Acknowledgments

The excellent technical assistance of Mrs. Y. Zaugg and Miss U. Müller is gratefully acknowledged. Thanks are due to Dr. H. Brandenberger, Gerichtlich-medizinisches Institut der Universität Zurich, who carried out the mercury determinations.

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ε-(γ-Glutamyl)lysine Cross-Linkage in Citrulline-Containing Protein Fractions from Hair*

Harry W. J. Harding and G. E. Rogers

ABSTRACT: The presence of ε-(γ-glutamyl)lysine as a cross-link in citrulline-containing protein fractions of medulla cells of hair and quill and the inner root sheath cells of hair follicles has been demonstrated by both chemical and enzymic methods. The chemical method determines the number of ε-amino groups of lysines that do not react with acrylonitrile under vigorous conditions. In the enzymic method the cross-link is measured directly by amino acid analysis of complete enzymic digests. Good agreement has been obtained between the two methods. As much as 13 moles of ε-(γ-glutamyl)-lysine/1000 moles of amino acid residues is present in the hair medulla protein. Lesser amounts are detected in the other proteins. The nature of the cross-link has been confirmed by isolation of the new compound appearing in a complete enzyme digest of hair medulla protein and its characterization as ε-(γ-glutamyl)lysine.

The proteins from the cells of inner root sheaths of hair follicles and medullae from hair and related structures such as porcupine quills have been shown to contain citrulline (Rogers, 1962, 1964; Bradbury and O'Shea, 1969). The cells in these tissues (Figure 1) are packed with the respective proteins as are the cortical cells with keratin. Recent work including limited amino acid sequence studies of peptides from porcupine quill medulla has demonstrated that the citrulline is covalently bound in peptide linkage in the proteins (Steinert *et al.*, 1969). In addition to containing citrulline these proteins are characterized by a very high glutamic acid content and a very low cystine–cysteine content and are therefore quite distinct from the surrounding keratin (Rogers, 1962).

The study of these proteins has consistently demonstrated their extreme insolubility in the usual protein solvents, including those used to extract keratin (Matoltsy, 1953; Rogers, 1964). In fact, methods for preparing medullary cells have relied upon this insolubility and have involved drastic conditions to remove the keratin (Matoltsy, 1953; Bradbury and O'Shea, 1969). Further, medullary cells that have been obtained from guinea pig hair using mild methods for the dissolution of the cortical keratin (Gillespie, 1964; Maclaren and Kilpatrick, 1969) have recently been shown to resist solution even under vigorous conditions such as extremes of pH and salt concentration, high concentrations of dissociating agents (urea and guanidine hydrochloride), reducing and oxidizing conditions, organic solvents, and detergents. Indeed, only reagents which cause peptide-bond cleavage, *e.g.*, dilute acid hydrolysis, will dissolve the protein even after the cell membrane has been disrupted (unpublished experiments).

The proteins are, however, readily solubilized by proteolysis (Stoves, 1945; Rogers, 1964), again in contradistinction to keratin. Rogers (1962, 1964) has taken advantage of this phenomenon and developed a method employing digestion with crystalline trypsin to release the proteins. The resultant digest contains a complex mixture of tryptic polypeptides which, although water soluble, have not proved to be very

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amenable to further fractionation. A crude fractionation yielding a citrulline-enriched fraction (formerly referred to as "tryptic core") can be achieved by acid precipitation (Rogers, 1962) but neither DEAE-cellulose chromatography nor gel filtration chromatography provide satisfactory fractionation. All fractions exhibit a large range of N-terminal amino acids. Gel filtration on calibrated Sephadex columns has indicated that many of the tryptic polypeptides from guinea pig hair medulla cells are similar in size with apparent molecular weights in the region of 10,000–20,000. Some are considerably larger and are excluded from Sephadex G-200, *i.e.*, having molecular weight greater than 500,000 (unpublished experiments).

In view of the large size of the tryptic polypeptides, their wide range of N termini and the insolubility of the original protein, it was postulated that the proteins were covalently cross-linked. Since the insolubility of the proteins in strong reducing and dissociating agents and the low value for half-cystine (about 10 residues/1000) excludes disulfide bonds as being significant in cross-linking, a search for other cross-links was undertaken. In initial studies (unpublished data) analyses of acid hydrolysates of samples that had been reduced with sodium borohydride by analogy with the method applied to collagen by Blumenfeld and Gallop (1966) and to elastin by Lent and Franzblau (1967), failed to reveal the presence of an aldehyde-type cross-link. However, the presence of a group blocking the ε-amino groups of some lysine residues was indicated since some of these residues failed to react when the tryptic polypeptides were treated with acrylonitrile. This technique has been recently used with success by Pisano *et al.* (1969) for the detection of ε-amino cross-links in insoluble fibrin.

This paper reports the finding of ε-amino cross-links in the citrulline-containing protein fractions of medulla cells from hair and quill and the inner root sheath cells of hair follicles. For identification of the cross-link, tryptic polypeptides of guinea pig hair medulla protein were extensively degraded with proteolytic enzymes and the products were separated by ion-exchange chromatography. A new compound, which initially was present as a shoulder on the trailing edge of the leucine peak, was purified and subsequently characterized as ε-(γ-glutamyl)lysine.

Materials and Methods

Materials. Hair was clipped from albino guinea pigs, cleaned by standard procedures of solvent and water extraction, and dried and ground in a Wiley mill to through −40 mesh size. The inner root sheaths of guinea pig hair follicles and the medulla tissue of porcupine quills were prepared as described previously (Steinert *et al.*, 1969).

Crystalline trypsin (minimal chymotrypsin content) was purchased from Mann Research Laboratories, New York, and α-chymotrypsin from Worthington Biochemical Corp., Freehold, N. J. Pronase (B grade) was obtained from Calbiochem, Los Angeles, Calif. Leucine aminopeptidase (hog kidney, type III), carboxypeptidase A (bovine pancreas), carboxypeptidase B (porcine pancreas), and pepsin (three-times crystallized) were all purchased from Sigma Chemical Co., St. Louis, Mo.

Synthetic γ-L-glutamyl-ε-L-lysine·H_2O (Grade 1) and α-L-glutamyl-L-glutamic acid acetate (Grade 1) were both obtained from Cyclo Chemical Corp., Los Angeles, Calif. Acrylonitrile was purchased from BDH, Poole, England, and triethylamine from Ajax Chemicals, Sydney, Australia. Pyridine was refluxed with ninhydrin before redistillation (Hill and Delaney, 1967).

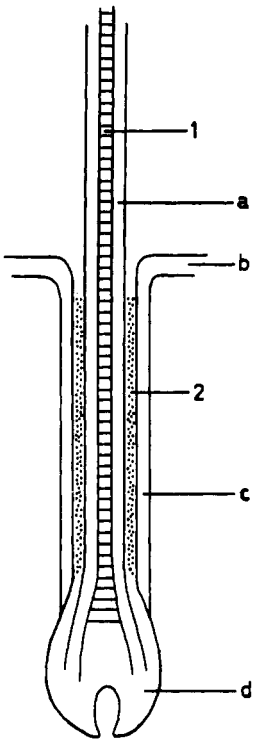

FIGURE 1: Diagrammatic representation of a hair follicle and emergent hair indicating the location of the cellular parts of the structure that were used in the present study: (1) medulla layer of the hair (originates in the follicle itself), (2) inner root sheath (present only in the follicle). a, b, c, and d, respectively, indicate the cortex of the hair, the epidermis of the skin, the follicle outer root sheath, and the follicle bulb.

Preparation of Tryptic Peptides. Ground hair was evenly suspended in water to a concentration of 5% (w/v) by degassing and then digested with trypsin (0.5%, w/w) in a pH-Stat (Radiometer, Model TTT Ic) at pH 8 and 37° using 1 N NaOH as titrant. At the completion of the reaction the insoluble keratin and cellular debris were removed by centrifugation. The supernatant was finally clarified by filtration through a 0.65 μ Millipore filter and freeze-dried.

The dried material was redissolved to a concentration of 1% (w/v) in aqueous pyridine (pH 7) and the acid-insoluble peptides precipitated by lowering the pH to 3.5 with glacial acetic acid. The precipitate was collected by centrifugation, washed with pH 3.5 pyridine–acetate buffer, and finally redissolved in aqueous pyridine and freeze-dried. The combined supernatants containing the pH 3.5 soluble tryptic peptides were also freeze-dried.

Porcupine quill medulla and guinea pig inner root sheaths were similarly digested with trypsin but no acid fractionation of the products was carried out in this study.

Digestion with Pepsin. The digestions with pepsin were carried out in 5% (v/v) aqueous formic acid at a substrate concentration of 0.5% (w/v) and an enzyme to substrate weight ratio of 1.5:100. Incubations were for 18–24 hr at 37°. The reactions were terminated by freeze-drying.

Digestion with Chymotrypsin. For the preparation of chymotryptic peptides for cyanoethylation experiments, substrates and enzyme were incubated at 37° at a weight ratio of 1:20 in 0.2 M *N*-ethylmorpholine acetate (pH 8.3).

TABLE I: Production of Buffer Gradients for Technicon Auto-Analyzer.[a]

Auto-grad Chamber	Routine Gradient (ml) pH of Buffer 2.96	3.80	5.00	Modified Gradient M4 (ml) pH of Buffer 2.96	3.80	5.00	10.00
1	70[b]			70[b]			
2	72[b]			72[b]			
3	75			75			
4	75			75			
5		70	5		75		
6	6	9	60		75		
7			75			75	
8			75			75	
9			75				75

[a] For details of buffer preparations, see text. [b] Chambers 1 and 2 also contained 5 and 3 ml of methanol, respectively.

The digests were freeze-dried after 26 hr. When chymotryptic digestion was used as a step in the complete enzymic digestion of the protein samples, the digestions were performed in 0.1 M Tris-Cl buffer (pH 8.0) at an enzyme to substrate weight ratio of 1:125.

Amino Acid Analyses. Acid hydrolysates of samples for amino acid analysis were performed in 6 N HCl *in vacuo* at 110° for 20 hr. The hydrolysate was evaporated to dryness in a rotary evaporator at 45°. Analyses were performed either on a Beckman 120C analyzer using the standard 4-hr system,[1] or on a single column Automatic Analyzer (Technicon) using gradient elution. The composition of the gradient routinely used is shown in Table I. The sodium citrate buffers were prepared as described by the manufacturer[2] except that the low pH buffer was adjusted to a pH of 2.96 rather than 2.875 and the composition of the pH 5.00 buffer was modified according to the procedure of Wells (1967).

Analyses for ε-(γ-glutamyl)lysine on the Technicon Analyzer were performed using the modified gradient M4 (Table I). The pH 10.00 buffer was prepared from pH 5.00 buffer by titration with anhydrous sodium carbonate. Norleucine was not used as an internal standard on the Technicon Analyzer in any analysis that involved carboxyethyllysine or ε-(γ-glutamyl)lysine since all these compounds eluted in the same region.

In these cases α-amino-β-guanidinopropionic acid was used as an internal standard.

Detection and Determination of Cross-Linking. REACTION WITH ACRYLONITRILE. This was carried out essentially according to the method of Pisano *et al.* (1969). The chymotryptic digests of the tryptic peptides were employed except in the case of hair medulla protein for which a peptic digest of the tryptic peptides was used. Samples (2–5 mg) were dissolved in 0.5–1 ml of aqueous triethylamine (3%, v/v). Acrylonitrile (0.2 ml) was added and the mixtures were incubated in sealed-glass tubes on a shaker at 37° for 100–144 hr. The contents were then evaporated twice at 50° under a stream of nitrogen and hydrolyzed for amino acid analysis. Controls used were native ribonuclease and the chymotryptic peptides of heat-denatured chymotrypsin.

COMPLETE ENZYMIC DIGESTION. For the direct determination of ε-(γ-glutamyl)lysine on an analytical level, approximately 3-mg samples of tryptic peptides were digested with pepsin and then chymotrypsin as described above. The chymotryptic digest (in 0.1 M Tris-Cl, pH 8.0) was then treated with pronase (2 mg) and Mg^{2+}-activated leucine aminopeptidase (0.2 mg) as described by Pisano *et al.* (1969). Following digestion with these enzymes, the mixture was made 0.2 M with respect to NaCl and 0.1–0.2 mg of carboxypeptidase A (solubilized according to Ambler, 1967) and 0.1 mg of carboxypeptidase B were added, and the digestion was continued at 37° for a further 24 hr. Each sample was then deproteinized with 1% (w/v) picric acid (Stein and Moore, 1954). After centrifugation to remove precipitated protein, the picric acid was removed from the supernatant on a small column of Dowex 2-X8 in 0.02 N HCl. The eluate was rotary evaporated to dryness.

Isolation of ε-(γ-Glutamyl)lysine. The pH 3.5 soluble tryptic peptides of guinea pig hair medulla were used for the isolation of ε-(γ-glutamyl)lysine. Enzymic digestion of 500 mg followed that described for the analytical determinations except that only one-half the relative amounts of pronase, leucine aminopeptidase, and carboxypeptidases A and B were used. The digest was deproteinized as above, rotary evaporated and redissolved in water. A control was run which contained all the enzymes but no substrates. Another control used native ribonuclease A as a substrate.

After samples had been removed for analysis, the solution was made 0.1 N with respect to HCl and applied to a 2.4 × 117 cm column of Bio-Rad AG 50W-X8 (100–200 mesh) equilibrated at room temperature in 0.2 M sodium citrate buffer (pH 2.96). Elution was carried out with 0.2 M sodium citrate (pH 3.85) at a flow rate of 150 ml/hr and 10-ml fractions were collected. Aliquots from each fraction were analyzed with ninhydrin. Although most of the material eluted as a large, tailed peak, amino acid analyses of aliquots from a number of fractions showed that some purification of the isodipeptide had been achieved. Fractions thus shown to contain this compound were desalted on a small Dowex 50-X8 column using 5 N NH_4OH. These eluates were rotary evaporated at 45°, dissolved in 0.1 N HCl and chromatographed on the Technicon amino acid analyzer column (Chromobeads type A, 0.6 × 130 cm) at 60°. The column had previously been calibrated with a standard mixture of amino acids. A sodium citrate gradient was used, similar to the M4 gradient already described for amino acid analysis except that chamber 9 of the Autograd contained pH 5.00 buffer and the citrate buffers themselves were made up without either Brij 35 or thiodiglycol. The total effluent was collected in 3-ml fractions and aliquots were removed for analysis with ninhydrin. Fractions containing the new ninhydrin-positive peak in the position of ε-(γ-glutamyl)lysine (after leucine) were pooled, desalted, and dried.

Trace impurities arising from the citrate buffer were removed by chromatography on a 0.6 × 68 cm column of Dowex 50-X8 (−400 mesh) operated at 50 ml/hr at 50°. Elution was carried out using a linear gradient of pyridine–acetic acid from pH 2.98 (0.19 M pyridine) to pH 5.0 (2.0 M pyridine). The buffers were adapted from those developed by Schroeder *et al.* (1962). After detection with ninhydrin,

[1] Procedures Manual, A-TB-033, 1966, Beckman Instruments, Palo Alto, Calif.

[2] Amino Acid Analyser Instruction Manual, AAA-1, 1967, Technicon Corp., Ardsley, N. Y.

TABLE II: Amino Acid Content of Guinea Pig Hair Proteins.[a]

Amino Acid	Residues/1000 Residues				
	Whole Fiber	Residue (Keratin)	Total Tryptic Digest of Medulla	Acid-Insoluble Tryptic Peptides	Acid-Soluble Tryptic Peptides
Cysteic acid		4.2			
Aspartic acid	49.2	47.4	68.1	70.9	65.7
Threonine	49.9	59.1	28.5	25.8	29.7
Serine	87.2	106.0	45.0	34.1	45.9
Glutamic acid	150.1	110.1	302.1	302.1	287.6
Citrulline[b]	23.5	trace	127.8	180.0	120.5
Proline	77.6	82.8	12.7	2.4	15.0
Glycine	72.2	84.4	48.1	40.0	51.3
Alanine	50.5	45.1	42.3	35.6	42.9
Valine	47.1	50.6	33.3	32.6	36.3
Half-cystine	137.0	175.8	7.6	12.7	9.0
Methionine	5.9	4.5	8.6	7.7	9.6
Isoleucine	25.9	28.1	20.3	24.9	21.0
Leucine	71.0	60.5	100.0	106.1	105.2
Tyrosine	26.0	28.9	17.5	16.6	17.1
Phenylalanine	22.4	20.5	28.5	39.1	28.5
Lysine	33.4	22.7	52.2	43.6	54.0
Histidine	12.0	10.5	13.4	12.2	14.4
Arginine	59.2	58.9	43.6	13.6	46.5
Tryptophan[c]	n.d.[d]	n.d.	7.8	9.1	6.3

[a] Uncorrected for hydrolytic losses, except as noted. [b] Corrected for decomposition to ornithine during hydrolysis by addition of the citrulline and ornithine values. [c] Colorimetric assay (Opiénska-Blauth *et al.*, 1963). [d] n.d., not determined.

fractions containing the purified material were pooled, rotary evaporated at 45°, and freeze-dried.

Dansylation.[3] Dansylation of synthetic and purified isolated ε-(γ-glutamyl)lysine was carried out using the conditions suggested by Gros and Labouesse (1969). Samples were incubated at room temperature for 1 hr and after drying *in vacuo*, hydrolyzed with 6 N HCl at 110° in sealed tubes for 4 hr. The dried hydrolysates were extracted with water-saturated ethyl acetate and the extracts and residues were examined separately by both high-voltage paper electrophoresis at pH 4.40 (Gray, 1967) and two-dimensional polyamide thin-layer chromatography using benzene–glacial acetic acid (9:1, v/v) and 1.5% aqueous formic acid as solvents (Woods and Wang, 1967). Samples of the dansylated compounds were also chromatographed and electrophoresed before hydrolysis.

Further Characterization of Isolated ε-(γ-Glutamyl)lysine. In addition to the two ion-exchange systems already described for amino acid analysis, the isolated ε-(γ-glutamyl)lysine was characterized by high-voltage paper electrophoresis at pH 4.0 (Kornguth *et al.*, 1963) and descending paper chromatography in a system of 1-butanol–acetic acid–water (4:1:5, v/v).

Results

Tryptic Digestion. Tryptic digestion of the hair was complete in 4 hr with 18% of its weight being solubilized. Amino acid analysis of the residual keratin showed that all the citrulline-containing material had been removed (Table II, column 2). The trypsin-solubilized medulla protein is enriched in aspartic and glutamic acids, leucine, and lysine as well as citrulline and contains reduced amounts of threonine, serine, and glycine and very low levels of proline and half-cystine when compared to the whole fiber (Table II, columns 1 and 3). The tryptic peptides insoluble at pH 3.5 are even further enriched in citrulline and leucine, while arginine especially is reduced (Table II, columns 4 and 5). Amino acid analyses of the tryptic peptides of porcupine quill medulla and guinea pig inner root sheaths have been published (Steinert *et al.*, 1969).

Chemical Determination of Cross-Links. The major reaction product of protein-bound lysine and acrylonitrile is hydrolyzed to ε-*N*,*N*-bis(β-carboxyethyl)lysine (Cavins and Friedman, 1967). The elution position of this compound on the Technicon Analyzer was determined using a marker prepared by reacting poly-L-lysine under the same conditions, including hydrolysis, as those used for the protein samples. Using the routine gradient, the product eluted between, but well separated from leucine and tyrosine, *i.e.*, in the position normally occupied by norleucine (internal standard). The color value was not calculated. The number of lysines involved in cross-links in the protein samples was therefore determined from the number of lysines remaining following alkylation with acrylonitrile and hydrolysis and the number of lysines expected from the control hydrolysate. Table III shows the results obtained for the tryptic peptides of guinea pig hair medulla, porcupine quill medulla, and guinea pig inner root sheaths. The values shown have been corrected using the average of the values found for the controls, chymotrypsin and ribonuclease, the absolute values of which are given.

Enzymic Determination of Cross-Links. Amino acid

[3] Abbreviation used is: dansyl, 1-dimethylaminonaphthalene-5-sulfonyl.

TABLE III: Comparison of Cross-Links Measured by Chemical and Enzymic Methods.

	Moles of Cross-Link/1000 Moles of Amino Acid Residues							
	Guinea Pig Hair Medulla							
	Total Tryptic Digest	Acid-Insoluble Tryptic Peptides[a]	Acid-Soluble Tryptic Peptides[b]	Porcupine Quill Medulla	Inner Root Sheath	Controls		
						Chymotrypsin	RNase	Enzyme
Chemical[c] (1)	16.2	20.7	18.3	0.8	1.3	2.3	1.2	
(2)	17.2	19.9	17.0			0.9		
Enzymic (1)	23.0[d]	19.9[d]	16.7[d]	2.0	2.2			0
(2)	13.2	14.5	12.6	2.3	2.8			0
(3)			15.4	2.0	2.1		0	0

[a] Tryptic peptides insoluble at pH 3.5: 12.5% (w/w) of total. [b] Tryptic peptides soluble at pH 3.5: 87.5% (w/w) of total. [c] Values corrected by the average of control values, except for control values, which are absolute. [d] Not completely resolved from leucine in these analyses.

analysis of aliquots of the enzymic digestions in general showed the appearance of only one new peak in significant amounts. This peak occurred as a shoulder on the trailing edge of the leucine peak using the routine Technicon gradient. By lowering the pH of the buffers in chambers 5 and 6 of the gradient (modified gradient M4) the new peak was made to appear slightly later, well resolved from all other amino acids. Chromatography of an authentic sample indicated that the new peak was indeed ε-(γ-glutamyl)lysine. The amount of this material in digests were therefore quantitated using the color value calculated from a known amount of the authentic compound. The results are shown in Table III. On the Beckman analyzer the new peak eluted between methionine and isoleucine.

The enzymic digest of the pH 3.5 soluble hair medulla tryptic peptides showed a second significant peak which was also present in trace amounts in the other digests. This peak, which appeared as a fairly broad peak immediately before valine in the Technicon analyses was shown by chromatography of an authentic sample to be α-L-glutamyl-L-glutamic acid. On the Beckman analyzer this compound eluted between valine and methionine.

The enzymic control digest showed no trace of peaks in the positions of either of the above peaks. Neither of the peaks was affected by dialysis of the various proteins prior to enzymic digestion. The components present in only trace amounts were not investigated.

Isolation and Characterization of the Cross-Link. The ε-(γ-glutamyl)lysine released by the complete enzymic digestion of 500 mg of pH 3.5 soluble tryptic peptides of hair medulla was initially isolated by chromatography on a large Dowex 50-X8 column using citrate buffer of constant pH and concentration. This procedure separated the isodipeptide from all the basic amino acids, tyrosine, phenylalanine, and the bulk of the acidic amino acids and citrulline. The ε-(γ-glutamyl)lysine was then purified from the major contaminant, leucine, by further chromatography on the Technicon column using a pH–concentration elution gradient of citrate buffers.

At this stage the product was essentially pure, but to remove small amounts of peptide-like material it was rechromatographed on a Dowex 50-X8 column using pyridine–acetic acid buffers. The product of the final purification step was pure as judged by chromatography on both the Technicon and Beckman amino acid analyzers.

The pure material cochromatographed with authentic ε-(γ-glutamyl)lysine in both analytical systems. It was identical to the synthetic material by high-voltage paper electrophoresis at pH 4.0 and by paper chromatography in 1-butanol–acetic acid–water (4:1:5, v/v). Acid hydrolysis of the isolated material in 2 N HCl at 110° for 2.5 hr yielded equal amounts of glutamic acid and lysine.

The dansylated product was identical with dansylated ε-(γ-glutamyl)lysine according to two-dimensional polyamide thin-layer chromatography and high-voltage paper electrophoresis at pH 4.40. Both compounds produced a major and a minor fluorescent spot. The nature of the minor spot has not been determined. Hydrolysates of the dansylated material showed dansylglutamic acid and α-dansyllysine by high-voltage paper electrophoresis.

Discussion

In common with other citrulline-containing proteins from hairs and quills (Rogers, 1962; Bradbury and O'Shea, 1969) the medulla protein of guinea pig hair has a high glutamic acid content and a very low proline and cysteine-cystine content (Table II). Except for the very high value for glutamic acid (almost one residue in three is glutamic acid) and the presence of citrulline, the analysis is similar to those of other structural proteins (Mazia and Ruby, 1968). By lowering the pH to 3.5, a fraction containing peptides enriched in citrulline and formerly referred to as the tryptic core can be precipitated from the tryptic digest (Rogers, 1962).

The presence of ε-amino cross-links in the citrulline-containing protein fractions has been demonstrated by both chemical and enzymic means. Acrylonitrile reacts with the free amino groups of amino acids to form the cyanoethyl derivatives which are converted into the acid-stable carboxyethyl derivatives by acid hydrolysis (Riehm and Scheraga, 1966; Cavins and Friedman, 1967). Thus, following reaction with acrylonitrile and hydrolysis, lysines not involved in an ε-amino cross-link would yield ε-*N*,*N*-bis(β-carboxyethyl)-lysine (Cavins and Friedman, 1967) whereas lysines that are

involved in such a cross-link would not react and would yield free lysine (Pisano *et al.*, 1969). Therefore, the amount of lysine remaining in such a hydrolysate is a measure of the amount of ε-amino cross-links in the original protein, provided that precautions are taken to see that the reaction of the lysines with acrylonitrile is not sterically hindered. Cross-linked lysines that are N terminal are a special case, producing α-*N*-(β-carboxyethyl)lysine, not free lysine, thus possibly introducing a small discrepancy. In the present work, either the peptic peptides or the chymotryptic peptides of the original tryptic peptides were used to ensure that all noncross-linked lysines were free to react. Even so, all the protein fractions tested showed the presence of nonreacting (*i.e.*, blocked) lysines, especially in the hair medulla (Table III). Since the citrulline-containing protein fractions are rich in glutamic acid residues it seemed likely at the outset that a cross-link between the γ-carboxyls of glutamic acid residues and the ε-amino groups of the lysine residues was responsible for the observed blockage of some of the lysine side chains.

Definitive evidence for the nature of the cross-link has been obtained by isolation of a new compound appearing in a complete enzymic digest of hair medulla protein and its characterization as ε-(γ-glutamyl)lysine. The presence of this cross-link has similarly been demonstrated to occur in two other citrulline-containing protein fractions, *viz.*, porcupine quill medulla and guinea pig inner root sheath protein (Table III). Good agreement is seen between the values obtained by both chemical and enzymic methods, indicating that all the lysines that are cross-linked are involved in the ε-(γ-glutamyl)lysine linkage.

The ε-(γ-glutamyl)lysine is present in hair medulla at very high levels compared to the other two proteins studied and to insoluble fibrin which has been shown by the same methods to contain about 1 mole of ε-(γ-glutamyl)lysine/1000 residues (Lorand *et al.*, 1968; Matačić and Loewy, 1968; Pisano *et al.*, 1969). There is some recent evidence that this cross-link occurs in collagen also (Bensusan, 1969). The value of 13 moles of ε-(γ-glutamyl)lysine/1000 moles of amino acid residues for hair medulla corresponds to 25% of the lysines residues being cross-linked. It may be noted here that the determination of cross-linked lysines in this case demonstrates the stability of the ε-(γ-glutamyl)lysine to hydrolysis by pepsin. Its resistance to several other proteolytic enzymes has already been reported (Kornguth *et al.*, 1963; Pisano *et al.*, 1969).

The presence of significant amounts of the dipeptide α-glutamylglutamic acid in the enzymic digest of the pH 3.5 soluble tryptic peptides of hair medulla is interesting yet perhaps not surprising in view of the high glutamic acid content of the proteins. The occurrence of this sequence in these proteins was predicted by Rogers (1962). It is probable that only small amounts of this dipeptide are seen in the digests of the other proteins studied because of the higher enzyme concentrations used.

The present results show directly that citrulline-containing fractions of porcupine quill medulla protein, guinea pig hair medulla protein and the inner root sheath protein of guinea pig hair follicles are cross-linked by the ε-(γ-glutamyl)lysine cross-link. The fact that no other unknown peaks were found at significant levels in analyses of enzymic digests of the protein fractions, suggests that this linkage is the major cross-link involved and is therefore, at least in part, responsible for the insolubility of these proteins.

At the present time neither the origin nor the function of the citrulline in these proteins is known (Rogers, 1964). However, this is not the case for the ε-(γ-glutamyl)lysine cross-links, to which the insolubility of the proteins can be ascribed. In addition, some evidence has been obtained (unpublished experiments) to indicate the enzymic origin of these cross-links. It has been shown that there is in hair follicle homogenates an enzyme (transamidase) which incorporates [^{14}C]glycine ethyl ester into casein by forming γ-glutaminyl derivatives. That such an enzyme would be capable of producing the ε-(γ-glutamyl)lysine cross-links is suggested by the demonstration that blood plasma transamidase forms these cross-links in insoluble fibrin (Matačić and Loewy, 1966, 1968). Of paramount interest is the fact that the hair follicle system possesses in closely associated cells, at least two mechanisms for cross-linking its proteins. One is the long-established disulfide bond that is typical of hair keratin, the other is the presently established γ link that occurs in the cells of the medulla and inner root sheath that adjoin the keratin-containing (cortical) cells of the hair (see Figure 1).

Acknowledgments

The authors thank Dr. J. P. E. Human, C. S. I. R. O., Wool Research Laboratories, Melbourne, for assistance in the preparation of the hair and Miss C. Hayles for assistance with the amino acid analyses.

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Isoelectric Focusing Behavior of Bovine Plasma Albumin, Mercaptalbumin, and β-Lactoglobulins A and B*

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ABSTRACT: The basic causes for the microheterogeneity of plasma albumin have remained obscure, and, in fact, there is not even an estimation of the number of species involved. This study was undertaken in the hope that isoelectric focusing might help answer these questions. To this end, crystallized bovine plasma albumin and mercaptalbumin were studied extensively by isoelectric focusing in a sucrose-stabilized column and in polyacrylamide gels. β-Lactoglobulin provided a standard of comparison because it focused well under all conditions employed, yielding two sharp, fairly symmetrical peaks corresponding to isoelectric pH values of 5.21 for the A and 5.34 for the B genetic variants. Difficulties were encountered with albumin samples which are attributed to the propensity of this protein to bind various trace impurities in the ampholyte mixture and in polyacrylamide gels. Focusing on washed gels yielded essentially the same results as were obtained on the column using sucrose density gradients. Monomeric, charcoal-defatted albumin yielded a main peak at pH 5.28 with shoulders on both high and low pH sides plus a broad peak at pH 4.8. Purified mercaptalbumin gave the main peak, essentially devoid of the shoulders, but also gave rise to the peak at 4.8.

Evidence is presented that the low pH component resulted from an interaction of the protein with a minor constituent of the ampholyte mixture. The results indicate that the purified mercaptalbumin is as homogeneous, by the electrofocusing criterion, as either of the β-lactoglobulin variants. The nonmercaptalbumin components clearly make a major contribution to the microheterogeneity of plasma albumin preparations.

Many investigators have demonstrated that plasma albumin preparations are not homogeneous. One aspect of the heterogeneity is associated with the nonintegral sulfhydryl content which has been shown to be due to the presence of two types of albumin: mercaptalbumin and nonmercaptalbumin. The nonmercaptalbumin has been shown to consist of mixed disulfides of cysteine and glutathione (King, 1961; Andersson, 1966). Another type of heterogeneity involves the state of aggregation of the albumin molecules. Plasma albumin can exist as monomer, dimer, trimer, and higher polymers. These forms can be readily separated by Sephadex exclusion chromatography as was first shown by Pederson (1962). Microheterogeneity has been demonstrated by electrophoresis at low pH (Sogami and Foster, 1963), by pH-solubility studies (Foster *et al.*, 1965; Petersen and Foster, 1965), and by salting out studies (Wong and Foster, 1969a, 1969b). Evidence has been presented that the presence of bound impurities (McMenamy and Lee, 1967; Sogami and Foster, 1968) and fluctuations in disulfide pairing (Sogami *et al.*, 1969) contribute to the observed microheterogeneity.

Isoelectric focusing (electrofocusing) in natural pH gradients, as described by Vesterberg and Svensson (1966), has proven valuable in studying the heterogeneity of myoglobin (Vesterberg and Svensson, 1966), cytochrome *c* (Flatmark and Vesterberg, 1966), and a number of other proteins (Vesterberg, 1968). Electrofocusing experiments have been performed in either sucrose-stabilized density gradients (Vesterberg and Svensson, 1966) or in polyacrylamide gels (Dale and Latner, 1968; Wrigley, 1968) where the pH gradient is established by a system of synthetic low molecular weight ampholytes called "carrier ampholytes." These experiments yield information on the isoelectric spectrum as well as the isoelectric pH of the sample.

Isoelectric focusing was used in this investigation in an attempt to examine the heterogeneities or microheterogeneities of charcoal-defatted monomeric bovine plasma albumin and of purified bovine mercaptalbumin. The β-lactoglobulin genetic variants A and B provided a convenient system by which to calibrate the electrofocusing technique. The reported isoionic points of the β-lactoglobulins are similar to that of plasma albumin, so that focusing in the same pH range was possible.

Experimental Section

Materials. Crystallized bovine plasma albumin, lot D71209, was obtained from Armour Pharmaceutical Co. This lot con-

* From the Department of Chemistry, Purdue University, Lafayette, Indiana 47907. *Received August 5, 1970.* This paper was taken from the thesis submitted in partial fulfillment of the requirements for the Ph.D. degree by Lawrence J. Kaplan, Purdue University, 1970. Presented in part at the 54th Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April 1970. Supported by Grants CA-02248 and T01 GM 01195 of the National Institutes of Health, U. S. Public Health Service.

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