Hersh, R. T., and Schachman, H. K. (1958), *Virology 6*, 234.
Hirs, C. H. W., Moore, S., and Stein, W. H. (1956), *J. Biol. Chem. 219*, 623.
Hjerten, S., Jernstedt, S., and Tiselium, A. (1965), *Anal. Biochem. 11*, 219.
Johnston, W., James, T. W., and Barber, A. A. (1967), *J. Comp. Biochem. Physiol. 22*, 261.
Marchelonis, J. J., and Edelman, G. M. (1968), *J. Mol. Biol. 32*, 453.
Pickett, S. M., Riggs, A. F., and Larimer, J. L. (1966), *Science 151*, 1005.
Sjoquist, J. (1960), *Biochim. Biophys. Acta 41*, 20.
Spackman, D. H., Stein, W. H. and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Spies, J. R., and Chambers, D. C. (1948), *Anal. Chem. 20*, 30.
Spiro, R. G. (1966), *Methods Enzymol. 9*, 3.
Stewart, J. E., Dingle, J. R., and Odense, P. H. (1966), *Can. J. Biochem. 44*, 1447.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Yphantis, D. A. (1960), *Ann. N. Y. Acad. Sci. 88*, 586.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

# Studies of Invertebrate Fibrinogen. II. Transformation of Lobster Fibrinogen into Fibrin*

G. M. Fuller† and R. F. Doolittle‡

ABSTRACT: Lobster fibrinogen is converted into a fibrin gel by the direct introduction of intermolecular ε-(γ-glutamyl)-lysine cross bridges. The number of cross-links per mole of starting fibrinogen was determined after total enzymic digestion of the fibrin followed by isolation and analysis of ε-(γ-glutamyl)lysine using an authentic radioactive tracer. The number of bridges was also estimated by the cyanoethylation procedure for quantifying blocked lysine ε-amino groups. We have also confirmed earlier notions that limited proteolysis is not involved in lobster fibrin formation by demonstrating that there is no change in amino-terminal residues during the fibrinogen–fibrin conversion. A series of electron micrographs is appended, which affords a dramatic visualization of the onset of the lobster gelation phenomenon.

The transformation of lobster fibrinogen into a fibrin gel is a phenomenon which has been studied by scores of investigators over a two-century period; its early history is well documented in a monograph by Glavind (1948). The conversion is catalyzed by a calcium-dependent enzyme found in certain lobster blood cells (hemocytes or coagulocytes) and muscle tissues. Vertebrate thrombins cannot substitute for the lobster clotting enzyme, and limited proteolysis, which is the basis of all vertebrate coagulation, is thought not to be involved. Instead, the formation of lobster fibrin appears to be directly dependent on the formation of intermolecular covalent bonds between fibrinogen molecules, resulting in a clot which cannot be dispersed by solvent systems known to disrupt weaker intermolecular forces.

The discovery (Doolittle and Lorand, 1962) that lobster fibrin formation is inhibited by certain glycine derivatives which also prevent the introduction of postgelation covalent bonds (cross-links) into vertebrate fibrin (Lorand *et al.*, 1962) led to renewed interest in the invertebrate coagulation scheme. Unfortunately, the prevailing theory at that time (Lorand *et al.*, 1962) about how vertebrate fibrin becomes reinforced by covalent bonds was erroneous in that it supposed the amino-terminal glycine residues of fibrin—exposed by the action of thrombin—to be incorporated into acceptor sites on companion molecules. Since lobster gelation was inhibited by the same glycine derivatives, it was natural to presume that the amino terminals of lobster fibrinogen were glycine and that they became incorporated intermolecularly during the clotting process. When we examined the amino terminals of lobster fibrinogen, however, we found them to be leucine (Fuller and Doolittle, 1971). This observation prompted us not only to reconsider our thoughts about how lobster fibrin is formed, but also to reevaluate notions as to how vertebrate fibrin became covalently cross-linked. We were subsequently able to demonstrate the involvement of ε-amino groups of lysine side chains in both kinds of cross-linking (Fuller and Doolittle, 1966). Independently, Lorand *et al.* (1966a,b) came to the same conclusions about the vertebrate system on the basis of experiments prompted by the observation that tosyl-L-lysine methyl ester is an inhibitor of vertebrate fibrin cross-linking (Lorand, 1965). Meanwhile, Matačić and Loewy (1966) had showed conclusively that the acceptor portions of the vertebrate fibrin cross-linking system were glutaminyl side chains. It only remained for Matačić and Loewy (1968) and Pisano *et al.* (1968) to independently and simultaneously isolate the ε-(γ-glutamyl)lysine isopeptides,

* From the Departments of Marine Biology and Chemistry, University of California, San Diego, La Jolla, California 92037. *Received May 15, 1970.* This investigation was supported by grants from the National Science Foundation (GB-4619 and GB-7332), and the National Institutes of Health (AI-07781 and HE-12759). During the period July 1966–Sept 1968, G. M. F. was supported by a National Institutes of Health predoctoral fellowship. This article is based in part on a Ph.D. dissertation submitted by G. M. F. to the University of California, San Diego. A preliminary account of portions of this study was given at The Pacific Slope Biochemistry Conference, June 1966. Dr. J. D. McLean, who coauthored the Appendix, was supported by the National Institutes of Health Program Project Grant GM 12027. His present address is: Division of Chemical Physics, CSIRO, Clayton, Victoria, Australia 3168.

† Present address: Department of Human Genetics, University of Texas Medical School, Galveston, Texas 77550; to whom to address correspondence.

‡ Career Development awardee, U. S. Public Health Service.

thereby demonstrating unequivocally the nature of covalent cross-linking in vertebrate systems.

In this article, we present evidence that the fundamental chemistry involved in the lobster fibrin formation is the same as occurs in the final stage of vertebrate fibrin reinforcement, involving as it does the condensation of glutaminyl and lysine side chains to form ε-(γ-glutamyl)lysine cross bridges. On the other hand, the involvement of the same types of amino acid side chains in the intermolecular bonding scheme is hardly evidence for common ancestry, and we argue, especially in the light of our physicochemical characterization of lobster fibrinogen (Fuller and Doolittle, 1971), that the two kinds of fibrin formation, vertebrate and invertebrate, are likely the result of a superficial parallel evolution. We have also confirmed earlier notions that the lobster-gelling phenomenon does not involve proteolysis and that the only enzyme involved is a calcium-dependent, sulfhydryl-type transglutaminase (transamidase) of the type isolated from mammalian liver by Waelsch and his coworkers (Clark *et al.*, 1959).

## Materials and Methods

Glycine methyl ester hydrochloride, glycine ethyl ester hydrochloride, and tosyl-L-lysine methyl ester hydrochloride were all purchased from Cyclo Chemical Corp., as was reference ε-(γ-glutamyl)lysine. In the latter case, aliquots of stock solutions were subjected to acid hydrolysis and their concentrations verified by measurement of the freed glutamic acid and lysine on a Spinco amino acid analyzer. A color coefficient for the unhydrolyzed substance was determined which was very close to the sum of the color coefficients of free glutamic acid and leucine. The reference ε-(γ-glutamyl)-lysine eluted at about 57 min when a pH 3.85, 0.2 N sodium citrate buffer was used on a standard 50-cm Spinco column.

[$^{14}$C]ε-(γ-Glutamyl)lysine was the generous gift of Dr. J. J. Pisano. Stock solutions were prepared which contained 0.036 μmole/ml and $3.6 \times 10^5$ cpm. All radioactivity measurements were performed in a Beckman scintillation counter using appropriate dispersing mixtures of scintillator (Benson, 1966).

Lobster fibrinogen was purified according to the scheme described in the preceeding paper (Fuller and Doolittle, 1971). Most experiments were carried out on both fibrinogens A and B as distinguished by chromatography on agarose A1.5. Fibrin preparations for subsequent chemical analysis were produced from either fibrinogen A or B by the addition of minimal amounts of lobster hemocyte extract (Fuller and Doolittle, 1971) and enough calcium chloride solution to bring the final concentration to 0.03 M. The protein contributed to the system by the coagulation enzyme extract was always less than five percent of the total protein. Fibrin clots were let stand a minimum of 50× the clotting time before washing or chemical determinations were undertaken.

Amino acid compositions and amino-terminal residues were determined as described in the preceding paper (Fuller and Doolittle, 1971). Fibrin preparations (10–30 mg each) were usually washed free of soluble contaminants contributed by the cell enzyme extract, although in those cases where the fibrin amino-terminal residues were determined directly on the mixture, the results were identical with washed preparations.

Amidination of lobster fibrinogen amino groups was achieved with 0.2 M ethyl acetimidate (Hunter and Ludwig, 1962) in a pH 8.5, 0.1 M sodium borate buffer, for 2 hr at 0°. Under these conditions Wofsy and Singer (1963) found that 62–65% of the lysine side chains of bovine γ-globulin were reacted.

The cyanoethylation procedure for measurement of blocked ε-amino groups of lysine residues was followed rather literally as described by Pisano *et al.* (1969). After tryptic digestion of lobster fibrinogen or fibrin preparations to ensure accessibility of the acrylonitrile reagent, 1.0 ml of the protein solution was mixed with 20 μl of triethylamine and 0.2 ml of acrylonitrile, the tubes sealed, and shaking allowed to take place for 72 hr at 37°. The tubes were opened and volatile materials evaporated, after which the residues were hydrolyzed with constant-boiling hydrochloric acid in the customary way. Residual lysine was measured on the short column of the Spinco amino acid analyzer.

Direct measurement of ε-(γ-glutamyl)lysine was achieved after total enzymic digestion of the fibrinogen and fibrin preparations. The protocol for total digestion was that suggested by Pisano *et al.* (1969) with the exception that the step which called for the use of prolidase was omitted.[1] The enzymes used for digestion included trypsin (Worthington), Pronase (Calbiochem), and leucine aminopeptidase (Worthington). After digestion was complete, small amounts (1.8 nmoles/preparation; 15,000 cpm) of [$^{14}$C]ε-(γ-glutamyl)-lysine were added as tracer substance. The digestion mixture was then subjected to chromatography on 0.9 × 30 cm Dowex 50-X4 columns equilibrated with 0.1 M ammonium acetate (pH 3.8). After 50 ml more of the pH 3.8 buffer was run through the column, a stepwise shift was made to pH 4.2 M ammonium acetate. Fractions of 1 ml were collected throughout and radioactivity monitored on 50-μl aliquots. The fractions containing the radioactivity were pooled and freeze-dried in round-bottom flasks. The residues were dissolved in distilled water and redried in test tubes. Usually, the samples were analyzed directly without further treatment on the Spinco amino acid analyzer employing pH 3.85 buffer. In some cases, a portion of the material was subjected to electrophoresis at pH 2.0 and the radioactive band eluted, concentrated, and subjected to total acid hydrolysis.

*Clotting Assays.* The effect of potential inhibitors of lobster clotting and/or the influence of chemical derivatization of lobster fibrinogen was investigated in a system of the following proportions: 0.2 ml of 1% fibrinogen solution, 0.2 ml of 0.1 M $CaCl_2$, 0.1 ml of additive at appropriate concentration, and 0.025 ml of cell enzyme extract. The clotting time was designated as that time when the system no longer flowed freely when the tube was gently tipped. The amount of protein incorporated in the clot was determined by measuring the $A_{280}$ of the liquid phase of the system before and after gel formation. All assays were carried out at 25 ± 1°.

## Results

*Amino Terminals and Amino Acid Analysis.* As in the case of lobster fibrinogen, the only free amino-terminal amino acid found in lobster fibrin was leucine. A large number of amino-terminal determinations was made employing a wide variety of conditions, since the recovery of some amino acids (*e.g.*, serine) is favored by certain milder treatments. As a consequence, not all the quantitative data are strictly comparable. Although there was considerable scatter in the quantitative data, as often occurs when performing end-group determinations on insoluble substances, the average amount of

[1] The prolidase acetone powder obtained by those authors from Worthington Biochemical Corp. is no longer available commercially.

TABLE I: Determination of Underivatized Lysines after Cyanoethylation (Moles of Lysine Recovered/420,000 Molecular Weight).

| Experiment | Fibrinogen A | Fibrin A | $\Delta A$[a] | Fibrinogen B | Fibrin B | $\Delta B$[a] |
|---|---|---|---|---|---|---|
| I | 2.8 | 7.5 | 4.7 | 2.3 | 8.7 | 6.4 |
| II | 5.4 | 9.5 | 4.1 | 4.6 | 11.9 | 7.3 |

[a] $\Delta A$ and $\Delta B$ represent the additional lysine residues unavailable to cyanoethylation in fibrin compared to fibrinogen and, by implication, involved in cross-linking through their ε-amino groups.

TABLE II: Determination of ε-(γ-Glutamyl)lysine Cross-Links after Total Enzymic Digestion.

| | Moles/420,000 Molecular Weight[a] | |
|---|---|---|
| | Fibrin A | Fibrin B |
| Experiment I | 3.6 | 3.4 |
| Experiment II | 2.5 | 2.5 |

[a] Corrected for losses during isolation by using tracer amounts of [$^{14}$C]ε-(γ-glutamyl)lysine and calculating per cent recovery of radioactivity.

leucine found by the Edman method was close to 2.0 residues/420,000 of fibrin, a value which was in close agreement with the number of amino-terminal leucines found in fibrinogen. Furthermore, similar results were obtained in fibrin systems completely dispersed by mercaptoethanol treatment in the presence of 5 M urea, in which cases the fibrin stayed in solution when pyridine was added volume for volume before the addition of phenyl isothiocyanate.

Usually the end-group determinations were performed on fibrin preparations washed free of the clotting enzyme. To be certain that no small molecular weight substances (which could conceivably have been produced by limited proteolysis) had been removed in the washing, several determinations were made on unwashed fibrin. These were not found to differ from the washed preparations. All observations were fully in accord with the notion that limited proteolysis is not involved in lobster fibrin formation.

The amino acid compositions of lobster fibrinogen and fibrin washed free of soluble contaminants were also found to be indistinguishable. This result was fully anticipated since more than 95% of the protein in these fibrinogen preparations became incorporated in the gel.

*Involvement of Lysine Side Chains.* Fibrin formation in the spiny lobster, like that reported earlier for another lobster species, *Homarus americanus*, (Doolittle and Lorand, 1962), is inhibited by the same glycine derivatives which block postgelation reinforcement in vertebrate fibrin systems (Lorand *et al.*, 1962). Glycine methyl ester and glycine ethyl ester are inhibitory to about the same degree as previously reported for *Homarus*, but the most effective inhibitor tried was tosyl-L-lysine methyl ester, a compound reported to be an inhibitor of cross-linking in vertebrate fibrin (Lorand, 1965). Gel formation in 0.5% lobster fibrinogen solutions is completely prevented at concentrations of tosyl-L-lysine methyl ester as low as $10^{-4}$ M. The powerful inhibition exhibited by the lysine derivative is consistent with the interpretation that the glycine derivatives are behaving as lysyl side-chain analogs in these situations (Doolittle, 1970).

Amidination of the most readily available amino groups in lobster fibrinogen with ethyl acetimidate completely blocks the gelation process. Under the same circumstances, control preparations of bovine fibrinogen remained completely clottable when exposed to thrombin, although they no longer were able to be cross-linked by factor XIII (Fuller and Doolittle, 1966).

Further proof of the involvement of lysyl side chains was established using the cyanoethylation procedure (Pisano *et al.*, 1969) whereby all lysines with free amino groups are irreversibly derivatized with acrylonitrile. Subsequent acid hydrolysis revealed that lobster fibrin contained significantly more free lysine after this treatment than did lobster fibrinogen, indicating that lysine amino groups became blocked during fibrin formation (Table I). The number of lysines which became inaccessible to the acrylonitrile as a result of fibrin formation amounted to 4–6/420,000. The fact that there was a significant amount of inaccessible lysine in the fibrinogen preparations indicates that cyanoethylation was not absolutely complete, however, and as a consequence the values may be somewhat high.

*Isolation of ε-(γ-Glutamyl)lysine.* Direct identification and quantitation of ε-(γ-glutamyl)lysine was made after total enzymic digestion of lobster fibrinogen and fibrin. Tracer amounts (1.8 nmoles/preparation) of [$^{14}$C]ε-(γ-glutamyl)-lysine were added to the digestions, which were then purified from the bulk of the free amino acids by chromatography on Dowex 50-X4. The radioactive pools were concentrated and analyzed directly on the amino acid analyzer at pH 3.85 (Table II). Corrections for procedural losses were readily calculated by counting the radioactivity in the final samples.

Direct identification of the ε-(γ-glutamyl)lysine was also made by further purifying the pools from Dowex 50-X4 on paper electrophoresis. The radioactive material was eluted, concentrated, and subjected to acid hydrolysis. Approximately equimolar amounts of lysine and glutamic acid were recovered in amounts close to that of the ε-(γ-glutamyl)lysine measured directly. In all cases the fibrin contained significant amounts (2.5–3.6 moles/420,000) of this isodipeptide, whereas the fibrinogen preparations from which they were prepared did not (Table II).

In these experiments, we also compared fibrinogens A and B with regard to inaccessible lysines and/or measurable amounts of ε-(γ-glutamyl)lysine, since it had been our conjecture that fibrinogen A was a stable dimer of fibrinogen B, perhaps representing an early, soluble form of fibrin (Fuller and Doolittle, 1971). There was no measurable amount of ε-(γ-glutamyl)lysine in either fibrinogen, however, and the fibrinogen A did not contain any more lysines which were inaccessible to cyanoethylation than did the fibrinogen B.

## Discussion

Fibrin formation in lobsters is the direct result of the enzymatic introduction of intermolecular covalent bonds between certain glutaminyl and lysyl side chains (Figure 1). Unlike the vertebrate process, limited proteolysis is not involved in the gelation phenomenon (Figure 2). The lobster clotting enzyme is an intracellular, calcium-dependent

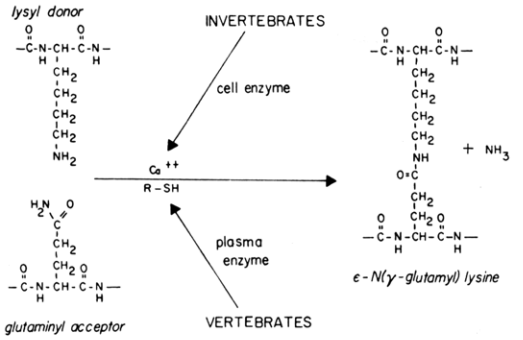


FIGURE 1: Proposed molecular mechanism for cross-linking in invertebrates and vertebrates.

transglutaminase which is released from ruptured hemocytes upon suitable provocation. It is very similar in its properties and action to a transglutaminase isolated from guinea pig liver by Clark *et al.* (1959). Indeed, Lorand *et al.* (1966a,b) have shown that lobster blood plasma can be gelled by the guinea pig enzyme. The same liver enzyme can also gel mammalian fibrinogen directly without the prior removal of the fibrinopeptides (Farrell and Laki, 1970).

In this report, we have definitively shown that ε-(γ-glutamyl)lysine cross-links are produced during the transformation of lobster fibrinogen into fibrin. Under the conditions employed, 2–6 cross-links were found for every 420,000 of fibrin. Of the two quantitative methods we used, one (cyanoethylation) has an experimental bias on the high side whereas the other (total enzymic digestion) would yield values, which, if in error at all, would be too low. Consequently, the average value (about four) is probably quite reliable.

On the other hand, it may be misleading to think that there is always a stoichiometric number of cross-links involved. The minimum number of cross-links per molecule necessary to produce a continuous molecular network is theoretically only two. In vertebrates, postgelation cross-linking catalyzed by activated factor XIII involves two different regions of the molecule in independent cross-linking situations (Chen and Doolittle, 1969), although both apparently depend on the formation of ε-(γ-glutamyl)lysine isopeptide linkages. The first of these to form results from the joining of overlapping carboxy-terminal segments of neighboring γ chains to yield γ–γ dimers (Chen and Doolittle, 1970). The second type,

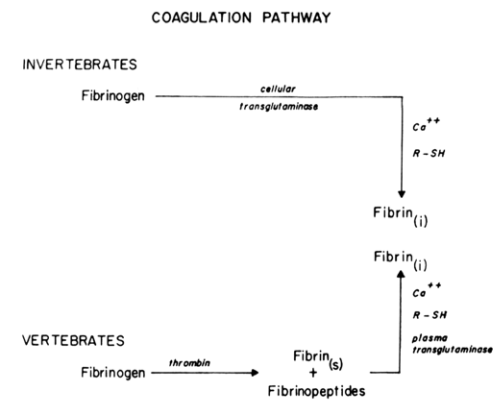


FIGURE 2: Coagulation pathways showing similarities and differences in the cross-linking reactions of vertebrates and invertebrates. Fibrin (s) = soluble fibrin. Fibrin (i) = insoluble fibrin.

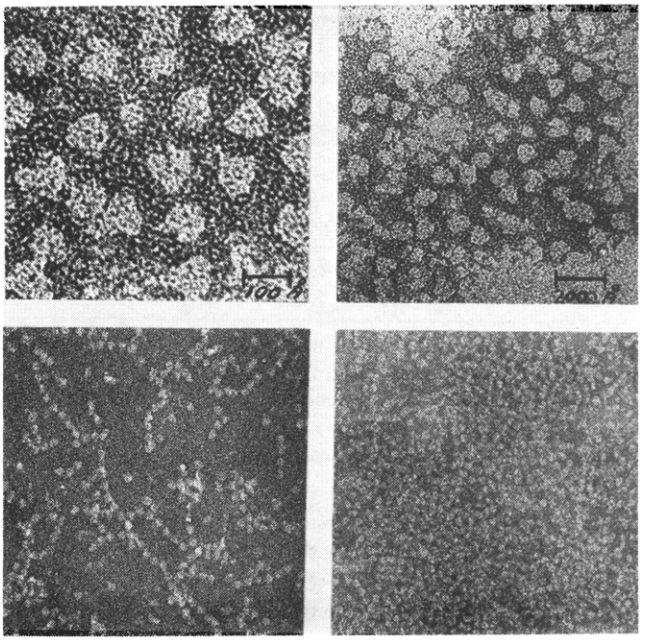

FIGURE 3: Electron micrographs of negatively stained preparations of lobster fibrinogen and fibrin. Upper left: high magnification of individual lobster fibrinogen molecules (1,400,000 ×). Upper right: larger field of lobster fibrinogen molecules (650,000 ×). Lower left: chains of lobster fibrinogen molecules shortly after exposure to the clotting enzyme (210,000 ×). Lower right: arrays of chains stacked side by side early in lobster fibrin formation (180,000 ×).

which is formed more slowly, has been shown to involve α chains exclusively and apparently results in a staggered system of α chain multimers (McKee *et al.*, 1970). As a consequence, the number of ε-(γ-glutamyl)lysine cross-links isolated per mole of vertebrate fibrin can vary with the exact clotting conditions, and reported values have ranged from 1.2 to 2.8 per mole (Matačić and Loewy, 1968; Pisano *et al.*, 1969). In theory, a minimum of four cross-links per mole should be possible if all sites are saturated, since each γ and each α chain has a minimum of one donor and one acceptor site, the vertebrate molecule also being a dimer. In the case of the lobster, our average value of four cross-links per mole would imply that there are at least two donor sites and two acceptor sites in each half-molecule of lobster fibrinogen.

Recent advances in our knowledge of postgelation cross-linking in vertebrate fibrin also indicate that the involvement of ε-(γ-glutamyl)lysine cross bridges in different systems is hardly evidence for common ancestry. At present there is no reason to believe that the γ and α chains of vertebrate fibrinogen stem from a common ancestor, and yet each is independently involved in postgelation cross-linking. Furthermore, the cross-linked systems produced are fundamentally different, one resulting in reciprocal dimer formation (γ–γ) and the other in unlimited multimer formation. Seen in this light, we tend to regard the involvement of ε-(γ-glutamyl)-lysines in lobster fibrin formation as a simple manifestation of parallel evolution. In line with our physicochemical characterization of lobster fibrinogen, there is no present basis for thinking that lobster fibrin formation has any direct evolutionary connection with the vertebrate process, even though it can be inhibited by the same agents which interfere with fibrin stabilization in vertebrates and involves the same kinds of amino acid side chains in the intermolecular covalent bonds which are formed.

Acknowledgment

We thank Dr. K. Tokuyasu for his help in printing some of these micrographs in preparation for publication.

Appendix

*Electron Microscopy of Lobster Fibrinogen and Fibrin*[2]

By G. M. Fuller, J. D. McLean, and R. F. Doolittle

As an adjunct to our chemical studies on the conversion of lobster fibrinogen into fibrin, a series of electron micrographs were made of lobster fibrinogen molecules and lobster fibrin preparations at various stages of gel development. The micrographs were made using a Phillips 300 electron microscope and represent preparations which were negatively stained with 1% phosphotungstic acid neutralized with potassium hydroxide. In all cases 0.1% fibrinogen solutions in 0.1 M Tris buffer (pH 7.5) were used as starting material. A drop of the protein solution was placed in 2.0 ml of 1% phosphotungstate and a carbon-coated copper grid (1500 mesh) dipped into the preparation and then dried. Fibrin formation was observed by adding appropriate amounts of calcium chloride and clotting enzyme to the fibrinogen dilute solution as described in the main text of this paper. The protein concentration was low enough that complete gelation could not occur.

The lobster fibrinogen molecule appears somewhat acorn-shaped when examined at high magnification (Figure 3, upper left plate). The average diameter of these molecules is approximately 100 Å, a value consistent with the hydrodynamic measurements made in the ultracentrifuge, since a perfect sphere with a density of 1.41 (partial specific volume 0.711) and a molecular weight of 420,000 has a theoretical diameter of 98 Å. A larger field of fibrinogen molecules at lower magnification (650,000 ×) is shown in the upper right plate of Figure 3.

The transformation into a fibrin gel begins with the formation of simple chains of fibrinogen molecules (Figure 3, lower left plate). These chains subsequently align themselves side by side into more or less regular sheetlike arrays (Figure 3, lower right plate), giving rise to the continuous molecular network which constitutes gelation.

[2] This portion of the study on the transformation of lobster fibrinogen into fibrin was conducted while Dr. J. D. McLean was affiliated with the Department of Biology, University of California, San Diego.

References

Benson, R. (1966), *Anal. Chem. 38*, 1353.
Chen, R., and Doolittle, R. F. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 420.
Chen, R., and Doolittle, R. F. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 472.
Clark, D. D., Mycek, M. J., Neidle, A., and Waelsch, H. (1959), *Arch. Biochem. Biophys. 79*, 338.
Doolittle, R. F. (1970), *Thromb. Diath. Haemorrh., Suppl. 39*, 25.
Doolittle, R. F., and Lorand, L. (1962), *Biol. Bull.*, 481.
Farrell, J., and Laki, K. (1970), *Blood 35*, 804.
Fuller, G. M., and Doolittle, R. F. (1966), *Biochem. Biophys. Res. Commun. 25*, 694.
Fuller, G. M., and Doolittle, R. F. (1971), *Biochemistry 10*, 1305.
Glavind, J. (1948), Studies on the Coagulation of Crustacean Blood, Copenhagen, Arnold Busck.
Hunter, M. J., and Ludwig, M. L. (1962), *J. Amer. Chem. Soc. 84*, 3291.
Lorand, J. B., Urayama, T., and Lorand, L. (1966a), *Biochem. Biophys. Res. Commun. 23*, 828.
Lorand, L. (1965), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 24*, 784.
Lorand, L., Konishi, K., and Jacobsen, A. (1962), *Nature (London) 194*, 1148.
Lorand, L., Ong, H. H., Lipinski, B., Rule, N. G., Downey, J., and Jacobsen, A. (1966b), *Biochem. Biophys. Res. Commun. 25*, 629.
Matačić, S., Loewy, A. G. (1966), *Biochem. Biophys. Res. Commun. 24*, 858.
Matačić, S., and Loewy, A. G. (1968), *Biochem. Biophys. Res. Commun. 30*, 356.
McKee, P. A., Mattock, P., and Hill, R. L. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 738.
Pisano, J. J., Finlayson, J. S., and Peyton, M. P. (1968), *Science 160*, 892.
Pisano, J. S., Finlayson, J. S., and Peyton, M. P. (1969), *Biochemistry 8*, 871.
Wofsy, L., and Singer, S. J. (1963), *Biochemistry 2*, 104.