Chemical and Enzymic Detection of Protein Cross-Links. Measurement of ε-(γ-Glutamyl)lysine in Fibrin Polymerized by Factor XIII*

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ABSTRACT: A chemical and an enzymic method were compared with respect to their ability to detect cross-links in human fibrin. The former method consisted of cyanoethylation followed by acid hydrolysis, a procedure under which ε-amino cross-linked lysine yields free lysine, whereas lysine elsewhere in the molecule yields an *N*-carboxyethyl derivative; 1–2 moles of ε-amino cross-linked lysine was found per mole of fibrin polymerized by factor XIII; much less was found when polymerization was competitively inhibited with glycine ethyl ester; very little was found when polymerization was prevented by removing Ca^{2+} with EDTA. Very low levels of ε-amino cross-linked lysine were detected in fibrin prepared from factor XIII poor human fibrinogen, but the level was greatly increased by the addition of human factor XIII. Amounts of ε-(γ-glutamyl)lysine separated from total enzymic hydrolysates of fibrin formed under each of these conditions were in quantitative agreement with those of ε-amino cross-linked lysine measured by the chemical method. In addition to providing a direct demonstration of the ε-(γ-glutamyl)lysine cross-link in polymerized human fibrin, this agreement indicated (1) that lysine is not cross-linked to any acceptor other than glutamate and (2) that the cyanoethylation technique is a valid procedure for detecting ε-lysyl cross-links in proteins.

The cross-linking of fibrin, which is the terminal step in blood clotting, is catalyzed by an enzyme known as active factor XIII. The nature of the cross-link(s) formed during this polymerization has been the subject of numerous investigations (see Loewy, 1968). According to earlier reports the cross-linking of fibrin monomer molecules involved a transamidation (or transpeptidation) reaction between glutaminyl (or asparaginyl) residues of the "acceptor" chain and free amino groups on the "donor" chain.

The basis for proposing such a reaction was the liberation of ammonia during cross-linking (Loewy *et al.*, 1964; Chandrasekhar *et al.*, 1964) and the earlier results obtained by the use of inhibitors (Lorand *et al.*, 1962, 1963). Cross-linking was found to be inhibited by various amines, including glycine ethyl ester (Lorand *et al.*, 1963), and by N-substituted amino acid esters or amides, *e.g.*, *N*-carbobenzoxyasparaginamide (Lorand and Jacobsen, 1964). This inhibition was interpreted as evidence that the amines competed with amino groups on the "donor" chain whereas N-substituted asparagine derivatives competed with the "acceptor" chain. Moreover, the report by Lorand *et al.* (1962) that there is a great reduction in the amount of N-terminal glycine when soluble fibrin is converted into insoluble fibrin and the findings that glycylglycine (Loewy *et al.*, 1964) and glycine ethyl ester (Lorand and Ong, 1966) could be incorporated into fibrin were considered as evidence that glycine was the "donor," its α-amino group being involved in the cross-linking.

Doolittle and Fuller (1967), however, showed unequivocally that the amounts of N-terminal glycine and tyrosine were not changed when soluble fibrin was converted into insoluble fibrin and thus eliminated the possibility that these α-amino groups could participate in the cross-linking. In contrast, these authors (Fuller and Doolittle, 1966) presented data to support a cross-linking mechanism involving the ε-amino group of lysine, and postulated that the cross-link was ε-(γ-glutamyl)lysine. Lorand *et al.* (1966), discounting their often-quoted glycine data (Lorand *et al.*, 1962) as well as other possible cross-links they had proposed, *e.g.*, β-aspartyl (Lorand and Jacobsen, 1964) or disulfide (Lorand *et al.*, 1959) bonds, also postulated the ε-(γ-glutamyl)lysine cross-link. Several lines of evidence could be marshalled to support this postulate. Changes in the amount of β-aspartylglycine during polymerization (Pisano *et al.*, 1966) had proved to be inconsistent,[1] and this linkage was ruled out by the measurements of Doolittle and Fuller (1967) described above. Various tissue transamidases catalyze a cross-linking of fibrin (Bruner-Lorand *et al.*, 1966; Tyler and Laki, 1967; Tyler, 1967). Furthermore, factor XIII was shown to have transamidase activity toward glutaminyl residues (Matačić and Loewy, 1966; Loewy *et al.*, 1966).

Despite all these studies, however, there was no direct demonstration of the ε-(γ-glutamyl)lysine cross-link until the simultaneous independent preliminary studies by Matačić and Loewy (1968) and Pisano *et al.* (1968). Lorand *et al.* (1968) have subsequently also claimed a

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[1] A full account of the experiments which ruled out the formation of β-aspartylglycine cross-links will be published in a supplement to *Thrombosis et Diathesis Haemorrhagica.*

direct demonstration of the cross-link. We wish to present here the complete enzymic study leading to the demonstration of the ε-(γ-glutamyl)lysine bond and the details of a chemical method utilizing cyanoethylation for detecting ε-aminolysine cross-links. It will be shown that the good quantitative agreement between the two methods, carried out on the same samples, not only confirms the formation of ε-(γ-glutamyl)lysine cross-links in fibrin polymerization, but also demonstrates that no "acceptor" other than glutamate participates in the formation of ε-aminolysine bonds during this process.

Materials and Methods

Comparison of Methods. The enzymic approach consisted of examining total enzymic digests of fibrin for ε-(γ-glutamyl)lysine. If this dipeptide represented the cross-link, it should have been readily demonstrable in fibrin which was cross-linked and minimal in that which was not. This experiment was possible because ε-(γ-glutamyl)lysine is not hydrolyzed by common peptidases and proteases (Kornguth *et al.*, 1963; J. J. Pisano, unpublished experiments) and thus would be expected to remain intact throughout the digestion procedure.

The chemical method took advantage of the fact that an acid-stable bond is formed when acrylonitrile reacts with the free amino group of an amino acid (Figure 1). Acid hydrolysis converts the cyanoethylamino acid into the corresponding carboxyethyl derivative. In the present experiments N-terminal lysine should yield (after cyanoethylation and acid hydrolysis) α,ε-dicarboxyethyllysine, and intrachain or C-terminal lysine should yield ε-carboxyethyllysine, whereas an ε-aminolysine cross-link involving a lysine residue at any position other than the N terminus should give rise to free lysine (Figure 1). Inasmuch as no N-terminal lysine is present in fibrin monomer, quantitative determination of the free lysine released should provide an index of the number of ε-aminolysine cross-links formed.

Materials. The human fibrinogen used was Blombäck fraction I-2 (Blombäck and Blombäck, 1956) which had been precipitated three times in the presence of ε-aminocaproic acid to remove plasminogen (Mosesson, 1962). It was more than 92% clottable and contained factor XIII as a contaminant. Factor XIII poor human fibrinogen was prepared by gradient elution chromatography on DEAE-cellulose (Mosesson and Finlayson, 1963). It was more than 97% clottable and was "free" of factor XIII when tested by the method of Loewy *et al.* (1961) in the presence of calcium and cysteine.

Leucine aminopeptidase (approximately 100–200 units/mg of protein) and prolidase acetone powder were purchased from the Worthington Biochemical Corp., Freehold, N. J. The latter enzyme was further purified by the method of Davis and Smith (1957) through ethanol precipitation (step 3) and had a specific activity of 250 units/mg of protein. Pronase (grade B) and trypsin were obtained from the California Corp. for Biochemical Research, Los Angeles, Calif. The trypsin had been treated with DFP and was used as a 1% solution in 0.001 M HCl. Crude human factor XIII (fraction 5 in the method of Loewy *et al.*, 1961) was isolated from the Blombäck fraction I-2 described above, and its activity was estimated according to Loewy *et al.* (1961). Human thrombin was prepared by

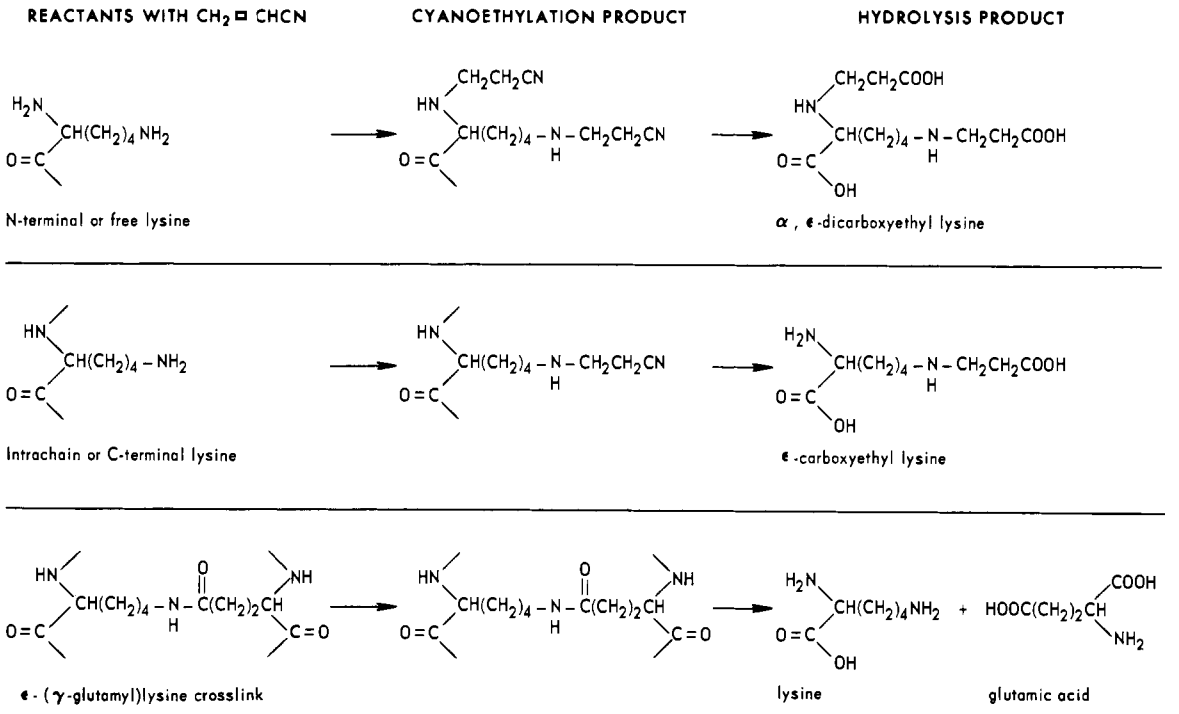

FIGURE 1: Peptidyllysine involved in an ε-amino cross-link will not react with acrylonitrile and yields free lysine after acid hydrolysis.

Dr. D. L. Aronson, Division of Biologics Standards, and was obtained as a gift from him.

Synthetic ε-(γ-glutamyl)lysine was prepared by catalytic hydrogenation of α-*N*-Cbz-ε-*N*-(Cbz-γ-L-glutamyl)-L-lysine dibenzyl ester purchased from Cyclo Chemical Corp., Los Angeles, Calif. It was tritiated by the New England Nuclear Corp., Boston, Mass., by the Wilzbach technique and purified by paper chromatography in a system of 1-butanol–acetic acid–water (4:1:5, v/v). This procedure was then repeated, and the material obtained was subjected to chromatography and rechromatography on the standard long column used in the amino acid analyzer (see below). It was then desalted on a column of Dowex 50. In this sequence of steps the ε-(γ-glutamyl)lysine had a constant specific activity of 4.4×10^8 dpm/μmole. [^{14}C]Lysine (180 μCi/μmole), purchased from New England Nuclear Corp., Boston, Mass., was purified by chromatography in the amino acid analyzer under the conditions described below for the measurement of lysine. The specific activity of the purified lysine was sufficiently high that the amount used in the cyanoethylation experiments (see below) was not detectable, other than by its radioactivity, in the amino acid analyzer. Triethylamine and acrylonitrile were purchased from Eastman Organic Chemicals, Rochester, N. Y., and were redistilled before use.

Preparation of Fibrin. Samples containing 22 mg of fibrinogen dissolved in 0.1 M Tris-Cl (pH 7.5) were placed in dialysis sacs (6-mm diameter). Various solutions prepared in the same buffer, and adjusted to pH 7.5 if necessary, were added to the sacs so as to achieve the following final concentrations in a total volume of 2.0 ml (samples containing fibrinogen contaminated with factor XIII) or 3.0 ml (samples containing factor XIII poor fibrinogen). All samples were 0.015 M in cysteine and all except those containing EDTA were 0.008 M in $CaCl_2$. To prevent cross-linking in certain samples, the Ca^{2+} ions required for the action of factor XIII were removed by adding sufficient EDTA to obtain a concentration of 0.008 M. Glycine ethyl ester, a competitive inhibitor of cross-linking, was added to certain other samples at a final concentration of 0.1 M. The concentrations of EDTA and glycine ethyl ester chosen were those which had been shown to give fibrin clots which were soluble in reagents such as 1% monochloroacetic acid, 2% acetic acid, and 8 M urea, which are commonly used to detect cross-linking (Tyler, 1966). Crude factor XIII, when used, was added back to factor XIII poor fibrinogen at the level of approximately 330 units (Loewy *et al.*, 1961) per ml.

The samples were then treated with thrombin (0.3–1.0 U.S. unit), whereupon each sac was tied, immersed in a solution with the same composition (except for protein) as the contents of the sac, and incubated at 37° for 90 min. They were then dialyzed at 5° against five changes of 0.1 M NH_4HCO_3. For the last change, $CaCl_2$ was added to the NH_4HCO_3 at a final concentration of 0.01 M even though the expected precipitation of some $CaCO_3$ occurred. Dialysis was necessary to remove the Tris which would interfere in the cyanoethylation experiments.

After dialysis each sac and its contents were cut into small pieces and allowed to fall into a conical centrifuge tube. Following the addition of 1 mg of trypsin to each sample, the tubes were centrifuged briefly to bring all material (tubing and clot fragments plus trypsin solution) together. The tubes were then closed with glass stoppers and incubated at 37° for 24–26 hr. This treatment solubilized the clots, served as a first step in the total enzymic digestion, and, it was hoped, rendered any "buried" lysine residues with free ε-amino groups more accessible to cyanoethylation. After the incubation the fragments of dialysis casing were removed and rinsed with water, whereupon the samples plus rinsings were freeze dried and redissolved in a measured volume of water. Residual $CaCO_3$ was removed by centrifugation. The solutions were held at −10° until analyzed.

Enzymic Method.[2] An aliquot (0.5 or 1.0 ml) amounting to half of each sample (*i.e.*, representing 11 mg of fibrinogen) was then buffered with 0.1 M Tris-Cl, treated with 2 mg of Pronase, incubated at 37° for 4 days, treated again with the same amount of Pronase, and incubated for another 2 days. (A small crystal of thymol was added to each sample at the beginning of the first incubation to inhibit microbial growth.) The pH was then raised to 8.0 with NaOH, 2–3 mg of prolidase and 1–2 mg of leucine aminopeptidase were added, and incubation was continued for 5 more days.

After the last incubation approximately 1×10^6 dpm (2.3 nmoles) of tritiated ε-(γ-glutamyl)lysine was added to each sample and the protein was precipitated by introducing sufficient trichloroacetic acid (24%, w/v) to achieve a final concentration of 12%. Following centrifugation, the clear supernatant fluid was diluted with five volumes of water and applied to a 1×10 cm column of Bio-Rad AG 50-X4 (200–400 mesh, H^+ form). The column was washed with about 30 ml of water, then eluted with 1 M pyridine until the radioactivity was recovered. (Usually about 45 ml of the 1 M pyridine was required.) The radioactive eluate was freeze dried, dissolved in a small volume of water, and applied to a 1.1×23 cm column of Bio-Rad AG 2-X8 (100–200 mesh, acetate form) which was then washed with water until the radioactive material was obtained (usually in the first 35 ml). This eluate was freeze dried, dissolved in pyridine acetate buffer (0.20 M pyridine, pH 3.23), and chromatographed in a Spinco Model 120C amino acid analyzer on an 18-cm column packed with PA-35 resin prepared according to instructions for treatment prior to chromatography with pyridine acetate buffers (Benson *et al.*, 1966). Elution was carried out at a flow rate of 60 ml/hr, and 1-ml fractions were collected; ε-(γ-glutamyl)lysine was eluted in fractions 51–57.

These fractions were pooled, freeze dried, and rechromatographed in the Spinco Model 120C amino

[2] The enzymic digestion, based on the procedure of Pisano *et al.* (1966), was carried out under conditions chosen to give the maximal yield of peptide. It differed from the published method by (1) the inclusion of the initial digestion with trypsin, (2) the use of twice as much of the other enzymes, and (3) a fivefold increase in the incubation time.

acid analyzer by the accelerated procedure for acidic and neutral amino acids (Hubbard, 1965), with the exception that a 0.20 M sodium citrate buffer of pH 3.83 was used without a buffer change. The amounts of ε-(γ-glutamyl)lysine, which under these conditions was eluted after 88.5 min (Figure 2), were calculated by the isotope dilution method.

Chemical Method. The remaining half of each sample of trypsin-digested fibrin was incubated with occasional shaking at 37° for 72–104 hr in a sealed-glass tube with 20 μl of triethylamine and 0.2 ml of acrylonitrile. After incubation the tubes were opened and placed in a 50° bath, and their contents dried with a stream of N_2. The residue was then treated with 0.3 ml of 6 N HCl where-

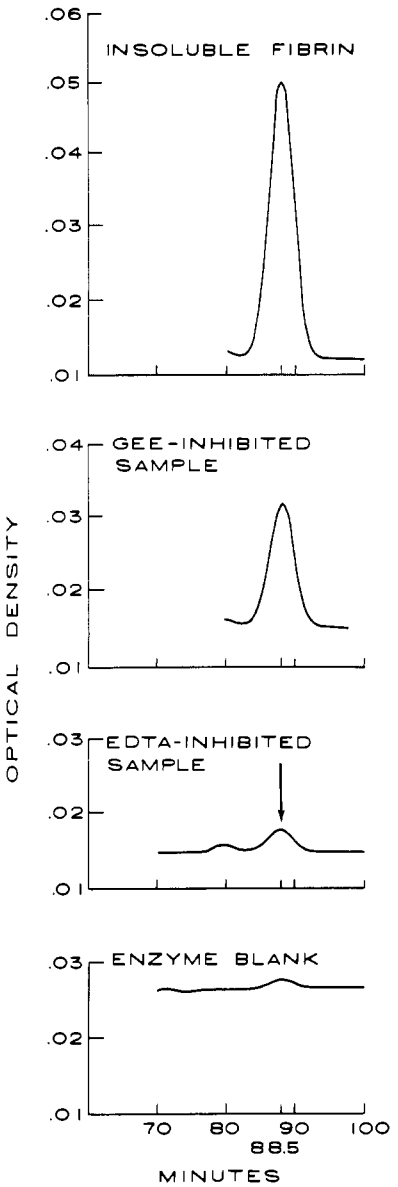

FIGURE 2: Chromatograms of ε-(γ-glutamyl)lysine isolated from total enzymic digests of fibrin formed under various conditions. Analyses were performed on the amino acid analyzer with columns maintained at 50° and developed with 0.20 M sodium citrate buffer (pH 3.83). The aliquots chromatographed (25–75% of the material recovered from the pyridine acetate chromatography step) contained 25.4, 17.5, and 0.6 nmoles, respectively.

upon the tubes were resealed and held at 110° for 24–48 hr. Following this hydrolysis the opened tubes were placed in a boiling water bath, and their contents were dried under a stream of N_2. Water was then added and the solutions were again dried with N_2. This procedure was carried out two more times.

After the samples had been redissolved in water, a tracer amount of [^{14}C]lysine was added. A small aliquot of each sample was taken for radioactivity measurements, and the remainder was placed on a column of Bio-Rad AG 50-X4 (200–400 mesh, hydrogen form) which had a volume of 1 ml. The columns were washed with 25 ml of water, then with 25 ml of 0.1 M collidine, and finally with 10 ml of 3 N NH_4OH. Measurements of radioactivity carried out at this point showed that 90–100% of the lysine was present in the NH_4OH eluates; therefore, no corrections were made for loss during chromatography.

The NH_4OH eluates were then freeze dried and redissolved in 1 ml of the sodium citrate buffer (pH 2.2) recommended as a diluent for the Spinco Model 120C amino acid analyzer, and the solutions were held at −10° until analyzed. Immediately prior to analysis, the samples were thawed and a 20-μl aliquot of each was taken for radioactivity measurement. A second aliquot (0.25 or 0.50 ml) was then chromatographed on an 18-cm column packed with PA-35 resin and developed with 0.35 M sodium citrate buffer (pH 4.04). Under these conditions lysine emerged at 125 min and was well separated from any other compounds still present. The elution time of lysine was very sensitive to pH, but it was not necessary to control the pH closely, as good results were obtained with elution times from 120 to 150 min provided the appropriate standard was used to identify the peak. As in the enzymic method, results were computed as moles (of lysine) per mole of fibrin, based on a fibrinogen molecular weight of 330,000.

Results

Figure 2 illustrates the separation of ε-(γ-glutamyl)lysine from hydrolysates of fibrin formed under various conditions. The elution time was constant, and no overlapping with other compounds occurred. The enzyme blank (prepared with no fibrinogen) exhibited only the expected small peak resulting from the addition of [^{3}H]ε-(γ-glutamyl)lysine; 1–2 moles of ε-(γ-glutamyl)lysine/mole of polymerized fibrin was found (Table I). Less than half of this amount was observed in soluble fibrin prepared with glycine ethyl ester; almost none appeared in that prepared with EDTA (Table I). Moreover, whereas the quantity of ε-(γ-glutamyl)lysine in fibrin prepared from factor XIII poor fibrinogen was approximately one-tenth of that found in polymerized fibrin, this value was in large measure restored by the addition of factor XIII (Table I).

Preparation of the samples for quantitative measurement of ε-(γ-glutamyl)lysine required four separate chromatographic procedures: (1) desalting of the digest on Bio-Rad AG 50 which also removed the basic

amino acids and peptides, (2) removal of acidic amino acids, peptides, and other substances on Bio-Rad AG 2 acetate, (3) further purification on a PA-35 resin column with a pyridine acetate buffer, and (4) quantitative measurement in the amino acid analyzer using a sodium citrate buffer (pH 3.83). The peptide recovered from step 3, although impure, was free of substances which would overlap the peptide peak obtained at step 4. Monitoring the purification and recovery was readily accomplished by the use of labeled ε-(γ-glutamyl)lysine.

Measurements carried out by the chemical method showed good agreement with the results of the enzymic method in all cases. Not only were 1–2 moles of ε-amino cross-linked lysine/mole of polymerized fibrin found, but the reduction in quantities of ε-amino cross-linked lysine formed in the presence of glycine ethyl ester and EDTA paralleled that of ε-(γ-glutamyl)lysine. Similarly, the low level of ε-amino cross-linked lysine in factor XIII poor fibrin was raised to 1.3 moles/mole by the addition of factor XIII (Table I).

TABLE I: Comparison of Fibrin Cross-Linking Measured by Chemical and Enzymic Methods.[a]

Samples	Experiment 1		Experiment 2	
	Moles of ε-(γ-Glutamyl)-lysine[b]/Mole of Fibrin	Moles of Cross-Linked Lysine[c]/Mole of Fibrin	Moles of ε-(γ-Glutamyl)-lysine[b]/Mole of Fibrin	Moles of Cross-Linked Lysine[c]/Mole of Fibrin
Polymerized fibrin	1.8	1.2	2.2	2.3
Polymerized fibrin	1.2	1.2	Lost	1.9
Soluble fibrin prepared with EDTA	0.07	0.26	0.02	0.08
Soluble fibrin prepared with EDTA			0.01	0.12
Soluble fibrin prepared with glycine ethyl ester	0.70	0.50	0.45	0.50
Soluble fibrin prepared with glycine ethyl ester			0.78	0.38
Factor XIII poor fibrin			0.18	0.24
Factor XIII poor fibrin + factor XIII			1.6	1.3
Factor XIII poor fibrinogen (no thrombin)			0.02	0.17
Control (no fibrinogen)	0	0		

[a] Reagents added in the preparation of fibrin were used at the following final concentrations: EDTA, 0.008 M; glycine ethyl ester, 0.1 M; and factor XIII, 330 units/ml. Values are based on a fibrinogen molecular weight of 330,000. [b] Detected by enzymic hydrolysis and measurement on amino acid analyzer. [c] Lysine unreactive with acrylonitrile; hence, ε-amino cross-linked lysine (see Figure 1).

Discussion

The data given here constitute a direct demonstration of the presence of the ε-(γ-glutamyl)lysine cross-link in polymerized fibrin. Moreover, the second criterion for acceptance of this cross-link, *viz.*, that the amount of ε-(γ-glutamyl)lysine should be reduced when cross-linking is inhibited, has also been fulfilled. Still another proof of this cross-link is the low level of ε-(γ-glutamyl)lysine in factor XIII poor fibrin, which was increased more than eightfold by adding factor XIII.

Just as the value of 0 obtained for the amount of ε-(γ-glutamyl)lysine in the enzyme blank showed that no spurious evidence of cross-linking was introduced by the enzymic digestion procedure, the virtual absence of this dipeptide in fibrinogen (as well as in fibrin prepared in the presence of EDTA) showed that this cross-link did not preexist but was formed during polymerization. The fact that a measurable, albeit minimal, quantity of ε-(γ-glutamyl)lysine was detected in fibrin prepared from fibrinogen[3] judged to be "free" of factor XIII is a reflection of the unreliability of solubility in 2% acetic acid as a criterion for the absence of cross-linking. Laki (1968) has shown that a urea-insoluble clot which was soluble in 2% acetic acid had undergone considerable polymerization. We found that fibrin prepared in the presence of sufficient glycine ethyl ester to yield soluble clots exhibited appreciable cross-linking (Table I).

Whereas in the present study 1–2 moles of ε-(γ-glutamyl)lysine/mole of polymerized fibrin was found in an all-human (*i.e.*, fibrinogen, thrombin, and factor XIII) system, the same cross-link was reported by Matačić and Loewy (1968) at a level of 2–3 moles/mole in an all-bovine system using more factor XIII

[3] The factor XIII poor human fibrinogen was chromatographic peak 1 (Mosesson and Finlayson, 1963). It is probable that "ascending peak 1" would provide a more nearly factor XIII free substrate.

and a longer incubation time. In a confirmatory study utilizing a bovine system, the value reported was 1–1.4 moles/mole (Lorand *et al.*, 1968). It is not known, of course, whether 3 moles/mole represents the maximum degree of cross-linking obtainable or whether the maximal extent of cross-linking is the same in both species. Furthermore, the minimum number of cross-links necessary to achieve a hemostatically effective clot has not been established.

Measurements of ε-amino cross-linked lysine yielded several types of information. First, the results obtained by the chemical method provide immediate substantiation of those obtained by the enzymic procedure. If either the cyanoethylation or the enzymic liberation of the dipeptide had been incomplete, more cross-links would have been observed by the chemical procedure. Second, the good quantitative agreement between the two methods indicates that lysine was not cross-linked to any "acceptor" other than glutamate (*e.g.*, aspartate), since under this condition higher values would be expected for cross-linked lysine than for ε-(γ-glutamyl)lysine.[4] Third, this agreement indicates that the cyanoethylation technique offers a potentially valuable tool for detecting unusual peptide linkages in proteins involving the ε-amino group of lysine.

The demonstration of the ε-(γ-glutamyl)lysine cross-link and the fact that lysine is cross-linked only to glutamate constitute partial justification for the term "plasma transglutaminase" which has been suggested as a synonym for active factor XIII (Loewy, 1968; Matačić and Loewy, 1968). The finding that no transglutaminase was detectable in the plasma or platelets of a factor XIII deficient patient (Loewy, 1968) lends support to this terminology. However, it has not been established that transamidation is the only reaction that occurs during fibrin polymerization catalyzed by factor XIII (Laki, 1968). Nevertheless, it is now permissible to suggest that the biochemical manifestation of factor XIII deficiency, *viz.*, a less stable fibrin clot (Duckert, 1964), results from an inability to form the ε-(γ-glutamyl)lysine cross-link. Proof of this suggestion awaits direct experimentation.

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[4] An exception to this statement would be the hypothetical situation in which all lysines bound in ε-lysyl linkages to nonglutamate acceptors had their α-amino groups bound to the α-carboxyl groups of lysine or arginine. In this case, trypsin digestion could free the α-amino groups for cyanoethylation, hence the lysines involved in the ε-lysyl linkages would not be detected as free lysine. A second, and even less likely, exception would be the situation in which the number of lysine residues cross-linked to nonglutamate acceptors was exactly equalled by the number of ε-(γ-glutamyl)lysine cross-links in which the lysine was adjacent to the carboxyl group of arginine or lysine.