**Molecular Properties of Lamprey Fibrinogen***

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Application of conventional methods not previously used for preparing lamprey fibrinogen has yielded preparations of very high purity and stability. These preparations made possible a detailed evaluation of molecular weights in normal and chaotropic buffers. The native molecule repeatedly gave a molecular weight of 352,000 to 358,000 via sedimentation equilibrium in citrate buffer; a molecular weight of 354,500 was obtained in guanidine buffer. An anomalous increase of the value of the measured apparent partial specific volume was observed in guanidine buffer. Molecular weight data of the reduced and alkylated subunits of lamprey fibrinogen, obtained by four different methods, have led us to assign molecular weights of 110,000 for \((A)\alpha, 72,000\) for \((B)\beta\), and 50,000 for \(\gamma\). Based upon these molecular weights obtained for the subunits, as well as that of the native fibrinogen molecule, the subunit composition can best be fitted to the formulation \([(A)\alpha, (B)\beta, \gamma]\) rather than the conventional \([(A)\alpha, (B)\beta, \gamma]\) which would yield a molecular weight of 464,000. Analysis of a stabilized clot induced by \(Ca^{2+}\) showed only \(\gamma\) dimers; \(\alpha\) subunit polymerization was undetectable. Cross-linking of lamprey fibrin in the presence of dansylcadaverine and \(Ca^{2+}\) results in fluorescent labeling of the \(\gamma\) chains and to a lesser extent the \(\gamma\) dimer. Differing from other reported vertebrate cross-linking systems, the lamprey fibrin \(\alpha\) subunit appears essentially unreactive in both polymer formation and dansylcadaverine incorporation. These distinct molecular properties may be reasonably attributed to the existence of the single \((A)\alpha\) subunit in the molecular structure of the molecule.

Lamprey (*Petromyzon marinus*), one of the most primitive vertebrate species, has a fibrinogen containing three distinct subunits, \((A)\alpha, (B)\beta,\) and \(\gamma\), similar to that found in higher ordered animals in the phylogenetic evolutionary scale (3). This contrasts sharply with coagulating proteins found in nonvertebrates such as “lobster fibrinogen” (4) and the coagulinogen of the horseshoe crab, *Limulus* (5). Certain major lamprey proteins which have been characterized also exhibit distinct properties from those of other vertebrate species, i.e., the subunit composition of the hemoglobin (6) and the immunoglobulins (7).

Different molecular weights have been reported for lamprey fibrinogen and its constituent subunits. A molecular weight of 440,000 was obtained postulating the conventional six-chain structure (8). The molecular weight of the \(\alpha\) chains was reported as approximately 105,000 by gel electrophoresis. Lamprey \(\beta\) chains were reported to be about the same size as human \(\alpha\) chains and \(\gamma\) chains about the same size as mammalian \(\gamma\) chains. Direct measurements on unreduced lamprey fibrinogen gave a molecular weight of about 400,000 (8). In a later publication, the molecular weights of the individual chains as determined by gel electrophoresis were reported as 100,000 for the \(\alpha\) chain, 55,000 for the \(\beta\) chain, and 47,000 for the \(\gamma\) chain (9). In this same publication, amino acid composition studies on \(\alpha, \beta,\) and \(\gamma\) chains from lamprey fibrin lead to a calculated molecular weight of 70,000 for the \(\alpha\) chains and a calculated molecular weight of 360,000 for intact lamprey fibrinogen.

The values of the molecular weights of the individual chains as obtained by gel electrophoresis are consistent with other reported values. Molecular weights of 50,000 for \(\gamma\) chains, 55,000 for \(\beta\) chains, 70,000 for \((B)\beta,\) chains, 110,000 for \((A)\alpha\) chains, and 210,000 for \(\alpha\) dimers have been reported (10). We have reported molecular weights obtained by gel electrophoresis of 112,000, 68,000, and 50,000 for the three subunits and a molecular weight of 326,000 for the intact molecule by sedimentation equilibrium on a sample purified by alcohol precipitation (2). The possibility of a single \(\alpha\) subunit as a means of reconciling the molecular weights of the subunits and the molecular weight of the intact molecule was suggested here. The present study utilizes lamprey fibrinogen of very high purity and stability and addresses itself to a detailed investigation of the properties of this molecule.

In order to perform physicochemical studies in aqueous solutions, we have developed modified methods for the isolation of lamprey fibrinogen in higher yields, high purity, and stability. Extensive molecular weight determinations of the isolated subunits (chains) were made by sedimentation equilibrium in 6 M guanidine buffers, gel filtration in 6 M guanidine buffers on Sepharose, and polyacrylamide gel electrophoresis to resolve these discrepancies. Concurrently, molecular weight determinations of the native and denatured lamprey fibrinogen were also performed by sedimentation equilibrium in citrate-NaCl and 6 M guanidine hydrochloride buffers. Based

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upon these studies, we have re-evaluated the molecular weight and subunit constitution of the lamprey fibrinogen.

Concomitant with these investigations, the mechanism of lamprey fibrin cross-linking was re-examined for possible reflection of a subunit influence. The negligible extent of polymer formation observed in the stabilized plasma clot as brought forth some pertinent observations that lamprey fibrinogen is different from fibrinogen of other vertebrates.

Our thorough investigation of the molecular properties has brought forth some pertinent observations that lamprey fibrinogens is different from fibrinogens of other vertebrates.

MATERIALS AND METHODS

Materials — Aminohydride, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylethylenediamine, 1-pyrroline, N,N,N'-tetramethyl-3-pyrrolidone, acrylamide, Acrylamide, N,N,N',N'-tetramethylethylenediamine, ammonium sulfate, and glycerol (reagent grade) were from Eastman. Sulfate salt was purchased from Calbiochem. EDTA, N,N,N',N'-tetrakis-ethylamino ethane sulfonic acid (TAE) was purchased from Merck. CM-52, suspended dansylcadaverine during fibrin formation.

Materials and Methods — Lamprey plasma was quickly thawed in a 37°C water bath taken to maintain the plasma at a temperature of 4°C. Transfer of the plasma into a cooling stage was continued as soon as possible. A single sample of plasma was used for all experiments. The blood was collected by the method of Lakl (11, 12). The fibrinogen 501utamS was subjected to electrophoresis on a cellulose acetate sheet (Whatman). The electrophoresis was performed at a constant current of 5 mA/cm^2 for 1.5 hours. The fibrinogen was located at the anode. The gel was stained with 2% aqueous solution of Coomassie blue R 185, washed three times with distilled water, and dried on the gel. A gel containing the fibrinogen 501utamS was sliced into 0.5 cm thick pieces and stored at 60°C. The fibrinogen 501utamS was subjected to electrophoresis on a cellulose acetate sheet (Whatman). The electrophoresis was performed at a constant current of 5 mA/cm^2 for 1.5 hours. The fibrinogen was located at the anode. The gel was stained with 2% aqueous solution of Coomassie blue R 185, washed three times with distilled water, and dried on the gel. A gel containing the fibrinogen 501utamS was sliced into 0.5 cm thick pieces and stored at 60°C.

Preparation of Lamprey Fibrinogen — Lamprey plasma was quickly thawed in a 37°C water bath taken to maintain the plasma at a temperature of 4°C. Transfer of the plasma into a cooling stage was continued as soon as possible. A single sample of plasma was used for all experiments. The blood was collected by the method of Lakl (11, 12). The fibrinogen 501utamS was subjected to electrophoresis on a cellulose acetate sheet (Whatman). The electrophoresis was performed at a constant current of 5 mA/cm^2 for 1.5 hours. The fibrinogen was located at the anode. The gel was stained with 2% aqueous solution of Coomassie blue R 185, washed three times with distilled water, and dried on the gel. A gel containing the fibrinogen 501utamS was sliced into 0.5 cm thick pieces and stored at 60°C.

Preparations of Lamprey Thrombin — Essentially a variation of the procedure described by Lakl (11, 12). For the isolation of factor X, a young plasma sample was used. The plasma was incubated at 4°C with a small amount of thrombin and a small amount of thrombin was added. The mixture was incubated at 4°C until the fibrinogen was completely converted into fibrin. The fibrin was precipitated by heating for 30 minutes at 50°C and after centrifugation at 5,000 rpm for 10 minutes, the supernatant was collected. The fibrin was redissolved in 0.9% NaCl solution and dialyzed against 0.9% NaCl solution at 4°C for 24 hours. The dialyzed solution was collected and stored at 4°C. The plasma sample was used for the isolation of factor X, a young plasma sample was used. The plasma was incubated at 4°C with a small amount of thrombin and a small amount of thrombin was added. The mixture was incubated at 4°C until the fibrinogen was completely converted into fibrin. The fibrin was precipitated by heating for 30 minutes at 50°C and after centrifugation at 5,000 rpm for 10 minutes, the supernatant was collected. The fibrin was redissolved in 0.9% NaCl solution and dialyzed against 0.9% NaCl solution at 4°C for 24 hours. The dialyzed solution was collected and stored at 4°C.

Preparations of Lamprey Leucocytes — A sample of the young lamprey (5-7 cm) collected was obtained for reduction and excretion. The lamprey plasma was prepared by freezing the plasma and storing it at 4°C.

1 Portions of this paper (including "Materials and Methods") are presented in miniprint as prepared by the author. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014, to the first four authors, and include a check for $2.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from the Washington Press.
The densities of the solvents and solutions were then measured, the protein concentrations were determined spectrophotometrically following the method of Lee and Timasheff (11), and the apparent partial specific volume, \( \bar{\rho} \), determined using the equation

\[ \bar{\rho} = \frac{\rho - \rho_s}{\rho - \rho_w} \]

where \( \rho \) is the solvent density in g/cm\(^3\), \( \rho_s \) is the difference between the solution and solvent densities, \( \rho_w \) is the solvent concentration in g/cm\(^3\), and \( \rho_s \) is determined by the relationship

\[ \rho_s = \frac{\rho - \rho_w}{\rho - \rho_w} \]

The measured apparent partial specific volume as defined above is the proper thermodynamic parameter which must be used if correct hydrodynamic molecular weights are to be obtained (24).

RESULTS

Yield and Purity—Using 400 ml of starting material of either of the designated plasmas, the yield of lamprey fibrinogen, 95% clottable, was usually in the order of 600 mg. The reduced preparations when examined on 5% or 7.5% SDS-polyacrylamide gels show three distinct bands (Fig. 1, zero time). No (A)\( \alpha \) doublet is discernible. Heat denaturation, as evidenced by precipitation, was notable at 31°C; the entire contents could be precipitated by heating for 30 min at 37°C. No differences were evident when this denatured material was reduced and examined on SDS-polyacrylamide gels. The results were identical with Fig. 1, zero time. Addition of either human thrombin (plasmin free) or lamprey thrombin to solutions of the highly purified lamprey fibrinogen produced a marked change in the mobility of the \( \beta \) subunit compared to the (B)\( \beta \) of the fibrinogen, Fig. 1, time 1 min. This has been ascribed previously to the removal of the large carbohydrate containing B-peptide (25).

Examination of the fibrin resulting from the action of plasmin-free human thrombin clearly shows the appearance of an \( \alpha \) subunit doublet as well as low molecular weight material (See Fig. 1). No mobility differences could be discerned in the \( \gamma \) subunits in the native fibrinogen or resulting fibrin formed via human thrombin. It would appear that the fibrin \( \alpha \) subunits as well as the \( \beta \) subunits are susceptible to further degradation by the action of human thrombin (Fig. 1). Lamprey thrombin removes a 6-residue A-peptide along with the 36-residue carbohydrate containing B-peptide (26). Our purest lamprey thrombin preparations rapidly clotted lamprey fibrinogen, but even in the presence of Trasylol, the fibrin clots lysed within short periods of time. These lysed preparations, when reduced and examined on SDS-polyacrylamide gels, showed major degradation of the \( \alpha \) subunits as well as degradation of \( \beta \) subunits. These studies will be reported in more detail in a subsequent publication.

Constituent Subunits—Fig. 2 illustrates the chromatography of the reduced and alkylated subunits on a column of CM-52 in 8 M urea and 0.1 M Tris-acetate buffer with a linear buffer gradient from pH 4.75 to pH 5.80. Identity of the subunit constituents was shown by running aliquots from the various numbered tubes on SDS-polyacrylamide gels. It is not possible to identify the first peaks (Tubes 33 and 36) as shown in the same mobilities with the subunit chains in the controls. In all, eight tubes, as numbered in Fig. 3, were assayed electrophoretically, showing a clear separation of the \( \gamma \), (B)\( \beta \), and (A)\( \alpha \) subunits, emerging in that order. Upon dialysis against distilled water, the \( \gamma \) and (B)\( \beta \) subunits rapidly precipitated; the (A)\( \alpha \) subunit precipitated more slowly. The horizontal bars in Fig. 2 indicate the combined fractions which were dialyzed and lyophilized. All of the above preparations were completely soluble in 6 M guanidinium-Tris buffers.

Amino Acid Compositions—The amino acid composition of our purest preparation is given in Table I. It is noteworthy that the molecule has an extremely high glycine content. Performic acid oxidation by the method of Hirs (16) followed by chromatographic analysis gave 49 residues of cysteic acid, indicative of 24 to 25 disulfide linkages. The molecular weight, as determined by amino acid analysis, is in the neighborhood of 360,000, a figure in close agreement with physical data.

Amino acid analyses of the isolated reduced and alkylated chains are also shown in Table I. These analyses were utilized for the calculation of the partial specific volumes of the native molecule and of the subunit chains according to the method of Lee and Timasheff (22).

Molecular Weight Determinations—Table II gives a com-
parison of the subunit molecular weights obtained by two methods, SDS-polyacrylamide gel electrophoresis and gel filtration chromatography on Sepharose CL-4B in 6 M guanidine HCl-Tris buffer (0.05 M), pH 7.5. Sedimentation equilibrium of the native molecule and subunit chains in aqueous and guanidine buffers will be presented in detail in a separate section.

Employing SDS-polyacrylamide gels of 5% and 7.5%, we consistently obtained molecular weights of 110,000, 78,000, and 50,000 for the (A)α, (B)β, and γ subunits, respectively. The α subunits of lamprey fibrin resulting from the action of lamprey (plasmin-free) human thrombin, even for relatively short reaction times, resulted in the appearance of a doublet of this subunit (see Fig. 1) with molecular weight of the order of 110,000 and 100,000. In addition, the molecular weight obtained for the β subunit of lamprey fibrin (following B-peptide removal either by lamprey or human thrombin) was in the order of 66,000 to 68,000, lower than expected for the removal of the 36-residue glycopeptide (21). Quantitation of the gels, assuming that all of the chains bind dye equally, gave the mass ratio of the (A)α subunit as 0.33 ± 0.010, the mass ratio of the (B)β subunit as 0.379 ± 0.010, and the mass ratio of the γ subunit as 0.288 ± 0.009.

These results are comparable to the molecular weights of 131,000 ± 13,600, 65,000 ± 3,200, and 53,000 ± 2,800 for (A)α, (B)β, and γ obtained from the KD values when a mixture of the reduced and alkylated lamprey fibrinogen in 6 M guanidine and 0.05 M Tris, pH 7.5, was percolated through a standardized column of Sepharose CL-4B equilibrated with the same buffer. As shown in Fig. 3, the logistic distribution curve, given by the equation

\[ K_D = \frac{1}{[1 + (M/M_c)^c]} \]

where b and c are fitting parameters, was fitted to the KD and molecular weight values of the standards, and the molecular weights of the lamprey subunits were calculated from the inverse equation

\[ M = c\left(1 - \frac{K_D}{K_D}ight)^{1/b} \]

TABLE I

| Amino acid composition of native lamprey fibrinogen and isolated reduced and alkylated chains | Tryptophan | Arginine | Histidine | Lysine | Tyrosine | Phenylalanine | Methionine | Isoleucine | Leucine | Serine | Threonine | Glutamic acid | Glycine | Alanine | Cysteine | Valine | Phenylalanine | Phenylalanine | Methionine | Isoleucine | Leucine | Tyrosine |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (A)α | 69.7 | 133.2 | 140.5 | 98.6 |
| (B)β | 61.8 | 66.1 | 66.7 | 79.6 |
| γ | 91.5 | 133.8 | 135.8 | 89.6 |
| Proline | 9.5 | 52.3 | 34.9 | 40.1 |
| Glycine | 228.2 | 100.0 | 102.6 | 261.4 |
| Alanine | 58.3 | 71.9 | 45.5 | 58.3 |
| Cysteine | 5.1 | 2.4 | 1.3 | 13.5 |
| Valine | 27.7 | 54.4 | 55.2 | 31.9 |
| Methionine | 17.7 | 34.9 | 16.5 | 18.0 |
| Isoleucine | 11.6 | 27.0 | 36.9 | 19.3 |
| Leucine | 48.0 | 63.2 | 77.3 | 51.2 |
| Tyrosine | 12.8 | 34.9 | 54.6 | 37.9 |
| Phenylyalanine | 17.2 | 22.2 | 33.8 | 23.9 |
| Histidine | 37.6 | 22.5 | 27.8 | 19.8 |
| Lysine | 30.4 | 45.8 | 53.3 | 30.1 |
| Arginine | 64.1 | 71.1 | 54.6 | 52.8 |
| Tryptophan | 16.6 |

* As aspartic acid/asparagine.
* As glutamic acid/glutamine.
* Determined by the method of Hirs (16), 49 ± residues for molecular weight 360,000.
* Determined by the method of Edelhoch (17), 62 residues for molecular weight 360,000.

Table II

| Molecular weights of isolated chains of lamprey fibrinogen |
|---|---|---|
| SDS-electrophoresis | SDS-electrophoresis | Gel filtration |
| (A)α | 110,000 | 118,000 ± 3,500 | 131,000 ± 13,600 |
| (B)β | 78,000 | 67,200 ± 3,500 | 65,000 ± 3,200 |
| γ | 50,000 | 51,000 ± 3,200 | 53,000 ± 2,800 |

* On 5% or 7.5% gels.
* In 6 M guanidine and 0.05 M Tris, pH 7.5, Sepharose CL-4B, see text for discussion of ± values.

Fitting gel filtration data requires making some assumptions regarding the distribution of pore sizes in the gel. The problems inherent in this have been very well discussed by Rodbard (15). While the most common procedure is to use a linear relationship between KD and log (M₀), it has been shown that this is significantly inferior to using either the log normal or logistic distribution functions. While the log normal and logistic distributions are essentially equivalent, the logistic function is much easier to use computationally since the log normal
distribution requires the use of either inverse error functions or integrals which cannot be evaluated analytically.

**Sedimentation Analysis: Sedimentation Coefficient**—The sedimentation coefficient, $s$, is defined by the equation

$$s = \frac{1}{\omega^2} \frac{d\ln r}{dt}$$  \hspace{1cm} (5)

where $\omega$ is the angular velocity of the rotor (radians/s) and $r$ is the radial position of the maximum ordinate. This equation may be integrated to give

$$r = r_0 \exp(\omega^2 st)$$  \hspace{1cm} (6)

where $r_0$ is the radial position of the meniscus. This form has the advantage that $s$ can now be a fitting parameter for the nonlinear least squares fitting of this equation to data of radial position of the maximum ordinate as a function of time. With the MLAB program, the quality of the fit of the data can be evaluated from the root mean square (RMS) error and from the standard errors of the fitting parameters.

The individual sedimentation coefficients were obtained at concentrations of 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 mg/ml, and the sedimentation coefficients and associated standard errors were corrected for buffer densities and viscosities to values of 20°C in water. The values of these sedimentation coefficients as functions of concentration were then used to obtain the sedimentation coefficient at infinite dilution by fitting these data with the equation

$$s_{20\text{w},\infty} = s_{20\text{w},0}(1 + kc)$$  \hspace{1cm} (7)

where $s_{20\text{w},0}$ and $k$ are now fitting parameters, and where the reciprocals of the variances of the individual $s$ values (variance = S.E.$^2$) are used to weight each point. With this procedure, $s_{20\text{w},0} = 7.88 \pm 0.08$ S was found for native lamprey fibrinogen in a buffer of 0.15 M NaCl, 0.05 m citrate, pH 7.5, and 1.0 mM EDTA. The addition of a $c^2$ term to Equation 7 did not improve the quality of the fit or significantly change the value obtained for $s_{20\text{w},0}$.

**Partial Specific Volume**—It is necessary to know the partial specific volume of the protein in order to make the corrections for solvent density in sedimentation velocity studies and to calculate the molecular weight in sedimentation equilibrium studies. We have calculated an apparent compositional partial specific volume based upon the amino acid composition and upon the assumption of 3% carbohydrate by weight with the specific volume of the protein in order to make the corrections.

**Molecular Weight of Native Lamprey Fibrinogen: Sedimentation Equilibrium**—The molecular weight of the native lamprey fibrinogen in a buffer of 0.15 M NaCl, 0.05 m citrate, pH 7.5, and 1.0 mM EDTA was determined by direct fitting of the data of concentration distribution as a function of radial position obtained from the ultracentrifuge. Concentration as a function of radial position for a single thermodynamically ideal macromolecular solute is given by the equation

$$c_r = c_0 \exp[AM(r^3 - r_0^3)]$$  \hspace{1cm} (8)

where $c_r$ is the concentration at radial position $r$, $c_0$ is the concentration at the cell bottom, $M$ is the molecular weight, and $A$ is given by

$$A = (1 - \bar{c}_0) \omega^2/2RT$$  \hspace{1cm} (9)

where $\bar{c}$ is the partial specific volume, $\rho$ is the solution density, $R$ is the gas constant, and $T$ is the absolute temperature. If more than one macromolecular component is present, then

$$c_r = \sum_{i=1}^{n} c_{oi} \exp[AM_i(r^3 - r_0^3)]$$  \hspace{1cm} (10)

In either case, the values of $M$ and $c_0$ or the values of $M_i$ and $c_{oi}$ are fitting parameters which are adjusted by the nonlinear least squares curve-fitting program in MLAB. Our experience with several samples of highly purified lamprey fibrinogen has been that these have been over 90% monomer, and that the highest molecular weight material present has, within the limits of precision of our measurements, always had a molecular weight which was an integral multiple of that of the fibrinogen monomer. Thus, Equation 10 could be written as

$$c_r = c_{01} \exp[AM(r^3 - r_0^3)] + c_{02} \exp[nAM(r^3 - r_0^3)]$$  \hspace{1cm} (11)

where $n$ could have the values 2, 3, or 4, depending upon whether one was considering the presence of a dimer, trimer, or tetramer, etc.

The relative quantity of each component is readily obtained by means of equations involving conservation of mass. Thus,

$$c_{oi}(r_i^3 - r_0^3) = \int_{r_0}^{r_i} c_{oi} \exp[AM_{oi}(r^3 - r_0^3)] \, dr$$  \hspace{1cm} (12)

Where $c_{oi}$ is the concentration of the $i$th component before distribution in the centrifugal field. Equation 12 then gives

$$c_{oi} = c_{oi}(1 - \exp[AM_{oi}(r_i^3 - r_0^3)])/AM_{oi}(r_i^3 - r_0^3)$$  \hspace{1cm} (13)

and since $\exp[AM_{oi}(r_i^3 - r_0^3)] << 1$ if the meniscus is well depleted, Equation 13 is well approximated by

$$c_{oi} = c_{oi}/AM_{oi}(r_i^3 - r_0^3)$$  \hspace{1cm} (14)

The relative amount of each macromolecular component is then given by

$$Q_i = c_{oi} \sum_{j=1}^{n} c_{oj}$$  \hspace{1cm} (15)

The possibility of thermodynamic nonideality has also been considered. The apparent molecular weight is given by the equation

$$M_{app} = M/(1 + BMc)$$  \hspace{1cm} (16)

where the subscripts indicate that the apparent molecular weight is a function of concentration and where $B$ is a virial coefficient. Equation 11 then becomes

$$c_r = c_0 \exp[AM(r^3 - r_0^3)/(1 + BMc)]$$  \hspace{1cm} (17)

and $B$ becomes an additional fitting parameter. Because $c_r$ appears on both sides of Equation 17, the root finder in MLAB must be used in order to fit the data when this equation is used.

Equations 8, 11, and 17 may be described as mathematical models for fitting the data from the ultracentrifuge. Three criteria were used in evaluating the fit of a given model to the data. First, the parameters obtained in fitting had to be physically meaningful, i.e., having nonnegative values. Second,
a minimum value of the root mean square error denoted a superior fit. Since the root mean square error obtained using MLAB takes the number of degrees of freedom of a model into account, the comparison of root mean square values for models having differing numbers of parameters is valid. Third, the deviation of experimental data points from the fitting line should not exhibit systematic error. If two models satisfy the first criterion and have essentially equal root mean square errors, a more random pattern of deviation can become an important selection criterion. Figs. 4 and 5 show examples of the fitting procedure. Fig. 6 illustrates a good example of the deviations from the fitting line. Fig. 7 shows how this can be used to discriminate between two models that have similar root mean square errors.

Six analyses of three preparations of highly purified native lamprey fibrinogen indicated that the solutions did not exhibit thermodynamic nonideality and that they had 3 to 6% tetramer present. The mathematical model we fit had molecular species from monomer through hexamer, subject only to the constraint that all fitting parameters had to be positive or zero. The concentration parameters of all species but monomer and tetramer were either zero or vanishingly small for all cases. Thus, the monomer-tetramer model satisfies the best fit criteria, but does not eliminate the small finite probability of the presence of dimer, trimer, etc. The monomer molecular weight was 352,000 ± 12,000 using the calculated compositional partial specific volume of 0.703 cm³/g and 358,000 ± 12,000 using the experimentally measured partial specific volume of 0.708 cm³/g.

The molecular weights of the (A)α, (B)β, and γ subunits of lamprey fibrinogen were also measured by equilibrium ultracentrifugation in 6 M guanidine buffer since they were not soluble in the citrate buffer. The apparent partial specific volumes of the subunits in this buffer were calculated from the amino acid composition by the method of Lee and Timashoff (22), assuming an equal weight per cent of carbohydrate of the (B)β and γ subunits. This gave calculated apparent partial specific volumes of 0.687, 0.700, and 0.722 cm³/g and resultant molecular weights of 78,000, 57,700, and 50,100 for the (A)α, (B)β, and γ subunits, respectively.

The considerable disparity between these values and the molecular weights obtained by gel filtration and by gel electrophoresis led us to suspect the validity of the values of these calculated apparent partial specific volumes. We measured the molecular weight of intact lamprey fibrinogen in the 6 M guanidine buffer in order to determine whether this disparity was due to the use of incorrect values for the apparent partial specific volumes or was due to false values obtained with the gel methods. Calculation of the apparent partial specific volume of the lamprey fibrinogen in 6 M guanidine buffer gave a value of 0.693 cm³/g. A molecular weight of 273,000 was obtained using this value for the apparent partial specific volume. Since there was no evidence for degradation of the lamprey fibrinogen in the 6 M guanidine buffer, this strongly

![Fig. 5. Ultracentrifugal equilibrium distribution of lamprey fibrinogen. Plot of concentration in optical density at 280 nm as a function of radial position for an equilibrium distribution of lamprey fibrinogen in 0.05 M citrate buffer, pH 7.8, 0.15 M NaCl, and 1 mM EDTA, at 6,000 rpm at 20°C for 7 days. The fitting line is for an ideal distribution of 97% fibrinogen monomer (M = 354,000) and 3% tetramer. The root-mean-square error of the fit is 0.007 optical density unit at 280 nm.](image)

![Fig. 6. Difference plot for assessing the quality of fit of a specific model. This difference plot shows the distribution of the data points about the fitting line for the concentration distribution shown in Fig. 5. The essentially random nature of the distribution and the small root mean square error demonstrate a good fit and an appropriate model.](image)
suggestions that the calculated apparent partial specific volume is incorrect. Calculation of the apparent partial specific volume necessary to give a molecular weight of 352,000, as obtained with the calculated compositional partial specific volume, gave a value of 0.734 cm$^3$/g. A value of 0.735 $\pm$ 0.003 cm$^3$/g was obtained experimentally from densimetric measurements on lamprey fibrinogen dialyzed against the 6 M guanidine hydrochloride buffer. An experimentally determined value for the extinction coefficient of $E_{1%}^{0} = 13.2$ was used to determine the protein concentrations. A value of 354,500 $\pm$ 7,500 was obtained for the molecular weight using this apparent partial specific volume. Application of this apparent partial specific volume to the individual subunits gives molecular weights of 104,700, 71,600, and 54,300 for the (A)$\alpha$, (B)$\beta$, and $\gamma$ subunits, respectively.

That this anomalous behavior is not unique to lamprey fibrinogen was then demonstrated when a molecular weight of 282,000 was obtained for bovine fibrinogen in 6 M guanidine buffer using the literature value of 0.718 cm$^3$/g for the partial specific volume (27). In order to obtain the cited value of 330,000 for the molecular weight, the partial specific volume had to be increased to 0.741 cm$^3$/g, an increase of 0.023 cm$^3$/g as compared to the increase of 0.031 cm$^3$/g for lamprey fibrinogen. It is of interest to note that solutions of fibrinogen and the constituent chains appeared to exhibit thermodynamic nonideality when insufficient time was allowed for equilibrium. The extent of the nonideality diminished with time and eventually disappeared. Equilibrium was assumed to be obtained when no nonideality could be demonstrated in two successive measurements taken 3 days apart. Times to equilibrium were approximately 2 weeks for the individual chains and 3 weeks for the intact molecule with column lengths of 6 mm. It is also of interest to note that the molecular weights obtained after correcting for nonideality did not differ very significantly from the final values obtained.

Cross-linking—Fig. 8 illustrates the inhibition of cross-linking of lamprey fibrin when dansylcadaverine is added to a mixture of lamprey fibrinogen, Ca$^{2+}$, and either lamprey or plasmin-free human thrombin. Only the $\gamma$ chains incorporate the fluorescent amine. It is also possible to discern minute formation of fluorescent $\gamma$ dimer probably caused by isopeptide bond formation prior to complete saturation of the acceptor sites by dansylcadaverine. It should be stressed that no extrinsic Factor XIII has been added. The $\alpha$ subunit showed no incorporation of fluorescence even after an 8-h incubation of the reaction mixture. These results are in contrast to similar studies reported with human fibrins and human plasma, which clearly demonstrated incorporation of dansylcadaverine into both $\alpha$ and $\gamma$ subunits with concurrent inhibition of cross-linking to $\alpha$ polymers and $\gamma$ dimers (28). Incorporation of dansylcadaverine into lamprey fibrin $\gamma$ subunits was Ca$^{2+}$- and thrombin-dependent. These results are in accord with previous reports which demonstrated that with lamprey fibrin as substrate, intrinsic Factor XIII mediated only the cross-linking of $\gamma$ subunits to $\gamma$ dimers with little or no cross-linking of $\alpha$ subunits to higher polymers (2, 8). Both incorporation of dansylcadaverine into $\gamma$ subunits or cross-linking of $\gamma$ subunits to dimers could be inhibited by iodoacetamide.

**DISCUSSION**

The problem confronting us now is 2-fold. What are the correct molecular weights of the individual chains and what is the structure of the intact molecule? The quality of the fibrinogen and its constituent subunits that we have been able...
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TABLE III

| Protein                  | $\bar{v}_{mol}^a$ | $M^a$   | $\bar{v}_{mol}^c$ | $M^c$   |
|--------------------------|-------------------|---------|-------------------|---------|
| Lamprey fibrinogen (native) | 0.703             | 352,000 ± 12,000 (6) | 0.708 | 358,000 ± 12,000 |
| Lamprey fibrinogen (6 M guanidine) | 0.693             | 273,000 ± 6,000 (10) | 0.735 | 354,500 ± 7,700 |
| Lamprey (A)a chain (6 M guanidine) | 0.687             | 78,900 ± 4,400 (10) | 0.735 | 104,700 ± 5,900 |
| Lamprey (B)β chain (6 M guanidine) | 0.700             | 57,700 ± 3,600 (6) | 0.735 | 71,600 ± 4,500 |
| Lamprey γ chain (6 M guanidine) | 0.722             | 50,100 ± 5,500 (6) | 0.735 | 54,300 ± 6,000 |
| Bovine fibrinogen (6 M guanidine) | 0.718             | 282,000 ± 7,200 (6) | 0.741f | 330,000 ± 8,400f |

$^a$ Calculated from amino acid composition and corrected for guanidine interaction.

$^b$ Calculated using $\bar{v}_{mol}$. The number of determinations is given in parentheses.

$^c$ Experimentally determined apparent partial specific volume.

$^d$ Calculated using $\bar{v}_{mol}$. Literature quoted value.

$^e$ Determined as needed to give the correct molecular weight for the intact fibrinogen.

$^f$ Literature value in aqueous solution.

The summation of the subunit molecular weights gives this structure a molecular weight of 355,500, comparing very well to the measured molecular weights in citrate and guanidine buffers. The conventional structure would have a molecular weight of 461,200. It should be noted that the (A)a subunit has a molecular weight much higher than that of the (A)a chains from other fibrinogens, implying the possibility of some internal linkage. This structure is also supported by the mass ratios obtained from quantitation of the gels. For the molecular weight of 354,500 for the intact molecule, the mass ratio of 0.333 for (A)a gives a molecular weight of 118,000; the mass ratio of 0.379 for (B)β gives a molecular weight of 134,400, corresponding to a molecular weight of 67,200 per (B)β; the mass ratio of 0.288 for γ gives a molecular weight of 102,000, corresponding to a molecular weight of 51,000 per γ (See Table III).

The calculation for the partial specific volume of the proteins in 6 M guanidine buffer by the method of Lee and Timasheff (22) is based upon the assumption that there is no significant volume change in the presence of the guanidine and that the amount of solvent components bound to the protein can be calculated. If the actual amount of guanidine bound is less or the hydration is greater than that which is assumed, the calculated partial specific volume will be too small. Calculation of the preferential interaction of component 3, guanidine hydrochloride, using the equation

$$\xi_i = \frac{\Delta g_i}{\rho_3} = \frac{\overline{\xi}_i - \phi_i}{1/\rho_3 - \bar{\overline{\xi}}_i}$$

(18)
gives a value of $\overline{\xi}_i = -0.26$ g of guanidine/g of protein (23). Here $\overline{\xi}_i$ is the calculated partial specific volume of the protein (0.706 cm$^3$/g), $\phi_i$ is the measured apparent partial specific volume (0.735 cm$^3$/g), $\rho_3$ is the density of 6 M guanidine (1.1418 g/cm$^3$), and $\bar{\overline{\xi}}_i$ is the partial specific volume of guanidine (0.763 cm$^3$/g). This negative value may be interpreted either as preferential exclusion of guanidine from the immediate vicinity of the protein or as preferential hydration. Additionally, the possibility of a volume increase must be considered as a possible explanation for the observed increase in apparent partial specific volume.

It may be argued that the intact fibrinogen in 6 M guanidine has a significant amount of structure, that the isolated chains are probably in a random coil configuration, and that the lower calculated values for the apparent partial specific volumes of the isolated chains are more appropriate to this configuration. Thus, it is suggested that the lower molecular weights for the isolated subunits are also more appropriate. This argument is countered by the molecular sizes obtained for the subunits by gel filtration in 6 M guanidine. It should be noted that the values of 131,000 ± 13,600, 65,000 ± 3,200, and 53,000 ± 2,800 for the (A)a, (B)β, and γ subunits, respectively, obtained by this method are in relatively good agreement with the values.
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of 104,000, 71,600, and 54,000 obtained ultracentrifugally. If the gel filtration values are too high, the most probable cause would be that the subunits were not in a random coil configuration. If this is the case, then the subunits going from a more ordered structure in the intact molecule to random coils when isolated cannot be proposed as a cause for decrease in the value of the apparent partial specific volume of the subunits. Conversely, if the subunits are in a random coil configuration, the agreement of the ultracentrifugal values and the gel filtration values argues that the apparent partial specific volumes are not significantly affected by a structural change. The validity of both sets of values is supported by the values obtained by gel electrophoresis where sodium dodecyl sulfate is the denaturant and by the molecular weight values from mass ratios.

Considering the fact that the apparent partial specific volumes of the subunits are probably not equal and considering the molecular sizes measured by gel filtration, gel electrophoresis, and mass ratios, we propose molecular weights of 110,000, 72,000, and 50,000 with a structure of \((A)_2\alpha, (B)_2\beta, \gamma_2\) giving a molecular weight of 354,000. We feel that this is strongly supported by the experimental evidence and by the most reasonable set of assumptions. Considering that the molecular weight and the sedimentation coefficient of lamprey fibrinogen are essentially comparable to those of other fibrinogens strongly suggests that the structure of lamprey fibrinogen closely resembles that of other fibrinogens. Taken in conjunction with the large molecular weight of the \((A)_2\alpha\) subunit, we feel that this subunit is probably either a single symmetrically folded chain or is composed of two chains covalently bound by noncysteine bonds. If the latter is the case, the phenomenon may have occurred during postribosomal assembly and processing of the fibrinogen molecule. We use the subscript \(n\) to suggest the possibility of more than one A-peptide per subunit. Correspondingly, the \((B)_2\beta\) and \(\gamma_2\) subunits would appear to be single chains. Either of these models appears to be consistent with all of our data and with the generally accepted structure of fibrinogens as a class.

It is now appropriate to consider how the cross-linking experiments and the dansylcadaverine incorporation data may be related to the postulated structure. There is virtually no polymer formation when cross-linking lamprey fibrin via thrombin-activated intrinsic Factor XIII. Comitantly, there is no dansylcadaverine incorporation into the \(\alpha\) subunit or into the traces of \(\alpha\) polymer. These results are obtained with either lamprey or human thrombin. It should be noted that only the lamprey thrombin removes the lamprey A-peptide, the human thrombin not being effective in this regard although both cause the removal of the B-peptide(s). In contrast to the unusual behavior of the \(\alpha\) subunit, the lamprey \(\gamma\) chains rapidly form dimers under the influence of calcium-dependent thrombin-activated intrinsic lamprey Factor XIII. Dansylcadaverine is incorporated into the \(\gamma\) chains and to a limited extent into the \(\gamma\) dimers. Thus, in this latter respect, and with respect to the fact that B-peptides are released, lamprey fibrinogen behaves in a manner analogous to all previously studied fibrinogens.

It is possible that cross-linking of lamprey fibrin may be regulated by the level of Factor XIII present in the plasma. Indeed, efforts to isolate lamprey Factor XIII from a large quantity of plasma were unsuccessful. The conventional Factor XIII assay using high specific activity \(^3\text{H}\)-labeled putrescine (29) under various conditions showed negligible activity in whole plasma as well as in concentrated fibrinogen solutions. However, from the cross-linking patterns observed on polyacrylamide gels, it is evident that a Factor XIII-like enzyme participates in stabilizing lamprey fibrin clots.

It is unlikely that the \(\alpha\) subunit does not cross-link because there are no cross-linking sites available to lamprey Factor XIII, since in previously reported experiments, lamprey \(\alpha\) subunits could be cross-linked by the addition of human Factor XIII (2, 10). Lamprey Factor XIII is activated by either human or lamprey thrombin and is Ca\(^{2+}\)-dependent. It is also inhibited by sulfhydryl reagents.

It is interesting to note that thermal stability of clottable proteins reflects the animals adaptation to its environment. Lampreys, which inhabit a cold environment, fall into this category, as does the Pacific salmon. Lamprey fibrinogen exhibits the lowest inherent stability of any of the vertebrate fibrinogens in terms of thermal denaturation, denaturing at 31°C as compared to salmon denaturing at 45°C and mammalian fibrinogens denaturing at 56°C. Thus, since lamprey fibrinogen differs significantly from other fibrinogens only with respect to the \((A)_2\alpha\) subunit and not with respect to the \((B)_2\beta\) and \(\gamma_2\) chains, this lower thermal stability may be yet another reflection of this unusual structure. This fibrinogen comes from one of the most primitive vertebrates and possesses certain properties which are distinct from those of the rest of the species examined in detail thus far. Nevertheless, this protein still performs the same function in the lamprey as its counterparts in higher vertebrates.

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21. In collaboration with Dr. J. W. Donovan, United States Department of Agriculture, Western Regional Research Laboratory, Berkeley, California, we have examined a number of our preparations of lamprey fibrinogen by differential scanning calorimetry. The protein exhibits two endotherms similar to a pattern reported from native bovine fibrinogen or a mixture of D and E fragments of the same molecule (30). However, the endotherm for the lamprey D domain appears at a lower temperature, 50°C, compared to 60°C for bovine. The endotherm for the E domain appears at higher temperatures, 105°C, compared to 100°C for bovine. The results would indicate that this primitive fibrinogen possesses regions of ordered structure that have remained relatively unchanged through evolution.
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