THE CONFIGURATION OF NATIVE AND PARTIALLY POLYMERIZED FIBRINOGEN

by

BENJAMIN M. SIEGEL

Department of Engineering Physics

AND

JOHN P. MERNAN AND HAROLD A. SCHERAGA

Department of Chemistry

Cornell University, Ithaca, N.Y. (U.S.A.)

Various physico-chemical methods are used to determine the configurations of protein molecules. It is important to recognize that these methods differ with respect to the information which they provide. For example, electron microscopy gives information about the *anhydrous* molecule whereas light-scattering data depend on the configuration of the *solvated* molecule in solution. Further, as has been pointed out recently[1], hydrodynamic measurements obtained from sedimentation, viscosity and flow birefringence may be interpreted in terms of an equivalent hydrodynamic ellipsoid, which may differ from the configuration of the molecule in solution. The extent of permeation of the molecule by the solvent, deviations of the configuration from ellipsoidal shape, electrostriction, selective adsorption in multicomponent systems, etc. will all enter into the determination of the equivalent ellipsoid for the solvated molecule and will determine how closely it corresponds to the actual configuration in solution.

In the work reported here a comparative study has been made of the results of several methods applied to the protein fibrinogen and to its intermediate polymers which are formed during the early stages of the thrombin-catalyzed fibrinogen-fibrin conversion[2]. Hydrodynamic[2-7] and light-scattering[8] data are available for bovine fibrinogen and its intermediate polymers, and recently developed techniques of electron microscopy have been successfully applied here to this protein. Thus, it is possible to compare the kinds of information obtainable from these various methods for the protein considered here.

Electron microscope observations on fibrinogen and on the early stages of fibrin formation have been reported[9,10] but satisfactory observations on the configuration of the fibrinogen molecule were not forthcoming. In the work presented here, it has been possible to obtain highly dispersed systems so that isolated particles could be observed and measured, and to avoid the formation of close packed monolayer films, the factor which made it impossible to give a definitive interpretation to the previous electron microscope observations. In the preparation of specimens the spray technique[11,12] was applied with several attendant advantages. The highly dilute solution sprayed in very

References p. 336.

small droplets (5–20 microns in diameter) allowed the particles to deposit without aggregation[11]. The rapid evaporation of solvent ($\sim$ 0.1 sec) minimized the effects of the great increase in salt concentration and pH change on drying, and the use of a volatile buffer[12] eliminated the necessity of washing the protein to remove salt.

EXPERIMENTAL

Materials. Armour bovine fraction I was refractionated according to LAKI's procedure[13], altered by dissolving the ammonium sulfate-precipitated fibrinogen in 0.3 *M* ammonium acetate (instead of in 0.3 *M* KCl), and dialyzing against this same solution at 2° C. This procedure yielded solutions containing 4–6 mg/ml of fibrinogen as determined by MORRISON's method[14]. Parke, Davis bovine thrombin (18 μ/mg) was used to effect clotting.

Methods. To investigate the unpolymerized fibrinogen, various dilutions up to 100,000 × were carried out on samples of the purified fibrinogen solution and the material was sprayed from a modified BACKUS AND WILLIAMS[12] gun on to standard collodion covered specimen screens.

To investigate the polymerized material, thrombin, dissolved in 0.3 *M* ammonium acetate, was added to solutions of fibrinogen in 0.3 *M* ammonium acetate and the mixture allowed to react at 25° C. The clotting conditions used throughout were: fibrinogen concentration, 2–3 mg/ml; thrombin concentration, 0.01 μ/ml; pH 7.0; ionic strength, 0.30. Under these conditions the clotting time was about 25 minutes. Immediately before the gel point, samples were withdrawn, diluted, and sprayed in the same manner as was used for the unpolymerized material. All dilutions of the reacting mixture were made with 0.3 *M* ammonium acetate. On some specimens polystyrene latex (Dow Chemical) was introduced for an internal calibration standard by spraying a dilute suspension of the particles (approximately 0.04 g/ml) after the fibrinogen solution droplets had dried.

Control specimens were prepared with only polystyrene and with salt solution of the same composition as the reacting mixtures.

All specimens were shadowed with uranium in calculated thicknesses of 3–5 A at shadowing angles of arc tan about 1:6. Electron micrographs were taken with an RCA type EMU-2B microscope.

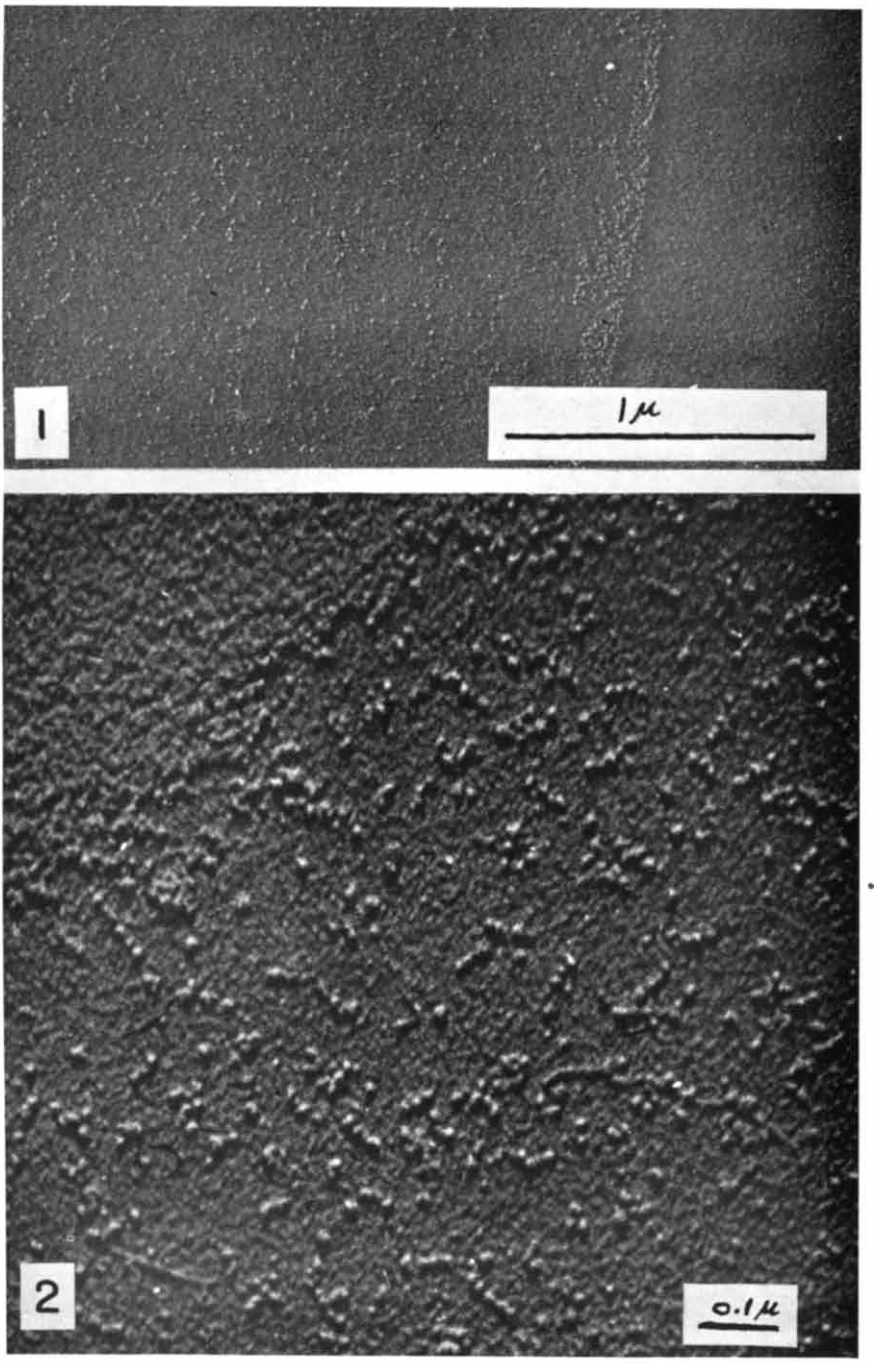
RESULTS

Observations on purified fibrinogen

Figs. 1 and 2 are typical electron micrographs showing areas near the drying edge of the droplet. In these preparations the purified fibrinogen solutions containing 2 to 3 mg/ml were diluted 10,000 × with 0.3 *M* ammonium acetate and sprayed immediately. If the concentration were higher before spraying, the material tended to deposit on the collodion surface in aggregates, making resolution of individual particles very difficult to achieve (greater dilution gave droplets that were extremely difficult to locate). Fig. 1 has the drying edge in the field and a direct comparison is obtained between the collodion substrate structure and the fibrinogen on the substrate. Fig. 2 shows at a higher magnification a section of a droplet closer to its center with increasing concentration of particles as the center of the droplet (upper left hand corner) is approached. The upper left hand area shows a close-packed film of fibrinogen similar to that reported by both HALL[9] and PORTER AND HAWN[10]. Even in this close-packed area it is still possible to pick out the nodose particles of the fibrinogen molecule as previously reported. On the other hand, in the center of Fig. 2 the discrete fibrinogen molecules are observable.

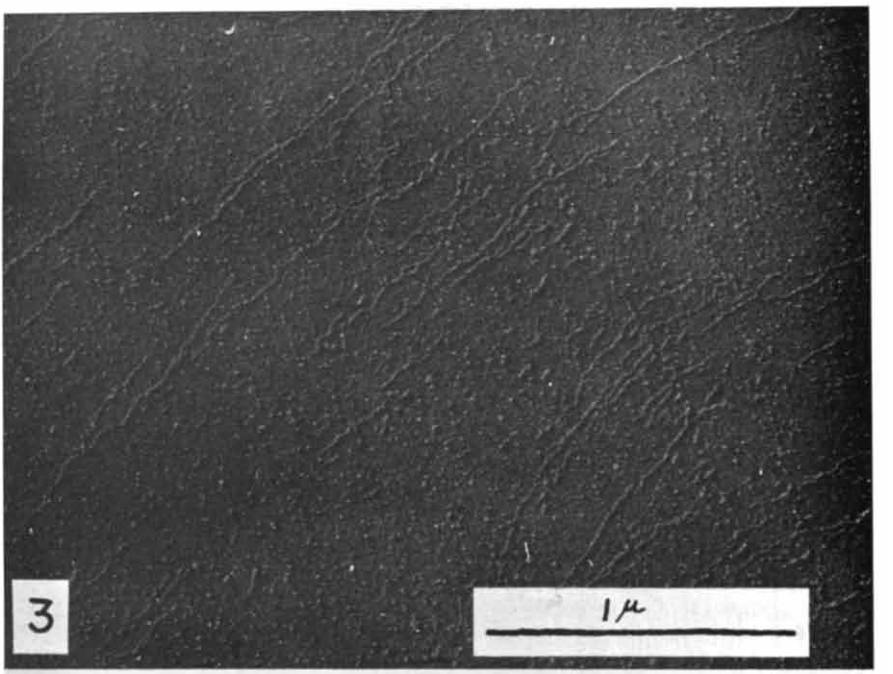
The dried unpolymerized fibrinogen molecules, as observed in the electron micrographs, are nodose particles approximately 60–80 A in width at the widest point, and of varying length. The actual width can certainly not be determined to better than 25 A in these shadowed preparations. Since measurements must be made on slightly "under focus" pictures to obtain good contrast, and "contamination" from the solutions or by oil from the diffusion pump used in shadowing is probably present, the measured values

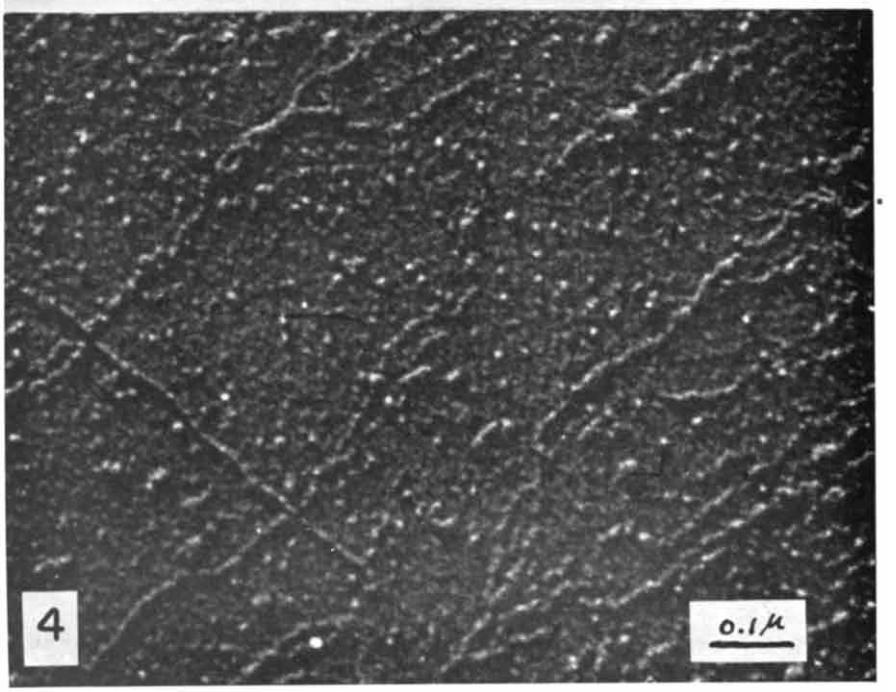
References p. 336.



Figs. 1 and 2. Native Bovine Fibrinogen. Sprayed from solutions diluted to approximately 0.2 γ/ml of fibrinogen in 0.3 *M* ammonium acetate. Shadowed with uranium.

Fig. 1. Mag. 40,000 ×. Drying edge of drop is visible in field; Fig. 2. Mag. 100,000 ×.





Figs. 3 and 4. Partially Polymerized Bovine Fibrinogen. Diluted 10,000 × and sprayed just before gel point. Shadowed with uranium.

Fig. 3. Mag. 40,000 × ; Fig. 4. Mag. 100,000 ×

are undoubtedly on the high side. The predominant length appears to be four "beads" somewhat widely spaced to give a total length of 500 A. However, a fewer longer and many shorter lengths are found, including many that appear as almost spherical particles 80–100 A in diameter.

Observations on polymerized fibrinogen

Figs. 3 and 4 are observations on the fibrinogen-thrombin reaction in which the samples were diluted 10,000 × with 0.3 *M* ammonium acetate just before the gel point and sprayed. The polymerization of the fibrinogen into filaments several thousand Angstroms long is readily apparent. The filaments are still approximately 60–80 A in width and exhibit the same nodose character of the shorter unpolymerized fibrinogen. Much of the shorter-length fibrinogen and some spherical particles are still in evidence. A study of the distribution of lengths of the intermediate polymers was not deemed meaningful since several factors in the preparative procedure influence the polymerization kinetics. Dilution of the solution just before spraying will cause depolymerization[5,8] while during the drying of the droplet the concentrating effects will cause the polymerization reaction to proceed again for an uncontrolled interval. Other effects in the drying of the droplet such as the great increase in salt concentration and the decrease in pH to about 5.0[12] will also alter the polymerization. Thus the point at which the electron microscope observations are made cannot be correlated with that obtaining in the flow birefringence or light scattering measurements made on the more concentrated solutions.

Observations on control specimens

Observations were carried out on uranium-shadowed specimens containing ammonium acetate and polystyrene but no protein. From these observations it can be concluded that none of the effects described herein can be attributed to salt or polystyrene, but, in fact, are characteristic of the protein. Actually the most satisfactory control is the observation of the drying edge of the droplet for here a direct comparison between the substrate and the protein is obtained and only contaminants introduced in the same solutions could interfere.

DISCUSSION

Sedimentation and viscosity measurements on purified fibrinogen indicate that in 0.3 *M* KCl and pH 6.8 the system is monodispersed, and the equivalent ellipsoid is prolate and has an axial ratio of about 5:1[3], assuming a molecular weight of 350,000 as given by light scattering[8]. Flow birefringence measurements also indicate a monodispersed system and the rotary diffusion constant gives a length of 560 A[3] for an axial ratio of 5:1. This would give a value of 112 A for the minor axis of the equivalent ellipsoid.

In considering light-scattering data, it should be kept in mind that the interpretation of such data (plotted in the usual manner as the reciprocal of scattering intensity vs. $\sin^2 \theta/2$, where θ is the angle between the incident beam and the scattered one) requires several assumptions[15]; these data can be used to obtain particle dimensions (for an assumed model) in solution only if the model used is characterized by a single parameter, such as the length of an infinitesimally thin rod or the radius of a sphere.

References p. 336.

If an ellipsoid of revolution (either prolate or oblate) of semi-axes a, a, c is used as a model, the axes are not determinable from the light-scattering data. In fact, a given experimental scattering curve would correspond to an infinite set of values of a and c, given by the equation

$$c^2 + 2a^2 = K$$

where K is a constant which depends on the initial slope of the scattering curve, plotted as indicated above. Thus, one can obtain any value for c from zero up to $K^{1/2}$ depending on the arbitrary choice of a. Because of this ambiguity in the configuration of the solvated molecule in solution from light-scattering data, an irrefutable comparison cannot be made with the equivalent hydrodynamic ellipsoid in order to determine the relative importance of the factors[1] entering into the determination of the equivalent ellipsoid. The light-scattering data quoted here have been interpreted[8] by *assuming* a value for the a-axis. Thus, a choice of a equal to zero (*i.e.* the infinitesimally thin rod approximation), or at least equal to a very small value[8], gave lengths from 500 to over 600 A for the axis $2c$. In this narrow range of assumed a-values, c does not vary significantly from $K^{1/2}$. It is seen that these assumptions* lead to a length which is similar to that obtained for the equivalent hydrodynamic ellipsoid.

The electron microscope pictures indicate that dried fibrinogen is nodose in character and *polydispersed*. The most frequent length consisted of four "beads" with a total length of 500 A and a thickness of 60 to 80 A at the widest part of the "beads". If we assume that this four "bead", 500 A particle is the molecular unit in solution (despite the fact that a *few* 5-bead particles are visible), then the dried protein particle is a fair approximation to the actual configuration as determined by light scattering (with the values assumed for a), which, as we have seen, has approximately the same length as the equivalent ellipsoid. Since the a-values were assumed, no conclusions can be drawn about swelling of the protein in going from the anhydrous to the hydrated state. It should also be noted that, in contradistinction to solution measurements, the electron microscope indicates a wide dispersion of observed lengths. It seems evident that the fibrinogen, as it exists in a solution of 0.3 M KCl at pH 6.8, must break into small unit particles as the fibrinogen is subjected to the rapidly changing environment in the drying process. This would indicate a relatively weak bonding between the spherical particles that string together to form the fibrinogen "molecule" observed in solution.

The interpretation of hydrodynamic data on the partially polymerized material involves some additional limitations (besides those indicated above) which make the conclusions based on such studies only qualitative at best. First of all, an average length is used to characterize the system which is actually polydispersed with an unknown distribution of sizes of intermediate polymers. Secondly, the particles are larger than 1000 A, the upper limit of validity of the quasi-static theory for propagation of light in dispersed media, upon which the PETERLIN-STUART theory of flow birefringence is based. The limitation of polydispersity also applies to the interpretation of light-scattering measurements.

* The density or volume of the molecule in *solution* is not determinable since, as previously pointed out[1], the volume is, in general, neither the partial specific volume nor the effective hydrodynamic volume. Choosing particular a-values for fibrinogen[8] is equivalent to the arbitrary assumption that the density is close to that of the solid protein or, in other words, that there is no significant amount of swelling in solution to lower the density of the protein.

References p. 336.

With these limitations in mind, the general picture from hydrodynamic and light scattering measurements made before the gel point is that a highly elongated "intermediate polymer" appears after some time (but before the gel point) in a system containing fibrinogen and thrombin[2]. The average length has been roughly estimated as 5000 A[4,5]. This "intermediate polymer" appears to be a distribution of particles of various sizes, the size distribution depending on the pH[7]. Further, it has been demonstrated that the formation of these polymers is reversible upon dilution with the solvent[5,8].

The electron microscope photographs of the polymerized material demonstrate clearly the existence of these elongated polymers in the anhydrous condition. These structures would appear to be fairly rigid, for light scattering indicates that their configuration in solution is highly elongated, and flow birefringence indicates that the phenomenon is predominantly an orientation effect[4–7]. The electron microscope also shows that the polymerization of activated fibrinogen to form these intermediate polymers is predominantly an end-to-end interaction since the thickness of the polymer is that of the monomer with the same nodose character. Since the width cannot be specified precisely, and drying effects are involved, this does not rule out a certain amount of overlapping of monomers rather than strictly end-to-end aggregation in forming the elongated polymer.

Our observations give no indication as to how the periodicity of 230 A observed in fibrin clots by both HALL[9] and PORTER AND HAWN[10] arises. HALL noted the nodose character of fibrinogen but could not correlate it with the periodicity in the fibrin strands. PORTER AND HAWN were led to postulate oblate ellipsoids for the fibrinogen which they believed would stack to give the elementary filaments of fibrin. However the hydrodynamic data can distinguish between a prolate and oblate equivalent ellipsoid[1], a prolate one being obtained for native fibrinogen[3]. From the similarity found between the light-scattering configuration and the equivalent ellipsoid (within the limitations imposed by the assumption introduced in the interpretation of the light scattering data), and also from the electron microscope observations reported here, it is possible to rule out the oblate ellipsoid configuration for the molecule in solution. While the fibrinogen molecule is probably thus an elongated one, it appears to have a substructure of weakly-bonded spheroidal units, the initial polymerization occurring by end-to-end bonding of the thrombin-activated molecule. The nature of the cross-linking aggregation that must occur to give the three dimensional fibrin gel is still to be determined.

SUMMARY

The configuration of dried specimens of fibrinogen and its intermediate polymers have been determined by electron microscopy. These observations have been compared with those reported on the basis of hydrodynamic and light-scattering methods. Reference has been made to the distinction between the equivalent hydrodynamic ellipsoid and the molecular configuration in solution. Further, it has been pointed out that the configuration of the solvated protein in solution cannot be determined from light scattering without the introduction of an assumption about the thickness of the molecule. Assumed values of the thickness of fibrinogen have been reported[8] which lead to a similarity in lengths between the light-scattering configuration and the equivalent hydrodynamic ellipsoid; there is also a similarity between the lengths of these ellipsoids and the predominant length observed in electron micrographs reported here.

Electron microscopy reveals a polydispersity of the anhydrous fibrinogen in contradistinction to the monodispersity indicated by the solution data, suggesting the existence of weakly-bonded sub-units in the fibrinogen molecule.

References p. 336.

The observations of the partially polymerized fibrinogen in solution have, within the limitations of a comparison of hydrated and anhydrous proteins, been confirmed by electron microscopy. Specifically the asymmetrical nature and approximate size range of the intermediate polymers have been demonstrated.

RÉSUMÉ

La configuration de spécimènes séchés de fibrinogène et de ses polymères intermédiaires a été étudiée au microscope électronique. Les résultats de cette étude ont été comparés à ceux obtenus par les méthodes hydrodynamique et de dispersion. La différence entre l'ellipsoïde hydrodynamique équivalent et la configuration moléculaire en solution a été mentionnée. De plus, il a été signalé que la configuration de la protéine solvatée en solution ne peut pas être déterminée à partir de la dispersion de la lumière sans que l'on n'introduise une supposition au sujet de l'épaisseur de la molécule. Des valeurs supposées de l'épaisseur du fibrinogène ont été rapportées préalablement[8]; elles conduisent à une similitude entre la longueur de la configuration établie par la méthode de les longueurs de ces ellipsoïdes et la longueur prédominante observée au microscope électronique et rapportée dans ce mémoire. Le microscope électronique révèle l'état polydispersé du fibrinogène anhydre, s'opposant à l'état monodispersé indiqué par l'étude des solutions, ce qui suggère l'existence de subunités faiblement liées dans la molécule de fibrinogène.

La microscopie électronique confirme, dans les limites d'une comparaison entre les protéines anhydres et hydratées, les observations sur le fibrinogène partiellement polymérisé en solution. En particulier, elle démontre l'asymétrie des polymères intermédiaires et permet d'établir l'ordre de grandeur de leur taille.

ZUSAMMENFASSUNG

Die Konfiguration von getrockneten Proben von Fibrinogen und seinen Zwischenpolymeren wurde elektronenmikroskopisch bestimmt. Diese Beobachtungen wurden mit jenen verglichen, die auf Grund von hydrodynamischen und Lichtstreuungsmethoden erhalten wurden. Es wurde der Unterschied zwischen dem äquivalenten hydrodynamischen Ellipsoid und der Molekularkonfiguration in Lösung erwähnt. Weiter wurde darauf hingewiesen, dass die Konfiguration des solvatisierten Proteins in Lösung nicht aus der Lichtstreuung bestimmt werden kann, ohne dass eine Annahme für die Dicke des Moleküls eingeführt wird. Angenommene Werte für die Dicke des Fibrinogen wurden berichtet[8], die zu einer Ähnlichkeit in den Längen zwischen der durch Lichtstreuung ermittelten Konfiguration und dem äquivalenten hydrodynamischen Ellipsoid führten; es besteht ebenfalls eine Ähnlichkeit der Längen dieser Ellipsoide und der vorherrschenden Länge, die in den hier berichteten Elektronenmikrobildern beobachtet wurde.

Die Elektronenmikroskopie zeigt eine Polydispersität des wasserfreien Fibrinogens im starken Gegensatz zur Monodispersität, wie sie sich aus den Werten in Lösung ergibt, was die Existenz von schwach gebundenen Untereinheiten im Fibrinogenmolekül vermuten lässt.

Die Beobachtungen von teilweise polymerisiertem Fibrinogen in Lösung wurden innerhalb der Grenzen eines Vergleichs zwischen hydratisierten und wasserfreien Proteinen durch elektronenmikroskopische Untersuchungen bestätigt. Es wurde besonders auf die asymmetrische Natur und den angenäherten Grössenbereich der Zwischenpolymeren hingewiesen.

REFERENCES

1 H. A. SCHERAGA AND L. MANDELKERN, *J. Am. Chem. Soc.*, 75 (1953) 179.
2 S. SHULMAN AND J. D. FERRY, *J. Phys. Colloid Chem.*, 55 (1951) 135.
3 H. A. SCHERAGA, J. K. BACKUS AND J. M. SAUNDERS, Abstracts of September 1952, A.C.S. meeting, p. 20C.
4 J. F. FOSTER, E. G. SAMSA, S. SHULMAN AND J. D. FERRY, *Arch. Biochem. Biophys.*, 34 (1951) 417.
5 H. A. SCHERAGA AND J. K. BACKUS, *J. Am. Chem. Soc.*, 74 (1952) 1979.
6 J. D. FERRY, S. SHULMAN AND J. F. FOSTER, *Arch. Biochem. Biophys.*, 39 (1952) 387.
7 J. K. BACKUS, M. LASKOWSKI, JR., H. A. SCHERAGA AND L. F. NIMS, *Arch. Biochem. Biophys.*, 41 (1952) 354.
8 S. KATZ, K. GUTFREUND, S. SHULMAN AND J. D. FERRY, *J. Am. Chem. Soc.*, 74 (1952) 5706, 5709.
9 C. E. HALL, *J. Biol. Chem.*, 179 (1949) 857.
10 K. R. PORTER AND C. VAN ZANDT HAWN, *J. Exptl. Med.*, 90 (1949) 225.
11 B. M. SIEGEL, D. H. JOHNSON AND H. F. MARK, *J. Appl. Phys.*, 19 (1948) 1187.
12 R. C. BACKUS AND R. C. WILLIAMS, *J. Appl. Phys.*, 21 (1950) 11.
13 K. LAKI, *Arch. Biochem. Biophys.*, 32 (1951) 317.
14 P. R. MORRISON, *J. Am. Chem. Soc.*, 69 (1947) 2723.
15 P. DOTY AND J. T. EDSALL, *Advances in Protein Chem.*, 6 (1951) 73.

Received November 22nd, 1952