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.

(21) V. I. Koenig, *Arch. Biochem.*, **25**, 241 (1950); K. Bailey and F. Sanger, *Ann. Rev. Biochem.*, **20**, 103 (1951).

(22) S. Shulman, unpublished experiments.

(23) H. A. Scheraga and L. Mandelkern, reported at the 121st Meeting of the American Chemical Society, April 3, 1952.

MADISON, WISCONSIN

[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

RECEIVED JUNE 16, 1952

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.

(2) S. Shulman and J. D. Ferry, *J. Phys. Colloid Chem.*, **55**, 135 (1951).

(3) J. F. Foster, E. G. Samsa, S. Shulman and J. D. Ferry, *Arch. Biochem. Biophys.*, **34**, 417 (1951).

(4) H. A. Scheraga and J. K. Backus, THIS JOURNAL, **74**, 1979 (1952).

(5) J. D. Ferry, S. Shulman and J. F. Foster, *Arch. Biochem. Biophys.*, **39**, 387 (1952).

length of fibrinogen, while sedimentation[2,6] shows a sharp peak whose sedimentation constant, combined with the birefringence data, corresponds to a cross-section area double that of fibrinogen. These data refer to the state of the intermediate in a solvent containing 0.50 *M* hexamethylene glycol at *p*H 6.2, ionic strength 0.45. Viscosity[2,6] and flow birefringence[4,5] studies indicate that the polymer dissociates with dilution, but these are not well adapted to quantitative calculations; in particular, the viscosity is complicated by non-Newtonian behavior.[6] Light-scattering measurements have now provided an independent determination of the molecular weight and length of the intermediate polymer, as well as quantitative information on the equilibrium and kinetics of its dissociation.

Materials and Methods

The fibrinogen used in these experiments was refractionated from Armour bovine Fraction I, Preparation 128-163, by two different procedures, as described in the preceding paper.[7] Fraction I-C contained 88% and Fraction I-L 96% clottable protein. The thrombin was a highly purified bovine preparation which was kindly given us by Dr. W. H. Seegers of Wayne University. It was dissolved in the presence of bovine plasma albumin as described elsewhere.[6] The hexamethylene glycol was redistilled.

The inhibited clotting systems were made up exactly as outlined in previous papers of this Series,[3,5] by mixing stock solutions of fibrinogen, hexamethylene glycol and thrombin, all of which contained 0.40 *M* sodium chloride plus 0.05 additional ionic strength as phosphate buffer, at *p*H 6.2. The final concentrations were 4 g. fibrinogen per l., one unit thrombin per ml. and 0.50 *M* hexamethylene glycol. The reaction was allowed to proceed for several days at 25°, the formation of intermediate polymer being followed qualitatively by viscosity measurements at an arbitrary velocity gradient[2] as well as ultracentrifuge measurements[2] with the Svedberg oil turbine apparatus. Samples were withdrawn at intervals and diluted for light scattering measurements with a solvent containing the same concentrations of glycol, salt, and buffer as the stock clotting mixture. In some cases, dilutions were made containing 200 g. glycerol per l. in addition to the other components, for comparison with flow birefringence measurements in glycerol.[3,5] As explained below, the light scattering was usually measured at various times up to about two hours after the moment of dilution. During this two-hour period, the temperature was that of the room (26 ± 1°).

The apparatus, calibration and technique for the light scattering measurements have been described in previous papers from this Laboratory.[7,8] All measurements were made at a wave length of 4358 Å.

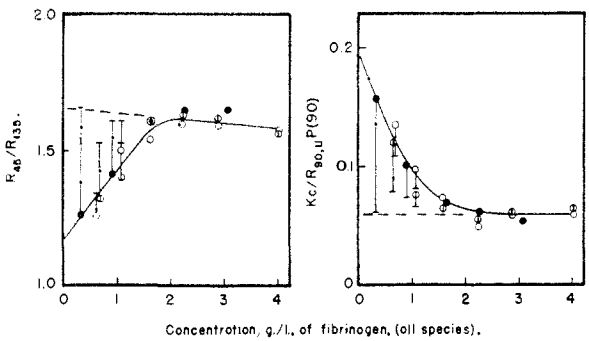

Fig. 1.—Dissymmetry (left) and $(Kc/R_{90,u})P(90) \times 10^5$ (right) plotted against concentration of fibrinogen (including all polymeric forms). Horizontal lines denote initial readings immediately after a change in concentration to the value of the abscissa, points final readings after 2 hr.: open circles, series 1, age 97 hr., decreasing concentration; black circles, series 2, age 121 hr., increasing concentration; slotted circles, series 3, age 165 hr., decreasing concentration.

Results

Preliminary Studies of Dissociation and Its Reversibility.—An inhibited clotting mixture was made up with Fraction I-C, and three series of light scattering measurements were made corresponding to ages (*i.e.*, times after addition of thrombin) of 97, 121 and 165 hr., respectively. It has been found previously[2,3] that the degree of polymerization changes relatively little over this time interval, the curve of viscosity against time becoming rather flat after 100 hr. The first and third series were made in the same manner as the viscosity[2] and birefringence[3] measurements, starting with the stock solution and adding increasing proportions of solvent to obtain successively decreasing concentrations. The second series was made in the manner more conventional for light-scattering measurements, starting with pure solvent and adding increasing portions of stock solution to obtain successively increasing concentrations. The scattered intensities were measured at 45°, 90° and 135° immediately after each addition of solvent or stock solution, respectively, and again at intervals up to about two hours, after which time the next concentration change was made. The dissymmetries were calculated, and the values of $(Kc/R_{90,u})P(90)$ were derived using the correction $P(90)$ estimated from the dissymmetry on the basis of a thin rod model. (The symbols have their conventional significance[8,9]; c always denotes the concentration of fibrinogen in g./ml. including all polymeric forms, *i.e.*, the concentration of the original stock (4 g./l.) divided by the factor of dilution.)

At concentrations above 2 g./l., whether reached from above or from below, there was little change in either absolute intensity or dissymmetry with time after an adjustment of the concentration. At lower concentrations, there was a progressive change with time, which became very slow, however, after two hours.

The final values of dissymmetry and of $(Kc/R_{90,u})P(90)$, attained after about two hours, are plotted against concentration in Fig. 1. The change with time while approaching the final value is indicated by a vertical line in each case, the first reading being denoted by a short horizontal line. These values have only approximate significance; no attempt was made in this preliminary experiment to extrapolate to zero time. The final values of both dissymmetry and scattering intensity decrease with dilution: thus both molecular weight and molecular length decrease, showing clearly that dissociation occurs. The initial values change hardly at all with dilution, however, showing that the dissociation is a rather slow process.

The virial coefficient A_2 is close to zero for fibrinogen in this solvent,[7] and the initial values on the right side of Fig. 1 suggest that the same is true for the intermediate polymer in the inhibited clotting system. On this basis, a horizontal extrapolation from the data at high concentrations, as indicated by the dashed line, provides an estimate of the weight average molecular weight in the inhibited clotting system, which is found to be about 2,800,000. (This average includes a substantial amount of material which is not polymerized at all, as shown by the ultracentrifuge.[2]) Extrapolation along the curve of final values to zero concentration provides a much more uncertain estimate of the average molecular weight which corresponds to complete dissociation of the polymer; this is found to be about 500,000, of the same order of magnitude as that of fibrinogen itself. It was noted some time ago that the intrinsic viscosity of an inhibited clotting system appears to approach that of fibrinogen with dilution.[2] No attempt is made to calculate lengths from these preliminary data; the dissymmetry roughly extrapolated from the final measurements does correspond approximately to the length of unpolymerized fibrinogen, while that extrapolated from the initial measurements corresponds to a much greater length. The dissociation product which resembles fibrinogen in size and length is presumably activated fibrinogen (f), the polymerizable molecule origi-

(6) P. Ehrlich, S. Shulman and J. D. Ferry, THIS JOURNAL, **74**, 2258 (1952).

(7) S. Katz, K. Gutfreund, S. Shulman and J. D. Ferry, *ibid.*, **74**, 5706 (1952).

(8) S. Katz, *ibid.*, **74**, 2238 (1952).

(9) P. Doty and J. T. Edsall, *Adv. Protein Chem.*, **6**, 37 (1951).

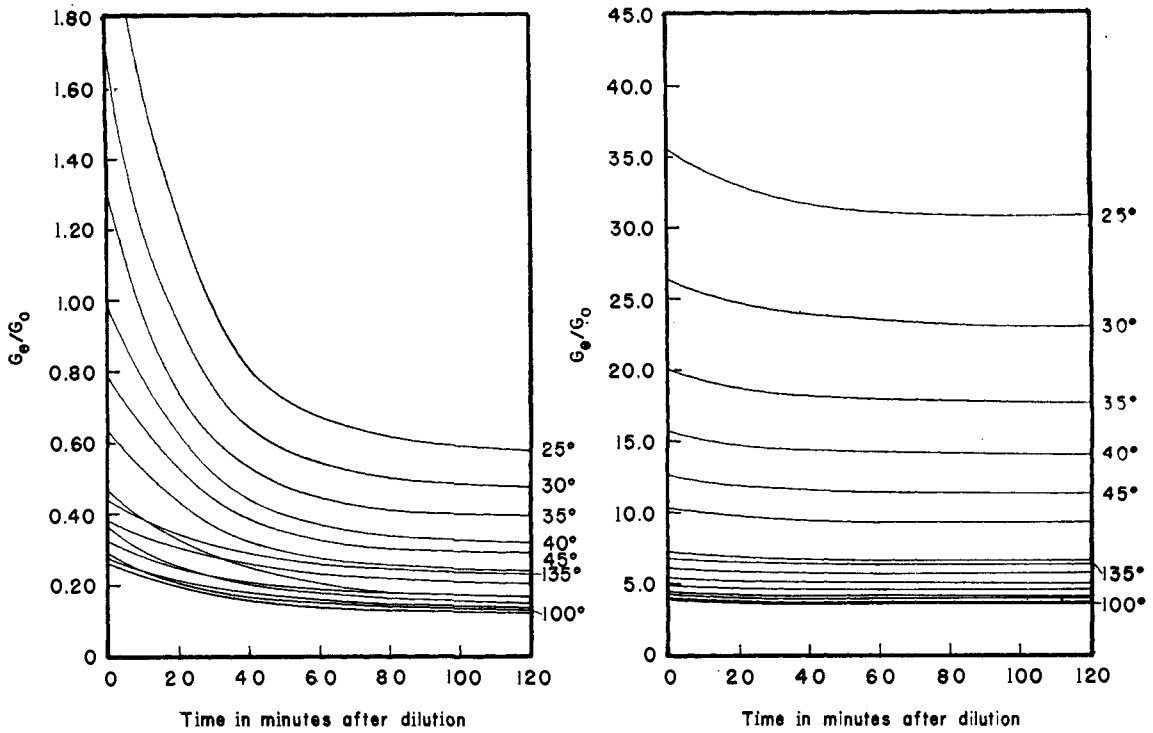

Fig. 2.—Galvanometer reading ratios corresponding to scattered intensities at various angles plotted against the time elapsed after dilution of an inhibited clotting system from 4 g./l. to 0.132 g./l. (at left) and to 1.75 g./l. (at right).

nally formed by the action of thrombin[10,11]; it is probably identical with the fragments formed by dissolving fibrin in urea and other reagents.[6]

The most important conclusion from the data of Fig. 1 is that the final state of the system at each concentration is roughly independent of the direction from which the concentration was reached, and that the dissociation is, to a first approximation at least, reversible. In all the remaining experiments, each concentration was made up separately by adding a single portion of stock solution to solvent, and scattering intensities were measured repeatedly at various angles from 25 to 135°.

Dissociation Followed by Angular Distribution Measurements.—An inhibited clotting mixture was made up with Fraction I-C. Aliquots were diluted from 4 g./l. to 0.132 g./l. and to 1.75 g./l. at ages of 95 and 100 hr., respectively. Concurrent ultracentrifuge measurements showed that 50% of the protein was associated with the fast sedimentation peak[2] and therefore presumably in the polymeric form. The change in scattering intensity with time after the moment of dilution is shown in Fig. 2, where the ratio of galvanometer readings G_θ/G_0 is plotted for various angles of observation, θ. (The curves for the higher angles fall close together and are not identified in the figure.) The sharp drop in intensity with time after dilution to 0.132 g./l. shows that dissociation of the polymer occurs at this concentration, apparently reaching equilibrium after two hours; at 1.75 g./l., however, there is little change with time. These results agree qualitatively with those of Fig. 1.

For analysis of the angular intensity distribution values of galvanometer ratios were interpolated from Fig. 2 at various time intervals and converted to $Kc/R_{\theta,u}$ by application of the appropriate filter factors, solvent correction, trigonometric factors, etc. Only the initial values (extrapolated to zero time) and the final values (at 2 hr.) are given here; they are plotted against $\sin^2(\theta/2)$ in Fig. 3. It will be assumed again that A_2 is nearly zero so that the intercepts of these plots are the reciprocals of the molecular weights and the shapes of the curves correspond approximately to those for the structure factors[9] $P^{-1}(\theta)$ extrapolated to infinite dilution. This is borne out by the close similarity of curves 1 and 3 measured at two very dissimilar concentrations before dissociation had begun, but representing the system in the molecular state which prevailed at 4 g./l. At 1.75 g./l., the apparent weight-average molecular weight of the system ($\bar{M}_w$) starts at 3,700,000 and drops with time to 3,080,000. At 0.132 g./l.. M_w starts at 3,230,000 and drops with time to 640.000—again of the same order as that of fibrinogen itself.

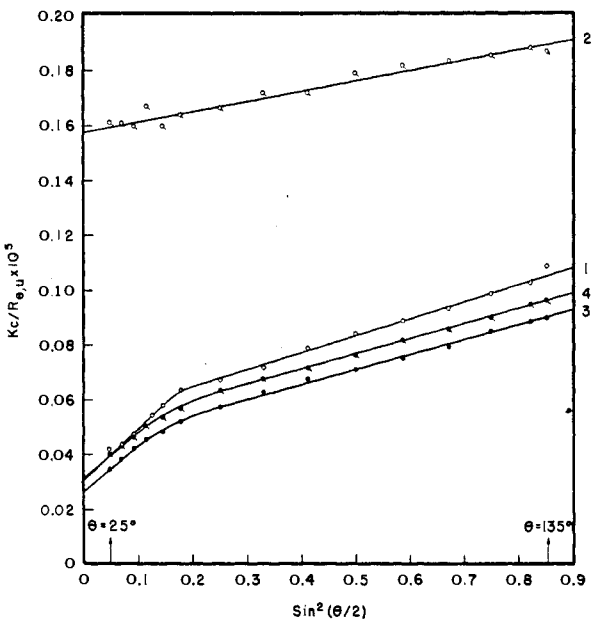

Fig. 3.—Angular distribution of scattered intensity derived from data of Fig. 2: curves 1 and 2, initial and final values after dilution to 0.132 g./l.; curves 3 and 4, initial and final values after dilution to 1.75 g./l.

The final curve at 0.132 g./l. is linear over the entire angular range, as found for unpolymerized fibrinogen[7] and

(10) K. Laki, *Arch. Biochem. Biophys.*, **32**, 317 (1951).

(11) The symbol **f** follows the uniform notation recently adopted by Dr. H. A. Scheraga and collaborators and ourselves.[5] Our previous[6] F′ is now **f**, and our Φ is $\mathbf{f}_n$.

expected for a rod or elongated ellipsoid of this length. To examine the angular distribution of scattering from the polymer, the data of curve 1 of Fig. 3 were normalized to obtain the function $P^{-1}(\theta)$ and then corrected for the presence of unpolymerized material in the amount of 50% of the total weight of protein. The function $P_2(\theta)$ for the polymer was obtained from the formula $P_2 = (\bar{M}_w P - w_1 M_1 P_1)/w_2 M_w$, where P is the average, observed function and P_1 is the corresponding function for unpolymerized fibrinogen[7] calculated for a rod of 520 Å. (or thin elongated ellipsoid of 650 Å.); $\bar{M}_w$ is the observed weight-average molecular weight, $M_1 = 340{,}000$, $w_1 = w_2 = 0.5$, and M_w, the weight-averge molecular weight of the polymeric species, is $(\bar{M}_w - w_1 M_1)/w_2$. The resulting curve $P_2^{-1}(\theta)$ (which actually differs only slightly from the observed curve $P^{-1}(\theta)$) is plotted in Fig. 4 together with the theoretical curve for a thin rod and a thin elongated ellipsoid.[9] The parameters of the theoretical plots are chosen to make the initial slopes coincide, and in this case the curves for the two models are very similar.

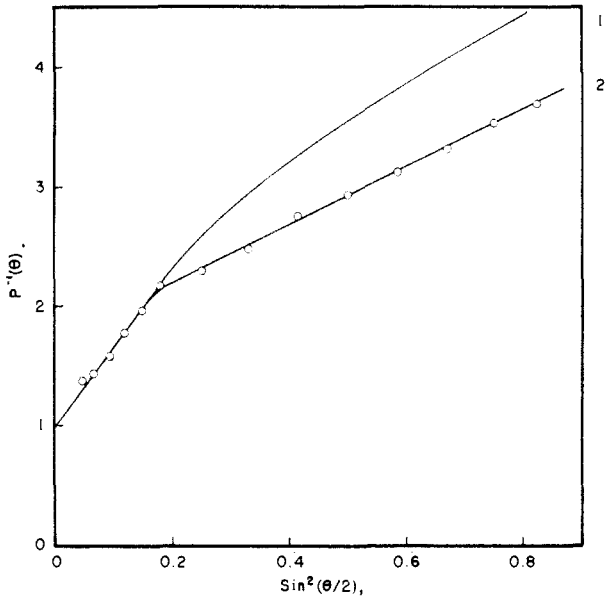

Fig. 4.—Theoretical curve for $P^{-1}(\theta)$ of a thin rod or ellipsoid (curve 1), compared with experimental function from curve 1 of Fig. 3 (curve 2).

The experimental curve is similar in shape to the theoretical one and certainly resembles it far more closely than the theoretical curve for a sphere or a random coil.[9] The rod or thin ellipsoid is thus the best choice of model for the intermediate polymer that can be made at the present time. Of the two, the rod seems more reasonable (especially in view of the remarkably uniform thickness of the typical fibrin strand as it appears in the electron microscope). The fact that the experimental curve falls below that for the model at higher angles cannot be accounted for by polydispersity (as shown by trial calculations), and it is not yet possible to offer an explanation for it. The effect of internal anisotropy, if assumed to have the same sign for the polymer as for fibrinogen itself,[12] would be a small deviation in the opposite direction.[13] The length calculated from the initial slope is 3850 Å. for a rod.

Dissociation in the Presence of Glycerol Followed by Angular Distribution Measurements.—Our first flow birefringence measurements[3] on the intermediate polymer were carried out in the presence of glycerol as well as the usual glycol, salt and buffer, and they gave the impression that dissociation occurred less readily in this solvent than in the presence of glycol, salt and buffer alone. A series of measurements similar to those shown in Fig. 3 was therefore made, in which the stock solution was first diluted with an equal volume of solvent containing 400 g. glycerol per l. together with the usual 0.50 M glycol and 0.45 ionic strength sodium chloride–phosphate; the resulting mixture, now containing 200 g. glycerol per l., was then added to a solvent containing glycerol at the level of 200 g./l. in the light scattering cell, so that the final fibrinogen concentration was of the order of 0.1 g./l.

The dilutions were made at two different ages of the stock inhibited clotting mixture—at 31 hr., when about 20% of the protein was associated with the fast peak in the ultracentrifuge, and at 103 hr., when about 35% was converted to the fast-sedimenting form. Angular distribution measurements were made repeatedly after dilution, and values of $Kc/R_{\theta,u}$ at different time intervals were calculated from interpolations on graphs such as Fig. 2. The initial values (extrapolated to zero time) and final values (1.5 or 2.5 hr. after dilution) are plotted against $\sin^2(\theta/2)$ in Fig. 5. The behavior is qualitatively very similar to that in the absence of glycerol. When the thrombin-catalyzed polymerization reaction has proceeded only 31 hr., the weight-average molecular weight (derived from the intercept of the initial curve, curve 1 of Fig. 5) is only 563,000; after dilution to 0.092 g./l., it drops to 524,000 (curve 2). At a later reaction time, when more of the polymeric form has accumulated, the average molecular weight is 1,250,000 (curve 3); after dilution to 0.092 g./l., dissociation brings it down to 490,000 (curve 4). Only curve 3 has the characteristic shape for a system containing substantial amounts of intermediate polymer; the others are straight lines within experimental scatter. From the similarity of curves 3 and 4 of Fig. 5 to curves 1 and 2 of Fig. 3, it is clear that glycerol does not interfere with the dissociation of the polymer upon dilution.[14] The previous uncertainty on this question[3] was probably due to the fact that a considerable time is required for the dissociation to reach equilibrium; moreover, the

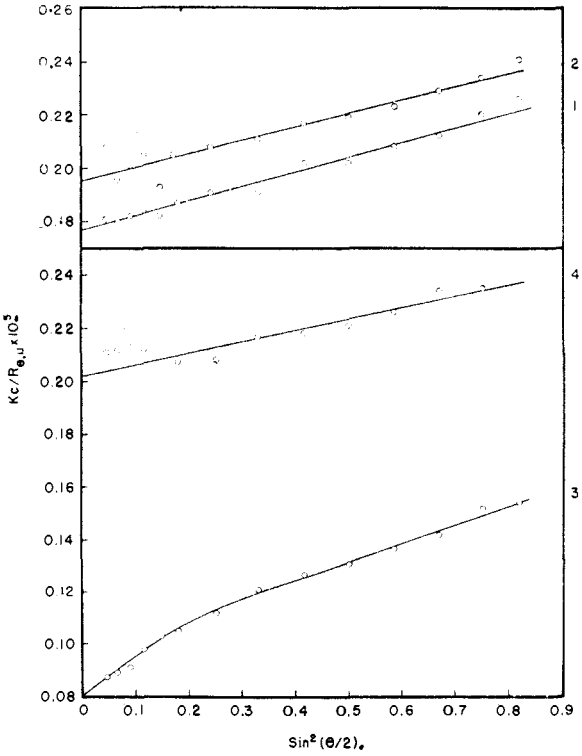

Fig. 5.—Angular distribution of scattered intensity for dilutions of inhibited clotting system to 0.092 g./l. in the presence of glycerol, concentration 200 g./l.: curves 1 and 2, initial and final (1.5 hr.) values after dilution at a reaction time of 31 hr.; curves 2 and 4, initial and final (2.5 hr.) values after dilution at a reaction time of 103 hr.

(12) C. S. Hocking, M. Laskowski, Jr., and H. A. Scheraga, THIS JOURNAL, **74**, 775 (1952).

(13) P. Horn, H. M. Benoit and G. Oster, *Compt. rend.*, **232**, 810 (1951).

(14) The dissociation is repressed, however, by small amounts of calcium, as shown by experiments which will be reported subsequently.

dissociation even at equilibrium is slight unless the dilution brings the concentration below a critical value, as will be shown in the next section.

Dissociation at Various Concentrations.—An inhibited clotting mixture was made up with Fraction I-L. Between the ages of 95 and 107 hr., when the ultracentrifuge showed a proportion of 50% of the protein in the fast-sedimenting form, aliquots were diluted to 0.098, 0.55, 0.92, 0.98 and 1.50 g./l., and intensity measurements were made repeatedly at various angles over a two-hour period after each dilution. From plots such as those of Figs. 2 and 3, the values of the weight-average molecular weight and the length (on the basis of a thin rod) were calculated, both extrapolated to zero time (the moment of dilution) and after two hours when the dissociation was essentially complete as shown by the plots against time (*cf.* Fig. 2). These initial and final values are given in Table I, together with other derived quantities.

TABLE I

DATA FROM LIGHT SCATTERING MEASUREMENTS OF DISSOCIATION OF INTERMEDIATE POLYMER

Stock clotting solution 4.0 g. fibrinogen per l., 1 unit thrombin per ml., *p*H 6.2, ionic strength 0.45, hexamethylene glycol 0.5 *M*, reaction time 95 to 107 hr.

Concn., g./l.	$\bar{M}_w$ × 10⁻⁶	Obsd. length, Å.	M_w × 10⁻⁶	ν_w	l	m	n/m
Initial values (moment of dilution)							
0.098	2.90	3560	5.46	16.1	3790	7.3	2.2
.55	2.13	2850	3.92	11.5	3030	5.8	2.0
.92	2.78	3040	5.22	15.4	3230	6.2	2.5
.98	2.88	3180	5.42	15.9	3380	6.5	2.4
1.50	3.02	3870	5.70	16.8	4110	7.9	2.1
Av.			5.13	15.1	3510	6.8	2.2

Concn.	$\bar{M}_w$ × 10⁻⁶	M_w × 10⁻⁶	n_w	α	Obsd. av. length, Å.
Final values (2 hr. after dilution)					
0.098	0.37	0.40	1.18	0.013	550
.55	0.46_5	0.59	1.74	.053	1060
.92	1.49	2.64	7.8	$.48_6$	2520
.98	1.41	2.48	7.3	.45	2205
1.50	2.30	4.26	12.6	.83	3720

The initial values of $\bar{M}_w$ are all close together (except for one low figure). It is concluded therefore that these represent the state of the system at 4.0 g./l., the concentration prevailing before dilution; and that the concentration dependence of reduced scattering intensity is slight, so these values, calculated on the assumption that $A_2 = 0$, represent the actual weight-average molecular weight at 4.0 g./l. Since 50% of the protein here had the same sedimentation constant as fibrinogen, values of M_w, the weight-average molecular weight of the *polymer* species, may be obtained by assuming that $\bar{M}_w$ includes a weight fraction of 0.5 with a molecular weight[7] of 340,000. These values are listed in the table, together with those for the degree of polymerization of the polymer species, n_w ($= M_w/340{,}000$).

The observed lengths are similarly treated to obtain the lengths of the polymer species, l, using the relationship[9] that the square of the average length is the Z-average of the squares of the lengths of the individual species. The end-to-end degree of polymerization, m, is calculated by dividing by the length of fibrinogen on the basis of a rod,[7] 520 Å. Finally, n/m gives the side-by-side degree of polymerization. The average values for n, m and n/m are about 15, 6.8 and 2.2, respectively.[15]

(15) Similar estimates of average degrees of polymerization in solutions containing fibrinogen and thrombin with no inhibitor, just before the moment of clotting, have been made by Steiner and Laki.[16] However, their values are averages over the *entire system*, and since there was undoubtedly a large proportion of unpolymerized fibrinogen present, they give no picture of the size and shape of the polymer species. It should be noted in particular that the presence of monomer weights the values of n and m differently.

(16) R. Steiner and K. Laki, *Arch. Biochem. Biophys.*, **34**, 24 (1951).

The final values of $\bar{M}_w$ are given in the table, and the corresponding values of $\bar{n}_w$, averaged over the entire system, are plotted in Fig. 6 against the logarithm of the concentration, together with the average of the initial values, which corresponds to the concentration of 4.0 g./l. The shape of the curve indicates that polymerization is leveling off at 2 to 4 g./l., so that practically all the material participating in the equilibrium is in the polymeric form. This leads to two important conclusions. First, the polymerization is not a simple condensation type[17] in which successive units add with the same intrinsic equilibrium constant; in such a case the weight-average molecular weight of the system should continue to increase with concentration (and should be approximately proportional to its square root, a situation very different from that of Fig. 6a). This conclusion has already been indicated, though not established, by the sharp sedimentation peak[2] and the moderate distribution of lengths shown in flow birefringence[3]; condensation polymerization equilibria provide a very wide spread of molecular weights. Second, the component represented by the slow sedimentation peak at 4 g./l. does not participate in the equilibrium. If it did, the average molecular weight of the system would be changing rapidly in the vicinity of this concentration, instead of leveling off. (Whether the slow component represents unchanged fibrinogen or some inactive derivative is not yet clear.)

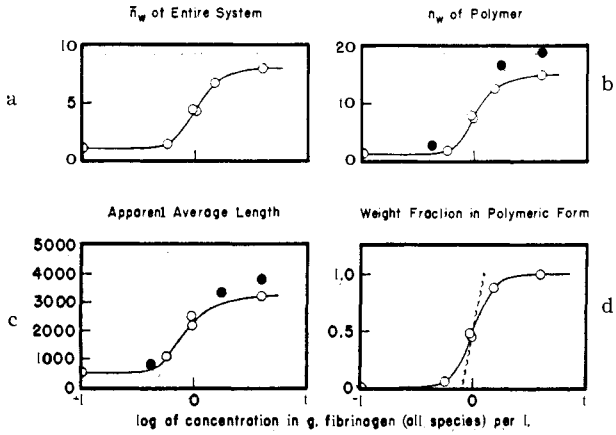

Fig 6.—Plots characterizing the equilibrium state of an inhibited clotting mixture as a function of the logarithm of concentration of fibrinogen (all species) in g./l.: a, weight-average degree of polymerization of entire system; b, weight-average degree of polymerization exclusive of inert fraction; c, apparent average length; d, weight fraction (α) of material in polymeric form, calculated as described in the text; open circles, data from Table I; solid circles, data from Fig. 3.

From the latter conclusion it follows that the equilibrium is characterized by the average molecular weight exclusive of the inert component—M_w rather than $\bar{M}_w$, obtained as in the preceding section. The corresponding values of n_w are listed and are plotted in Fig. 6b, and show again that the average degree of polymerization is approaching a limiting stage at higher concentrations. The same effect is apparent in Fig. 6c, where the observed average length is

TABLE II

CHANGE IN AVERAGE MOLECULAR WEIGHT AND LENGTH AFTER DILUTION FROM 4.0 TO 0.132 G./L.

Time after dilution, min.	M_w × 10⁻⁶	Average length, Å.
0	3.23	3850
10	1.89	2530
20	1.37	1960
30	1.04	1370
40	0.87	1090
80	0.66	820
120	0.64	790

(17) P. J. Flory, *J. Chem. Phys.*, **12**, 425 (1944).

plotted against the logarithm of the concentration; it increases rather abruptly within a small concentration range and approaches a limiting value. In Figs. 6b and 6c, points derived from the data of Fig. 3 are also included. Figure 6d will be discussed below.

Rate of Dissociation.—From the data of Fig. 2, the weight-average molecular weight of the system and the observed average length were evaluated by interpolating at various time intervals after the moment of dilution, and making plots similar to those of Fig. 3. The resulting values are given in Table II. Although no detailed kinetic analysis is attempted at present, it is clear that the process extends over a period of an hour or more.

Discussion

Size and Shape of the Intermediate Polymer.—The data from the initial values of Table I show gratifying agreement with the results of other experimental methods. The average length of 3500 Å. conforms to the conclusion from flow birefringence of a range of lengths in the general neighborhood of 4000 Å. A rod of length 3500 Å. and cross-section area double that of fibrinogen, insofar as it can be approximated by an ellipsoid of the same molecular weight and cross-section area, should have a sedimentation constant of 25 *S*, close to the experimental value[18,20] of 23 *S* (extrapolated to infinite dilution and corrected to water at 20°). The side-by-side value of two suggests that the fundamental process of attachment is lateral dimerization with partial overlapping, which when repeated leads to two parallel end-to-end chains with staggered junctions.[23] This is the most specific geometrical picture which has been proposed thus far; it implies that the loci of attraction are on the sides rather than the ends of the fibrinogen molecule. It would lead to an intermediate polymer strictly homogeneous with respect to width but with the possibility of heterogeneity with respect to length. The nature of the charged groups which may lead to this configuration has been discussed elsewhere.[23]

Heterogeneity of the Intermediate Polymer.—The flow birefringence data indicate that the polymer is somewhat but not grossly heterogeneous. Further information can be obtained from more detailed analysis of the data of Table I and Fig. 6.

If it is assumed that the polymer is homogeneous and consists only of 15-mers, the proportions of 15-mer and monomer can be calculated from n_w. The weight fraction of the protein participating in the equilibrium which is in the polymeric form is given by $\alpha = (n_w - 1)/(n - 1)$, where $n = 15$. The resulting values of α are listed in Table I and plotted in Fig. 6d. The shape of the latter curve is in fact characteristic of an equilibrium of the type $n\mathbf{f} \leftrightarrows \mathbf{f}_n$, where no intermediates are present between monomers and n-mers, and n is a fairly large number. The larger the value of n, the more sharply[24] α rises from 0 to 1. From the equilibrium expression $m_n/m_1^n = K_1$ where the m's are concentrations in moles per liter, it can be shown that the slope of a plot of α against the logarithm of concentration (in any units), at the point where $\alpha = 1/2$, is equal to $2.303(n - 1)/6$. For $n = 15$, the predicted value is 5.4, and a dotted line with this slope is drawn in Fig. 6d. The experimental slope is a little less, as would be expected for a certain distribution of values around 15, but the fact that it is of the same order of magnitude is further evidence against gross heterogeneity.[23]

Thermodynamic Calculations.—Because of the rapid change in α with concentration, and the large value of n, the equilibrium constant cannot be calculated from the data of Fig. 6d with much accuracy except near the middle of the curve. We make the calculation at the point where $\alpha = 1/2$, calling this the critical concentration by analogy with the similar treatment of micelle equilibria.[24,25] In terms of total fibrinogen the critical concentration is 1.0 g./l., but since half the protein has been concluded to be inert with respect to the equilibrium the value is taken as 0.5 g./l. From this, the equilibrium constant for association to the 15-mer is found to be 0.44×10^{85} and the standard free energy change is $-120{,}000$ cal. or -8000 cal. per fibrinogen unit.

Mechanism of Stabilization of the Intermediate Polymer.—It has been difficult to understand why the polymerization is stabilized at an intermediate stage of about 15 under the conditions of these experiments with very little material of sizes between this and the monomer. The similarity between the characteristics of this equilibrium and that of soap micelles suggests a mechanism for this stabilization.[23] Since fibrinogen[27] and its activated form[28] carry a net negative charge, the growth of the polymer requires electrostatic work whose magnitude per monomer unit increases with the value of n. As in a micelle,[26] the net free energy of association goes through a minimum with increasing n,

(18) The earlier figure[2] of 24.5 *S* has been revised by applying a temperature correction for the oil turbine ultracentrifuge, using the observed ratio of extrapolated sedimentation constants of fast and slow peaks and assuming that the true value of the latter is equal to that of fibrinogen. The sedimentation constant of bovine fibrinogen is 8.0 ± 0.1 *S* when determined by both oil turbine and Spinco ultracentrifuges with proper corrections.[19]

(19) S. Shulman, unpublished experiments.

(20) It might be argued that the experimental sedimentation constant cannot be related to the size and shape of the polymer if this is in rapid equilibrium with dissociation products. Considerations similar to those of Singer and Campbell[21] for antigen-antibody complexes show this argument to be inapplicable. Equilibrium within a time very short compared with that of a centrifuge experiment is excluded by the direct kinetic observations (Table II) and by the fact that the sedimentation constant increases with dilution,[2] instead of decreasing as it does in certain insulin solutions, for example.[22] If slow equilibration behind the $\mathbf{f}_n$ boundary occurred, it would tend to form additional $\mathbf{f}_n$ by association of **f**. Because of the characteristics of the equilibrium, however, with its sharp dependence on concentration, the concentration of **f** remaining will be inadequate to form any appreciable quantity of $\mathbf{f}_n$.

(21) S. J. Singer and D. H. Campbell, THIS JOURNAL, **74**, 1794 (1952).

(22) J. L. Oncley and E. Ellenbogen, *J. Phys. Chem.*, **56**, 85 (1952).

(23) J. D. Ferry, *Proc. Natl. Acad. Sci.*, **38**, 566 (1952).

(24) G. S. Hartley, "Aqueous Solutions of Paraffin Chain Salts," Hermann & Cie., Paris, 1936.

(25) The existence of a critical concentration region within which dissociation occurs explains the earlier observation[2] of a fall in the reduced specific viscosity with dilution with no concomitant change in the sedimentation pattern. The drop in apparent reduced specific viscosity from a concentration of 4 g./l. down to 2 g./l. was primarily due to increased non-Newtonian effects with increasing flow rate[6]; while the relative areas in the sedimentation pattern did not change because the critical concentration had not been attained. At 1 g./l., the viscosity was dropping rapidly because of the onset of dissociation; but no sedimentation measurements were made at this low a concentration.

(26) P. Debye, *J. Phys. Colloid Chem.*, **53**, 1 (1949).

(27) S. Shulman and J. D. Ferry, *ibid.*, **54**, 66 (1950).

(28) E. Mihályi, *Acta Chem. Scand.*, **4**, 351 (1950).

resulting in stabilization. In the absence of inhibitors, the free energy loss corresponding to formation of local bonds far outweighs the electrostatic work and the polymerization is unlimited. In the system studied here, the hexamethylene glycol interferes with the formation of local bonds,[29] so that the local and electrostatic contributions are of the same order of magnitude, and stabilization occurs at a rather low value of n. The degree of distribution about the mean value of n would be determined by the sharpness of the minimum in the free energy of association.

Although the value of n will no doubt depend on the composition of the solvent, and under normal physiological conditions there may be no stabilization of this sort at all, nevertheless the fundamental geometry of polymerization is probably unaffected by the artificial conditions under which these experiments are carried out; so that a detailed study of the geometry and structure of our intermediate polymers can elucidate the mechanism of conversion of fibrinogen to fibrin in nature.

Acknowledgments.—We are indebted to Professor J. W. Williams for use of the Svedberg oil turbine ultracentrifuge, and to Miss Carol Braatz for technical assistance.

(29) J. D. Ferry and S. Shulman, THIS JOURNAL, **71**, 3198 (1949).

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[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY,[1] CALIFORNIA INSTITUTE OF TECHNOLOGY]

A Theory of Antibody–Antigen Reactions. I. Theory for Reactions of Multivalent Antigen with Bivalent and Univalent Antibody[2]

BY RICHARD J. GOLDBERG[3]

RECEIVED JULY 18, 1952

The most probable distribution of species is calculated for a system composed of univalent and bivalent antibody and f-valent antigen. This distribution is used as the basis for a theory of antibody–antigen reactions. The critical extent of reaction, which has been described previously by Flory and Stockmayer, is the point at which the system changes from one composed chiefly of small aggregates into one composed chiefly of relatively few exceedingly large aggregates. This point is interpreted to be the point at which precipitation commences in certain antibody–antigen systems. Since the fraction of reacted antibody sites and the fraction of reacted antigen sites cannot individually exceed unity, the ratio of bivalent antibody to f-valent antigen in the system must lie between specified limits in order for the system to attain the critical point. These limits are functions of the antigen valence and the fraction of antibody sites belonging to bivalent antibody. This theory, consequently, suggests a mechanism by which inhibition to precipitation is achieved. Limits may also be computed for points other than the critical point. Expressions for various antibody–antigen ratios are derived and are compared with the Heidelberger–Kendall equation, which is easily obtainable from the theory presented here. The aggregation as a function of the extent of reaction is compared to that described for a system containing f-valent antigen and univalent antibody only. Contrary to a previous belief, the possibility of large aggregate formation has a predominant effect on the system at small extents of reaction. Previously obtained experimental values for (1) the positions of the inhibition zones, and (2) the antibody–antigen ratios of the precipitates, of four antibody–antigen systems are compared with the values calculated by the theory. Two of the systems contain horse antibody and two contain rabbit antibody. Good agreement is obtained. The theory appears to have withstood a difficult set of tests. The calculations indicate that the apparent lack of antibody-excess inhibition in the systems containing rabbit antibody results chiefly from the relatively small solubility of the rabbit antibody. Results of Rh agglutination tests are in qualitative agreement with the theory in regard to the variation of the positions of inhibition zones with the combining power of the antigen.

Part A. Introduction

For a long time it has been attractive to consider antibody–antigen reactions as involving the combination of specific sites by which very large aggregates are attained. To require the existence of aggregates of this kind, one must necessarily assume that the antibody and antigen molecules responsible for the size of the aggregate are multivalent with respect to each other. If one is to require further that the antibody–antigen molecular ratio of these very large aggregates be variable and no less than unity, then he must consider antibody molecules to be bivalent and antigen molecules to be greater than bivalent. It should be noted that the existence of univalent antibody molecules in the system is still permitted. They cannot, however, be responsible for the specific growth of the aggregate to a size involving more than one antigen molecule, since wherever they occur they end chains which might otherwise have grown longer than they are.

A general theory presenting the features of a system involving reactions between bivalent antibody molecules and multivalent antigen molecules has not yet been achieved. It is the purpose here to present such a theory, which, it is hoped, will create a greater understanding of antibody–antigen reactions through the interpretation of serological data.

Although theoretical treatments have been developed in the past, they have not been sufficiently general to predict the common characteristics of the precipitin reaction. Variable antibody–antigen ratios of the precipitate, which depend on the preparation of the system, are of fundamental importance to a good theory. Inhibition to precipitation in regions of antigen excess and also antibody excess should be accounted for without relying on artificial assumptions of solubility. One should attempt to describe the relative amounts of precipitate corresponding to the composition of the system. One should be able to explain the relative differences of the system arising from the manner in which composition is varied. The quantitative function of blocking antibody molecules has yet to be described for the common systems.

(1) Contribution number 1668.

(2) This work was supported by a grant from the U. S. Public Health Service.

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