sentative value, and since there are 6.6×10^9 diffusion-controlled encounters per sec. in a liter of molar solution at room temperature in water, then if each encounter is effective (*i.e.*, optimal efficiency) the lifetime will be about 10^{-4} sec. The constancy, within a factor of two of the retardation constants of the halogenated members suggests that the lifetimes of the metastable states of such molecules changes very little with the nature or the extent of the halogenation. Some direct measurements of phosphorescence lifetimes of halogenated benzenes[12,13] in rigid media indicate that the lifetimes are practically independent of the extent of halogenation, although a decreased lifetime is obtained when chlorine is replaced with bromine. For the best retarders Fl shows a markedly lower retardation constant than the other dyes, indicative of a shorter lifetime. On the other hand, visual comparison of the phosphorescences of fluorescein (Fl) and eosin (FBr_4) in glycerol at $-180°$ indicates that the latter dye has the shorter lifetime. This may be explained on the basis of the lifetimes of the halogenated dyes changing less rapidly with viscosity[14] than that of fluorescein because of a protecting effect of the halogens. This protecting effect (fewer non-radiative transitions with increasing halogenation) has been noted by McClure, *et al.*,[12] for the bromobenzenes.

The retardation by the ground state dye (step 8), by other dyes of the family, and by the other foreign materials noted above is not correlated with the absorption and emission properties of the dye being faded or the retarder, but can be explained simply by a diffusion-controlled quenching mechanism. Mechanisms of quenching based on intermolecular energy transfer[15] are probably unlikely here since the retarders have a diversity of absorption and emission characteristics. Further, substances having these characteristics in common show widely dissimilar effects (*e.g.*, 4,4′-diaminostilbene-2,2′-disulfonic acid is effective while quinine sulfate is not; aniline is effective but bromobenzene is not, etc.).

Lewis, *et al.*,[16] have identified the β-phosphorescence of fluorescein as arising from the transition from the lowest triplet level of the dye to its ground state. It is not unlikely that this is also the origin of the β-phosphorescence of the halogenated fluorescein derivatives. Our phosphorescence quenching experiments reported above in which there is a parallelism between the efficiencies of retardation and of phosphorescence quenching[17] indicates strongly that the metastable species deduced from the kinetic data on the photoreduction is indeed the triplet state of the dyes.

Acknowledgments.—This work was supported by the Photographic Branch of the Signal Corps (Contract No. DA-36-039 sc-42463) and by the Air Research and Development Command of the Air Corps (Contract No. AF 18(600)-1182).

(12) P. P. Dikun, A. A. Petrov and B. Ya. Svishnikov, *Zhur. Eksptl. Teoret Fiz.*, **21**, 150 (1951).

(13) D. S. McClure, N. W. Blake and P. L. Hanst, *J. Chem. Phys.*, **22**, 255 (1954).

(14) Compare, B. Ya. Sveshnikov and P. P. Dikun, *Doklady Akad. Nauk, S.S.S.R.*, **60**, 571 (1948). See also G. Porter and M. W. Windsor, *Disc. Faraday Soc.*, No. 17, 178 (1954).

(15) See T. Förster, *Ann. Physik*, **2**, 55 (1948).

(16) G. N. Lewis and M. Kasha, THIS JOURNAL, **66**, 2100 (1944); G. N. Lewis and M. Calvin, *ibid.*, **67**, 1232 (1945); for review, see M. Kasha, *Chem. Revs.*, **41**, 401 (1947).

(17) Compare, S. Boudin, *J. chim. phys.*, **27**, 285 (1930); H. Kautsky and A. Hirsch, *Chem. Ber.*, **64**, 2677 (1931).

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The Conversion of Fibrinogen to Fibrin. XIX. The Structure of the Intermediate Polymer of Fibrinogen Formed in Alkaline Solutions[1]

BY EDWARD F. CASASSA

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The enzymatic action of thrombin results in a high degree of conversion of bovine fibrinogen to a soluble polymer in a solvent of *p*H 9.5 and molar ionic strength 0.45, but fibrin gel does not appear provided the protein concentration is sufficiently low. Conventional treatment of light scattering data from the polymer solutions, by extrapolation of the intensity to zero scattering angle, is shown to lead to meaningless values of molecular weight and radius of gyration. However, the experimental results do conform to the scattering behavior of thin rod-like particles for the asymptotic limit of indefinitely great length; and it is possible to determine the ratio of molecular mass to length though neither can be determined separately. Comparison of this ratio with that of monomeric fibrinogen leads to the conclusion that the polymer cross-section is double that of the monomer.

Introduction

When acted upon by thrombin, the plasma protein fibrinogen polymerizes to form fibrin, a three-dimensional network structure. The process comprises several steps, reversible at least under some conditions, as shown by the scheme[2,3]

$$F \overset{th}{\rightleftharpoons} f \rightleftharpoons f_n \rightleftharpoons \text{fibrin}$$

An enymzatic reaction with thrombin th converts fibrinogen F to an activated form f; an intermediate polymer f_n then appears and this participates in the fibrin gel formation. The rates and equi-

(1) This investigation was supported by the Office of Naval Research, United States Navy, under Contract N7onr-28509.

(2) In previous publications from this Laboratory[3] the first and third steps of the reaction sequence were not indicated as reversible. Recent work of Donnelly, Laskowski, Notley and Scheraga,[4] however, has demonstrated the reversibility of these processes.

(3) J. D. Ferry, *Proc. Nat. Acad. Sci.*, **38**, 566 (1952).

(4) T. H. Donnelly, M. Laskowski, Jr., N. Notley and H. A. Scheraga, *Arch. Biochem. Biophys.*, **56**, 369 (1955); T. H. Donnelly, M. Laskowski, Jr., and H. A. Scheraga, paper presented at the 128th meeting of the American Chemical Society, Minneapolis, Minnesota, September, 1955.

libria for the various stages depend upon the ionic strength, *p*H and concentrations of the reactants, and certain substances capable of inhibiting the last step entirely under some conditions.

The intermediate polymer of bovine fibrinogen has been studied earlier by flow birefringence,[5,6] sedimentation,[7] viscosity,[7] electron microscopy,[8] and light scattering.[9] These investigations have demonstrated that the polymer is composed of greatly elongated, probably rod-like or ellipsoidal, particles. In particular, from the light scattering study carried out in this laboratory it was concluded that the aggregate—formed at *p*H 6.2 in the presence of hexamethylene glycol as a gelation inhibitor[10,11]—though not necessarily strictly homogeneous in size, contains at least fifteen fibrinogen residues and has an average cross-section roughly twice that of fibrinogen. Inhibition also occurs[6,12] at *p*H 9.5, but if the protein concentration is low enough at this *p*H, a high degree of conversion to intermediate polymer can be attained in the absence of inhibitor without gelation taking place. Dilute systems of this type, which form the subject matter of this paper, are of special interest as the solute is sufficiently dilute to exhibit ideal thermodynamic behavior; and, therefore, considerable simplification can be achieved in the interpretation of light scattering data.

Experimental Procedures

Materials and Preparation of Solutions.—The refractionation of fibrinogen (from Armour bovine material Fraction I, Lot 210) was carried out by precipitation by ammonium sulfate following procedure II described in the preceding paper of this series.[13] The products contained about 90% clottable fibrinogen determined by gravimetric assay. The thrombin used was highly purified bovine material, of activity about 850 units per mg., obtained through the generosity of Dr. W. H. Seegers of Wayne University. A stock solution containing 60 units of thrombin per ml. was made up in 0.45 *M* sodium chloride to which was added 2 mg./ml. of bovine serum albumin to minimize loss of enzyme activity through adsorption on glass. This solution was frozen quickly in test-tubes in 5 ml. lots by immersion in Dry Ice and acetone mixture and then stored for future use at −15°. The solvent for fibrinogen was in every experiment a buffer of *p*H 9.5 made up as 0.100 *M* in glycine, 0.0500 *M* in sodium hydroxide, 0.400 *M* in sodium chloride.

For removal of dust particles preparatory to light scattering measurements, fibrinogen solutions and the thrombin stock solution, diluted to 20 units per ml. with the protein solvent buffer,[14] were centrifuged separately at 20,000 *g* for two hours at a temperature below 25°. Then the fibrinogen and enough thrombin solution to bring the concentration of enzyme to 0.5 unit per ml. were pipetted into a light scattering cell[16,17] and the mixture was allowed to stand at 25° for the 4 to 6 hours required until the scattered light intensity no longer changed significantly with time; *i.e.*, until the polymerization reaction was apparently no longer progressing.

Measurements of Light Scattering.—The photometer used in this study was that described by Katz[16] with the addition of a thermostat[17] to maintain the temperature of the sample at 25 ± 0.15° during all experiments. To determine the angular distribution of scattered intensity, measurements were made at 17 or 18 values of the scattering angle θ (measured relative to the direction of the incident beam) between 25 and 140°, the limits imposed by the thermostat window. All measurements were made with unpolarized light at 4358 Å. As in earlier work in this laboratory[9,13] the reference standard for scattered intensity was a certain sample of polystyrene dissolved in butanone for which the absolute scattering has been determined by Carr and Zimm.[15,18] The specific refractive index increment[13] for fibrinogen in the glycine buffer was taken to be 0.195. The total protein concentration obtained spectrophotometrically,[13] rather than the amount of clottable protein, was used in all light scattering calculations.

Apparent Angular Dependence of Light Scattering.—In the previous paper of this series corrections were discussed for errors falsifying the angular distribution of scattered intensity when the scattering envelope does not depart greatly from that expected for particles small compared to the wave length of light. For very large particles, as in the present case, where the intensity distribution is not symmetrical about 90° and the variation of intensity with angle may be very rapid it becomes necessary to consider additional sources of error.

If the intensity distribution is sufficiently asymmetric about 90°, considerable error can be introduced from scattering excited by backward reflections of the emergent incident light beam.[19] The apparatus used in this work was designed to minimize the effect of such reflections. Because of the inclination of the walls of the light scattering cell (which has the conical form of an erlenmeyer flask[16,17]) direct reflections from the glass–liquid interfaces where the exciting beam passes out of the cell into the surrounding water-bath in the thermostat are deflected downward out of the solution volume viewed by the detector for scattered radiation. No significant reflection arises in connection with the outer glass–air interface at the exit window through which the incident beam leaves the thermostat since this window is made of a glass which transmits but five per cent. of the incident radiation; hence only about 0.01% of the original intensity is reflected[20] back through the solution. The only reflection of the incident beam which need be considered arises at the inner glass–water interface at the exit window of the thermostat. With the refractive index of glass 1.513 and that of water 1.340 the amount of light reflected at this point is 0.4% of the incident intensity.

With the method of calibration used for the photometer, a measured intensity of scattering is based, ultimately, on the observed scattering at right angles from the standard solution in the same experimental arrangement. Therefore, readings at right angles are automatically corrected for the amount of light scattered by back reflection of the incident beam; but at other angles a correction must be made to the observed intensity $I(\theta)_{obs}$ to obtain the true value $I(\theta)_{cor}$ given by

$$I(\theta)_{cor} = \frac{I(\theta)_{obs} - kI(180 - \theta)_{obs}}{1 - k}$$

where k is the fraction of the incident beam reflected. In the most extreme cases encountered in this work, the reflectance error caused the observed intensity to be too large

(5) J. F. Foster, E. G. Samsa, S. Shulman and J. D. Ferry, *Arch. Biochem. Biophys.*, **34**, 417 (1951); J. D. Ferry, S. Shulman and J. F. Foster, *ibid.*, **39**, 387 (1952).

(6) H. A. Scheraga and J. K. Backus, THIS JOURNAL, **74**, 1979 (1952); J. K. Backus, M. Laskowski, Jr., H. A. Scheraga and L. F. Nims, *Arch. Biochem. Biophys.*, **41**, 354 (1952).

(7) S. Shulman and J. D. Ferry, *J. Phys. Colloid Chem.*, **55**, 135 (1951); P. Ehrlich, S. Shulman and J. D. Ferry, THIS JOURNAL, **74**, 2258 (1952).

(8) P. Kaesberg and S. Shulman, *J. Biol. Chem.*, **200**, 293 (1953); B. M. Siegel, J. P. Mernan and H. A. Scheraga, *Biochim. Biophys. Acta*, **11**, 329 (1953).

(9) J. D. Ferry, S. Shulman, K. Gutfreund and S. Katz, THIS JOURNAL, **74**, 5709 (1952).

(10) J. D. Ferry and S. Shulman, *ibid.*, **71**, 3198 (1949).

(11) S. Shulman, *Arch. Biochem.*, **30**, 353 (1951).

(12) I. Tinoco, Jr., and J. D. Ferry, *Arch. Biochem. Biophys.*, **48**, 7 (1954).

(13) E. F. Casassa, *J. Phys. Chem.*, **60**, 926 (1956).

(14) At this stage the concentration of serum albumin in the thrombin solution was tenfold greater in earlier practice.[15] The reduction did not affect thrombin activity.

(15) J. D. Ferry, M. Miller and S. Shulman, *Arch. Biochem. Biophys.*, **34**, 424 (1951).

(16) S. Katz, THIS JOURNAL, **74**, 2238 (1952).

(17) E. F. Casassa and S. Katz, *J. Polymer Sci.*, **14**, 385 (1954).

(18) C. I. Carr and B. H. Zimm, *J. Chem. Phys.*, **18**, 1616 (1950).

(19) H. Sheffer and J. C. Hyde, *Can. J. Chem.*, **30**, 817 (1952).

(20) For normal incidence, the fraction of light reflected from an interface between media of refractive indices n_1, n_2 is $(n_2 - n_1)^2/(n_2 + n_1)^2$.

by about 0.6% at the largest scattering angles employed and too low by 0.3% at the smallest angles.

As with reflection of the incident beam, direct reflections of scattered radiation from the cell walls are deflected out of the field of view of the detector by the conical form of the scattering cell. Diffuse reflections of the scattered light from the inner surface of the thermostat vessel may contribute to the observed intensity, but since this surface is dull black we assume that such reflections are a negligibly small part of the total intensity. We also neglect possible errors arising from secondary scattering.

If the intensity of scattering varies rapidly with the scattering angle it may be necessary to take account of the fact that an experimental measurement at the angle θ' actually gives an average over the intensity distribution through a finite range of angles. With unpolarized incident light, the intensity of scattering which would be observed through slits of infinite resolution from a system exhibiting no depolarization at right angles is (except for a proportionality constant)

$$I(\theta') = \frac{1 + \cos^2\theta'}{\sin\theta'} P(\theta')$$

where $P(\theta)$ is the angular distribution function for scattered intensity[21]; but the intensity actually measured is

$$<I(\theta')>_{av} = \int I(\theta) g(\theta,\theta') d\theta \qquad (1)$$

g being a normalized weighting factor determined by the geometry of the optical system. If the form of g is known, the difference between $I(\theta')$ and $<I(\theta')>_{av}$ can be investigated for forms of $P(\theta)$ of interest—most conveniently perhaps by expanding the integrand of equation 1 in Taylor's series about the point θ' and integrating term by term. Taking (as an approximation for the apparatus used in this work) $g = 1/2\delta$ over the range $\theta' - \delta$ to $\theta' + \delta$ and zero elsewhere with δ one degree, we find for $P(\theta)$ inversely proportional to $\sin(\theta/2)$ that $<I(\theta')>_{av}$ differs from $I(\theta')$ by less than 0.2% at 25°, the smallest angle at which scattering measurements were made, where the error is greatest. Since the experimental angular dependence to be discussed conforms approximately to the $P(\theta)$ used in the calculation, we conclude that the slit correction may safely be neglected.

Results and Discussion

Interpretation of Experimental Data.—At sufficiently low solute concentration, the reduced excess intensity of scattering $R(\theta)$, the scattering from the solution less that from the solvent, is proportional to the solute concentration. For a system of solute particles heterogeneous with respect to size and shape we write

$$R(\theta) = Kc\Sigma M_i P_i(\theta) w_i = KcM_w P(\theta) \qquad (2)$$

where c is the concentration of solute in units of weight/volume, w_i is the weight fraction of the solute species i, and $K = 2\pi^2 n^2 (dn/dc)^2/\lambda^4 N$, λ being the wave length of light *in vacuo*, and N Avogadro's number. We assume that the specific refractive index increment dn/dc is the same for all solute species. As given here $R(\theta)$ represents a scattered intensity divided by the factor $1 + \cos^2\theta$ appearing with unpolarized incident light. Since the angular distribution functions for intensity designated by $P_i(\theta)$ and the average $P(\theta)$ defined[22] by equation 2 are unity at $\theta = 0$, extrapolation of results to zero angle yields the weight average molecular weight M_w. This operation is usually carried out using a plot of $Kc/R(\theta)$ against $\sin^2(\theta/2)$ since the curve must become linear at sufficiently small angles with an initial slope simply related to the radius of gyration of the solute molecule.[23] If, in addition, $P(\theta)$ can be shown to correspond to a function calculated for a particular molecular model, it is possible to obtain molecular dimensions[23-25] and molecular size distributions.[25,26]

When the angular dependence of $R(\theta)$ is large, the primary question to be considered in the evaluation of experimental data to establish $P(\theta)$ and molecular weight is whether measurements have been made at small enough angles for the extrapolation to zero angle to be reliable. One simple way of investigating this matter is to plot log $Kc/R(\theta)$ against log $\sin(\theta/2)$ on transparent graph paper and attempt a superposition of the experimental data upon curves of log $P^{-1}(\mu L)$ *versus* log μL for plausible molecular models, where L is a characteristic dimension of the model and $\mu = (2\pi/\lambda')\sin(\theta/2)$, λ' being the wave length of light in the medium. If there is a point of superposition for the experimental and theoretical plots, with the axes held parallel, the assumed model is at least a possible one, and M and L are determined by the relative displacement of the coordinates.

For a system of identical randomly oriented cylindrical rods of length L and thickness small compared to λ', the scattering function[27] is

$$P(\mu L) = \mathrm{Si}(2\mu L)/\mu L - (\sin(\mu L)/\mu L)^2 \qquad (3)$$

in which $\mathrm{Si}(u)$ is the integral sine function $\int_0^u (\sin x/x)dx$. In Fig. 1 we show a comparison of logarithmic plots of equation 3 with the experimental results from a representative solution of polymerized fibrinogen. It is evident that the two plots do coincide fairly well but only along a linear portion of the theoretical curve, representing the limiting behavior of $P(\theta)$ for very long rods, where there is no single point of superposition. We conclude that the experimental data do not extend to small enough angles for a unique extrapolation of $c/R(\theta)$ to zero angle to be possible. *Therefore, such an extrapolation cannot be expected to determine a meaningful molecular weight or size.*

In spite of this serious difficulty, important information can be obtained from the experiments by evaluating the data with reference to the asymptotic form of the light scattering equation for very long thin rods[28]

$$\frac{Kc}{R(\theta)} = \frac{2\Sigma M_i w_i/L_i^2}{\pi^2(\Sigma M_i w_i/L_i)^2} + \frac{4\sin(\theta/2)}{\lambda'\Sigma M_i w_i/L_i} + O(M^{-1}L^{-1}) \qquad (4)$$

in which it is implied as before that $c/R(\theta)$ is independent of concentration. Two points are of note concerning equation 4: (1) in this limit, a plot of $Kc/R(\theta)$ against $\sin(\theta/2)$ is a straight line[29] the slope of which is determined by the weight average

(21) The inverse proportionality to $\sin\theta$ appears in $I(\theta)$ because the illuminated volume of solution viewed by the detector varies in this manner with the angle θ, provided the exciting beam is well collimated.

(22) For both fibrinogen and the intermediate polymer the angular intensity distribution is the same experimentally as would be obtained with vertically polarized incident light because the depolarization effect is far too small to affect sensibly the total intensity.

(23) P. Debye, *J. Phys. Chem.*, **51**, 18 (1947).

(24) B. H. Zimm, *J. Chem. Phys.*, **16**, 1093 (1948); **16**, 1099 (1948), B. H. Zimm and W. H. Stockmayer, *ibid.*, **17**, 1301 (1949).

(25) H. Benoit, *J. Polymer Sci.*, **11**, 507 (1953).

(26) M. Goldstein, *J. Chem. Phys.*, **21**, 1255 (1953).

(27) T. Neugebauer, *Ann. Physik*, **42**, 509 (1943).

(28) E. F. Casassa, *J. Chem. Phys.*, **23**, 596 (1955).

(29) The different angular dependence of the analogous asymptotic relation for long chain polymers derived by Benoit[25] arises from the random flight statistics describing the molecular configuration.

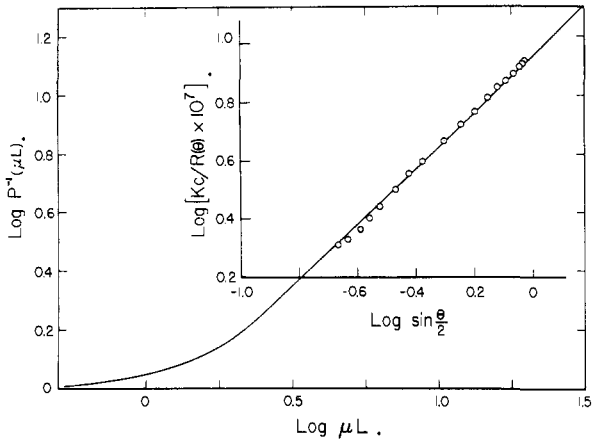

Fig. 1.—Comparison of the theoretical scattering functions for randomly oriented thin cylindrical rods with experimental results (expt. B-I) for fibrinogen polymerized at *p*H 9.5 without inhibitor.

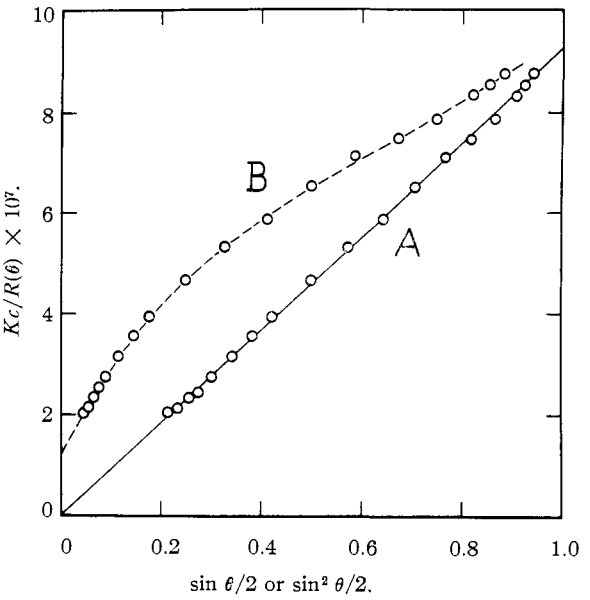

Fig. 2.—Light scattering from polymerized fibrinogen (expt. B-I). The abscissa is sin(θ/2) for curve A; sin² (θ/2) for curve B.

of M/L; (2) the intercept at $\theta = 0$ gives a molecular weight, the number average if the rod thickness and density are the same for all species. If the molecular weight is sufficiently high, the intercept is zero (within experimental error) and the observed scattering depends neither on molecular weight or length nor on their distribution, but only on the molecular cross-section determining M/L.

Figure 2 illustrates the typical agreement between the asymptotic relation and the experimental data for the intermediate fibrinogen polymer. It is evident that $Kc/R(\theta)$ over the entire range of angles studied is a nearly linear function of sin $\theta/2$ with an intercept indistinguishable from zero; hence a molecular weight cannot be established though a tentative lower limit of 10 to 20 million might be set if the rod model is correct.[30] In contrast a plausible appearing empirical extrapolation of $Kc/R(\theta)$ plotted against $\sin^2(\theta/2)$, also shown in Fig. 2, gives a weight average molecular weight of the order of only 8 million.

While the linear relationship of equation 4 is in reasonable agreement with the experimental results, minor deviations have led us in fitting the data to add a small cubic term; *i.e.*, omitting the insignificant constant term we replace equation 4 by

$$\frac{Kc}{R(\theta)} = \frac{4 \sin(\theta/2)}{\lambda' \Sigma M_i w_i / L_i} \left(1 + \gamma \sin^2 \frac{\theta}{2}\right) \qquad (4a)$$

γ being chosen to give the best agreement with the experimental points. The solid curve of Fig. 2 represents an expression of this form.

The modification of equation 4 by a cubic term can be justified (at least in a qualitative sense) in that the influence of details of the particle structure would be expected in some circumstances to affect the angular scattering distribution in precisely this way. At smallest angles the angular dependence of scattering is determined by the over-all form of the molecule. With increasing angle the scattering behavior of subsidiary structural features gradually influences the intensity function, but whether such effects are measurable depends on the size of the dimensions in question and the wave length of the incident light. The complete scattering function can then often be expressed approximately as a product of the original function and terms containing dimensions characterizing the structural details; for example, the function for a long cylinder of radius R is[32]

$$P(\theta) = P(\theta)_{\text{rod}} \left(\frac{J_1(2\mu R)}{\mu R}\right)^2 = P(\theta)_{\text{rod}} (1 - \mu^2 R^2 + \ldots) \qquad (5)$$

where $P(\theta)_{\text{rod}}$ is the intensity function of equation 3 and $J_n(x)$ designates the Bessel function of order n. A more interesting case to consider in connection with the present study is an aggregate of two parallel cylinders in contact, each of radius R, for which the function derived by Oster and Riley[33] is

$$P(\theta) = P(\theta)_{\text{rod}} \left[\frac{J_1(2\mu R)}{\mu R}\right] [1 + J_0(4\mu R)]$$
$$= P(\theta)_{\text{rod}} (1 - 3\mu^2 R^2 + \ldots) \qquad (6)$$

Quantitative Results.—In Table I are listed the molecular parameters[34] obtained by graphical fitting of equation 4a to experimental data by plotting $Kc/R(\theta)$ against sin $(\theta/2)$ as shown in Fig. 2. Fibrinogen at 0.405 and 0.420 g./l. (expts. A, B-I) was polymerized as described above and when the

(30) The linear form of equation 4 does not pertain uniquely to the cylindrical rod model: $P(\theta)$ for thin prolate ellipsoids[31] also yields an asymptotic relation for $Kc/R(\theta)$ linear in sin $(\theta/2)$ but with intercept zero and slope $10(3\lambda'\Sigma M_i w_i/L_i)^{-1}$, L_i being the ellipsoid length. Consequently a molecular weight is not obtained while an experimental slope determines a value of $\Sigma M_i w_i/L_i$ which is 5/6 that given by equation 4. Although we evaluate data in terms of the rod model, considering it the more plausible physically, the ellipsoid would evidently be equally acceptable in terms of agreement with experiment.

(31) L. C. Roess and C. G. Shull, *J. Appl. Phys.*, **18**, 308 (1947).

(32) G. Porod, *Acta Phys. Austriaca*, **2**, 255 (1948).

(33) G. Oster and D. P. Riley, *Acta Cryst.*, **5**, 272 (1952).

(34) Because of a redetermination of calibration constants and use of equation 4a rather than the simple linear form of equation 4, the results given in Table I differ slightly from those reported for some of the experiments in a preliminary communication[22] concerning this work.

TABLE I

LIGHT SCATTERING FROM FIBRINOGEN POLYMERIZED WITHOUT INHIBITOR AT *p*H 9.5

Experiment	A	B-I	B-II[a,b]	B-III[a,c]	B-IV[a,c]	C	D
Protein concn., g./ml.	0.405	0.420	0.210	0.210	0.114	0.840	1.30
Intercept of $Kc/R(\theta) \times 10^7$ *vs.* $\sin(\theta/2)$	0.0	0.05	0.0	0.1	0.0	0.0	−0.6
$M/L \times 10^{-11}$, g./cm.	1.38	1.35	1.34	1.27	1.28	1.38	2.14
γ	0.032	0.021	0.021	0.022	0.030	0.043	0.043
Cross-section, as fibrinogen monomer units	2.09	2.05	2.03	1.92	1.94	2.09	3.24

[a] Dilutions of the solution polymerized at 0.42 g./l. [b] Measurements extrapolated to moment of dilution. [c] Data at equilibrium state of polymer dissociation.

reaction had apparently stopped, the angular distribution of scattering was measured. It is evident that $Kc/R(\theta)$ is almost identical for both experiments, each graph giving an intercept not significantly different from zero and a value for the average M/L, determined by the initial slope, of 1.37×10^{11} g./cm. The curvature at large angles determined by γ is small and positive. The experimental points for angles below 90° appear to fall on a curve of barely discernible sigmoid character which we have not attempted to duplicate in curve fitting. If not simply an experimental artifact, this effect might conceivably be a manifestation of details of the scattering function for thin rods given by equation 3; but it appears profitless to speculate further on this point.

The polymer solutions containing 0.42 g./l. or less protein were stable, exhibiting no evidence of gel formation for at least 24 hours after addition of thrombin. We show for comparison the results for solutions in which the polymerization was carried out at higher protein concentrations (expts. C, D) in which fibrin gel did appear. In the system with 0.840 g./l. of fibrinogen, indications of gelation could be seen about two hours after the addition of thrombin although a tenacious clot did not form.[35] It will be noted that $Kc/R(\theta)$ for this weak gel is the same as for the soluble polymers.

The polymerization of fibrinogen at 1.30 g./l., on the other hand, leads to somewhat different results. The experimental data still obey the nearly linear form of equation 4a, but a negative constant term—which might be interpreted as arising from non-random distribution of rod-like scattering units—has to be added. The initial slope of $Kc/R(\theta)$ *vs.* $\sin(\theta/2)$ is smaller than is the case for the soluble polymers and determines a value of M/L 56% greater if the same interpretation is applicable in both instances.

To determine whether the intermediate polymer was dissociable by dilution, portions of the system polymerized at 0.420 g./l. were diluted to 0.210 and 0.114 g./l. and after about 14 hours, when equilibrium with respect to any dissociation of polymer had presumably been established, the scattering from the diluted solutions was measured. In addition, the change in scattering of the solution diluted to 0.210 g./l. was followed for a period of time immediately after dilution, and the data were used to determine by extrapolation the scattering at the moment of dilution. Since the decrease in scattering with time was very small, the extrapolated values are probably reliable. From the experimental results shown in Fig. 3 it is evident that $R(\theta)/c$ did not change upon dilution before dissociation occurred. This finding is a justification for the assumption made in the theoretical development that $R(\theta)/c$ is independent of c, at least over the range of concentrations of the soluble polymers.

The equilibrium values of $R(\theta)/c$ are smaller as is demonstrated by Fig. 3, than the values before dilution, but only by about 6%. The decrease may be interpreted as indicating either the dissociation of that amount of polymer into small fragments, though not necessarily into monomer, or as a rearrangement resulting in a smaller average cross-section for the polymers. This behavior is in contrast to that reported for systems polymerized at 4 g./l. at *p*H 6.2 with inhibitor[9] where dilution to concentrations below 0.5 g./l. was followed by nearly complete dissociation to monomer within two hours. Contrary to expectations, the dilution to 0.114 g./l. apparently did not cause a greater dissociation than the dilution to 0.210 g./l.; however the changes are so small as to be near the limits of experimental error. The parameters of equation 4a for the diluted systems are given in Table I.

It should be noted that the assumption, made in treating the light scattering data, of identifying the protein concentration with the polymer concentration, may introduce an error making the calculated value of M/L too low because the presence of small amounts of unpolymerized material would not contribute materially to $R(\theta)$. Since the ultracentrifuge could not be used for detecting the presence of low molecular weight material[7,9] at the low concentrations used here, there is no direct confirmation that polymerization is complete. We can, however, exclude the possibility that large quantities of monomeric fibrinogen are present by considering that at 25°, the smallest angle of observation used, $R(\theta)$ for polymer is about 4 times greater than at 135°, the largest angle; but for monomer the scattering is nearly independent of angle and amounts to approximately one third the polymer scattering at 135°. Therefore, considerable amounts of monomer would cause the curve of $Kc/R(\theta)$ against $\sin \theta$ to be concave downward at large angles, but experimentally the curvature is always in the opposite direction. If the equilibrium between fibrinogen and the intermediate polymer is similar to that in solutions of micelles the almost negligible dissociation of polymer upon dilution by a factor of 2.8 would indicate that the polymers were formed

(35) These observations would lead to the conclusion that the minimum fibrinogen concentration for clot formation is roughly 0.5 g./l. However, we attach no quantitative significance to this figure because clotting experiments under the same conditions have led to variable results. It appears that the stability of solutions of the intermediate polymer depends critically on the purity of the fibrinogen used. Some demonstrably impure samples have given polymer of abnormally great clotting tendency.

well above the critical monomer concentration and hence that conversion to polymer was nearly complete.

Dividing the experimental values of M/L for polymer by M/L for fibrinogen, found to be 0.66×10^{11} in earlier light scattering studies,[13,36] on the assumption of a cylindrical rod as the appropriate molecular model, we obtain a ratio of molecular cross-sections fairly near 2 in all the experiments on ungelled solutions listed in Table I. From these results we conclude that the structure of the intermediate polymer formed under the conditions of our experiments is characterized by lateral dimerization of fibrinogen units combined with far more extensive aggregation in the longitudinal direction. Studies of polymers formed under different conditions by light scattering and other methods[7-9] have led to the same general conception of the polymer structure,[37] but the evidence cited here is perhaps the most direct and least equivocal thus far obtained.

The differences in the quantity γ given in Table I for the various solutions studied are probably not experimentally significant; hence for comparison with equations 5 and 6 we take an average value of 0.025. Identifying $\gamma \sin^2(\theta/2)$ with μ^2R^2 for the single cylinder we obtain a radius R of 81 Å.; however using $M/L = 1.37 \times 10^{11}$ and the density determined by the partial specific volume[38] 0.71 for fibrinogen, we obtain a radius R of only 23 Å. If we employ the more plausible double cylinder model and calculate R from equation 6, we obtain a radius of 47 Å. for each of the cylinders. This may be compared with the 16 Å. radius for fibrinogen obtained from $M/L = 0.66 \times 10^{11}$ and the partial specific volume.

Undoubtedly molecular volumes, and hence the radii, computed from the partial specific volume are too small because the protein certainly imbibes some solvent and occupies a greater volume than that of the protein matter alone; but since a doubled effective volume would only increase the cylinder radius by a factor of $\sqrt{2}$, it appears that quantitative agreement with the measured γ cannot be obtained by invoking any reasonable amount of swelling. There is however one modification of the model which might justify a somewhat larger radius: Shulman[39] has suggested from hydrodynamic evidence that the mass of the fibrinogen molecule is not uniformly distributed, the actual shape partaking somewhat of the character of a string of beads. If then the protuberances of adjacent fibrinogen units were opposed in the lateral dimerization process, the effective radius would be increased. It seems unlikely on the basis of the present experimental results that any more definite interpretation of the cubic term in equation 4a can be proposed. It is possible that small angle X-ray scattering which covers a higher range of the variable μ than is accessible by scattering of visible light would yield more significant information than we have been able to obtain.

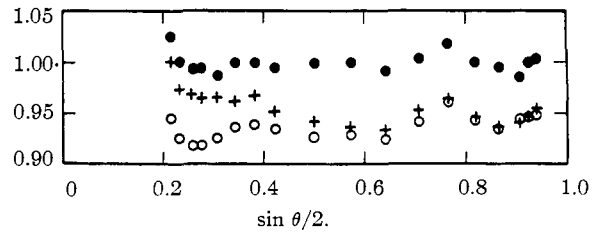

Fig. 3.—Ratio of $R(\theta)/c$ for fibrinogen polymer after dilution to that before; dilutions from 0.420 g./l. to 0.210 g./l. (circles) and 0.114 g./l. (crosses). Solid circles are results at moment of dilution; other points are from measurements 14 hours later.

Conclusion

The results reported above raise a question as to the reliability of molecular weights and lengths, obtained earlier[9] by the conventional analysis of light scattering data, for fibrinogen polymers prepared at *p*H 6.2 in the presence of hexamethylene glycol. A brief re-examination of the experimental data has failed to demonstrate clearly whether the asymptotic behavior for long rods is manifested. It seems likely that the polymers formed at *p*H 6.2 have a cross-section of about two monomer units, as do the polymers at *p*H 9.5, but that they may be of somewhat lower molecular weight—low enough for the reported molecular weights and lengths to be correct, at least in magnitude. In the case of polymers formed at *p*H 6.2 in the presence of small amounts of calcium,[40] it appears that the asymptotic light scattering formulas apply and yield average cross-sections of about three fibrinogen monomers.

Apart from the elucidation of structural features of the fibrinogen polymer, the investigation described here serves to illustrate difficulties generally attending the interpretation of light scattering data when angular dependence of scattered intensity is very large: it underlines the need for reliable measurements extending to angles as small as possible if great uncertainty in empirical extrapolation to the limiting intensity at zero scattering angle is to be avoided, and shows *a fortiori* the dubious nature of extrapolations based only on 90° scattering and a dissymmetry ratio $R(45)/R(135)$. Though it has not always been adequately considered, the question of possible errors in determining $R(0)$ may well be important in connection with certain substances of very high molecular weight—*e.g.*, viruses, nucleic acids, muscle proteins, pectins, dextrans, as well as some inorganic polymers—which are of considerable current interest in chemical and biological studies.

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