The larvae of *Chauliognathus* are carnivorous (*7*) and have glands that are apparently homologous to those of the adult (*8*). Nothing is known about the chemistry of their secretion, which is white and sticky like that of the adult (*9*).

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10. Aided by NIH grant AI-02908. We thank W. J. Dress and P. A. Hyypio for identifying the composites, Rosalind Alsop for collecting the secretion, and S. Schrader of Cornell University's Mass Spectrometer Facility (NIH grant FR 00355). Also we thank N. A. Sörensen for 8-*cis*-dihydromatricaria acid and for providing ultraviolet-absorption data on the previously unknown *yne-ene-yne* chromophore, which is quite different in character. Report No. 22 of our series Defense Mechanisms of Arthropods.

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Cross-link in Fibrin Polymerized by Factor XIII: ε-(γ-Glutamyl)lysine

Abstract. *ε-(γ-Glutamyl)lysine has been isolated from enzymatic hydrolyzates of cross-linked human fibrin. This compound was not detected in "non-cross-linked" fibrin prepared with ethylenediaminetetraacetic acid, which inhibits factor XIII; intermediate amounts were observed when the fibrin was prepared with glycine ethyl ester, which inhibits factor XIII competitively. These and ancillary experiments furnish conclusive evidence that ε-(γ-glutamyl)lysine cross-links form in human fibrin during polymerization catalyzed by factor XIII.*

The cross-linking of fibrin is the terminal step in blood clotting. This reaction is catalyzed by an enzyme; the inactive precursor of this enzyme is now designated factor XIII, though it has been known as serum factor, Laki-Lorand factor (LLF), fibrinase, fibrin-stabilizing factor (FSF), and plasma transglutaminase (*1*, *2*).

Earlier reports indicated that cross-linking occurred through the joining of fibrin monomer molecules by a transamidation (or transpeptidation) reaction involving glutaminyl (or asparaginyl residues of the "acceptor" polypeptide chain and free amino groups on the "donor" chain. This concept was based on (i) reduction in the number of amino-terminal glycine residues in cross-linked fibrin, (ii) inhibition of cross-linking by certain glycine derivatives, and (iii) liberation of ammonia during cross-linking (*2*).

There is evidence that the lysine of the "donor" chain rather than glycine inhibits the cross-linking (*3*). Fuller and Doolittle (*4*) and Lorand *et al.* (*5*) postulated that the cross-link is ε-(γ-glutamyl)lysine. Since active factor XIII has transamidase activity toward glutaminyl residues (*6*) and various tissue transamidases catalyze a cross-linking of fibrin (*7*), the postulated ε-(γ-glutamyl)lysine bond seemed reasonable and highly tenable; however, neither group proposing this cross-link was able to demonstrate it directly.

We now report that the ε-(γ-glutamyl)lysine bond (Fig. 1) is in fact the cross-link in fibrin polymerized by factor XIII. ε-(γ-Glutamyl)lysine was isolated from total enzymic hydrolyzates of polymerized fibrin, and the amount of this dipeptide was compared with that in hydrolyzates of fibrin monomer. This experiment was feasible because ε-(γ-glutamyl)lysine is not hydrolyzed by common peptidases and proteases (*8*).

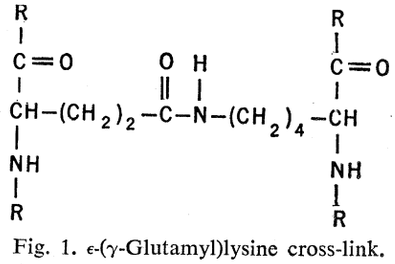

Fig. 1. ε-(γ-Glutamyl)lysine cross-link.

The human fibrinogen used [Blombäck (*9*) fraction I-2, precipitated three times in succession in the presence of ε-aminocaproic acid to remove plasminogen (*10*)] was more than 92 percent clottable; the amount of factor XIII present as a contaminant was enough to yield a totally acid-insoluble clot (*11*). Samples containing 22 mg of fibrinogen were dissolved in 0.1*M* tris-(hydroxymethyl)aminomethane chloride (tris) buffer, *p*H 7.5, and were placed in small dialysis sacs. Solutions prepared in the same buffer (and adjusted to *p*H 7.5 if necessary) were added to obtain the desired final concentrations in a total volume of 2 ml. All samples were adjusted to 0.015*M* with respect to cysteine and all except those containing ethylenediaminetetraacetic acid (EDTA) were made up to contain 0.008*M* $CaCl_2$; EDTA (0.008*M*) was used in certain samples to prevent cross-linking by removing Ca^{++} ions required for the activity of factor XIII; glycine ethyl ester (GEE), a competitive inhibitor of factor XIII (*2*), was used in others at a concentration of 0.1*M*. (The EDTA and GEE concentrations were chosen to give fibrin clots soluble in 1 percent monochloroacetic acid, 2 percent acetic acid, and 8*M* urea, reagents which are used to detect cross-linking.)

The samples were clotted with human thrombin (*12*), incubated at 37°C for 90 minutes, and dialyzed at 5°C, first against 0.1*M* NH_4HCO_3, then against 0.1*M* NH_4HCO_3 containing 0.01*M* $CaCl_2$. After dialysis, each sac was cut into small pieces; the mixture of tubing and clot fragments was incubated with 1 mg of trypsin at 37°C for 24 hours, and the samples were freeze-dried and dissolved in 2.00 ml water. Half of each sample was then digested successively with pronase (1.33 mg/ml), prolidase (460 unit/ml), and leucine aminopeptidase (110 unit/ml), essentially by the procedure of Pisano *et al.* (*13*). After the last incubation, tritiated (*14*) synthetic ε-(γ-glutamyl)lysine was added to each sample, and the protein was precipitated with an equal volume of 24 percent trichloroacetic acid. After centrifugation, the supernatant was diluted with five volumes of water and applied to a column (1 by 10 cm) of Bio-Rad AG 50-X4 (200 to 400 mesh; H^+ form). The column was washed with 30 ml of water, then eluted with 1*M* pyridine until the radioactivity was recovered (about 45 ml). The radioactive eluate was freeze-dried, dissolved in

Table 1. Presence of ε-(γ-glutamyl)lysine in enzymic hydrolyzates of cross-linked fibrin; its absence in soluble fibrin prepared by removing essential calcium with EDTA; its reduction in soluble fibrin prepared with competitive inhibitor GEE.

Sample	ε-(γ-Glutamyl) lysine (moles per mole of fibrin*)
Polymerized fibrin	1.8
Polymerized fibrin	1.2
Soluble fibrin with EDTA	0.07
Soluble fibrin with GEE	0.7
Control (no fibrinogen)	0

*Based on a fibrinogen molecular weight of 330,000.

water, and put on a column (1.1 by 23 cm) of Bio-Rad AG 2-X8 (100 to 200 mesh, acetate form) which was then washed with water (about 35 ml) until virtually all the radioactive material had been eluted. This eluate was freeze-dried, dissolved in pyridine acetate buffer (0.20*M* pyridine, *p*H 3.23), and chromatographed in a Spinco model 120C amino acid analyzer on a column (18 cm) packed with PA-35 resin prepared according to instructions for treatment prior to chromatography with pyridine acetate buffers (*15*). Fractions (1 ml) were collected; those containing ε-(γ-glutamyl)lysine (fractions 51-57) were pooled, freeze-dried, and chromatographed in the amino acid analyzer by the accelerated procedure for acidic and neutral amino acids (*16*), with the exception that a 0.20*M* sodium citrate buffer (*p*H 3.83) was used without a buffer change. Amounts of ε-(γ-glutamyl)lysine, which were eluted after 88 minutes under these conditions, were calculated by the isotope dilution method.

A direct demonstration (Table 1) of ε-(γ-glutamyl)lysine was substantiated by the values for ε-amino-cross-linked lysine determined by subjecting a portion of each sample to cyanoethylation. The procedure (*17*) may offer a generally applicable method for detecting ε-lysyl linkages in proteins, especially insoluble proteins such as fibrin and collagen. The technique consists essentially of treating the protein with acrylonitrile, which reacts with residues having free amino groups to form cyanoethyl amino acids (*18*). When the protein is subsequently hydrolyzed with acid, these are converted to carboxyethyl derivatives (such as carboxyethyllysine). Lysine residues bound in ε-amino cross-links have no free amino group to react with acrylonitrile, hence upon hydrolysis they appear as free lysine. Lysine measured in the amino acid analyzer then serves as an index of the number of cross-links. This procedure not only confirmed the above results but also proved of great value in supporting experiments. It revealed that little cross-linked lysine was formed when the substrate was human fibrinogen free of factor XIII (*19*), whereas 1.3 moles per mole of fibrin were found when a preparation of human factor XIII (*11*) was added to this substrate.

Cross-linking of fibrin is usually determined by its solubility in acid. Now it is apparent that this is not a reliable test as evidenced by the fact that even though the amount of glycine ethyl ester used was sufficient to yield an acid-soluble clot ("non-cross-linked" fibrin by this criterion), the amounts of ε-amino–cross-linked lysine and of ε-(γ-glutamyl)lysine were reduced by only half. Similarly, data obtained with the analytical ultracentrifuge showed that cross-linked fibrin may be acid soluble (*20*).

The good agreement between the amount of cross-linking measured by enzymic and chemical methods indicates that lysine is cross-linked only to glutamate and not to any other acceptor (such as aspartate). The possibility of other, completely different types of cross-links is not ruled out, nor is it certain that transamidation is the only reaction occurring during fibrin polymerization (*20*). Nevertheless, our results provide the first direct demonstration that ε-(γ-glutamyl)lysine constitutes a cross-link of polymerized fibrin (*21*).

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Mutant of Bacterium paracoli 5099 with an Altered DNA: Identification as a Flavobacterium

Abstract. *A difference in the base composition of the DNA of* Bacterium paracoli *5099 and of a "mutant" (No. 1975) derived from it was found. This is in accordance with the finding of others. However, biochemical tests revealed that the "mutant" was a* Flavobacterium, *whereas the parent strain belonged to a species of* Escherichia. *The base composition of the DNA of the "mutant" is similar to that reported for the DNA of* Flavobacterium.

Our current concepts of the chemical basis of heredity equate a mutation with a change in a single nucleotide in the material (DNA or RNA) which constitutes the genome of the system under observation. The present-day techniques are not discriminatory enough to permit us to differentiate between the DNA's isolated from a wild bacterial strain and its mutants, although a start in this direction was made by the demonstration of a slight shift in buoyant density of the DNA of an *Escherichia coli* mutator strain (*1*). Therefore, the report by Gause and his collaborators (*2, 3*) of significant differences in base composition of the DNA derived from *Bacterium paracoli* 5099 and some of its mutants was of considerable interest and warranted confirmation by other laboratories. We now