where one calcium can apparently bind two molecules of cephalin (Feinstein, 1964). Evidence in favor of the role of phospholipids in calcium binding is afforded by preliminary experiments (E. J. Harris, B. Chance, and L. Mela, unpublished data) on the effect of a local anesthetic, such as butacaine, on the H^+/Ca^{2+} values at both low Ca^{2+} concentrations and at higher concentrations where other materials of the membrane or soluble anions might be involved.

The H^+/Ca^{2+} values allow a tentative identification of the nature of the anion involved; values in the region of 0.8 are likely to be due to phosphate, while considerably lower values (approximately 0.2) would be due to a permeant anion such as acetate (Rasmussen *et al.*, 1965). Since we observe consistent values in excess of 1, we tend to attribute the initial binding and the "membrane buffer capacity" to phospholipid components (see also Chappell *et al.*, 1963).

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β-Aspartyl Peptides in Enzymatic Hydrolysates of Protein*

Edward E. Haley, Betty J. Corcoran, Frederic E. Dorer, and Donald L. Buchanan

ABSTRACT: In a search for the source of β-aspartyl oligopeptides in urine we have analyzed exhaustive enzymic hydrolysates of human hemoglobin, porcine pepsin, human and bovine Achilles tendon collagen, and egg white lysozyme. β-Aspartylglycine was present in all hydrolysates and β-aspartylalanine was found in digests of hemoglobin. There was no increase in the β-aspartylglycine content of human or bovine tendon collagen with aging *in vivo* nor of lysozyme with aging *in vitro*. The data suggest that, in intact proteins, β-aspartyl linkages do not form spontaneously from α-aspartyl or asparaginyl linkages.

We have previously described the isolation and identification of several β-aspartyl di- and tripeptides from human urine (Buchanan *et al.*, 1962a). β-Aspartylglycine (Asp(Gly))[1] was the most abundant. The belief that β-aspartyl peptides are metabolic end products, less readily cleaved by tissue peptidases and more susceptible to renal excretion than α-aspartyl peptides, was supported by results of experiments in which human subjects were given glycine-labeled Asp-Gly or Asp(Gly), or labeled glycine intravenously (Buchanan *et al.*, 1962b). Most of the β peptide was quickly excreted, while the metabolic pattern of the injected α peptide was similar to that found when free glycine was administered. An explanation of the occurrence of these urinary peptides may be the spontaneous or even catalyzed conversion of asparaginyl or α linkages during *in vivo* metabolism. Isomerization occurs with α- and β-aspartyl peptides in acid solution and the β isomer is more stable (John and Young, 1954; Swallow and Abraham, 1958; Bryant *et al.*, 1959). Our present data show that conversion of Asp-Gly to Asp(Gly) occurs spontaneously in pH 7.4 buffer

* From the Veterans Administration Hospital, West Haven, Connecticut, and the Department of Biochemistry, Yale University, New Haven, Connecticut. *Received April 26, 1966.* This work was supported in part by a research grant (AM-1277) from the National Institutes of Health.

[1] Abbreviations used: Asp-Gly, α-L-aspartylglycine; Asp(Gly), β-L-aspartylglycine; Asp(Ala), β-L-aspartylalanine; Asn-Gly, L-asparaginylglycine. It is assumed that the peptides in the protein hydrolysates are of the L configuration. Other abbreviations: TCA, trichloroacetic acid; VK, Viokase; PRN, Pronase; COL, collagenase; LAP, leucine aminopeptidase; PRL, prolidase; CA, carboxypeptidase A; CB, carboxypeptidase B.

at 37°, and that, under these conditions, Asn-Gly converts to Asp(Gly) more readily than does Asp-Gly. We considered the possibility that even in protein molecules these linkages might convert. Upon endogenous breakdown of protein the β linkages would be resistant to cleavage leaving the β-aspartyl peptides to be eliminated in the urine.

This report describes the finding of β-aspartyl oligopeptides in enzymic hydrolysates of human hemoglobin, porcine pepsin, human and bovine Achilles tendon collagen, and egg white lysozyme. Asp(Gly) was found in all hydrolysates in amounts ranging from 0.009 mole/mole of protein for lysozyme to 1.5 moles/mole of bovine collagen. Asp(Ala) was found in hemoglobin hydrolysates but in lower concentration than Asp(Gly).

Materials and Methods

Materials. Twice-crystallized human hemoglobin was purchased from Mann Research Laboratories. We also prepared once-crystallized, salt-free, lyophilized hemoglobin by the procedure of Drabkin (1946) from 56-day-old bank blood. A sample of this hemoglobin (100 mg) was incubated by Dr. A. G. Loewy with 20 mg of plasma transglutaminase and [1-^{14}C]glycine ethyl ester (Loewy *et al.*, 1966). Radioactivity measurements of the TCA-precipitated protein (659 cpm/mg) indicated the binding of 5 moles of glycine ethyl ester/mole of hemoglobin. Porcine pepsin, twice crystallized, was purchased from Nutritional Biochemicals Corp. Bovine Achilles tendon was from Worthington Biochemical Corp. Other specimens of bovine Achilles tendon were taken from freshly killed calves, 2- to 3-days old, and from cows 5- to 6-years old, whose carcasses had been refrigerated for 2 days after slaughter. Specimens of human tissue were taken at autopsy. The infant specimen of tendon collagen came from seven autopsies of newborn and stillborn infants. The ages were from the eighth month *in utero* to 1 week *post partum*. The adult specimen was from a 67-year-old male. All collagen was purified by the method of Einbinder and Schubert (1951).

Lysozyme, twice crystallized, collagenase, leucine aminopeptidase, prolidase, carboxypeptidase A, and carboxypeptidase B were from Worthington. Viokase (4× N. F. pancreatin) was obtained from the Viobin Corp., and Pronase, B grade, from Calbiochem. Labeled Asp-Gly and Asp(Gly) and unlabeled Asp(Ala) were prepared as described elsewhere (Buchanan *et al.*, 1966). The radioactive peptides were prepared with [^{14}C]glycine and each had a specific radioactivity of 2.5 μc/mg. Asn-Gly, mp 204–205° dec (lit. 205–206°), was prepared from carbobenzoxy-L-asparagine and glycine ethyl ester hydrochloride (Woodward *et al.*, 1961; Miller and Waelsch, 1952).

Analytical Methods. Thin layer chromatography was performed on glass plates coated with Whatman Chromedia, CC-41, and developed with *n*-butyl alcohol–acetic acid–water (3:1:1). Strips for radioactive scanning were of Whatman No. 3 paper developed in a similar solvent (4:1:1). Two-dimensional chromatograms were on the same paper with pyridine–acetic acid–water (10:7:3) ascending, and *t*-butyl alcohol–formic acid–water (14:3:3) descending. After they were sprayed with 0.2% ninhydrin in ethanol, developed plates and papers were heated to 120° for 5 min, this temperature being essential for the production of the characteristic blue color of β-aspartyl peptides (Le Quesne and Young, 1952).

Analytical chromatography was done with a Technicon amino acid analyzer. For Asp(Gly) the column was developed with phosphate buffer, pH 1.82, and 0.5 M in Na^+ (Dorer *et al.*, 1966). With Asp(Ala), dilution of this buffer with an equal volume of water caused the peptide to separate from an unidentified ninhydrin-reactive compound which was mixed in the peak when the buffer was not diluted. In all analyses about 20% of the eluent went to the analytical system and the rest was collected as 5-min fractions.

The progress of enzymatic hydrolyses was followed with the modified Van Slyke α-amino nitrogen method (Koch, 1929) or with ninhydrin and the Technicon AutoAnalyzer. Radioactivity was measured in a gas-flow counter and by a recording radioactivity scanner.

Enzymatic Digestions. The conditions of each incubation are given in Tables I and II; all incubations and preincubations were at 37°. Pepsin, hemoglobin, and lysozyme were 1% solutions in 0.05 M Tris chloride buffer, pH 8.0.[2] Manganese chloride was added to 0.0025 M in the pepsin solution and to 0.005 M in the others. Finely divided collagen was put into 0.5% suspension in 0.067 M Tris chloride with 0.08 M NaCl, pH 7.4, buffer. A few crystals of thymol were added to each digest and most also contained tetracycline hydrochloride (Tetracyn, Pfizer) at 10 μg/ml. Glassware and buffers were autoclaved and sterility was tested by culture on blood agar at the end of each incubation.

Enzymes were added sequentially, and not until a given enzyme had caused the degree of hydrolysis that had been found to be nearly maximal was the next added. Except for collagen, which was first treated with collagenase, proteins were first digested with Viokase. Pronase followed in most instances, and then came a mixture of leucine aminopeptidase and prolidase. The final enzyme mixture added was that of carboxypeptidases A and B. Viokase and Pronase were put into the digests as solids. Leucine aminopeptidase (0.1 mg/ml) was preincubated in 0.1 M Tris chloride, pH 8.0, and 0.002 M $MnCl_2$ for 3 hr. Prolidase (1.0 mg/ml) was preincubated in the same buffer with 0.02 M $MnCl_2$ for 1 hr. Carboxypeptidase A was first dissolved (1.0 mg/ml) in 10% LiCl and carboxypeptidase B (0.5 mg/ml) in 0.05 M Tris chloride, 0.11 M NaCl, pH 7.5. Before the carboxypeptidase solutions were added, the concentration of the digestion mixture was brought to 0.1 M NaCl and 0.005 M zinc acetate with the solid salts, and the pH lowered to 7.6 with HCl.

Either at the beginning or at the completion of some

[2] The pH values for Tris buffers are for 37°.

TABLE I: Enzymatic Hydrolyses of Hemoglobin and Lysozyme and the β-Aspartyl Peptide Content[a] of the Hydrolysates.

Protein (g)	Enzyme Addn[b] (mg)	Cumulative Digestion Time (hr)	Hydrolysis[c] (%)	Total ^{14}C Recov (%)	Asp(Gly) Content: Moles/Mole of Protein	Asp(Gly) Content: % Theory[d]	Asp(Ala) Content: Moles/Mole of Protein	Asp(Ala) Content: % Theory[e]
Hemoglobin, 2 × cryst, 1.0	VK; 50, 25	147	82		0.021	1.1	0.014	0.14
	LAP, 1; PRL, 10	192	82					
	CA, 5; CB, 0.5	261	84					
Hemoglobin, 1 × cryst, 1.0	VK; 25	116	64	94[f]	0.077	3.9	0.011	0.11
	PRN; 26, 26	209	90					
	[g]LAP, 1.1; PRL, 10	280	90					
	CA, 5; CB, 0.5	305	95					
Hemoglobin, 1 × cryst, 1.0	VK; 25, 25, 25	69	71	100[f]	0.008	0.4		
1 × cryst, 0.084, treated with transglutaminase and glycine ethyl ester	VK, 2.6	47	42		0.074	3.7		
	PRN; 2.5, 2.5	166	74					
	LAP, 0.1; PRL, 1.1	210	84					
	CA, 0.5; CB, 0.025	282	88					
Hemoglobin, cryst liquor,[h] 0.5	VK, 25	116	78		0.051	2.6	0.012	0.12
	PRN; 13, 13	238	88					
	[g]LAP, 0.5; PRL, 5	260	94					
	CA, 2.5; CB, 0.25	278	*i*					
Lysozyme, 0.8	VK; 20, 20	92	9	104[f]	0.0083	0.21		
	PRN, 10	194	39					
	LAP, 1; PRL, 10	219	50					
	CA, 4; CB, 0.4	239	51					
Lysozyme, 1.0, aged at −20°, 69 days	VK; 25, 25	94	11	85[f]	0.0093	0.23		
	PRN, 10	194	38					
	LAP, 1.3; PRL, 10	219	46					
	CA, 5; CB, 0.5	239	52					
Lysozyme, 1.0, aged at 37°, 69 days	VK; 25, 25	94	22	86[f]	0.011	0.28		
	PRN, 10	194	46					
	LAP, 1.3; PRL, 10	219	53					
	CA, 5; CB, 0.5	239	53					

[a] All analytical values corrected for sampling aliquots. Enzyme blank values subtracted. [b] LAP added was 100–150 units/g of substrate; CB, 45–60 units/g. [c] Followed with ninhydrin reaction (AutoAnalyzer) or with Van Slyke α-amino nitrogen method. Complete hydrolysis taken as that obtained with 6 N HCl at 120° for 18 hr. [d] Based on two -Asp-Gly- sequences per molecule of hemoglobin, or four -Asp-Gly- sequences per molecule of lysozyme. [e] Based on four -Asp-Ala- and six -Asn-Ala- per molecule of hemoglobin. [f] Asp([^{14}C]Gly) added after digestion. [g] Digest heated (100°, 5 min) to inactivate Pronase. [h] Protein in liquor of crystallization. [i] Analytical sample lost.

digestions (*cf.* Tables I and II) β-L-aspartyl[^{14}C]glycine (0.27 μmole and 1.26 × 10^6 cpm/g of substrate) was added. Enzyme blank incubations were carried out to match the sequence, timing, and concentrations of all reagents except that no protein substrates were present.

Protein Aging in Vitro. To study the possibility that, in intact protein, β-aspartyl linkages might form spontaneously *in vitro*, lysozyme (2 g) was dissolved in 200 ml of 0.05 M Tris buffer, pH 7.4. One-half of the solution was sterilized by filtration with a Morton, ultrafine, fritted-glass filter and kept at 37° for 69 days. The remaining one-half was frozen and stored at −20° for the same period. Both solutions were then brought to pH 8.0 with NaOH, digested with enzymes as described, and analyzed.

To compare these results with those obtained with small peptides 0.25-μmole quantities of α-L-aspartyl-[^{14}C]glycine (1.9 × 10^5 cpm) were placed in sterile test

TABLE II: Enzymatic Hydrolyses of Collagen and Pepsin and the β-Aspartylglycine Content[a] of the Hydrolysates.

Protein (g)	Enzyme Addn[b] (mg)	Cumulative Digestion Time (hr)	Hydrolysis[c] (%)	Total ^{14}C Recov (%)	Asp(Gly) Content (moles/mole of protein)
Bovine[d] collagen, 1.0	COL; 10, 10, 10, 10	96	35	85[e]	1.3
	PRN; 30, 30	192	72		
	LAP, 1.4; PRL, 10	216	89		
	CA, 5; CB, 0.25	234	91		
Cow collagen, 1.0	COL; 10, 10, 10	73	30	102[e]	0.83[f]
	PRN; 30, 30, 10	168	63		0.85
	LAP, 1; PRN, 10	191	81		
	CA, 5; CB, 0.25	210	82		
Calf collagen, 1.0	COL; 10, 10, 10	73	29	104[e]	0.82[f]
	PRN; 30, 30, 10	168	64		0.86
	LAP, 1; PRL, 10	191	79		
	CA, 5; CB, 0.25	210	80		
Human adult collagen, 0.3	COL; 5, 5, 5, 5	94	31	99[e]	0.76[f]
	PRN; 10, 10	165	47		0.80
	LAP, 0.75; PRL, 5	192	80		
	CA, 2.5; CB, 0.25	212[g]	86		
Human infant collagen, 0.3	COL; 5, 5, 5, 5	94	38	99[e]	1.1[f]
	PRN; 10, 10	165	58		1.1
	LAP, 0.75; PRL, 5	192	89		
	CA, 2.5; CB, 0.25	212	89		
Pepsin, 0.5	VK; 13, 13	121	69		0.12
	LAP, 0.5; PRL, 5	165	79		
	CA, 2.5; CB, 0.25	256	*h*		

[a] All analytical values corrected for sampling aliquots. Enzyme blank values subtracted. [b] LAP added was 100–150 units/g of substrate. CB, 45–60 units/g. [c] Followed with ninhydrin reaction (AutoAnalyzer) or, with pepsin, the Van Slyke α-amino nitrogen method. Complete hydrolysis taken as that obtained with 6 N HCl at 120° for 18 hr. [d] Commercial source. [e] Asp([^{14}C]Gly) added after digestion. [f] Duplicate chromatographic analyses. [g] Undissolved residue of 20.7 mg (dry) could not be solubilized by further treatment with collagenase. [h] Analytical sample lost.

tubes and treated overnight with 0.4 ml of 70% ethanol to sterilize the peptide. After the alcohol had been taken off *in vacuo* 1 ml of sterile 0.1 M Tris buffer, pH 7.4, was added to each tube. Tubes were kept at 37° and at −20° until they were checked for sterility and analyzed by paper chromatography and strip scanning.

Similar incubations were performed with 2.5 μmoles of Asn-Gly/tube. These were analyzed semiquantitatively by comparison of the ninhydrin spots with those of Asp(Gly) and Asn-Gly chromatographed on the same paper.

Separation and Analyses. Digests of hemoglobin and of Achilles tendon from a 67-year-old man, which contained sediments, were centrifuged; the precipitates were washed with water and the washings were added to the supernatant fluid. All digests were desalted on Dowex 50 and the acidic ampholytes were then isolated with Dowex 2 columns as previously described (Buchanan *et al.*, 1962a) except that the fraction of interest, hereafter designated as the acidic fraction, was displaced with 1 M formic acid. To reduce in bulk the two predominant compounds of the acidic fraction, aspartic and glutamic acids, the material displaced with formic acid was taken to dryness and dissolved in a medium containing: KH_2PO_4, 0.035 M; NaCl, 0.02 M; and $MgSO_4$, 0.002 M. The concentration of aspartic plus glutamic acids was approximately 1 mg/ml. After the pH had been adjusted to 7.0 with NaOH, the solution was sterilized with a Morton filter and inoculated with a strain of chromobacteria of the pseudomonad type. This strain, designated in this laboratory as organism 12, is one of several microorganisms selected from a soil sample by their ability to proliferate on L-aspartic acid as the sole carbon and nitrogen source when cultured in the inorganic medium described above. This strain also metabolizes and destroys several other amino acids including glutamic acid, but does not attack Asp(Gly). It will neither

grow on solutions of the peptide in the inorganic medium, nor reduce the ninhydrin color or radioactivity of the compound. Slant cultures on the above inorganic medium plus 0.5% L-aspartic acid and 2% agar are transferred every 2 weeks.

In each instance the inoculum, representing about 0.1 ml of packed organisms, was prepared by suspending two 48–72-hr slant cultures in 4 ml of sterile 0.9% NaCl. After incubation of the acidic fraction at room temperature for about 65 hr the organisms were removed by centrifugation and washed once with isotonic saline. The supernatant fluid and washings were desalted as before. The microorganisms reduced the aspartic and glutamic acid concentrations by more than 90%.

The amino acid and peptide mixture was dissolved in water or dilute aqueous pyridine and passed through a 0.6 × 30 cm column of Dowex 2-X8 (acetate) eluted with 0.4 M acetic acid at 0.5 ml/min. Forty 5-ml fractions were collected and each was taken to dryness and dissolved in 0.1 ml of water. Each (1 μl) was subjected to thin layer chromatography, and to ensure the inclusion of all of the peptide, aliquots of fractions adjacent to visible Asp(Gly) were counted. The fractions that contained Asp(Ala) were also pooled, the two peptides having separated completely on Dowex 2.

Aliquots of the pooled fractions, representing 20–33% of the digest, were subjected to analytical column chromatography. Glycine (0.50 μmole) was added as an internal standard. The fractions containing the peptides of interest and the standard were appropriately combined and desalted. Each compound was found to be homogeneous by thin layer chromatography and identical with its standard by analytical and thin layer chromatography. Acid hydrolysis of each peptide yielded equimolar quantities of the constituent amino acids by analytical chromatography. Analytical values were corrected for sampling aliquots, to complete recovery of the added radioactivity, for the added labeled peptide, and for the enzyme blank.

Results

Hemoglobin. In preliminary experiments with crude, red cell lysate and with twice-crystallized hemoglobin, the acidic fraction was incubated with organism 12 and the desalted mixture was then subjected to two-dimensional chromatography. The predominating blue ninhydrin spot was Asp(Gly); another was shown to be Asp(Ala), and a third remained unidentified.

When hemoglobin incubations were analyzed quantitatively (Table I), from 0.02 to 0.08 mole of Asp(Gly) was found/mole of protein (mol wt 63,000) or 0.3–1.2 μmoles/g of protein. Hemoglobin has but one aspartylglycyl sequence in each of its two β chains and has no asparaginylglycyl units (Konigsberg *et al.*, 1962, 1963). If the Asp(Gly) that was found originates from this sequence in hemoglobin the percentage of the aspartyl linkage in question that exists as, or is converted to, the β configuration may be 4% or more. The enzymic hydrolyses were never complete and there appears to be some correlation between the peptide found and the extent of hydrolysis.

The amount of Asp(Ala) present was always less than that of Asp(Gly). There are four aspartylalanyl and six asparaginylalanyl sequences per molecule of hemoglobin. Only 0.12–0.24% of the total was isolated with the β configuration by these methods. The β-aspartyl peptide content of the protein in the mother liquor of the hemoglobin crystallization was not significantly different from that of the crystals, evidence that, if the β linkage exists prior to hydrolysis, the molecules that contain it are not excluded during crystal formation.

Collagen. This protein was studied mainly to test the effect of *in vivo* aging on Asp(Gly) content. From 0.8 to 1.9 moles of Asp(Gly) was found/mole (mol wt 300,000) of collagen, or 2.6 to 6.2 μmoles/g of protein (Table II). There were no significant differences between the tendons of young and old cattle or humans. In one experiment where labeled Asp(Gly) was added before and in another where it was added after the digestion, the recoveries of the radioactive peptide were 86 and 85% and the amounts of Asp(Gly) found were 1.3 and 1.5 moles/mole of collagen. Tendon collagen has a high content of glycine (Neuman, 1949; Eastoe, 1955) but because no information is available on the number of aspartyl- and asparaginylglycyl linkages in the molecule a calculation of the "yield" of Asp(Gly) is not possible.

Lysozyme. The digests of this protein had the lowest Asp(Gly) content of any of the proteins tested (Table I). Based on four aspartylglycyl sequences per molecule, and no asparaginylglycyl linkages (Canfield, 1963), the recovery as the β-peptide was 0.6–0.8 μmole/g of protein, or 0.2–0.3% of the total possible. The extent of hydrolysis of lysozyme was considerably less than that of the other proteins. *In vitro* aging for 69 days did not increase the measured quantity of Asp(Gly), while in the dipeptide control experiments there was substantial isomerization.

Pepsin. On two-dimensional chromatography, the pepsin digest revealed Asp(Gly) and two other blue ninhydrin spots. Quantitative analysis revealed only 0.12 mole of Asp(Gly)/mole of pepsin (3.3 μmoles/g of protein) (Table II) making it unlikely that β-aspartyl linkages contribute to the acidic nature of pepsin.

Control Experiments. Hydrolysis of enzyme proteins in the absence of substrates yielded small amounts of Asp(Gly). For the enzymes used to digest each protein, the control levels were equivalent to 0.0054 mole/mole of hemoglobin, 0.085 mole/mole of collagen, and 0.00049 mole/mole of lysozyme. The values in Tables I and II for experiments with protein substrates have had these quantities subtracted. It is noteworthy that at least one, and perhaps more, of the enzyme proteins release Asp(Gly) when hydrolyzed. On a weight basis, the recovery of this peptide from the mixed enzyme protein was close to that from hemoglobin.

When Asp-Gly was incubated for 6 and 34 days, under the same conditions as those used to study

the aging of lysozyme, 3 and 17% converted to Asp-(Gly). No detectable conversion occurred at −20°. With Asn-Gly approximately 20% converted to Asp-(Gly) in 4 days.

To test the extent of isomerization of Asp-Gly that might occur as this fragment is released, we added α-L-aspartyl[^{14}C]glycine (0.67 μmole and 5.3 × 10^6 cpm) to 1 g of crystalline hemoglobin before incubation. More than 90% of the radioactivity was found in the fraction that contained glycine and only 0.46% was isolated with Asp(Gly). This conversion is approximately 10% of that found with hemoglobin and consistent with nonenzymatic isomerization.

We have no evidence that Asp(Gly) is loosely bound to hemoglobin. The peptide was not detected in the TCA-soluble fraction of erythrocyte lysate nor was any removed by passage of the lysate through Dowex 50. More conclusive was the failure to detect Asp(Gly) with either analytical chromatography of hemoglobin after 30% hydrolysis with Viokase or with two-dimensional chromatography after 63% hydrolysis with Pronase. Finally, we dialyzed erythrocyte lysate against 0.9% NaCl for 45 hr at 5°, desalted the dialysate, and isolated the acidic fraction. No Asp(Gly) was found in a sample which, if hydrolyzed by enzymes, would have yielded 75 times a detectable quantity. The possibility that Asp(Gly) cannot be separated from hemoglobin by dialysis was excluded by adding 0.26 μmole and 2.08 × 10^6 cpm of tracer peptide to 10 ml of lysate (12% hemoglobin). Dialysis against two 190-ml changes of saline reduced the radioactivity of the lysate below 500 cpm.

The formation of β-aspartylglycyl linkages in hemoglobin by transamidation between asparagine residues and glycine ethyl ester was not catalyzed by plasma transglutaminase (Table I) (Loewy *et al.*, 1966). Although 5 moles of labeled glycine ethyl ester was transferred/mole of hemoglobin, no radioactivity was found in the Asp(Gly) isolated from the enzymatic hydrolysate. The analytical value for Asp(Gly) agrees with the other experiments.

Discussion

Our primary aim has been to explain the presence of the β-aspartyl di- and tripeptides in urine (Buchanan *et al.*, 1962a). Although individuals getting no protein foods excreted substantial quantities of β-aspartyl peptides, especially Asp(Gly), the addition of protein to the diet increased the daily excretion of the β-aspartyl peptides that can be measured quantitatively (Dorer *et al.*, 1966). This suggests that these peptides may arise during protein hydrolysis, the endogenous portion resulting from protein turnover and the exogenous increment accompanying the digestion of protein food. If tissue and dietary proteins are a major source of these compounds the question of greatest interest is whether β-aspartyl linkages exist in the protein or are formed during its degradation by hydrolytic enzymes.

The data presented here do not support the stoichiometric existence of β-aspartyl bonds in any of the proteins studied. It seems likely that if enzymes are capable of releasing any Asp(Gly) they should be able to free the major portion on prolonged incubation. Even more convincing are the analytical results (Konigsberg *et al.*, 1962, 1963; Canfield, 1963) on the aspartylglycyl sequences of hemoglobin and lysozyme. But if only small fractions of aspartylglycyl or other aspartyl sequences exist with the β configuration, they could have been missed by the methods employed. The hypothesis that proteins might be formed with predominantly α- but with occasional β-aspartyl linkages is not attractive. That α-aspartyl and -asparaginyl bonds, once formed, may convert to β-aspartyl linkages at least has precedent with oligopeptides. Asn-Gly, under conditions regarded as physiological, converts to Asp(Gly) at about 5%/day. More relevant to the findings with lysozyme, which has no asparaginylglycyl sequence, was the observation that Asp-Gly isomerized to Asp(Gly) at about 0.5%/day while aging of the protein caused no measurable change.

Collagen has been shown to have a very slow rate of turnover (Neuberger and Slack, 1953; Thompson and Ballou, 1956). That the enzymatic breakdown of tendon collagen from a 67-year-old man liberated no more Asp(Gly) than that found in the same tissue at birth suggests that isomerization does not occur in the intact protein. However, it is still possible that other changes take place as collagen ages (Harrington and Von Hippel, 1961) that make the liberation of β-aspartyl peptides by enzymatic hydrolysis more difficult.

Yet another possibility for the presence of β-aspartyl linkages in protein is the adventitious attachment by transamidation *in vivo* of free α-amino groups of small metabolites to the amide group of asparaginyl residues. It is conceivable that during the original purification small numbers of protein molecules with short β-aspartyl side branches could have cocrystallized with the unaltered protein. Enzymatic hydrolysis by our procedures would again cleave all but the β-aspartyl bond and liberate the dipeptide. Precedent for this type of reaction is found in the formation of γ-glutamyl linkages between glutamine residues of glucagon and glycine ethyl ester catalyzed by plasma transglutaminase (Loewy *et al.*, 1966). That this enzyme catalyzes also transamidation between the amino-terminal glycine amino groups of one fibrin monomer and the carboxylamide group of asparagine or glutamine of another has been suggested (Loewy *et al.*, 1964). Our analysis of a sample of crystalline hemoglobin that had, in Loewy's laboratory, incorporated considerable radioactivity when incubated with labeled glycine ethyl ester in the presence of plasma transglutaminase showed no radioactivity in the isolated Asp(Gly). It is still possible that other enzymes exist which can promote analogous reactions with asparaginyl residues. Pisano (1966) has found Asp(Gly) in enzymatic digests of insoluble fibrin as well as in collagen. He did not report analyses of fibrinogen.

Definitive answers will depend on better methodology.

That β-aspartyl bonds seem refractory to attack by several endo- and exopeptidases improves the chance of isolating and characterizing pertinent segments of protein molecules. Purely chemical procedures, such as esterification and reduction or amide formation and hypochlorite oxidation, before hydrolysis (Chibnall and Rees, 1958; Kandel *et al.*, 1954) or hydrazinolysis (Akabori *et al.*, 1952), may require conditions drastic enough to produce isomerization or other artifacts.

Whether β-aspartyl linkages exist in proteins or are by-products of enzymic hydrolysis, it is reasonable that the quantities of β-aspartyl peptides excreted in urine could originate from body protein turnover and from dietary protein. With an erythrocyte life span of 120 days the expected daily output of Asp(Gly) from hemoglobin alone by an individual with 6 l. of blood would be 7 μmoles. The actual daily excretion by individuals receiving no protein is 45–90 μmoles. Similarly one-tenth of the daily output of 5–15 μmoles of Asp(Ala) could arise from erythrocyte turnover alone.

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