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The Glutamyl Linkages in Collagen*

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ABSTRACT: In the preceding paper (Bensusan, 1969) it was shown that the sequential digestion of collagen with collagenase, papain, and prolidase plus leucine aminopeptidase resulted in the release of essentially all of the glutamic acid residues as free glutamic acid. None of these enzymes is known to hydrolyze γ-glutamyl linkages. Since the presence of a relatively large number of such linkages has been reported previously (Franzblau *et al.*, 1963), a resolution of the apparent discrepancy was deemed necessary. Preliminary experiments with the enzymes showed them incapable of hydrolyzing the γ-glutamyl peptide linkage in γ-glutamylalanine, γ-glutamylglutamic acid, or glutathione. The free carboxyl groups of ichthyocol gelatin were modified by coupling them with L-alanine methyl ester. When this derivative was subjected to the enzymic hydrolysis, it was found that only 2.6 residues out of a possible 50 residues of glutamic acid appeared in the digest. This demonstrated not only that the γ-carboxyl groups are free in ichthyocol but that the enzymes do not hydrolyze γ-glutamyl linkage. The method used by Franzblau *et al.* (1963) to demonstrate the presence of γ-glutamyl linkages in ichthyocol was then applied to ichthyocol, oxidized ribonuclease, oxidized insulin A, and oxidized insulin B. The results with ribonuclease and insulin B chains indicated the presence of γ-glutamyl linkages. Insulin A chains gave no reaction. A similar method based on the report by Hoare *et al.* (1968) was investigated using ichthyocol and salt-soluble collagen. The results showed that all reacting free carboxyl groups of glutamyl residues were the γ-carboxyl groups. It was concluded that essentially all the glutamyl residues in collagen are involved in the normal α-glutamyl linkages.

Gallop *et al.* (1960) first reported the possible existence of γ-glutamyl linkages in collagen. Their technique involved the esterification of the free carboxyl groups and then the conversion of the esters into hydroxamates with neutral hydroxylamine. The hydroxamates were treated with 1-fluoro-2,4-dinitrobenzene (FDNB)[1] to form the dinitrophenylhydroxamates, which, when heated in strong alkali, undergo the Lossen rearrangement to yield an amine and carbon dioxide. An α-linked glutamyl residue would be expected to yield 2,4-diaminobutyric acid by this treatment, whereas a γ-linked glutamyl residue would yield succinic semialdehyde which could be identified as the 2,4-dinitrophenylhydrazone derivative. These investigators found that 35 residues of glutamic acid had reacted but that only 8.4 residues of 2,4-diaminobutyric acid had been produced. A positive identification of succinic semialdehyde was made. They cautiously recognized the possibility that the treatment of the esterified gelatin with hydroxylamine could result in an intramolecular carboxyl group exchange which would give false results. With this in mind, they formed the hydroxamates of the free carboxyl groups at approximately pH 4 by treating the gelatin with a water-soluble carbodiimide in the presence of hydroxylamine (Franzblau *et al.*, 1963). When the dinitrophenylation and Lossen rearrangement were carried out on these hydroxamate derivatives of ichthyocol, they found that 25 residues of glutamic acid were lost and that 4 residues of 2,4-diaminobutyric acid and 22 residues of succinic semialdehyde were formed on acid hydrolysis, indicating that a large majority of the glutamyl residues are involved in γ-glutamyl linkages.

Joseph and Bose (1960) used a sodium hypobromite oxidation of trypsin digests to investigate possible γ-glutamyl linkages. The succinic acid which would be produced from the oxidation of N-terminal glutamyl residues involved in γ-linkages was determined. These investigators found 1.3 and 0.14 residues of succinic acid in buffalo hide collagen and alkali-processed gelatin, respectively.

Bensusan (1969), using an enzymic digestion technique

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[1] Abbreviations used are: CMC, 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)carbodiimide metho-*p*-toluenesulfonate; EDC, 1-ethyl-3-dimethylaminopropylcarbodiimide; FDNB, 1-fluoro-2,4-dinitrobenzene.

involving collagenase, papain, prolidase, and leucine aminopeptidase, found that all the glutamic acid was free in the hydrolysates. Since these enzyme are not known to hydrolyze γ-glutamyl bonds, the results suggest the absence of such bonds. However, the possibility exists that enzyme impurities present in the enzyme preparations could result in the hydrolysis of γ-glutamyl linkages either by promoting a carboxyl group interchange or by effecting the hydrolysis of such bonds directly.

This study was undertaken in an attempt to determine whether or not γ-glutamyl linkages do play a significant role in the primary structure of the collagen chains.

Material and Methods

Material. The collagen samples and enzymes were the same as in the preceding paper (Bensusan, 1969). The 1-cyclohexyl-3-[2-morpholinyl-(4)-ethyl]carbodiimide metho-*p*-toluenesulfonate (CMC) was purchased from the Aldrich Chemical Co. and 1-ethyl-3-dimethylaminopropylcarbodiimide (EDC) was obtained from the Pierce Chemical Co. Peptides were purchased from the Cyclo Chemical Co.[2] and the insulin and ribonuclease were obtained from Mann Research Laboratories. All other chemicals used were the best obtainable.

Enzymic Hydrolysis. The enzymic hydrolysis was carried out as previously described (Bensusan, 1969). When the glutamyl peptides were used, the substrate:enzyme ratio was calculated to be equivalent to that used in the digestion of collagen based on a glutamic acid content of 5%.

Alanylation of Carboxyl Groups. Ichthyocol gelatin (10 mg/ml) was treated with 1 M L-alanine methyl ester and 0.4 M EDC at pH 4.75 using a Radiometer pH-Stat for 4 hr at room temperature as described by Hoare and Koshland (1967). The reaction product was dialyzed for 18 hr in small diameter casing against 10° running tap water and then for 6 hr against deionized water. A portion was removed and hydrolyzed in constant-boiling HCl for 18 hr. The remainder was digested enzymically. An untreated control was digested under the same conditions at the same time as the alanylated sample.

Reactions with Hydroxylamine and Carbodiimides. METHOD 1. The series of reaction steps with hydroxylamine and CMC was carried out on ichthyocol gelatin, and the oxidized derivatives of ribonuclease, insulin A chains, and insulin B chains by following the method described by Franzblau *et al.* (1963) exactly. The single exception was that the protein concentration in each case was 6 mg/ml instead of 200 mg/ml in the initial reaction.

METHOD 2. Ichthyocol gelatin was warmed to 50° to liquify the gel and was then cooled to room temperature. The solution was made 1 M in hydroxylamine hydrochloride and 0.4 M in CMC or EDC by adding the solid reagents. The mixture was stirred at room temperature for 2 hr. The reaction mixture was then dialyzed extensively and hydrolyzed in constant-boiling HCl. Note that no attempt was made to control the pH of the reaction.

METHOD 3. This method was based on the investigations of Hoare *et al.* (1968) using model compounds. Liquified ichthyocol or salt-soluble calf gelatin (50 mg in 5 ml of water) was adjusted to pH 4.75 in a tube attached to a Radiometer pH-Stat. Solid hydroxylamine hydrochloride (5 mM) was added and dissolved quickly using a magnetic stirrer. The solid carbodiimide (2 mM) was added a small amount at a time, taking about 1 hr to add the first 25%, then the remainder was added quickly. The reaction was allowed to proceed for an additional 3 hr. The pH was maintained by the automatic addition of 1 M NaOH throughout the entire procedure. At the end of the reaction time, the product was dialyzed and hydrolyzed as before. Both CMC and EDC were used with ichthyocol gelatin and EDC was used with salt-soluble calfskin gelatin.

Amino Acid Analyses. Small aliquots (150–250 μl) of the dialyzed samples from methods 1–3 were diluted with 3 ml of constant-boiling point HCl and prepurified nitrogen was bubbled through the solution which was then frozen in a Dry Ice–acetone mixture. The tube was evacuated with a high-vacuum oil pump and sealed. Hydrolysis was carried out at 100° for 18–24 hr. Amino acid analyses were performed on a Technicon amino acid analyzer with the 5.5-hr gradient system of Thomson and Miles (1964). This gradient system was also used for the enzymic digests although the resolution of the peptide fractions was not as complete as with the 21-hr gradient system (Bensusan, 1969).

Analyses of the Products of the Lossen Rearrangement. The formation of the 2,4-dinitrophenylhydrazone derivative of aldehyde (presumably succinic semialdehyde) and the subsequent isolation and quantification were carried out as described by Franzblau *et al.* (1963). The 2,4-diaminobutyric acid was determined on the amino acid analyzer. A standard sample of 2,4-diaminobutyric acid was added to a sample of standard amino acids for the location of the peak and determinations of the color yield.

Results and Discussion

Hydrolysis of Glutamic Acid Peptides. The effect of the various enzymes on γ-glutamyl bonds was investigated. Glutathione, γ-L-glutamyl-L-alanine, γ-L-glutamyl-L-glutamic acid, and α-L-glutamyl-L-alanine were treated in the same way as was the gelatin in the enzymic digestion (Bensusan, 1969). The products were analyzed on the amino acid analyzer. The results showed that no free glutamic acid or alanine was produced from the two γ-glutamyl dipeptides. Free glycine, but no free glutamic acid, was seen in the enzymic digests of glutathione, whereas both alanine and glutamic acid were quantitatively liberated from the α-glutamyl peptide.

Enzymic Digestion of Alanylated Gelatin. The above demonstration that the enzymes will not hydrolyze γ-glutamyl linkages in peptides does not eliminate the possibility that they might hydrolyze these linkages in a polypeptide or protein. In order to test this possibility, the free carboxyl groups of ichthyocol gelatin were alanylated by the treatment of the protein with L-alanine methyl ester and EDC. Thus, if there are γ-glutamyl linkages in the gelatin which can be hydrolyzed by the enzyme mixture, their hydrolysis and the hydrolysis of the alanine methyl ester groups attached to free α-carboxyl groups should liberate all the glutamic acid. On the other hand, if the glutamyl residues are involved in α linkages and if the enzymes are unable to hydrolyze the γ-

[2] Lot 10840 which was labeled γ-L-glutamyl-L-alanine was the α-glutamyl peptide, whereas lot 10835, labeled α-L-glutamyl-L-alanine was the γ-glutamyl peptide as determined by titration studies.

linked alanine methyl ester groups, no free glutamic acid should be liberated in the enzymic hydrolysates. The results of this experiment are given in Table I.

The analysis of the acid hydrolysate of the alanylated ichthyocol (second column, Table I) shows that 81 extra alanine groups are present which is consistent with the value of 74 found for the sum of the glutamic acid and aspartic acid residues found in the enzymic digests as seen in Table VI of the preceding paper (Bensusan, 1969). Some of these alanine groups may be the result of a small amount of the alanine methyl ester which was not removed by dialysis. The enzymic digestion of the untreated control resulted in the liberation of 27 aspartic acid and 49 glutamic acid residues, in good agreement with our previous values (Bensusan, 1969). However, only 2.6 residues of glutamic acid were liberated in the enzymic digests of the alanylated ichthyocol. The ninhydrin-positive material eluting with hydroxyproline in the enzymic digest was isolated, acid hydrolyzed, and analyzed. This fraction was found to contain alanine, glutamic acid, and aspartic acid, presumably resulting from the hydrolysis of the methyl esters of β-aspartyl-L-alanine and γ-glutamyl-L-alanine. The results of this experiment demonstrate that at least 95% of the glutamyl residues in ichthyocol have free γ-carboxyl groups and that the enzymes used in the enzymic digestion *cannot* hydrolyze γ-glutamyl bonds.

Investigation of Reactions with Hydroxylamine and Carbodiimides. Gallop *et al.* (1960) and Franzblau *et al.* (1963) demonstrated the possible existence of γ-glutamyl linkages on the basis of a chemical technique. With the advent of a similar technique (Hoare *et al.*, 1968), a reinvestigation of the use of hydroxylamine and carbodiimides in the determination of glutamyl linkages appeared necessary. As a first step in this direction, ichthyocol and oxidized samples of ribonuclease, insulin A, and insulin B were treated by the method described by Franzblau *et al.* (1963). The results with ichthyocol were in good agreement with their values. A loss of 24 residues of glutamic acid was only partially accounted for by the appearance of 6 residues of 2,4-diaminobutyric acid. Approximately 20–25 residues of the 2,4-dinitrophenylhydrazone of "succinic semialdehyde" (*vide infra*) apparently accounted for the remaining glutamyl residues. In addition, 7 residues of threonine, 5 residues of serine, and all the tyrosine were lost as a result of the treatment. Insulin A chains showed no significant reaction of any kind despite repeated attempts. The results of the treatment using oxidized ribonuclease and oxidized insulin B are given in Table II.

The results in Table II show that the loss of 1.2 residues of the 5 residues of glutamic acid (not counting glutamine residues) in ribonuclease is accompanied by the production of 1.8 total residues of 2,4-dinitrophenylhydrazone derivatives. The loss of 0.3 residue out of a possible 2 residues of glutamic acid in insulin B is accompanied by the production of 0.3 residue of dinitrophenylhydrazones, indicating γ-glutamyl linkages, since no 2,4-diaminobutyric acid was found in either case as would be expected from α-glutamyl linkages. These data indicate that their technique is not reliable for the determination of glutamyl linkages if one assumes that these proteins contain no γ-glutamyl linkages. Although they did show a loss of serine and threonine in their determinations, they did not consider the differences to be significant. The loss of tyrosine was considered significant. The results of Table II show significant losses of the amino acid residues containing hydroxyl groups. Such losses are consistent with the demonstration by Carraway and Koshland (1968) that tyrosyl residues will form *O*-arylisoureas with carbodiimides which are partly resistant to cleavage with hydroxylamine and very resistant to acid hydrolysis. The possible production of 2,3-diaminopropionic acid was not determined because of its low ninhydrin color yield and its incomplete resolution from lysine. The remaining losses are accounted for by the formation of dinitrophenyl derivatives of the N-terminal and ε-amino groups of lysine residues and by the reaction of the free carboxyl group of the C-terminal residue. The reason for the low value of cysteic acid in the control sample of ribonuclease is unknown.

Thin-layer chromatography of the 2,4-dinitrophenylhydrazones of the aldehydes produced on acid hydrolysis of treated samples of ribonuclease and insulin B showed a major hydrazone which cochromatographed with the single hydrazone from ichthyocol (R_F of 0.47 in 1-butanol saturated with

TABLE I: Enzymic Digestion of Ichthyocol after Alanylation of the Free Carboxyl Groups.

	Content (μmoles/1000 μmoles)			
	Acid Hydrolyzed		Enzyme Hydrolyzed	
	Control	Alanylated[a]	Control[b]	Alanylated[b]
Hydroxyproline	78	85	52	*c*
Aspartic acid	46	47	27	6.8
Thr + Ser + amide	63	64	92	92
Glutamic acid	80	79	49	2.6
Proline	118	113	41	56
Glycine + (1)[d]	327	314	276	290
Alanine + (2)	135	216	140	174
Valine + (4)	14	13	21	21
Methionine	12	12	14	13
Isoleucine + (9)	8.2	7.2	12	13
Leucine + (10)	20	20	28	27
Tyrosine + (15)	2.9	2.1	7.4	4.6
Phenylalanine	14	14	12	12
Lysine	24	27	25	21
Histidine	3.5	3.4	4.8	4.0
Arginine	49	51	48	49
Hydroxylysine	5.9	6.8	5.4	3.9
Ornithine	1.8	1.2	2.2	1.9
Gly-Pro-Hyp			33	31
Arg peptides			3.2	2.2

[a] The value of alanine was assumed to be 135 residues/1000 residues in arriving at the total number of micromoles. [b] The values of μmoles/1000 μmoles were calculated by assuming the arginine (including that in peptides) to be 51 μmoles/1000 μmoles. [c] Contaminated by additional fraction (see text). [d] The number in parentheses refers to the peptide (Bensusan, 1969) accompanying the amino acid in the analyses of enzymic digests.

TABLE II: Products of Ribonuclease and the B Chain of Insulin Treated According to Franzblau *et al.* (1963).

	Content (Residues/Chain)					
	Ribonuclease			Insulin B		
	Control	Exptl	Diff	Control	Exptl	Diff
Cysteic acid	5.4	7.4	+2.0	1.94	1.99	
Aspartic acid	14.4	14.9		1.09	0.70	−0.39
Threonine	9.5	8.8	−0.7	1.01	0.56	−0.45
Serine	13.9	13.9		1.10	0.65	−0.45
Glutamic acid	13.2	12.0	−1.2	3.16	2.86	−0.30
Proline	4.1	4.5		1.08	1.16	
Glycine	3.8	3.6		2.97	3.54	+0.54
Alanine	12.4	12.2		1.98	1.72	−0.26[a]
Valine	7.0	6.4	−0.6[a]	3.00	3.00	
Methionine	3.6	4.0				
Isoleucine	1.4	1.2				
Leucine	2.2	2.6		3.98	3.96	
Tyrosine	5.7	1.5	−4.2	1.95	1.79	−0.16
Phenylalanine	2.8	2.5		2.90	2.55	−0.35[b]
Lysine	11.3	8.8	−2.5[b,c]	0.96	0.29	−0.67[c]
Histidine	2.9	2.7		1.86	1.80	
Arginine	3.9	3.9		0.96	0.99	
DNP-lysine		2.5	+2.5		ND	
2,4-Diaminobutyric acid		0			0.04	+0.04
2,4-Dinitrophenylhydrazones		1.8	+1.8		0.27	+0.27
Unknown		2.8	+2.8		0	

[a] C-Terminal amino acid reacting as a free carboxyl group. [b] N-Terminal amino acid reacting with FDNB. [c] Free ε-amino groups reacting with FDNB.

water). In addition, ribonuclease gave an additional hydrazone which cochromatographed with the 2,4-dinitrophenylhydrazone of succinic semialdehyde (R_F of 0.75). The 2,4-dinitrophenylhydrazone of succinic semialdehyde, synthesized by the method of Hendler and Anfinsen (1954), had the reported melting point (201°) and a mixture melting point of 160–180° with 2,4-dinitrophenylhydrazine (mp 194°). The isolated hydrazone from ichthyocol had a melting point of 111°. The term "2,4-dinitrophenylhydrazone" was used in Table II because of the uncertainty of its true identity. Although I could not establish an identity between the hydrazone isolated as a product of the Lossen rearrangement and the hydrazone of succinic acid semialdehyde, it is entirely possible that Franzblau *et al.* (1963) did indeed recover this aldehyde.

Hoare *et al.* (1968) showed that carbodiimides could effect the Lossen rearrangement of hydroxamic acids at pH 4.75 with the resultant formation of the amine derivative or an aldehyde in the case of the free amino acid hydroxamate. These results could afford a possible explanation of the unusual results obtained by Franzblau *et al.* (1963). Thus, when they treated ichthyocol with hydroxylamine and CMC, the hydroxamates formed would spontaneously undergo the Lossen rearrangement to form γ-amino groups from the glutamyl residues. When they next treated the reaction product with FDNB, the DNP derivative would be formed and would then be missed in the acid hydrolysates. For this reason, experiments were conducted treating ichthyocol with hydroxylamine and CMC or EDC without controlling the pH of the reaction mixture (method 2) as in their experiments. The results of the analysis of the acid hydrolysates of the products are given in Table III, second and fourth columns. It can be seen that no significant reaction occurred with CMC but that 6 residues of glutamic acid are lost and 8.5 residues of 2,4-diaminobutyric acid are formed when EDC was used. Since CMC was the carbodiimide employed by Franzblau *et al.* (1963), it is difficult to determine whether or not the explanation offered above is a reasonable one.

The results varied in the experiments in which the pH was maintained at 4.75, as described for method 3 in the Methods section. According to the results shown in the third column of Table III, maintenance of a constant pH did not improve the extent of the reaction involving CMC. However, when EDC was the carbodiimide used, the loss of glutamic acid was accompanied by the production of an equivalent amount of the expected 2,4-diaminobutyric acid from both ichthyocol (fifth column) and salt-soluble collagen (last column). With ichthyocol and EDC (fifth column) approximately 40–50% of the 49 available glutamic acid residues (Table I) reacted to completion. In a personal communication, D. E. Koshland, Jr. (1969) stated that similar yields were obtained in his laboratory with ribonuclease and lysozyme in the presence of urea. In any event, these results show that there are no appreciable γ-glutamyl linkages in collagen, in agreement with the results obtained by enzymic digestion.

TABLE III: Products of the Reaction of Salt-Soluble Collagen and Ichthyocol Treated According to Hoare *et al.* (1968).

	Content (Residues/1000 Residues)						
	Ichthyocol					Salt Soluble	
	Control	CMC[a]	CMC[b]	EDC[a]	EDC[b]	Control	EDC[b]
Hydroxyproline	89	84	88	87	87	103	105
Aspartic acid	51	47	49	49	41	47	40
Threonine	29	28	29	31	30	18	20
Serine	40	38	43	40	41	36	38
Glutamic acid	80	80	79	74	56	82	67
Proline	100	112	101	101	102	129	114
Glycine	318	314	321	310	322	300	321
Alanine	129	128	127	131	129	113	117
Valine	13	13	13	14	18	17	17
Methionine	14	12	12	12	12	5.9	4.3
Isoleucine	7.5	7.1	7.6	8.1	6.6	9.3	9.4
Leucine	21	22	20	22	23	26	25
Tyrosine	3.2	3.1	2.3	2.1	2.1	2.2	1.3
Phenylalanine	14	15	14	14	11	14	10
Lysine	27	28	26	29	30	30	31
Histidine	3.4	3.6	3.9	3.1	3.5	4.9	4.1
Arginine	52	55	50	53	55	52	52
Hydroxylysine	6.8	6.7	6.7	7.3	5.7	7.9	7.5
Ornithine	0.8	0.9	1.0	1.5	1.0	1.0	1.0
2,4-Diaminobutyric acid		1.0	1.0	8.5	21		15

[a] The pH of the reaction was not stabilized (method 2). [b] The pH of the reaction was stabilized at pH 4.75 (method 3).

The results of the experiments presented in this and the preceding paper (Bensusan, 1969) do not eliminate the possibility that there may be a small number of, perhaps as many as three, γ-glutamyl linkages in collagen. On the other hand, there is no unequivocal evidence at this time to suggest that such linkages do exist.

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