compensate for the water content of the formic acid solution. The titers of the formic acid solutions were obtained by titration with the hydroxide solutions. Only *freshly* prepared solutions were utilized. All volumetric ware was calibrated. Titrations were carried out in an atmosphere of nitrogen.

In the experiments with concentrated solutions, the above method of preparing MOH solutions resulted in phase splitting. Therefore, the procedure was as follows. Saturated aqueous MOH was added to a known amount of HA in 70% dioxane, pure dioxane was added to compensate for the water content of the MOH, and the resulting solution, which contained a slight excess of HA, made up to volume. Since the amount of MOH was not measured accurately, the resultant mixture was standardized by titration, the HA with standard base to brom thymol blue, and the MA with standard HCl in 70% dioxane to methyl orange. To our knowledge, the latter titration has not been reported previously. The analytical determinations for MA plus HA were within 0.08% of the synthetic value, based on the amount of HA taken.

E.M.F. Measurements.—E.m.f. measurements were made with the equipment as described earlier,[5,10] but with a modification in technique which eliminated the V_p measurement as described in procedure II of the experimental part of ref. 10. Equilibrium e.m.f. readings were obtained in the normal manner by adjustment of the "helipot" of the Model GS *p*H meter. The "helipot" was then locked at its position and the output terminals of the Rubicon potentiometer connected across the input terminals of the *p*H meter. The actual e.m.f. was obtained by balancing the output of the Rubicon potentiometer against the potentiometer circuit of the *p*H meter. In this connection the galvanometer of the *p*H meter served as the null indicator. The accuracy of e.m.f. measurements obtained by this technique is estimated to be ±0.02 mv. Glass electrode "C" of ref. 5 was used in all measurements.

Cell Techniques.—In all experiments at the lower concentrations, mixtures of MCl, MA and HA were prepared by adding the required amount of MOH to an appropriate mixture of HCl and HA. In the experiments at the higher concentrations, the required amount of HCl was added to a solution containing a known amount of HA and MA. In the experiments devised to measure the acetic acid activity and in those devised to measure K_{NaAc} with c_{NaAc}/c_{NaCl} ratios not equal to one (see Table IV), the e.m.f. measurements were made on individually prepared solutions in beaker-type cells.[5]

Dilution cell techniques[5] were employed in all the other measurements. In the $K_{HA} - K_{MA}$ determinations, the initial carboxylic acid concentration was varied from *ca.* 0.005 to 0.01 *M*. The initial electrolyte concentration was *ca.* 0.002 *M*.

Throughout the course of the experiments E° for cell (4) was checked frequently by obtaining e.m.f. measurements in the HCl–HA mixtures prior to the addition of MOH and by e.m.f. measurements in HCl solution. In all measurements E^* was found to be -0.63180 ± 0.00005 v.

All e.m.f. measurements for duplicate solutions were found to be reproducible to within at least ±0.1 mv.

TALLAHASSEE, FLORIDA

(10) A. L. Bacarella, E. Grunwald, H. P. Marshall and E. Lee Purlee, *J. Org. Chem.*, **20**, 747 (1955).

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

The Conversion of Fibrinogen to Fibrin. XXI. Polymer Structure and Thermodynamic Interactions in Alkaline Clotting Systems Inhibited by Hexamethylene Glycol[1]

BY EDWARD F. CASASSA[2] AND IRWIN H. BILLICK[3]

RECEIVED OCTOBER 19, 1956

Ultracentrifugation and light scattering studies have been made on solutions of partially polymerized fibrinogen in a sodium chloride–glycinate buffer at *p*H 9.5 and ionic strength 0.45 with added hexamethylene glycol at 0.5 *M* to suppress the formation of fibrin gel. Sedimentation shows that in 12 hours at 25°, the enzymatic action of thrombin at a concentration of 1 unit/cc. results in the conversion of about 80% of the fibrinogen in solutions originally containing about 4 g./l. to polymeric form. When corrected for the effect of thermodynamic non-ideality, the angular distribution of 4358 Å. light scattered by the polymeric fraction through angles from 25 to 140° agrees quite closely with that theoretically required for a system of cylindrical rods of length indeterminate, but at least of the order of 5000 Å., and mass/length ratio 2.3 times that for monomeric fibrinogen, also presumed to be rod-like. Thermodynamic interactions between solute components—ordinarily obtained from the variation of the second virial coefficient with composition—are here deduced from the change in concentration dependence of scattered intensity with scattering angle. The net interaction between polymer molecules is one of repulsion, quantitatively somewhat greater than is accounted for by the mutually excluded volume for two impenetrable cylinders. The effective monomer–polymer intermolecular force is, however, one of attraction; *i.e.*, the presence of monomeric particles is favored in the statistical environment of a polymer molecule.

Introduction

Paper XIX[4] in this series concerned a study by light scattering of the soluble polymer (or polymers) of bovine fibrinogen formed by the enzymatic action of thrombin at *p*H 9.5. That investigation involved only polymer formed at very low protein concentration, less than 0.5 g./l., since at higher concentrations further reaction occurs and this intermediate polymer is converted to fibrin gel. In this paper we discuss polymers prepared in the same buffer system but stabilized at much higher protein concentration by the addition of hexamethylene glycol, one of a number of substances capable of inhibiting gelation,[5] at least within certain ranges of ionic strength, *p*H and concentration of protein and inhibitor.

In the earlier work the task of interpreting light scattering data from the very dilute solutions was simplified by two factors: the conversion of fibrinogen to polymer apparently was substantially complete—and was assumed to be so—and the concentration was sufficiently low for the solute to be considered thermodynamically ideal (*i.e.*, the scattered intensity due to solute was directly proportional to concentration). In the present study of inhibited clotting systems neither of these simplifications holds. Thus we use the velocity ultracentrifuge to obtain an estimate of the extent of conversion of fibrinogen to polymer and evaluate

(1) This investigation received support from the Office of Naval Research, United States Navy, under Contract N7onr-28509.

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(3) Union Carbide and Carbon Fellow in Physical Chemistry, 1954–1955.

(4) E. F. Casassa, THIS JOURNAL, **78**, 3980 (1956).

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

light scattering results in accordance with the theory for multicomponent systems. Since the solutions are not ideal, it is necessary to determine the effect of thermodynamic interactions by performing a suitable extrapolation of data to infinite dilution; however, this operation is complicated by the circumstance that the polymer dissociates when diluted. The aim of the analysis undertaken here is a resolution of this somewhat involved combination of phenomena and a determination of the relevant physical and thermodynamic quantities.

Materials and Procedures

The fibrinogen used in this work was fractionated from Armour bovine Fraction I, Lot 210, by Procedure II described in Paper XVIII of this series.[6] The solvent for the polymerized systems with which we are concerned was a sodium chloride–glycinate buffer, ionic strength 0.45 and *p*H about 9.5, of the composition given earlier,[6] containing 0.500 *M* hexamethylene glycol. These materials and the bovine thrombin used were as specified in references 4 and 6.

Concentrations of total protein and clottable fibrinogen were determined, respectively, by absorption of ultraviolet light[6] and gravimetric clot assay.[6,7] Concentrations used in this paper are always based on total protein; the amount of clottable fibrinogen was determined only as a criterion of the quality of the various refractionated preparations.

For the preparation of inhibited clotting systems, protein and thrombin[4] solutions were centrifuged[6] separately to remove dust particles, then mixed in a dust-free light scattering cell[8] which was allowed to stand in a thermostat at 25° for 12 hours. At the end of this period, when the turbidity was no longer changing appreciably, light scattering and sedimentation measurements were made, as nearly as possible at the same time.

In sedimentation experiments, the Svedberg oil turbine ultracentrifuge was used except in one instance (expt. G40 in which a Spinco centrifuge was used). The schlieren photographs obtained were enlarged by projection and traced before analysis. The sedimentation constants for the fast (polymer) and slow (monomer) peaks were obtained in the usual fashion from slopes of plots of the logarithm of the boundary distance from the axis of rotation *versus* time, while the relative amounts of monomer and polymer present were determined from the areas under the respective peaks (above the base line given by the solvent alone) measured planimetrically. Temperature corrections to data from the Svedberg apparatus were obtained from calibrations[9] made by observation of the melting behavior of diphenyl ether in the centrifuge during operation.[10] Finally, sedimentation constants were expressed, as is customary in Svedberg units, relative to a solvent with the density and viscosity of water at 20°. In the determination of the composition of the system, corrections in the form developed by Trautman, *et al.*,[11] were made for the dilution occurring as a sedimentation boundary moves through the radial sector cell and for the effect first considered by Johnston and Ogston,[12] arising from interaction between components in a system comprising two distinct sedimenting boundaries. This calculation involves sedimentation constants for monomer and the intermediate polymer extrapolated to infinite dilution, for which the values used were, respectively, 7.95 reported by Shulman[13] for fibrinogen and 22.4 from the present investigation. The weight fractions of polymer in the inhibited systems listed in Table I are averages of results obtained in this way from several photographs taken at different stages during a sedimentation experiment.

In addition to the sedimentation experiments on the polymerized systems, in three cases measurements were also carried out on solutions diluted, after polymerization, to half the original solute concentration and allowed to stand for about 24 hours at 25°, when equilibrium with respect to polymer dissociation presumably had been achieved. Sedimentation data on systems diluted by larger factors were not obtained because at low concentrations the refractive index gradients produced are too small to give sufficiently well-defined schlieren patterns.

The light scattering photometer and the thermostatic device used with it have been described elsewhere.[8,14] The calibration of photometer readings in terms of absolute light intensity, experimental procedures in scattering measurements, and the methods of correcting the original data for various instrumental errors, were the same in essential respects as were described in earlier publications of this series.[4,6] Measurements of scattered intensity covered a range of scattering angles from 25 to 140° and were made at a temperature of 25° with unpolarized incident light of wave length 4358 Å. After scattering had been measured from the original solution of intermediate polymer, portions of it were diluted in light scattering cells with differing amounts of the salt–buffer–glycol solvent; and then the change of scattering was observed over a period of time as any dissociation of the diluted polymer proceeded. In determining the scattered intensity from solutions at equilibrium, at least three readings at each angle were averaged. The kinetic data obtained immediately after dilution when the scattering was changing most rapidly were necessarily less precise, being limited by the frequency of readings possible without an automatic recording device. To establish the dependence of the scattering behavior of the undissociated polymer upon concentration, it was necessary to extrapolate the data from the diluted solutions back to the moment of dilution. This extrapolation, of which an example is shown in Fig. 1, was carried out in entirely empirical

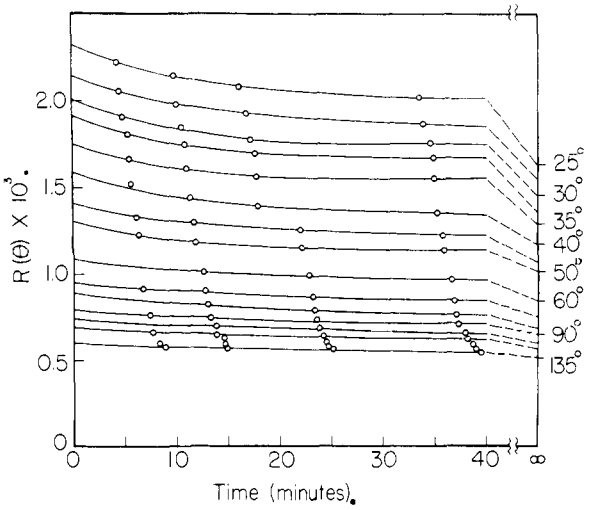

Fig. 1.—Change in light intensity scattered at various angles from an inhibited clotting system (G47) after dilution from 3.81 g./l. total protein to 0.95 g./l. The points presumed to represent equilibrium were obtained 24 hours after dilution.

fashion, no assumption being made as to the functional form of the time dependence of scattering. Some scattering data, as is indicated by Fig. 1, were obtained from clotting systems at equilibrium after dilution to low concentrations at which correlative sedimentation measurements could not be made; but no quantitative discussion of these results, nor of the data obtained during the course of the polymer dissociation process, will be given in this paper since interpretation offers some problems not covered by our present treatment.

In this paper we designate the excess intensity (the scattering from the solution less that from the solvent alone) scattered through an angle θ by $R(\theta)$, defining this quantity by $I(\theta)s^2/I_0v(1 + \cos^2\theta)$ where I_0 and $I(\theta)$ are, respectively,

(6) E. F. Casassa, *J. Phys. Chem.*, **60**, 926 (1956).
(7) J. D. Ferry and P. R. Morrison, THIS JOURNAL, **69**, 388 (1947).
(8) E. F. Casassa and S. Katz, *J. Polymer Sci.*, **14**, 385 (1954).
(9) Determined by R. L. Baldwin, private communication.
(10) S. Shulman, *Arch. Biochem. Biophys.*, **44**, 230 (1953).
(11) R. Trautman, V. N. Schumaker, W. F. Harrington and H. K. Schachman, *J. Chem. Phys.*, **22**, 555 (1954).
(12) J. P. Johnston and A. G. Ogston, *Trans. Faraday Soc.*, **42**, 789 (1946).
(13) S. Shulman, THIS JOURNAL, **75**, 5846 (1953).
(14) S. Katz, *ibid.*, **74**, 2238 (1952).

the irradiances (energy sec.$^{-1}$ cm.$^{-2}$) of incident light and of light scattered from a small irradiated element of volume v at a distance s from the point of observation. In dividing $I(\theta)$ by $1 + \cos^2\theta$ we eliminate the angular intensity dependence, which is of no interest to us, arising from the use of unpolarized light. We shall also have occasion to use the angular intensity distribution function[15] $P_i(\theta)$ for a solute component i defined by $P_i(\theta) = R_i(\theta)/R_i(0)$ at infinite dilution. In this limit, which we indicate by subscript 0, the scattering from the components of a heterogeneous solute is additive; and we may write the familiar relation

$$[Kc/R(\theta)]_0 = 1/\Sigma_i M_i P_i(\theta) w_i \qquad (1)$$

in which w_i is the weight fraction of component i in the solute and M_i is its molecular weight.[16] It is implied by the form of equation 1 that all solute components have the same specific refractive index increment[6]; and for our calculations we assume this to be the case taking the constant K, reported elsewhere[6] for fibrinogen, as 5.996×10^{-7} consistent with the solute concentration c in g./cc.

The extrapolation to infinite dilution required to obtain $P(\theta)$ is conveniently carried out in conjunction with an extrapolation on the same plot of the angular data at each concentration to zero angle in the manner advocated by Zimm.[15] The graph shown as Fig. 2 is a conventional Zimm

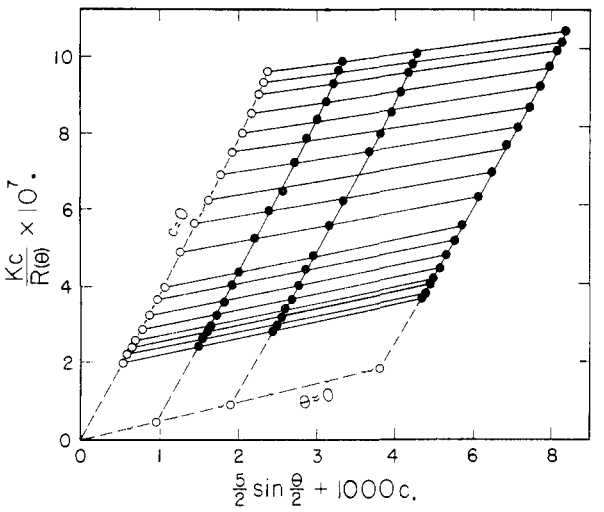

Fig. 2.—Angle and concentration dependence of scattering from an inhibited system (G47) in which no dissociation of intermediate polymer has occurred.

plot except that the angular abscissa is $\sin(\theta/2)$ rather than $\sin^2(\theta/2)$. The points for the highest concentration are for the inhibited clotting system while the two sets of points at the left represent scattering, *at the moment of dilution*, from the same system diluted by factors of two and four. Results for solutions diluted below about 1 g./l. total protein were not used since the points calculated showed a very erratic course as a function of concentration. It is likely that polymer dissociation took place too rapidly for the data obtained initially after addition of solvent to determine an accurate extrapolation to the time of dilution. There is indeed no direct proof that the extrapolated values at higher concentration are correct, but the linear character of the concentration dependence of $Kc/R(\theta)$ found in the several experiments supports the validity of the procedure.

The plot of $Kc/R(\theta)$ extrapolated to infinite dilution in Fig. 2 represents the light scattering behavior at this limit of an inhibited clotting system *in which no dissociation of intermediate polymer has occurred*. To obtain the scattering from the polymer fraction alone, we must correct the total scattering for the small relative contribution due to monomeric fibrinogen. Explicitly, the subscripts 2 and 4 denoting monomer and polymer, respectively, it follows from equation 1 that

$$\left[\frac{R_4(\theta)}{Kc_4}\right]_0 w_4 = \left[\frac{R(\theta)}{Kc}\right]_0 - M_2 P_2(\theta) w_2$$

in which the weight fractions w_2, w_4 may be determined from the sedimentation data. The value of $M_2P_2(\theta)$ is expressed adequately by $4.31 \times 10^5 \times (1 - 0.187 \sin^2(\theta/2))$ obtained earlier.[6]

Interpretation and Discussion of Results

Sedimentation.—Except for the instances to be noted presently, the schlieren photographs obtained from the inhibited clotting systems exhibited two distinct symmetrical peaks. We take this character as evidence that the molecular weight distribution is bimodal. We do not claim, however, that the polymer is necessarily homogeneous: since the sedimentation constant for a rod-like particle is much more sensitive to the thickness than to the length, the existence of a fairly wide range of lengths is not excluded; but it does appear that the molecular cross-section may be fairly uniform. It has been suggested[17] that the two peaks could arise from a distribution of molecular sizes initially broad and continuous through changes in composition during sedimentation to satisfy the equilibria between molecular species at each point in the centrifuge cell. However, from our results, which show that most of the fibrinogen is converted to polymer (except for experiment G37 as recorded in Table I) and that the amount does not apparently change greatly in the course of sedimentation, it is evident that polymerization of monomer in front of the sedimenting boundary, presumably the most important effect to be considered, cannot seriously falsify the interpretation of data. Another indication that the slow peak, at least, does not reflect the effect of an equilibrium between species, is that its sedimentation constant agrees fairly well with that observed for fibrinogen alone[13] even though the individual results given in Table I are unaccountably erratic.

TABLE I

SEDIMENTATION OF INHIBITED CLOTTING SYSTEMS

Expt.	Assay, % clottable protein	Protein concn., g./l.	Thrombin concn., g./l.	pH	Sedimentation consts., Svedberg units $s_{20,w}$ slow	$s_{20,w}$ fast	Wt. fraction polymer
G37	91.4	2.19	0.5	9.60	9.0	16.1	0.41 ± 0.08
G38	90.6	3.28	1.0	9.49	7.1	19.0	.77 ± .10
G38[a]		1.64		9.51	8.8	14.1	.74 ± .11
G39	92.4	4.25	1.0	9.50	7.6	16 9	.76 ± .03
G39[a]		2.13		9.50	6.8	19.9	.72 ± .04
G40	90.7	4.54	1.0	9.5	5.9	16.0	.78 ± .03
G40[a]		2 27		9.5	7.3	20.6	62 ± .01
G47	92.2	3.81	1.0	9.45	7.5	16.8	.81 ± .07

[a] Clotting system diluted to 50% of the original protein concentration and allowed to equilibrate for 24 hours.

(Sedimentation constants for both slow and fast boundaries are in agreement with earlier observa-

(15) B. H. Zimm, *J. Chem. Phys.*, **16**, 1093 (1948).

(16) In this paper we make the approximation of regarding the solvent as a single component, thus neglecting in the formulation we use, possible preferential sorting of solvent components by the solute.[6] This simplification affects in no significant degree the conclusions reached. Similarly no distinction is made between native fibrinogen and the enzymatically activated form, nor is any account taken of the relatively low molecular weight polypeptide released by the action of thrombin.

(17) J. K. Backus, M. Laskowski, Jr., H. A. Scheraga and L. F. Nims, *Arch. Biochem. Biophys.*, **41**, 354 (1952).

tions[18] on inhibited systems.) If one accepts the suggestion of Ferry, *et al.*,[19,20] that the polymers of fibrinogen are constituted by a double row of monomer units arranged longitudinally with end-to-end junctions staggered, the first polymer formed would presumably be a unit 1.5 times as long as fibrinogen with a hydrodynamically effective thickness greater by perhaps the same factor. The presence of a large amount of a component of this type would certainly affect the sedimentation pattern since its sedimentation constant would be considerably higher than that of fibrinogen. We conclude then that the distribution of polymer species at equilibrium does not include an important fraction of the protein in the lowest polymers, *i.e.*, that the distribution may be regarded as discontinuous, comprising monomer and a range of extent not definitely known in which fall the polymeric species.

Two experiments (G37, G38) constitute partial exceptions to this description in that the schlieren patterns have a skewed appearance indicative of some heterogeneity. This is probably reflected also in the rather large deviations from the mean in the several determinations of the composition of the system averaged to give the values indicated in Table I. In addition, the sedimentation constants for the slow peak appear somewhat high.

Two features of the results given in Table I are noteworthy: the high extent of conversion of fibrinogen to intermediate polymer, averaging 78% in agreement with earlier work,[18] in systems polymerized at protein concentrations above 3 g./l., and the small amount of dissociation of polymer after dilution to 2 g./l. The rather small and gradual decrease of scattered light intensity (see Fig. 1) occurring after fourfold dilution of an inhibited system may be cited in support of the conclusions from the sedimentation experiments.

It is certain that the polymerization reaction is reversible[19,21,22] but it may not be assumed that the monomer–polymer distribution determined from sedimentation is the equilibrium composition since it is not certain that all of the monomeric material is participating in the equilibrium. One pertinent consideration is that about 9% of the protein present is incapable of clotting—whether it is foreign material or, more likely, partly denatured fibrinogen is unknown—although there is evidence[6] that part of this fraction can enter into polymer formation. It is also conceivable that thrombin may be denatured fairly rapidly at high *p*H in which case some of the fibrinogen might not polymerize through failure to be activated. The low polymer content accompanying the low thrombin concentration in one case, G37, suggests this possibility.

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

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

(20) J. D. Ferry, *Proc. Natl. Acad. Sci. U. S.*, **38**, 566 (1952); J. D. Ferry, S. Katz and I. Tinoco, Jr., *J. Polymer Sci.*, **12**, 509 (1954).

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

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

Light Scattering: Structure of the Intermediate Polymer.—The angular dependence at infinite dilution of $c/R(\theta)$ shown in Fig. 2 and $[c_4/R_4(\theta)]_0$ shown in Fig. 3 has the same general character

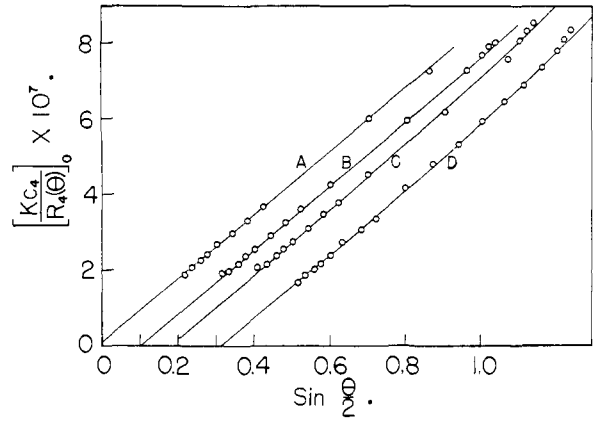

Fig. 3.—Light scattering from the polymeric fraction of inhibited clotting systems corrected for thermodynamic interactions. Curves A, B, C, D are derived, respectively, from systems G38, G39, G39[a], G47 listed in Table I. For clarity, plots B, C, D are shifted one, two and three scale units to the right.

as that reported[4] earlier for very dilute solutions of intermediate polymer without inhibitor in which $c/R(\theta)$ did not vary with concentration: *i.e.*, $[c_4/R_4(\theta)]_0$ in the inhibited systems is a nearly linear function of $\sin(\theta/2)$ except at large angles where some upward curvature is evident, and the intercept at $\theta = 0$ is zero within experimental uncertainty. It has been shown[4,23] that an angular intensity distribution of this type is produced (though not uniquely) by randomly oriented thin cylindrical rods of sufficiently great length in relation to the wave length of incident light that experimental measurements over a particular range of angles employed determine only the limiting behavior of the scattering function $P(\theta)$ for indefinitely long rods. Under such circumstances, conventional empirical extrapolation of $[Kc_4/R_4(\theta)]_0$ *versus* $\sin^2(\theta/2)$ to $\theta = 0$ leads to meaningless results for molecular weight and radius of gyration; but a suitable analysis of the data does yield the weight average ratio of the molecular weight to length M/L and possibly a rough independent estimate of the particle thickness. The data conform quite well to the theoretical expression[4] of the form

$$\left[\frac{Kc_4}{R_4(\theta)}\right]_0 = \left(K' + \frac{4 \sin(\theta/2)}{\lambda' \Sigma_i \overline{M_i w_i / L_i}}\right)(1 + \gamma \sin^2(\theta/2)) \quad (2)$$

where λ' is the wave length of light in the medium and the coefficient γ depends on the rod cross-sections. Since, as noted above, the angle-independent term K' is very small, no interpretation of it has been attempted (and in the calculations described in the section following it is taken as zero).

The values for the average M/L and for γ given in Table II are derived from the curves plotted in Fig. 3. Dividing the experimental M/L based on

(23) E. F. Casassa, *J. Chem. Phys.*, **23**, 596 (1955); A. Holtzer, *J. Polymer Sci.*, **17**, 432 (1955).

TABLE II

LIGHT SCATTERING FROM THE POLYMERIC FRACTION OF INHIBITED CLOTTING SYSTEMS

Experiment	G38	G39	G39	G47
K'	0.07	0.00	0.18	−0.12
$\Sigma_i M_i w_i / L_i \times 10^{-11}$, g./cm.	1.47	1.49	1.48	1.49
γ		0.017	0.048	0.060
Cross-section of polymer as fibrinogen monomer units	2.26	2.29	2.27	2.30

the rod model by that for fibrinogen, 0.65×10^{11} g./cm. in the salt–glycine–glycol solvent,[6] we obtain a polymer cross-section of 2.28 expressed in terms of monomer units. Though still fairly near two monomers, this cross-section appears significantly larger than the value 2.09 found in the study of very dilute uninhibited clotting systems.[4] Although no definite information as to the molecular weight of the polymer can be obtained, the fact that the asymptotic behavior of the assumed scattering function is obeyed for 4358 Å. light down to 25°, the smallest angle accessible with our apparatus, indicates that the length of a polymer molecule is at least of the order of 5000 Å.

Probably the simplest interpretation of the curvature of the plots shown in Fig. 3 is to ascribe it to the effect of finite particle thickness on the scattering function: *e.g.*, if the molecule can be approximated as a circular cylinder, $\gamma = (2\pi R/\lambda')^2$ where R is the cylinder radius.[4,24] Taking γ as 0.04, the average of the very discordant figures presented in Table II, we obtain a radius of 100 Å. which may be compared with 24 Å. calculated for a cylinder having the experimental M/L of the polymer and the partial specific volume, 0.71, of fibrinogen.[25] Since the dissolved protein is undoubtedly considerably swollen, the latter radius is surely too small; but the discrepancy between the two figures is so large it does not seem possible to account for it by any reasonable degree of swelling. The disagreement may be even worse than appears here, for the experiment from which the largest value of γ was obtained (G47) provided probably the most reliable data. Similar lack of agreement between γ and M/L was found in dilute uninhibited systems and the observations made for those systems apply as well in this case. In particular, introduction of a more complex (and presumably more realistic) molecular model, two parallel cylinders in contact, fails to account for the large γ in either case. In the present work errors in extrapolation of data to infinite dilution can be large enough to affect seriously the apparent value of γ. It is also quite possible that small deviations of the actual molecular structure from the straight rod form we have assumed may be involved. It is expected that bent configurations would give rise to upward curvature in plots of $[c/R(\theta)]_0$ against $\sin(\theta/2)$: for instance, in the extreme case of the random coil, $[c/R(\theta)]_0$ is asymptotically linear[26] in $\sin^2(\theta/2)$.

(24) For the very rough comparisons made here, we take no account of the fact that γ is actually a complicated average over the distribution of intermediate polymer species.

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

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

Light Scattering. Thermodynamic Interactions.—It will be noted that in the plot reproduced as Fig. 2, the slope of $Kc/R(\theta)$ for the inhibited system with respect to concentration is not independent of the scattering angle: evidently the slope decreases as the angle increases. In this section we develop an interpretation of this effect.

For a multicomponent solute, a simple extension of a treatment given originally by Zimm[15] for long-chain flexible polymers leads to the result that excess scattering as a function of concentration and angle is given by the relations[27]

$$\frac{Kc}{R(\theta)} = \frac{1}{\Sigma_i M_i P_i(\theta) w_i} + 2A_2(\theta)c + \ldots \qquad (3)$$

and

$$A_2(\theta) = \frac{\Sigma_i \Sigma_j B_{ij} M_i P_i(\theta) w_i M_j P_j(\theta) w_j}{(\Sigma_i M_i P_i(\theta) w_i)^2} \qquad (4)$$

We have designated the coefficient of $2c$ in equation 3 by $A_2(\theta)$ to indicate that it will generally depend on θ. Since $P_i(0)$ is unity, the thermodynamic second virial coefficient is $A_2(0)$, the same as that obtained from osmotic pressure measurements except for the average involved. There are limitations, which we will not discuss here, on the applicability of equation 4; but it can be shown to be valid for systems of thin rod-like particles.

For a two component solute consisting of a monomer 2 and a polymer 4 we rewrite equation 4 in a form convenient for calculation

$$A_2(\theta) = \frac{B_{44}g^2r^2 + 2B_{24}gr + B_{22}}{(1 + gr)^2} \qquad (5)$$

where $g = M_4P_4(\theta)/M_2P_2(\theta)$ and $r = w_4/w_2$. To determine the thermodynamic quantities B_{ij} in the inhibited clotting system in which no polymer has dissociated, we use equation 5 recognizing that the treatment of the polymer, and even the monomer, as a single species is an approximation and hence that $B_{24}gr$, $B_{44}g^2r^2$, and gr may actually represent averages. It is worth noting, however, that the B_{ij} are independent of molecular length for thin rigid rods.[28] Hence, if a distribution of lengths is the only factor differentiating the various polymer species and if $MP(\theta)$ is the same for all monomer species, equation 5 is adequate as written in that the thermodynamic and angle dependent quantities, whether averages or not, are separable as products.

It is apparent from Fig. 2 that at any θ, $c/R(\theta)$ is a linear function of concentration. This is indeed what we should expect from theory as well; for it has been shown[29] for suspensions of thin rods that the contribution of the term in c^2 to the equation of state in the form of equation 3 is small compared to that in c up to relatively high concentrations.

The thermodynamic interaction constants can now be obtained from the experimental values of $A_2(\theta)$ in the following way. Since $M_2P_2(\theta)$ is known independently and $(M_4P_4(\theta))^{-1} = [Kc_4/R_4(\theta)]_0$ can be determined as shown above, g can be calculated for each θ. Then, the composition ratio r being determined by sedimentation, the B_{ij} can be chosen to

(27) H. E. Stanley, Doctoral Thesis, Massachusetts Institute of Technology (1949); J. J. Blum and M. F. Morales, *J. Chem. Phys.*, **20**, 1822 (1952).

(28) B. H. Zimm, *ibid.*, **14**, 164 (1946).

(29) L. Onsager, *Ann. N. Y. Acad. Sci.*, **51**, 627 (1949).

provide the best agreement between the calculated and experimental $A_2(\theta)$. In carrying out the determination it is convenient to substitute in equation 5 an analytical expression for gr, derived by the analysis described in the last section, and then to choose B_{24} and B_{44} by trial and error to obtain the optimum fit of the calculated function with experiment. Two such comparisons performed graphically, are shown in Fig. 4. Since $A_2(\theta)$ is very insensitive to any reasonable value of B_{22}, no attempt was made to determine this quantity from the experimental data and it was simply assumed to be 1.8×10^{-5}, the second virial coefficient for fibrinogen[6] in the same solvent. This assumption may be in error as the unpolymerized fraction is presumably, at least in part, activated fibrinogen rather than fibrinogen; but the computed values of B_{24} and B_{44} are not significantly affected.

The expedient we have used of determining the B_{ij} from an analysis of the angular variation of $A_2(\theta)$ is rather unusual; but it is made necessary by the asymptotic character of the observed scattering behavior of the polymer as well as by the experimental impracticability of varying the composition of the solute in order to determine, in orthodox fashion, $A_2(0)$ at several different compositions.[30] Because an asymptotic relation for $M_4P_4(\theta)$, invalid for sufficiently small angles and becoming infinite at $\theta = 0$, was substituted in equation 5, the curves of $A_2(\theta)$ plotted in Fig. 4 do not extrapolate to $A_2(0)$ at zero angle but rather give the interaction constant B_{44}. The distinction is not of practical importance, however, in the solutions studied here in which a large part of the protein is in the polymeric form, for the weighting of the B_{ij} in equation 5 causes $A_2(0)$ to be determined overwhelmingly by the influence of B_{44}.

In the interpretation of the experimental results for the thermodynamic interactions given in Table III it is useful to consider the formal expression for

TABLE III

THERMODYNAMIC INTERACTIONS

Expt.	$B_{22} \times 10^5$ (cm.^{3} g.$^{-2}$)	$B_{24} \times 10^5$	$B_{44} \times 10^5$
G37			3.0 ± 0.5[b]
G38	(1.8)[a]	−8.9 ± 0.7[b]	2.3 ± .1
G39	(1.8)	−7.0 ± .7	2.44 ± .1
G47	(1.8)	−6.0 ± .7	2.49 ± .1

[a] The value of B_{22} was assumed in calculating B_{24} and B_{44}. [b] The limits of error shown are estimated uncertainties of the curve fitting procedure, but the exact values of the interaction constants given for G39 and G47 determine the curves plotted in Fig. 4.

B_{ij} from the general statistical mechanical theory of fluids[28,31]

$$B_{ij} = -\frac{N}{2\bar{M}_i\bar{M}_jV}\int[F_2(i,j) - F_1(i)F_1(j)]\,d(i)\,d(j) \quad (6)$$

in which $V^{-2}F_2(i,j)d(i)d(j)$ is the probability that a pair of solute molecules of types i and j in a system of macroscopic volume V have configurations within the differential element $d(i)d(j)$ of configuration space, position and orientation being specified by sets of coördinates for both molecules symbolized by (i), (j); and similarly $V^{-1}F_1(i)d(i)$ is the probability that the coördinates of an isolated molecule i are in $d(i)$. Avogadro's number is denoted by N. At large intermolecular distances, where there is no correlation between molecular pairs, $F_2(i,j) = F_1(i)F_2(j)$ and the integrand of equation 6 vanishes. Consequently if there exists an effective molecular repulsion, *i.e.*, a decreased probability of finding other molecules in the statistical environment of a reference molecule, $F_2(i,j) < F_1(i)F_1(j)$ on the average and B_{ij} is positive. Conversely a net molecular attraction gives a negative B_{ij}. In thermodynamic language, $2B_{ij}M_iM_j$ is (to the approximation that equation 3 terminated at the term in c is adequate to represent the equation of state) essentially the derivative of the logarithm of an activity coefficient for component i with respect to the concentration of component j.[32,33]

The positive sign of the experimental B_{44} would be expected from the excluded volume effect for interaction between particles mutually impenetrable, at least to some extent, in the absence of long-range attractive forces. Assuming that the intermediate fibrinogen polymer behaves as a rigid, impenetrable, circularly cylindrical rod with cross-section determined by the experimental values of M/L and partial specific volume $\bar{v}$, we calculate a theoretical interaction constant of 1.0×10^{-5} cm.^{3} g.$^{-2}$ from the equation

$$B_{44} = \left(\frac{N\pi\bar{v}L^3}{4M^3}\right)^{1/2}$$

which is equivalent to an expression derived by Zimm.[28] Since the model employed is oversimple, among other respects in that it does not allow for swelling of the dissolved protein, it is not surprising that this result is smaller than the experimental value 2.4×10^{-5}.

The more interesting result, however, is the negative B_{24}. According to the interpretation given above, this signifies an effective attraction between monomer and polymer so that the average density of monomer particles in the region near a polymer molecule is greater than the macroscopic concentration. In the absence of intermolecular attraction B_{24} would be positive and, if monomer and polymer

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(31) W. G. McMillan, Jr., and J. E. Mayer, *J. Chem. Phys.*, **13**, 276 (1945).

(32) W. H. Stockmayer, *ibid.*, **18**, 58 (1950).

(33) It is perhaps advisable to meet at this point the possible objection that the interpretation given here, in which equilibrium thermodynamics (or statistical mechanics) has been presupposed, is inapplicable to a system such as this which is not at equilibrium with respect to a chemical reaction. Actually the proper criterion of validity of our reasoning is that the reaction shall not proceed measurably on the time scale of the experimental measurement. In principle, light scattering is determined by the instantaneous state of the system (within limits set by quantum mechanics); and for our treatment of the inhibited clotting systems to be correct, we require only that the polymer dissociation reaction proceed much more slowly than the time of regression of concentration fluctuations, *i.e.*, that there exists statistical equilibrium with respect to Brownian motion. Evidently this condition obtains in the present case; and by extrapolation of data from diluted solutions back to the moment of dilution we have determined the scattering from the undissociated system. It must of course be recognized that the statistical equilibrium state involved is not, except for the undiluted solution, the state of minimum free energy. It may be noted that the type of information obtained here by light scattering is generally inaccessible by osmotic pressure measurements owing to the long time required to achieve osmotic equilibrium.

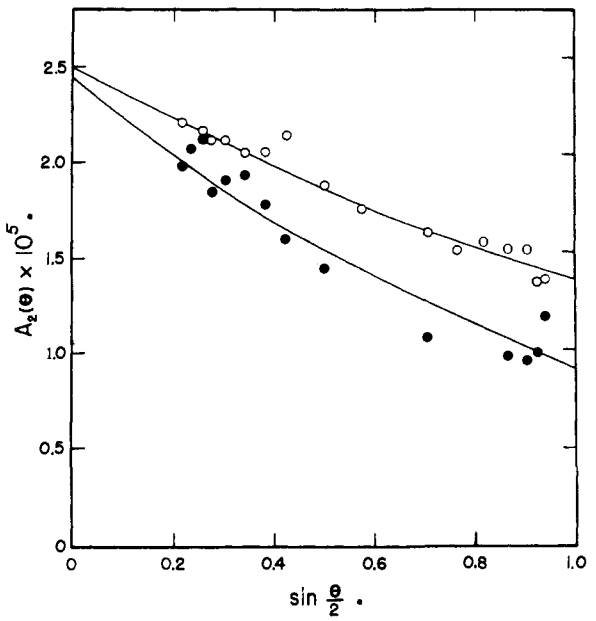

Fig. 4.—Angular dependence of $A_2(\theta)$ in undissociated systems G39 (solid circles) and G47 (open circles). The curves represent equation 5 with the interaction coefficients listed in Table III and with gr given by

$$\left(\frac{11.88}{\sin(\theta/2)} + 2.22 \sin\frac{\theta}{2}\right)\left(1 - 0.017 \sin^2\frac{\theta}{2}\right) \text{ for G39, and by}$$

$$\left(\frac{8.80}{\sin(\theta/2)} + 1.64 \sin\frac{\theta}{2}\right)\left(1 - 0.060 \sin^2\frac{\theta}{2}\right) \text{ for G47.}$$

behaved as mutually impenetrable particles, larger than B_{44}.

Conclusion

The interpretations advanced above prompt some comparisons with an earlier light scattering study by Ferry, *et al.*,[19] of inhibited clotting systems prepared under the conditions we have employed except that the solutions were phosphate buffered at *p*H 6.2.

There are obvious differences in the polymerization. The conversion of fibrinogen to intermediate polymer in the systems at lower *p*H amounts to but 50% compared to nearly 80% obtained in the present study; and it also appears, although adequate data are lacking, that the polymerization is more rapid at *p*H 9.5 than at *p*H 6.2.[22,34]

The conventional treatment of light scattering data to obtain the molecular weight and length of the polymer, which was used by Ferry, *et al.*, is only applicable if the polymer rods are somewhat shorter than those formed at *p*H 9.5. As we have noted elsewhere,[4] the data do not show unequivocally whether this analysis is adequate; but it seems likely that the rod length at *p*H 6.2 is very near the limit for its applicability.

An important point of difference between the results of the two studies is the far greater magnitude of thermodynamic interactions at *p*H 9.5. In the evaluation of data at *p*H 6.2, $A_2(\theta)$ was taken as zero; and although a more detailed investigation of the matter might be desirable, there is internal evidence in the results published (*cf.* Fig. 1, reference 19) that the second virial coefficient is in fact unimportant at the lower *p*H. Since the isoelectric points[35] of fibrinogen and its thrombin activated form are in the vicinity of *p*H 6, it would perhaps be natural to correlate the greater thermodynamic interaction at *p*H 9.5 with increased electrostatic forces at high *p*H. However, the fact that such an effect is not observed in solutions of monomeric fibrinogen[6] in the two solvents concerned seems to preclude the simple explanation. Furthermore, the possible influence of the neutralization reaction between the protein and the different buffers would obviously also have to be considered.

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MADISON, WISCONSIN

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