of 0.01 M salt which has been previously shown to suppress metachromasy also suppresses dye binding measured by equilibrium dialysis. This would indicate two different mechanisms of suppression of metachromasy. From the results of our previous work, we believed that high chromotrope concentrations destroyed metachromasy by acting as a salt. Equilibrium dialysis experiments indicate that this idea may not be correct. Of particular interest in looking for a connection between dye binding and metachromasy is the case of methylene green, a dye similar to methylene blue but showing almost no deviations from Beer's law and almost no metachromatic effect. In equilibrium dialysis the extent of its binding by chondroitin sulfate is only about 30% of that of methylene blue or crystal violet as indicated by the ratios in the last column of Table II. The extent to which methylene green becomes concentrated in the bag may be considered as a measure of the Donnan effect. The increased concentration effect with methylene blue or crystal violet over that of methylene green may then be a measure of the binding of these dyes by chromotrope. The work of Kurnick and Mirsky[8] using dialysis shows binding of methyl green by desoxyribonucleate. Methyl green, a close relative of crystal violet, also obeys Beer's law and seems to show no metachromasy.

Another factor which must be considered in the interaction of dye and chromotrope is the formation of precipitates such as that of toluidine blue by heparin.[9] This precipitation occurs at dye and chromotrope concentrations higher than those used in the present study. It is important to note that the dye–chromotrope complex prepared in our laboratory, when washed and redissolved in water, does not produce a metachromatic spectrum but one similar to that of a dilute toluidine blue solution. This precipitate of dye and heparin thus has spectral properties which may account for the rise in the α- and β-bands of metachromatic dyes at high chromotropic concentrations. It might be assumed that two separate conditions of the dye can exist which produce the same spectrum.

It seems possible to correlate the metachromatic properties of polysaccharides and detergents in the more dilute solutions, as follows. At chromotrope concentrations 10^{-6} and below there is no marked evidence for interaction between dye and chromotrope. At these low concentrations the individual polysaccharide molecules are assumed to be linearly disposed and the detergent molecules to be separated and ionized. In the concentration range from 10^{-6} to about 10^{-4} where α- and β-bands are depressed and μ-bands rise, it is assumed that micelle formation of the chromotropes occurs to produce in the solution micro regions of high anion density. The occurrence of such micelles in soap solutions has been extensively studied and the critical concentration of micelle formation has been found[7,10] for various soaps to lie in the range from 10^{-4} to 0.5 M. The work of Fuoss and Strauss[11] similarly indicates that high molecular weight linear polyelectrolytes change their molecular shapes with increasing concentration from one of extended chains to one of highly folded or coiled chains. Thus, in more concentrated solutions of both anionic detergents and polysaccharides, micelle-like configurations seem to be formed. Such micelles may account for the results of both metachromatic and dialysis experiments though the mechanism is still obscure.

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(9) L. B. Jacques, *Biochem. J.*, **37**, 189 (1943).
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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

The Conversion of Fibrinogen to Fibrin. X. Light Scattering Studies of Bovine Fibrinogen[1]

BY SIDNEY KATZ, KURT GUTFREUND, SIDNEY SHULMAN AND JOHN D. FERRY

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Light scattering measurements have been made on solutions of bovine fibrinogen, using two types of purified fractions and three different solvent compositions. The angular scattering distribution was measured in a conical cell, and the dissymmetry and absolute intensity were measured with high precision in a semi-octagonal cell. From the results, the molecular weight is calculated to be 340,000 ± 20,000; the length is 520 Å. on the basis of a thin rod and 650 Å. on the basis of a thin ellipsoid. These values are not perceptibly affected by the presence of 0.50 M hexamethylene glycol.

Introduction

The arrested polymerization of fibrinogen by thrombin in the presence of certain inhibitors has been studied by sedimentation,[2,3] viscosity,[2,3] and flow birefringence.[4–6] Much valuable additional

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(3) P. Ehrlich, S. Shulman and J. D. Ferry, THIS JOURNAL, **74**, 2258 (1952).
(4) J. F. Foster, E. G. Samsa, S. Shulman and J. D. Ferry, *Arch. Biochem. Biophys.*, **34**, 417 (1951).
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information concerning this process can be obtained from light scattering measurements, as described in a subsequent paper.[7] Before reporting studies of polymerized fibrinogen, however, we present here some light scattering measurements on fibrinogen itself. The results lead to a somewhat lower molecular weight and length than those derived from light scattering by Steiner and Laki[8] and by Hocking, Laskowski and Scheraga.[9]

Materials and Method

The fibrinogen used in these experiments was refractionated from Armour bovine Fraction I, Preparation 128–163, following two different procedures. In the first,[6] a precipitate was removed at 0° at low ionic strength and the supernatant solution was shell frozen and thawed; the precipitate remaining after thawing was washed and recovered as Fraction I-C, which contained 88% clottable protein. The second procedure, following Laki,[10] employed the same initial cold precipitation; the supernatant solution was brought to 23% of saturation with ammonium sulfate, and the resulting precipitate was recovered as Fraction I-L. The clottabilities of different preparations of I-L varied from 88 to 97%. All preparations were free from fibrinolysin.

The hexamethylene glycol was kindly furnished by E. I. du Pont de Nemours and Co. It was redistilled under reduced pressure.

Stock solutions of fibrinogen were adjusted to the desired ionic strength and *p*H by dialysis, and given a preliminary clarification by filtration through washed filter pads. Concentrations of fibrinogen were determined by gravimetric assay,[11] and concentrations of total protein by Kjeldahl or by dry weight with a blank correction for salt. In some experiments, hexamethylene glycol was added to the stock fibrinogen solution, in the form of an equal volume of a 1 *M* solution containing the same proportions of salt and buffer, which had been previously filtered through sintered glass. The final stock solution, and a portion of the corresponding solvent, were then clarified by centrifugation in a Servall Model SS-2 centrifuge at 20,000 g. for two to three hours.

Light scattering measurements were made in an apparatus previously described.[12] Three wave lengths of light were employed; in addition to 4358 and 5461 Å., obtained from the mercury arc by using conventional filters, the 3650 Å. line was isolated (with probably some contribution from 3655 Å.) by Corning filters[13] 738 and 5860.

Some of the experiments were made in a hand-blown conical cell similar to that previously described,[12] the scattered intensity being measured at angles from 25 to 135°. The cell geometry was checked by measuring the angular intensity distribution from a dilute solution of fluorescein. When corrected for the solvent scattering and for the angular dependence of the volume viewed, the intensity was found to be radially constant within 1% from 25 to 135°. The conical cell was calibrated against a standard square cell, using both fluorescein in water and fluorescein in a solution containing 0.50 *M* hexamethylene glycol and buffer at ionic strength 0.45. The factors obtained were used for calculating absolute reduced intensities from measurements on fibrinogen solutions with and without glycol, respectively. Other experiments were made in a semi-octagonal dissymmetry cell, with intensity measurements at 45, 90 and 135°.

In view of the difference between our results on fibrinogen and those of other workers,[8,9] the instrument calibration was repeatedly checked with Debye standard polystyrene[14] dissolved in butanone. The butanone employed was dried with calcium chloride and redistilled (b.p. 79–80°) from a 30-plate Oldershaw still with automatic take-off. Three calibration runs checked to somewhat better than 1%.

For calculations of molecular weights and lengths, the refractive indices of the solvents were measured for the sodium D line with an Abbe refractometer and corrected to the desired wave lengths by the Cauchy dispersion formula with constants obtained from the same instrument. The following values were obtained: for salt solution 0.40 *M* in sodium chloride with 0.05 additional ionic strength contributed by phosphate buffer at *p*H 6.2 (a solvent used in many previous investigations[2-4]), 1.3534 at 3650 Å., 1.3456 at 4358 Å. and 1.3389 at 5461 Å.; in the same solvent containing 0.50 *M* hexamethylene glycol,[4,6] 1.3536 at 4358 Å. and 1.3463 at 5461 Å. For salt solution 0.40 *M* in sodium chloride with 0.05 additional ionic strength contributed by veronal at *p*H 8.2, the values were as assumed to be the same as in the corresponding solution with phosphate buffer. The value of dn/dc for fibrinogen at 4358 Å. was taken as 0.197 ml./g., estimated by Dandliker,[15] and assumed to be the same in all solvents. This is close to the values employed by Steiner[8] (0.195) and Scheraga[9] (0.199).

Results

Angular Dependence.—Figure 1 shows plots of $Kc/R_{\theta,u}$ against $\sin^2(\theta/2)$ for Fraction I-C in salt buffer and Fraction I-L in glycol–salt buffer. In

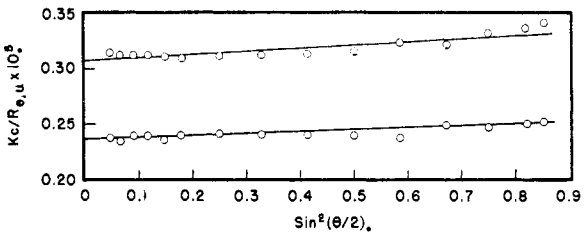

Fig. 1.—Angular distribution of scattered light from fibrinogen, measured at 4358 Å.: lower curve, in 0.40 *M* NaCl with 0.05 ionic strength phosphate buffer at *p*H 6.2 (Expt. IV-1); upper curve, in the same solvent with 0.50 *M* hexamethylene glycol (Expt. IX-1).

the former, each point is the average of measurements at ten different fibrinogen concentrations between 0.06 and 3.0 g./l.; in the latter, each is the average for five different concentrations between 0.7 and 2.1 g./l. The points were averaged because there was very little, if any, dependence on concentration. The symbols have their conventional significance.[12,18] The plots are nearly flat and linear within experimental error. They cannot be used to differentiate among the various shape models of rods, ellipsoids, cylinders, etc., all of which[18,19] give linear plots when the characteristic molecular dimension is rather small compared with the wave length of the light. They do, however, show that our fibrinogen preparations exhibit no evidence of polydispersity as found by Hocking, Laskowski and Scheraga[9]; the peculiar angular dependence found by the latter workers, and attributed by them to large spherical particles of low density, is completely absent. Reliable determinations of molecular weight and length may thus be made from dissymmetry measurements. In

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(15) We are indebted to Dr. W. B. Dandliker for this value, which was derived from dn/dc for the D line reported by Armstrong[16] and dispersion data of Perlmann and Longsworth.[17]

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TABLE I

DISSYMMETRY MEASUREMENTS

Expt.	Fraction	Clottability	*p*H	3650 Å.	4358 Å.	5461 Å.
IX-3	I-L	96	6.2	1.121 ± 0.004	1.086 ± 0.002	1.072 ± 0.006
X	I-L	97	6.2	1.110 ± .003	1.084 ± .003	1.070 ± .001
XI	I-C	87	6.2	1.117 ± .001	1.091 ± .005	1.068 ± .005
IX-2	I-L	96	8.2	1.103 ± .004	1.074 ± .003	.....
IX-1	I-L	96	6.2[a]	.....	1.097 ± .004	.....
Average at *p*H 6.2				1.116	1.087	1.070
Ellipsoid length, Å.				630	660	760
Rod length, Å.				510	530	590

[a] In 0.50 *M* hexamethylene glycol (conical cell).

principle, the molecular length and weight can be determined from the slope and intercept of a plot such as in Fig. 1, but because of the small degree of angular dependence, a dissymmetry cell with plane windows provides more accurate data.

Dissymmetry Measurements.—In Fig. 2, the dissymmetry, R_{45}/R_{135}, is plotted against the concentration of fibrinogen at three different wave lengths, for the three best experiments: two on Fraction I-L (clottability 96–97%) and one on Fraction I-C (clottability 88%), all measured in the dissymmetry cell in 0.45 ionic strength sodium chloride–phosphate at *p*H 6.2. The error circles represent 0.25%. The three experiments agree and, in view of the small dissymmetry, show gratifying precision. Since there is no perceptible dependence on concentration, the values have been averaged at different concentrations for each experiment and are presented in Table I together with another set of data in sodium chloride–veronal at *p*H 8.2 and a set in 0.50 *M* hexamethylene glycol, *p*H 6.2. The mean deviations are indicated. Finally, the averages of the three experiments (without glycol) at *p*H 6.2 are listed; the values at *p*H 8.2 and in the presence of glycol differ very little from these. The molecular lengths are also given, calculated on the basis of an elongated ellipsoid of revolution of minor axis 40 Å. (practically equivalent to an infinitesimally thin ellipsoid) and of an infinitesimally thin rod. The values from measurements at 3650 and 4358 Å. are in satisfactory agreement, and are considered to be more reliable than those at 5461 Å. The

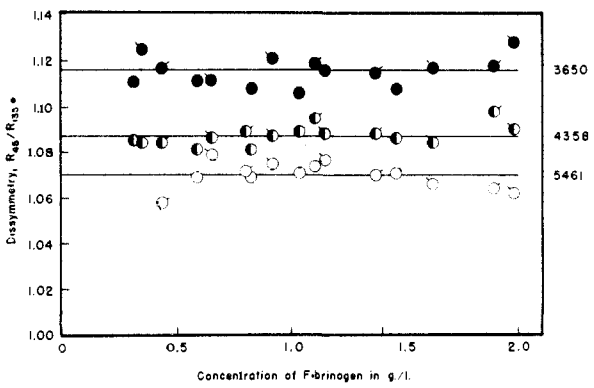

Fig. 2.—Dissymmetry, R_{45}/R_{135}, plotted against concentration of fibrinogen at three wave lengths, denoted by figures at right margin: untagged and left tags, Expts. X and IX-3, with Fraction I-L; right tags, Expt. XI, with Fraction I-C.

lengths calculated for a thin cylinder[19] are identical with those for a thin rod.

Absolute Scattering Intensity.—A plot of $Kc/R_{90,u}$ against concentration of fibrinogen is given in Fig. 3, for five concentration series, of which two are in the presence of 0.50 *M* hexamethylene glycol; four are at *p*H 6.2, and one at *p*H 8.2.

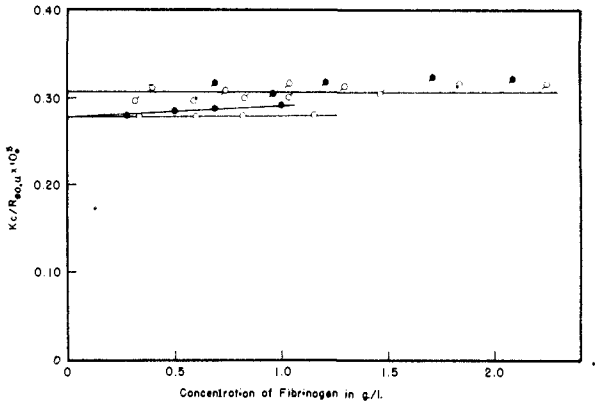

Fig. 3.—Reduced scattering intensity at 90°, measured at 4358 Å., plotted against concentration of fibrinogen: open circles, salt-buffer; solid circles, salt-buffer with 0.50 *M* hexamethylene glycol; untagged, Expt. XIII (*p*H 6.2); downward tags, Expt. IX (*p*H 6.2 with glycol, 8.2 without); upward tags, Expt. X (*p*H 6.2).

The measurements were made at 4358 Å. Except perhaps for one series in glycol, there is no slope within experimental error, so the thermodynamic coefficient A_2 is zero. The molecular weights calculated from the intercepts together with the correction factor $P(90)$ derived from the experimentally measured dissymmetries are given in Table II, where the preparations are identified, together with two additional values from supplementary experiments. (Both rod and ellipsoid models give a value of 0.95 for $P(90)$, and this was used for all preparations.) The molecular weights for the preparations of highest purity are near 340,000; the others are near 400,000. Since the concentrations used in the calculations are those of fibrinogen alone, and the contribution of nonclottable protein to light scattering has been ignored, the apparent molecular weights from the less pure fractions are obviously too high. Assuming that a clottability of 97% corresponds to 100% fibrinogen,[20] the values for the purer fractions should be very close to the correct molecular

(20) L. Lóránd, *Nature*, **167**, 992 (1951).

weight of fibrinogen, which may be taken as 340,000 ± 20,000.

TABLE II

MOLECULAR WEIGHTS

Expt.	Fraction	Clottability	pH	Molecular weight
VIII	I-L	88	6.2	(403,000)
IX-1	I-L	96	6.2, HMG[a]	337,000
IX-2	I-L	96	8.2	335,000
X	I-L	97	6.2	353,000
XI	I-C	87	6.2	(400,000)
XIII-1	I-L	88	6.2	(385,000)
XIII-2	I-L	88	6.2, HMG[a]	(394,000)

[a] In the presence of 0.50 *M* hexamethylene glycol.

The table shows clearly that the presence of 0.50 *M* hexamethylene glycol does not affect the apparent molecular weight within experimental error. This result is to be expected, even if there is moderate binding of glycol by the protein, since the influence of the glycol on the refractive index is slight.

Discussion

A molecular weight of 340,000 is smaller than the values of 540,000 found by Steiner and Laki[8] and 407,000 by Scheraga and collaborators.[9] The latter involved the use of a dissymmetry correction calculated indirectly from the length as derived from flow birefringence.

The rod length of about 520 Å. is also considerably smaller than the value of 840 Å. given by Steiner and Laki, which is based on a dissymmetry of 1.23, very much larger than that found in the present investigation. Our ellipsoid length of about 650 Å. is, however, in excellent agreement with the value of 670 Å. derived by Scheraga and collaborators from flow birefringence measurements. The latter workers did not place any reliance on their length estimated from light scattering, recognizing it to be too high.

By combining our values for molecular weight and ellipsoid length, assuming a partial specific volume[21] of 0.71, the axial ratio is found to be 19. For an unhydrated ellipsoid, this corresponds to an intrinsic viscosity of 0.25, in excellent agreement with the experimental value of 0.25 reported by Scheraga[9] and also determined recently in this Laboratory for more highly purified fibrinogen than employed previously.[2] However, the molecular weight and axial ratio correspond to a sedimentation constant of 9.6×10^{-13}, which is considerably higher than the experimental values of 7.9 and 8.1×10^{-13}, obtained in the Spinco and Svedberg oil turbine ultracentrifuges, respectively, in an extensive series of measurements at the University of Wisconsin.[22] Possibly better correlation of these physical constants can be achieved through a different approach to the molecular hydrodynamics.[23]

By combining our values for molecular weight and rod length, and assuming the shape of a circular cylinder, the axial ratio is found to be 17. There are no theoretical equations available, however, for relating the shape of a cylinder to intrinsic viscosity or sedimentation constant.

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(22) S. Shulman, unpublished experiments.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

The Conversion of Fibrinogen to Fibrin. XI. Light Scattering Studies on Clotting Systems Inhibited by Hexamethylene Glycol[1]

BY JOHN D. FERRY, SIDNEY SHULMAN, KURT GUTFREUND AND SIDNEY KATZ

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Light scattering measurements have been used to study the size, shape and dissociation equilibrium of the intermediate polymer formed by the action of thrombin on fibrinogen in 0.50 *M* hexamethylene glycol, *p*H 6.2, ionic strength 0.45. About half the protein is converted to a polymer with a weight-average degree of polymerization of 15, a length of about 3500 Å., and a width double that of fibrinogen. It is postulated that the polymerization proceeds by lateral dimerization with partial overlapping, giving two parallel end-to-end chains with staggered junctions. The polymer dissociates with dilution, the dissociation occurring rather sharply at a critical concentration; the characteristics of the equilibrium are similar to those for micelle formation. The standard free energy of formation of the 15-mer is estimated to be −120,000 calories per mole. It is suggested that the mechanism of stabilization of the intermediate polymer is similar to that of micelle formation, involving a balance between long-range repulsive electrostatic forces and local attractive forces.

Introduction

Hexamethylene glycol and a number of other substances inhibit the clotting of fibrinogen by thrombin so that an intermediate polymer accumulates.[2] Flow birefringence measurements[3-5] indicate for this polymer (or sequence of polymers) a range of lengths from roughly 6 to 10 times the

(1) This is Paper No. 16 of a series on "The Formation of Fibrin and the Coagulation of Blood" from the University of Wisconsin, supported in part by research grants from the National Institutes of Health, Public Health Service. This work was also supported in part by the Office of Naval Research, United States Navy, under Contract N7onr-28509, and by the Research Committee of the Graduate School of the University of Wisconsin from funds supplied by the Wisconsin Alumni Research Foundation. This work was presented in part at the 120th and 121st Meetings of the American Chemical Society, September 4, 1951, and April 3, 1952.

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