Studies of Invertebrate Fibrinogen. I. Purification and Characterization of Fibrinogen from the Spiny Lobster*

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ABSTRACT: The fibrinogen of an invertebrate, the California spiny lobster (*Panulirus interruptus*), has been purified and a number of its physical and chemical properties have been determined.

The molecular weight of this fibrinogen was determined to be 420,000. The sedimentation coefficient, $s^0_{20,w}$, of the native fibrinogen was found to be 14.5 S and the diffusion coefficient 2.9×10^{-7} cm^2 sec^{-1}. Treatment with sodium dodecyl sulfate lowers the sedimentation coefficient to 7.1 S, suggesting dissociation into half-molecules. Subsequent reduction and alkylation results in a further decrease in the sedimentation coefficient to 4.0 S. These observations are consistent with a half-molecule composed of three polypeptide chains linked by disulfide bridges. The only free amino-terminal amino acid found was leucine, although quantitation of recoveries indicated that some of the amino terminals may be blocked.

The hemolymph (blood) of certain crustaceans contains a soluble protein which can be transformed into a solid gel upon suitable provocation. This protein, generally referred to as fibrinogen, has been purified previously from the bloods of several species of lobster, and preliminary physicochemical characterization has shown it to be quite different from the vertebrate protein of the same apparent function (Duchateau and Florkin, 1953; Stewart *et al.*, 1966). These observations were consistent with early reports (Glavind, 1948) that lobster fibrinogen is not clotted by vertebrate thrombins, but is readily gelled in a single-step process by an enzyme found in its blood cells (variously referred to as hemocytes or coagulocytes) and muscle tissue.

The discovery (Doolittle and Lorand, 1962) that the transformation of lobster fibrinogen into fibrin can be inhibited by certain glycine derivatives which also prevent postgelation cross-linking in vertebrate fibrin, led to renewed interest in the invertebrate clotting mechanism. In order to investigate the potential similarities and differences in fibrin formation in vertebrates and invertebrates, we have developed an improved purification scheme for lobster fibrinogen and characterized several of its physicochemical properties. None of these leads us to believe that the invertebrate fibrinogen molecule has any direct evolutionary ancestry in common with vertebrate fibrinogen molecules. In a separate article (Fuller and Doolittle, 1971) we deal with the enzymatic transformation of the lobster fibrinogen molecule into fibrin gels.

Material and Methods

Blood Collection. Live, fresh lobsters (*Panulirus interruptus*) were obtained from the Scripps Aquarium, La Jolla, Calif. Hemolymph was collected by inserting a 0.75-in. 13-gauge needle into the anterior-most ventral soft segment. Usually 25–50 ml of blood was drawn into a 50-ml plastic syringe containing from 5 to 10 ml of 0.1 M sodium citrate, although the amounts of blood obtained depended on the size of the lobster. The blood from several lobsters was pooled and immediately stored on ice. The citrated blood was centrifuged for 15 min at 20,000 rpm at 4° in order to collect the white cells (hemocytes) normally found in the hemolymph fluid. The plasma could then be stored in plastic bottles at 4° for several weeks without loss of clotting activity. When sodium azide (2–5 drops of a 1% solution/100 ml of plasma) was added, the storage time at 4° could be extended for several months. Furthermore, the plasma retained its clotting activity indefinitely when stored at −70°.

Preparation of Clotting Enzyme. The cellular mass from the initial centrifugation was washed with 0.25 M sucrose containing 0.01 M cysteine and adjusted to pH 7.5. The cells were then drained and transferred to a Potter-Elverhelm glass homogenizer and thoroughly disrupted using 2–3 ml of the sucrose–cysteine buffer. The homogenate was centrifuged for 20 min at 20,000 rpm at 4°, and the supernatant fluid containing the active lobster coagulating enzyme recovered. The crude enzyme extract was stable indefinitely when stored at −70°. A 25-μl quantity of the enzyme extract (A_{280} *ca.* 35) prepared in this manner could routinely clot 0.2 ml of lobster plasma in 20–30 sec after addition of 0.2 ml of 0.1 M calcium chloride. For comparison purposes, tissues from other organs, including tail muscle, were homogenized in the same way; the enzyme activity found in these preparations was not nearly as great as that from the extracted hemocytes.

Clotting assays were routinely performed as follows: 0.2 ml of plasma or fibrinogen was pipetted into a 0.5 × 8.0 cm clotting tube and 0.025 ml of enzyme solution added. Finally, 0.2 ml of 0.1 M calcium chloride was added, the contents mixed quickly, and timing begun. The tube was gently tilted to test for the formation of a solid clot, and the clotting time measured as the time when the gel would stand

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rigidly when the tube was horizontal. The per cent clottable protein was measured by comparing the A_{280} of the original mixture with that of the residual fluid after removal of the fibrin gel on a glass rod.

Polyacrylamide Electrophoresis. ANALYTICAL GELS. Procedures described by Davis (1964) were used in preparing and running acrylamide gels. Tray buffer consisted of 0.19 M glycine and 0.025 M Tris (pH 8.4). A 6% acrylamide gel concentration was used because it minimized the protein sticking to the top of the gel as well as shortening the running time. The sample gel or large pore gel described by Davis was replaced by the "ion-focus" condition described by Hjerten *et al.* (1965). Electrophoresis was carried out for two hours at 2.5 mA/tube.

Preparative Electrophoresis. Preparative acrylamide electrophoresis was accomplished on a Polyprep instrument (Büchler Instrument Corp.). As in the case of the analytical gels, a 6% running gel was used in the preparative separation of fibrinogen B from fibrinogen A. The upper buffer was 0.039 M glycine–0.05 M Tris; the lower buffer contained 0.1 M Tris, 50 ml of 0.25 N HCl, and 10^{-4} M EDTA. The membrane chamber buffer consisted of 0.5 M Tris titrated to pH 9.0. A sample volume of 1.0 ml containing 50–70 mg of protein was layered on top of the gel. The protein was eluted from the membrane chamber by a constant buffer flow of 15 ml/hr. The separation was performed at 4° and usually took 20 hr.

Analytical Ultracentrifuge. SEDIMENTATION VELOCITY. Sedimentation velocity and sedimentation equilibrium determinations were performed in a Spinco Model E analytical ultracentrifuge using schlieren optics and a RTIC temperature unit. Sedimentation velocity studies were performed at 59,780 rpm in 12-mm sectored cells with aluminum or Kel-F centerpieces. Sedimentation velocities were measured in 0.1 M Tris buffer (pH 8.0). Sedimentation coefficients were calculated from a plot of time *vs.* log of the distance of migration of the refractive index peak to the center of rotation. All determinations were corrected to standard conditions. Some studies of sedimentation coefficients were made in 0.1% sodium dodecyl sulfate–0.1 M Tris buffer (pH 8.0).

DIFFUSION COEFFICIENT. The diffusion coefficient measurements were determined in the ultracentrifuge according to the technique described by Ehrenberg (1959). The procedure is similar to a synthetic boundary determination for sedimentation equilibrium studies. Several protein concentrations were used and extrapolated to zero concentration.

SEDIMENTATION EQUILIBRIUM. Molecular weights were determined by means of the high-speed equilibrium method described by Yphantis (1960) using a modified multichannel cell (Yphantis, 1964). The partial specific volume was determined by pycnometry and by calculation from the amino acid composition. Both methods gave comparable values, 0.711 cm^3/g.

Extinction Coefficient. The extinction coefficient was calculated from determinations of total nitrogen. Total protein nitrogen was measured by the micro-Kjeldahl procedure. The $E_{1\text{ cm}}^{1\%}$ at 280 mμ was determined to be 12.5.

Amino Acid Analysis. Amino acid analyses were performed on a modified (6.6-mm light-path cuvet) Spinco Model 120B amino acid analyzer (Spackman *et al.*, 1958). Samples were prepared for analysis by exhaustive dialysis against distilled water followed by lyophilization in tarred hydrolysis tubes. Dry weights were determined and constant-boiling HCl added; tubes were evacuated, sealed, and maintained at 105–107° for 24 or 48 hr. Cysteine was measured as cysteic acid on performic acid oxidized samples according to the method of Hirs *et al.* (1956). Tryptophan was determined according to the method of Spies and Chambers (1948).

Reduction and Alkylation. Disulfide reduction was accomplished according to the procedure outlined by Fleischman *et al.* (1962). During the procedure the lobster fibrinogen was maintained in protein-solubilizing solvents, such as 8 M urea, 6 M guanidine hydrochloride, or 0.1% sodium dodecyl sulfate.

Amino-Terminal Analysis. Amino-terminal analyses were performed, for the most part, using the phenyl isothiocyanate procedure of Edman (1950). A variety of modifications in coupling, washing, and cleavage conditions were tried inasmuch as the recovery of different amino-terminal residues is favored by different conditions with this procedure. Once it had been established that the only PTH[1] residues ever found were PTH-leucine (or isoleucine), most of the quantitation was determined using the procedure described by Blombäck and Yamashina (1958). PTH-amino acids were quantified by elution after paper chromatography according to the method of Sjoquist (1960). A value of 16,700 was used for $\epsilon_{269}^{1\text{ cm}}$ for PTH-leucine. Identification of the amino-terminal residue as leucine, as opposed to isoleucine, was established using a new coupling reagent, thioacetylthioglycollic acid, which readily regenerates the free amino acid after cleavage from the protein (G. A. Mross and R. F. Doolittle, unpublished procedure). Leucine was then readily distinguished from isoleucine on the Spinco amino acid analyzer.

Carbohydrate and Lipid Determinations. Total carbohydrate was estimated by the anthrone reaction. Protein samples were tested directly without prior hydrolysis by measuring the furfural derivatives formed in concentrated sulfuric acid in the presence of anthrone (Spiro, 1966). Qualitative determination of sugars and sugar derivatives was made after hydrolysis of the protein in 2 N HCl for 6 hr at 100°. Hydrolysates were applied to Whatman No. 1 paper and chromatographed for 40 hr in 1-butanol–pyridine–0.1 N HCl (5:3:2, v/v).

Total lipid was estimated by dry weight measurements after extraction of protein samples with acetone (acetone–1% protein solution, 9:1, v/v). Both the acetone extracts and the residual protein pellets were freed of acetone and brought to constant weight in a vacuum oven containing P_2O_5, and the per cent lipid calculated. Qualitative identification of the carotenoid pigment astaxanthin in the lipid fraction was made by thin-layer chromatography.

Results

Purification of Fibrinogen. Traditionally fibrinogen purification procedures are gauged by the amounts of clottable protein they yield. Using this criterion several investigators have produced lobster fibrinogen preparations of high purity by relatively simple precipitation techniques (Duchâteau and Florkin, 1953; Stewart *et al.*, 1966). We have confirmed that the isoelectric precipitation procedure introduced by Glavind (1948) effectively yields a fibrinogen preparation of high clottability. The material exhibits several components in gel electrophoresis, however, including a band which corresponds to hemocyanin. Furthermore, gel filtration on agarose A1.5 revealed two clottable components in addition to the hemocyanin. We refer to these two clottable components as lobster fibrinogens A and B. On the basis of arguments presented in the discussion, we regard the smaller of these

[1] Abbreviation used is: PTH, phenylthiohydantoin.

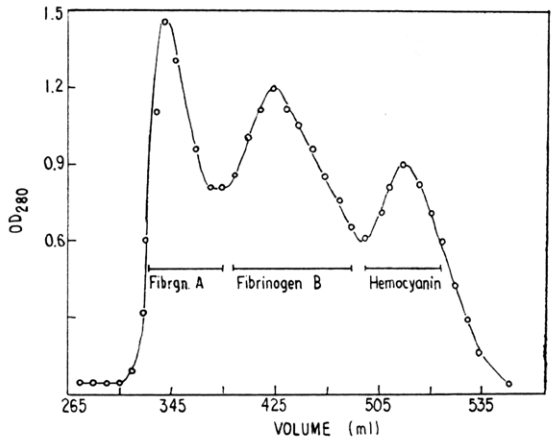

FIGURE 1: Gel filtration of crude lobster fibrinogen on a 5.5 × 50 cm agarose A1.5 column equilibrated with 0.05 M glycine containing 10^{-3} M EDTA (pH 10.0).

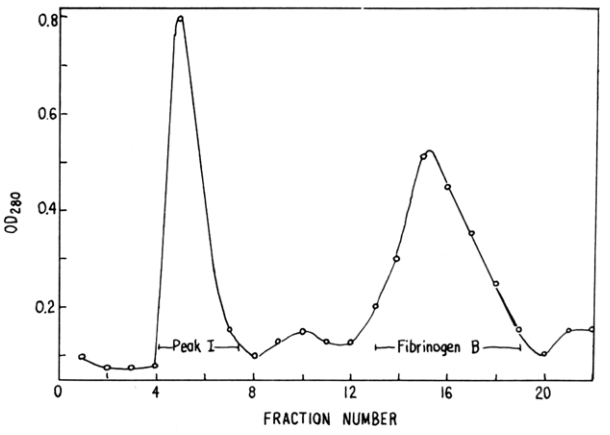

FIGURE 2: Elution profile of lobster fibrinogen B from preparative polyacrylamide electrophoresis. Peak I represents a nonprotein contaminant of the buffer system, which absorbs at 280 mμ. Peak II is lobster fibrinogen B. Fibrinogen A is retarded by the gel and does not elute even after extensive running.

two fibrinogens, fibrinogen B, as the primary coagulable protein synthesized in lobster systems. The following scheme yields a fibrinogen B preparation which is not only entirely clottable, but also gives a single band upon analytical gel electrophoresis.

Preparation of Crude Fibrinogen by Isoelectric Precipitation. Usually 100 ml of citrated plasma was dialyzed against several changes of 0.05 M Tris buffer (pH 8.0) at 4°. The cold plasma was then brought to pH 5.0 by the dropwise addition of 1 M sodium acetate buffer (pH 4.6). A dense precipitate forms when the pH nears pH 5; the slurry was stored on ice for 1 hr before being centrifuged at 15,000*g* for 15 min. The supernatant fluid, which contained no clottable protein, was discarded. The precipitate, which is generally bright orange, is then redissolved in a volume of 0.1 M Tris buffer (pH 8.0) roughly equivalent to 20% of the starting plasma volume. It is then redialyzed against 0.05 M Tris and reprecipitated with the sodium acetate buffer. Although the bulk of the hemocyanin is removed by these two precipitations, electrophoretically detectable traces remain. The clottability of this preparation, which we refer to as crude fibrinogen, is usually greater than 90%. The average yield of clottable protein from 100 ml of citrated plasma is about 0.4 g.

Agarose Gel Filtration. Previously Pickett *et al.* (1966) and Johnston *et al.* (1967) demonstrated that lobster hemocyanin can be dissociated into subunits at high pH and low ionic strength in the presence of EDTA. Since the molecular size of native lobster hemocyanin is close to that of one of the observed lobster fibrinogens, separation by gel filtration was impractical at neutral pH. By dissociating the lobster hemocyanin into subunits, however, this difficulty was largely overcome. Accordingly, the crude lobster fibrinogen preparations were dialyzed against a 0.05 M glycine buffer (pH 10.0), containing 10^{-3} M EDTA, and chromatographed on a 5.5 × 50 cm agarose A1.5 (100–200 mesh) column equilibrated with the same buffer (Figure 1). All operations were conducted at 4°.

Three major peaks were always obtained, the first two of which were fully clottable and are termed lobster fibrinogens A and B. Both of these components were readily concentrated by isoelectric precipitation at pH 5.0 or by the use of a Dia-flo apparatus. Acrylamide gel electrophoresis showed fibrinogen A to be a heterogeneous preparation giving several bands near the origin. The fibrinogen B, however, displayed a major, faster moving, band, although it still contained some of the heterosperse fibrinogen A material.

Preparative Gel Electrophoresis. Final separation of lobster fibrinogen B from fibrinogen A was achieved using a Büchler preparative acrylamide gel electrophoresis apparatus (Figure 2). During an ordinary 20-hr run only the faster moving fibrinogen B was eluted. The preparation, which was fully clottable, gave only one band upon analytical gel electrophoresis. The analytical gel electrophoresis patterns obtained at various stages of lobster fibrinogen purification are summarized in Figure 3.

Molecular Weight Determinations. SEDIMENTATION VELOCITY. Sedimentation velocity determinations on lobster crude fibrinogen preparations always exhibited two major components. The faster moving material had a sedimentation coefficient, $s^0_{20,w}$ = 19.4 S, whereas the slower moving peak

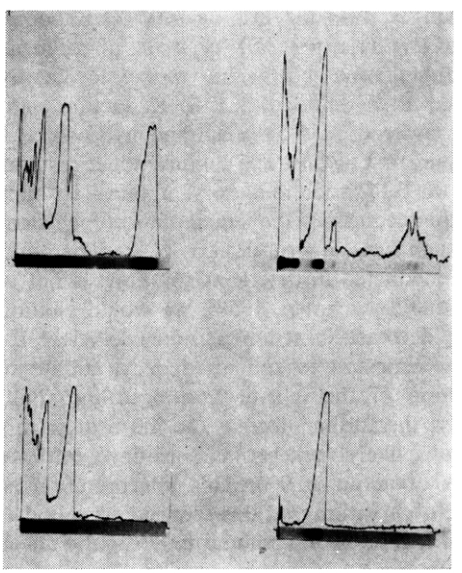

FIGURE 3: Analytical polyacrylamide gel electrophoresis of lobster fibrinogen preparations at successive steps of purification. Upper left, lobster plasma, upper right, after isoelectric precipitation, lower left, after agarose gel chromatography; lower right, after preparative acrylamide electrophoresis.

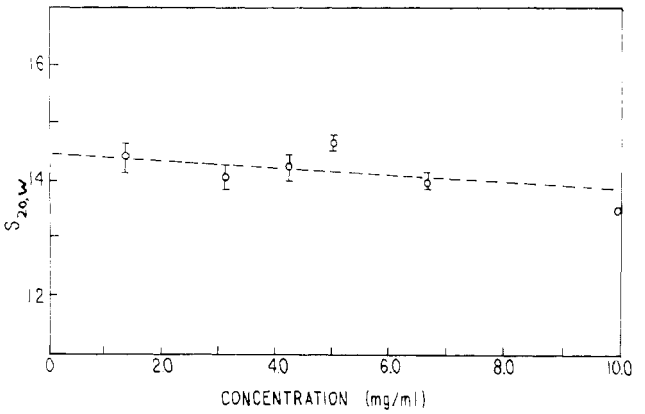

FIGURE 4: Concentration dependence of sedimentation coefficient, $s^0_{20,w}$, for lobster fibrinogen B.

had $s^0_{20,w}$ = 14.5 S. Experiments on pure fibrinogen B over a wide range of concentration gave a single peak with a sedimentation coefficient equal to 14.5 S (Figures 4 and 5). The faster moving peak in the crude fibrinogen preparations is clearly fibrinogen A and is consistent with the larger size indicated by its earlier elution position on agarose gels.

DIFFUSION COEFFICIENTS were estimated from time-dependent boundary spreading observed during ultracentrifuge runs using a synthetic boundary cell. Four different concentrations of lobster fibrinogen B were used; extrapolation to infinite dilution gave a value for $D^0_{20,w} = 2.90 \times 10^{-7}$ cm^2 sec^{-1} (Figure 6).

The partial specific volume of lobster fibrinogen B was determined by pycnometry and found to be 0.711. When this value is combined with the observed diffusion and sedimentation coefficients in the classical Svedberg equation, a molecular weight of 417,000 is obtained.

SEDIMENTATION EQUILIBRIUM determinations of several concentrations of lobster fibrinogen B were studied. The average molecular weight determined was 420,000 (Figure 7), a value in good agreement with that calculated from the sedimentation velocity and diffusion data.

Dissociation of Lobster Fibrinogen Molecule. When lobster fibrinogen B was dialyzed against 0.1% sodium dodecyl sulfate–0.1 M Tris (pH 8.0) for 24 hr at room temperature, the sedimentation coefficient measured in that solvent decreased to a value of 7.1 S. Reduction and alkylation of this material in 6 M guanidine hydrochloride followed by dialysis, first against 1% sodium dodecyl sulfate and then against the 0.1% sodium dodecyl sulfate–Tris buffer, resulted in a further decrease in the sedimentation coefficient to 4.0 S.

Although any interpretation of sedimentation velocity studies conducted in detergent solutions is not without risk (Hersh and Schachman, 1958), we would cautiously submit that the decrease in sedimentation coefficient from 14.5 to 7.1 after exposure to sodium dodecyl sulfate represents a dissociation of the lobster fibrinogen into half-molecules. Similarly, the further decrease to 4.0 S after reduction and alkylation likely reflects dissociation into fundamental subunits of about 70,000. This interpretation is consistent with the observation that this reduced and alkylated material emerged from a G-200 column only slightly ahead of bovine serum albumin.

Amino Acid Analysis. The amino acid analyses of lobster fibrinogens A and B are virtually indistinguishable (Table I) and not very different from values reported by Stewart *et al.* (1966) for a crude lobster fibrinogen preparation from a related lobster species (*Homarus americanus*). The major difference found was in the amount of tryptophan, which those authors found to be absent and which we found to be present in substantial amounts. The most noteworthy feature of lobster fibrinogen is its high concentration of leucine (10 mole %). The amino acid composition of lobster fibrinogen is very different from published compositions for lobster hemocyanin (Pickett *et al.*, 1966).

TABLE I: Amino Acid Compositions of Lobster Fibrinogens A and B.

Amino Acid	Fibrinogen A (Mole %)[a]	Fibrinogen B (Mole %)[b]	Residues/ 420,000 g[c]
Lysine	3.90	4.18	145
Histidine	4.06	4.16	145
Arginine	4.78	4.75	165
Aspartic acid	9.27	9.90	344[d]
Threonine	8.00[e]	7.12[e]	248
Serine	8.86[e]	8.19[e]	285
Glutamic acid	11.44	10.91	379[d]
Proline	5.55	5.25	183
Glycine	5.98	6.17	215
Alanine	5.74	5.46	190
Half-cystine	1.28[f]	1.31[f]	46
Valine	7.36[g]	6.90[g]	240
Methionine	1.49	1.69	59
Isoleucine	4.97[g]	5.12[g]	178
Leucine	10.43	9.60	334
Tyrosine	2.89	3.18	111
Phenylalanine	4.15	4.10	143
Tryptophan[h]		1.94	67

[a] Average of two 24-hr and two 48-hr hydrolysates. [b] Average of four 24-hr and two 48-hr hydrolysates. [c] Calculated for lobster B only. Assumes 5% of molecular weight contributed by carbohydrate and lipid. [d] Aspartic acid and glutamic residues include contributions from asparagine and glutamine also. [e] Threonine and serine values based on 24-hr hydrolysates only and corrected for 5 and 10% losses, respectively. [f] Half-cystine values based on performate oxidized samples only. [g] Valine and isoleucine values from 48-hr hydrolysates only. [h] Tryptophan was determined independently on a sample of pure fibrinogen B.

Amino-Terminal Analysis. Leucine was found to be the only amino-terminal amino acid in both lobster fibrinogens A and B. Occasionally a small amount of aspartic acid was identified, but this was attributable to the presence of slight contamination with hemocyanin, the end group of which we found to be aspartic acid. Similarly, occasionally we detected small amounts of residual glycine remaining from the agarose gel filtration buffer. Further dialysis of these preparations resulted in the disappearance of the glycine.

A variety of conditions was used for the Edman endgroup determinations since some amino acids (for example, serine) are elusive in situations where other amino acid derivatives are best recovered. Consequently, not all of our quantitative data are comparable. The average number of leucine end groups found was 1.8 for every 420,000 molecular weight when conditions were used which are optimum for measuring end groups in mammalian fibrinogen (Doolittle and Fuller,

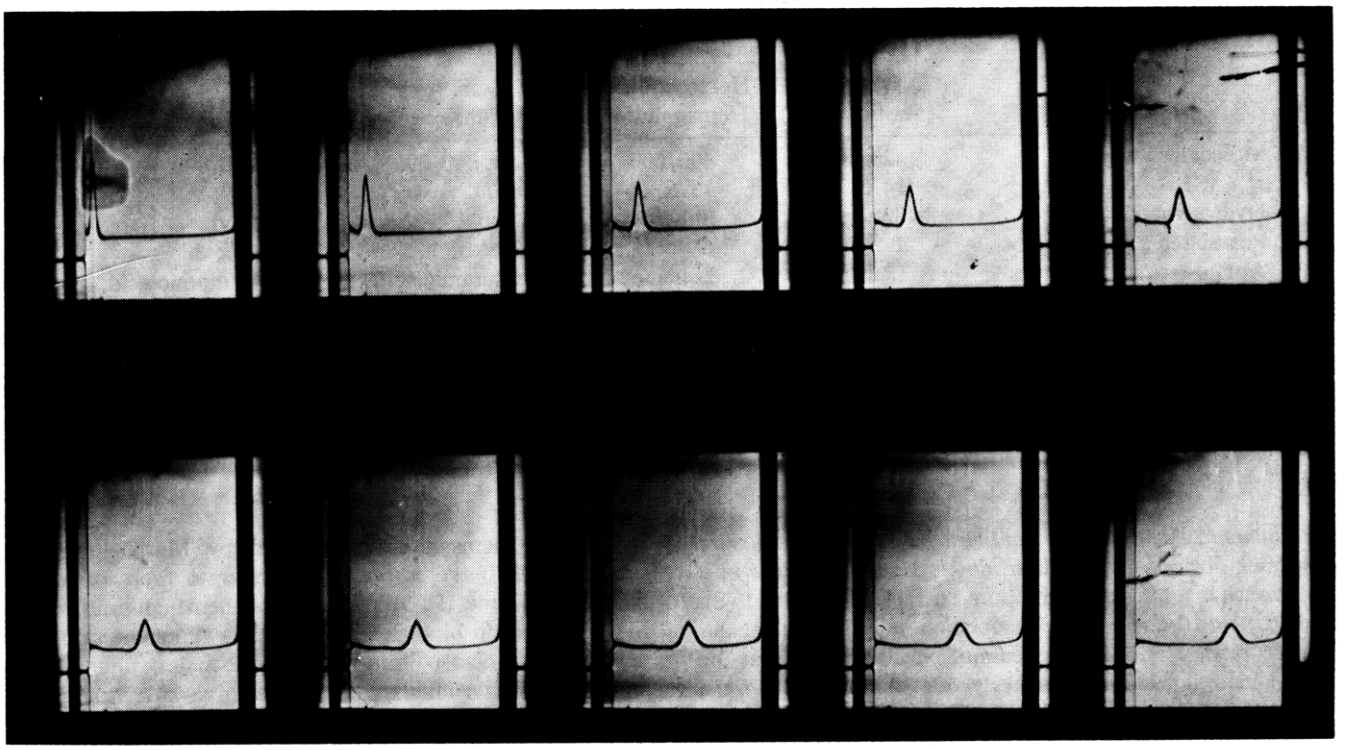

FIGURE 5: Schlieren photograph of pure lobster fibrinogen B. Initial photograph taken when speed of 59,780 rpm was attained Successive frames at 4.0-min intervals.

1967). The 1.8 value is not corrected for procedural losses. Bovine fibrinogen controls were usually run simultaneously.

Carbohydrate and Lipid Contents. Crude lobster fibrinogen preparations consisting of more than 90% clottable protein were found to contain about 3% carbohydrate. Chief among the sugars found after acid hydrolysis were glucose, mannose, and glucosamine.

The lobster fibrinogen was also found to have a significant content of acetone-extractable lipid, amounting to as much as 5% by weight. One of the major components of this associated lipid is the carotenoid pigment astaxanthin; it is likely that this adduct is responsible for the bright orange color of lobster fibrinogen.

Discussion

Lobster Fibrinogens A and B. Previous physicochemical characterizations of crude lobster fibrinogen preparations have always revealed two components in the ultracentrifuge. Duchâteau and Florkin (1963), studying fibrinogen from *Homarus vulgaris*, found two peaks with sedimentation coefficients of 21 and 16.9 S, respectively, whereas Stewart *et al.* (1966) reported values of approximately 21 and 18 S for *Homarus americanus*.

Because both lobster fibrinogens A and B are fully clottable, have indistinguishable amino acid compositions and have identical amino-terminal residues, we tend to regard fibrinogen A as a stable dimer of fibrinogen B. It has a substantially higher $s^0_{20,w}$ in our hands (19.4 S compared to 14.5 S for fibrinogen B), precedes fibrinogen B on agarose A1.5 gel chromatography, and only slightly permeates 6% acrylamide gels.

At first we speculated that the fibrinogen A might be a partially clotted form of the native B molecule, but subsequent analysis has failed to reveal any of the covalent bonds involved in lobster fibrin formation (Fuller and Doolittle, 1971).

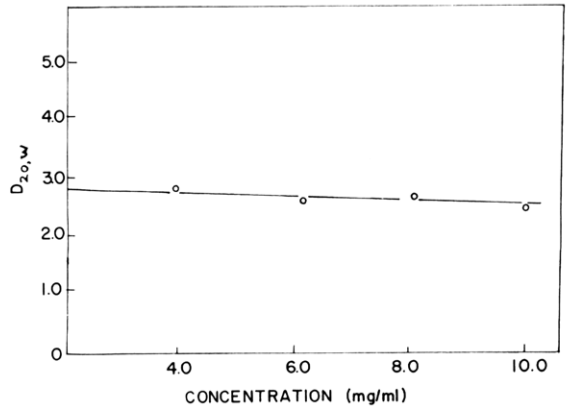

FIGURE 6: Concentration dependence of diffusion coefficient, $D_{20,w}$, for lobster fibrinogen B.

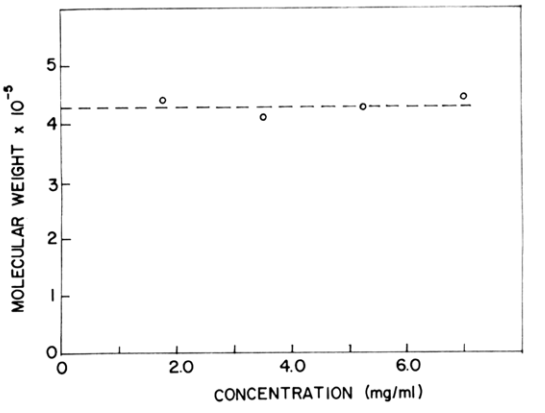

FIGURE 7: Sedimentation equilibrium determinations of molecular weight of lobster fibrinogen at several concentrations.

TABLE II: Comparison of Lobster Fibrinogen to Some Other Proteins of Interest.

	Lobster Fibrinogen (B)	Horseshoe Crab Hemagglutinin[a]	Lamprey Eel Fibrinogen[b]	Bovine Fibrinogen[b]
Molecular weight	420,000	400,000	*Ca.* 340,000	340,000
$s^0_{20,w}$ (S)	14.5	13.5	8.0	7.9
Hydrodynamic shape	Sphere	Sphere	Rod	Rod
Postulated subunits	2 × 3	6	2 × 3	2 × 3
Amino-terminal residues	Leucine	Leucine	Glutamic acid, aspartic acid, and serine	Glutamic acid, pyrrolidone-carboxylic acid, and tyrosine

[a] Marchelonis and Edelman (1968). [b] Detailed references to original data are reviewed in Doolittle (1970).

Postulated Subunit Structure of Lobster Fibrinogen. Exposure of lobster fibrinogen to sodium dodecyl sulfate results in a decisive change in sedimentation coefficient (14.5–7.1 S), leading us to believe that dissociation into half-molecules has occurred. The possibility cannot be discounted that the change in sedimentation is merely due to unfolding of the molecule, of course, although Hersh and Schachman (1958) successfully applied this approach in the analysis of bushy stunt virus.

Complete reduction and alkylation of the lobster fibrinogen, combined with exposure to sodium dodecyl sulfate resulted in a further decrease in sedimentation coefficient to 4.0 S and the production of material which emerges from G-200 columns only slightly ahead of bovine serum albumin. This latter observation lends support to a model of lobster fibrinogen in which the half-molecules consist of three polypeptide chains of about 70,000 being held together by disulfide bridges. The half-molecules are evidently held together only by weak forces. The fact that we only recovered approximately 2 moles of leucine/420,000 molecular weight suggests that some of the chains may have blocked amino terminals. On the other hand, quantitative end-group analysis on large molecular weight proteins has a history of unreliability, and we cannot entirely rule out the possibility that there are only four subunits per molecule and that they all end in leucine. Further experiments using sodium dodecyl sulfate acrylamide gel electrophoresis (Weber and Osborn, 1969) are under way in an effort to resolve this problem.

Relation of Lobster Fibrinogen to Other Proteins. Certainly one of the overriding aims of this study is to find if there is any common ancestry between the blood coagulation event as it occurs in invertebrate animals like the lobster and the hemostatic process which exists in vertebrates. Beyond the fact that lobster fibrinogen is a large molecule composed of subunit polypeptides, the data accumulated so far do not indicate that the lobster protein has any more in common with vertebrate fibrinogen molecules than it does with any other protein. In fact, vertebrate fibrinogen molecules, whether from primitive fish like the lamprey eel or recently evolved mammals (*e.g.*, bovine or human) have very similar physicochemical characteristics, including rodlike hydrodynamic behavior attributable to great asymmetry (Table II). The lobster fibrinogen, on the other hand, exhibits hydrodynamic behavior in the ultracentrifuge indicative of minimal asymmetry. In fact, lobster fibrinogen has much more in common with another invertebrate protein which is not even involved in blood clotting. The properties of horseshoe crab (*Limulus polyphemus*) hemagglutinin reported by Marchelonis and Edelman (1968) are surprisingly similar to those of lobster fibrinogen (Table II). Horseshoe crab hemagglutinin, which has an uncertain biological function,[2] is about the same size and shape as lobster fibrinogen, has six major subunits with leucine amino terminals, and is distinctive in having a high leucine content (10.2 mole %). It is not at all implausible that horseshoe crab hemagglutinin and lobster fibrinogen share common ancestry. In contrast, lobster fibrinogen has no apparent correspondence to any of the properties reported for lobster hemocyanin (Pickett *et al.*, 1966). Eventually comparative amino acid sequence studies should definitively settle the origin and ancestry of all of these interesting proteins.

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[2] It is generally accepted that coagulation in horseshoe crabs is confined to a cellular agglutination which results in a gelatinous mass and that there is no transformation of a soluble blood protein (*i.e.*, fibrinogen) into a fibrin gel (Glavind, 1948).

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

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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,

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