0, water)	$C_{11}H_{17}N_3O_6$	45.99	5.96	14.63	45.76	5.96	14.51
α-L-Aspartylglycyl-L-valine	188	+12.0 (*c* 2.0, water)	$C_{11}H_{19}N_3O_6$	45.67	6.62	14.52	45.70	6.66	14.55
β-L-Aspartylglycyl-L-valine·CH_3OH	150	−19.9 (*c* 2.0, water)	$C_{12}H_{23}N_3O_7$	44.85	7.21	13.07	45.22	7.17	13.22
α-L-Aspartylglycyl-L-asparagine·C_2H_5OH	119	+17.1 (*c* 2.0, water)	$C_{12}H_{22}N_4O_8$	41.14	6.33	15.99	41.10	6.31	15.95
β-L-Aspartylglycyl-L-asparagine·H_2O	229	−7.2 (*c* 2.0, water)	$C_{10}H_{18}N_4O_8$	37.27	5.63	17.36	37.58	5.60	17.36

contained only traces of peptides and were discarded. Pure fractions of each compound (Table I) were combined and poured through a 2.2 × 5 cm column of Dowex 50-X8 (hydrogen) and displaced with 1 M aqueous pyridine into weighed test tubes. These were dried on a test tube rotary evaporator. When syrups and gums formed these were put into solution and the water was removed azeotropically by repeated addition of absolute alcohol until only white solid remained. This was dried *in vacuo* to constant weight. The crude yields of Table I were calculated from these weights corrected for solvents of crystallization and, in a few

cases, for less than quantitative recovery of the crystals plus the dried residue of the mother liquor of the first recrystallization. Each peptide was recrystallized three times or more. Table I gives the conditions and recovery for a single typical crystallization. Table II gives decomposition points, specific rotations, and microanalyses.

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