fibrinogen concentration of about 0.1 g./liter, a *p*H of about 7.3, and a relatively high thrombin concentration, both incomplete precipitation of fibrinogen and occlusion of other proteins probably occur. On the basis of this study, values for the fibrinogen content of normal human plasma in the literature may well be too high.

In view of the very high occlusion of the lipoproteins, Fractions IV-1 and III-0, reports of elevated fibrinogen levels in pathological conditions involving concomitant increases in lipoprotein should be viewed with some circumspection. Recent reports[28] of a protein of high molecular weight and considerable asymmetry, which may occur in concentrations up to 50–70% in pathological plasma are pertinent in this connection. At such concentrations a protein with an occlusion factor of only 10 would cause apparent yields 2 to 3 times greater than the actual fibrinogen content.

Acknowledgments.—We are indebted to Miss Susan G. Miller for her assistance in many of these analyses and to Drs. E. J. Cohn, J. T. Edsall, and J. D. Ferry for their generous advice.

Summary

1. Analytical procedures for fibrinogen are briefly summarized and several convenient modifications of technique are described.

2. The yield of fibrin obtained by the action of human thrombin on human fibrinogen, and its modification under various conditions of *p*H and concentration, and by the presence of other proteins, has been studied.

3. At a *p*H of 6.3 complete yields were obtained at fibrinogen concentrations between 0.5 and 2.0 g./liter and at thrombin concentrations between 0.05 and 0.20 unit/cc. Reduced yields were obtained at both higher and lower concentrations of both reactants.

4. Other proteins were carried down by fibrin in amounts which varied widely with their nature. These ranged from a negligible occlusion of serum albumin to a 10–25% occlusion of certain lipoproteins and an almost complete occlusion of several enzymes, including thrombin. In general, the occlusion of each added protein was proportional to its concentration. Certain plasma components were shown to be occluded in amounts sufficient to introduce a considerable error into analyses of plasma fibrinogen.

5. A study of the effect of the fibrinogen and thrombin concentration and of the *p*H on occlusion revealed that in each case the condition which favored occlusion also favored the formation of a "fine" clot. This, and the fact that proteins of high molecular weight and asymmetry are strongly occluded, while smaller, more asymmetrical ones are not, indicates that physical entrapping is important, although the polar and nonpolar interactions of the molecules may also play a part.

6. Optimal conditions for complete yields and minimal occlusion are defined, and a procedure for estimating the extent of occlusion is described.

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(28) (a) K. O. Pedersen, "Ultracentrifugal Studies on Serum and Serum Fraction," Uppsala, 1945, p. 39, 121. (b) J. Waldenström, in "The Svedberg, 1884 30/8 1944," Uppsala, 1944, p. 558–573.

[CONTRIBUTION FROM THE DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

Studies on Double Refraction of Flow. III. Human Fibrinogen and Fraction I of Human Plasma[1]

BY JOHN T. EDSALL, JOSEPH F. FOSTER[2] AND HERBERT SCHEINBERG

Fibrinogen, as the major constituent involved in the clotting of blood, is a protein of particular chemical and biological interest. In the past, however, its instability has rendered difficult the study of its physico-chemical properties. The large-scale fractionation of blood plasma, employing ethanol at low temperature and low ionic strength,[3] has made possible the separation of fractions rich in fibrinogen, in active and relatively stable form.[4] Such preparations can be dried from the frozen state and thus preserved for months or years. Further subfractionation has yielded preparations in which as much as 96 to 98% of the protein is clottable with thrombin.[5] The high intrinsic viscosity of fibrinogen in solution,[6] and the readiness with which it is converted by thrombin into the complex network of the fibrin clot, indicate that the molecule is elongated

(1) This paper is Number 61 in the series "Studies on the Plasma Proteins" from Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross, and Number XVI in the series "Preparation and Properties of Serum and Plasma Proteins" from the same laboratory. The preparations of Fraction I, purified fibrinogen, and other fractions studied in this work were carried out under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

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(3) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, THIS JOURNAL, **68**, 459 (1946).

(4) J. T. Edsall, P. R. Morrison and J. D. Ferry, in preparation.

(5) P. R. Morrison, J. T. Edsall and S. G. Miller, in preparation.

(6) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. & Colloid Chem.*, **51**, 184 (1947).

and highly asymmetrical. A study of double refraction of flow, which yields data particularly adapted to the determination of the lengths of asymmetrical molecules, has therefore been carried out on solutions of these fibrinogen-containing fractions of human plasma. Some other protein components of plasma, which tend to precipitate in association with fibrinogen but are not clottable with thrombin, have also been studied and found to consist of quite elongated molecules.

Experimental

Apparatus.—The apparatus used in this investigation has been described in detail in the previous papers of this series.[7,8] It consists of concentric cylinders of radius 2.5 cm. and 7.0 cm. high. The annular gap between the cylinders is 0.25 mm. wide. The cylinders themselves, and all metal parts of the apparatus which come in contact with the solution being studied, are constructed of Carpenter No. 4 MO cold-drawn stainless steel, which was found to be superior to other varieties of stainless steel in its inertness with respect to the catalysis of protein denaturation. The inner cylinder serves as the rotor and can be driven with adequate power at speeds between 100 and 2500 r. p. m. The velocity gradient in the flowing solution may be taken as essentially uniform across the entire width of this narrow gap. The gradient in sec.$^{-1}$ is numerically equal to 10.5 times the speed in r. p. m. The light source employed is a mercury arc, with filters which transmit the 5460 Å. line. The extinction angle χ, which is a measure of the angle between the optic axis of the flowing liquid and the direction of the stream lines, is determined with the aid of a pair of coupled and crossed Nicol prisms, which can be rotated until the position for light extinction is noted in an observing telescope. Measurements of double refraction are made with the aid of a Sénarmont compensator (quarter wave plate).

Fibrinogen Preparations.—Fraction I is separated from human plasma[3] by addition of ethanol to a final concentration of 8–10% by volume, at *p*H near 7 and at a temperature near −3°. The protein in this fraction (from human plasma) commonly consists of fibrinogen to the extent of 60–65%, as judged from the amount of protein clottable with thrombin.[9] The remainder is approximately 7% albumin, 8% α-globulin, 15% β-globulin and 7% γ-globulin. The Fraction I precipitate is commonly taken up in sodium citrate buffer, approximately 0.05 *M*, at *p*H 6.3. The resulting solution can be filtered through clarifying filters and used directly for study; otherwise it may be frozen and dried from the frozen state. Purified fibrinogen (Fraction I-2) was separated from Fraction I by methods to be described elsewhere.[5] The procedure involves the removal of the more soluble components by precipitation at a *p*H near 5.1; and the subsequent precipitation by cooling, at a higher *p*H, of a fraction with a very high temperature coefficient of solubility (Fraction I-1). The protein remaining in the supernatant liquid, under these conditions, is nearly pure fibrinogen. One typical preparation obtained in this manner (Run 167 Fr. I-2) contained 96–98% of protein which was clottable with thrombin. However, after drying from the frozen state and redissolving, only 93% of the protein was clottable. It was this material which was used for the experiments subsequently described in this paper.

The non-clottable protein of Fraction I was studied in certain runs. This material was obtained from the synergetic liquid extruded from clotted Fraction I.

Solvent.—Glycerol–water–salt systems were used as solvents in all runs reported here. The function of the glycerol was to increase the viscosity of the solvent, thereby decreasing the rate of rotary Brownian movement of the fibrinogen molecules sufficiently to permit adequate orientation at the velocity gradients employed. Studies were made over a range of glycerol concentrations between 38 and 63% (by weight) corresponding to a four-fold variation in viscosity. Additional variation was attained through adjustment of temperature over the range 5 to 25°. The viscosities of glycerol–water mixtures have been determined, over a considerable temperature range, by Cocks[10] and Sheely.[11] It was found, on study of their data, that log η, for any given glycerol–water mixture, was very nearly a linear function of temperature between 0 and 30°. Log η was therefore plotted against t for a series of glycerol–water mixtures, of compositions ranging from 20 to 70% by weight, and the appropriate value of η for any given solution under study was derived from this chart. For the highest glycerol concentrations and the lowest temperature studied, a short extrapolation of the experimental curves had to be made in order to estimate the viscosity. The small effect of the salt present in our solutions on the viscosity was neglected in making the calculations, since an error below 2% in the viscosity of the solvent leads to an error considerably below 1% in the calculated dimensions of the protein molecule. This is small in comparison with other sources of uncertainty in the experiments.

The presence of glycerol was found, in all cases, to be perfectly compatible with the formation of clear fibrinogen solutions. In fact a noticeable reduction in turbidity invariably was observed upon addition of the glycerol (probably due in part to the increased refractive index of the medium) and the solutions were rendered more stable, and less liable to spontaneous clotting.

Preparation of Solution.—The fibrinogen powder was weighed into a small bottle and dissolved in a weighed amount of sodium citrate solution of ionic strength 0.3 (approximately 0.05 *M*). The *p*H of this resulting solution was approximately 6.3 (no studies were made of the effect of variation of *p*H). Solution of the protein took place rapidly. The calculated amount of glycerol was then added, the solution thoroughly mixed by gentle agitation, and filtered through a filter paper under gravity into a small suction flask to remove any trace of undissolved material. This flask was then evacuated, using a water aspirator, and maintained under vacuum until the solution was run into the apparatus. This evacuation procedure proved effective in preventing formation of air bubbles in the solution between the cylinders.

Method of Measurement.—The experimental procedure was identical with that employed in the study of zein.[8] In particular, the precautions which were necessary in order to minimize errors due to reflections from the cylinder walls were observed in the present studies, as in the work on zein. Disturbances from this source appeared to be somewhat less troublesome in the measurements on fibrinogen.

Discussion of Results

The principal results obtained in these investigations are summarized in Figs. 1, 2 and 3 and in Table I. The figures all deal with a single fibrinogen preparation of high purity (Fr. I-2, run 166). The effects of varying protein concentration, of varying solvent composition and viscosity, and of varying temperature were all systematically studied.

Concentration Dependence.—Figures 1 and 2 summarize results obtained at three different protein concentrations from 0.12 to 0.50%, the solvent being 54% glycerol in all cases. In Fig. 1, the extinction angle χ is plotted as a function of $G\eta$ (velocity gradient in sec.$^{-1}$ times viscosity of

(7) J. T. Edsall, C. G. Gordon, J. W. Mehl, H. Scheinberg and D. W. Mann, *Rev. Sci. Instruments*, **15**, 243 (1944).

(8) J. F. Foster and J. T. Edsall, THIS JOURNAL, **67**, 617 (1945).

(9) The quantitative assay of fibrinogen is described by P. R. Morrison, *ibid.*, **69**, 2723 (1947).

(10) L. V. Cocks, *J. Soc. Chem. Ind.*, **48**, 279T (1929).

(11) M. L. Sheely, *Ind. Eng. Chem.*, **24**, 1060 (1932).

TABLE I

SUMMARY OF RESULTS ON FRACTION I, PURIFIED FIBRINOGEN AND RELATED FRACTIONS[a]

Expt.	Preparation	% Clot[b]	G. % prot. concn.	Glycerol concn.	Viscosity of solvent, poises	Temp., °C.	Range of $G\eta$	Value at low $G\eta$: $\eta\Theta/T$	Value at low $G\eta$: Length, Å.	Value at high $G\eta$: $\eta\Theta/T$	Value at high $G\eta$: Length, Å.	$\left[\frac{\Delta}{G\eta c}\right]_0$
1	167 Fr. I-2	93	0.12	53.6	0.078–0.080	17.8–18.1	750–1470	0.79	800	1.09	730	0.17
2	167 Fr. I-2	93	.12	53.6	.132– .135	4.5– 4.8	624–2020	.87	775	1.39	675	.17
3	167 Fr. I-2	93	.25	54.0	.078– .081	17.7–18.7	610–1200	1.24	700	1.40	670	.18
4	167 Fr. I-2	93	.25	54.0	.132– .136	4.6– 5.5	300–2040	1.09	730	1.39	695	.17
5	167 Fr. I-2	93	.50	53.6	.129– .135	4.2– 5.3	240–2030	0.38	(1020)	1.17	710	.19
6	167 Fr. I-2	93	.32	38.7	.036	20.4–20.6	337– 672	1.20	700	1.14	720	.21
7	167 Fr. I-2	93	.32	38.7	.057	7.2– 7.3	268– 855	0.45	(970)	0.85	760	.21
8	167 Fr. I-2	93	.26	63.1	.145– .146	17.9–18.0	548–2190	1.28	690	1.50	655	.28
9	167 Fr. I-2	93	.26	63.1	.233– .250	5.5– 6.0	735–3770	1.0	740	1.83	615	..
10	167 Fr. I-1	60	.25	54.0	.078– .079	19.4–19.7	370–1180	1.82	615	1.24	685	0.15
11	167 Fr. I-1	60	.25	54.0	.129– .137	4.5– 5.7	488–2050	0.91	760	1.25	695	.17
12	Lederle IT-386	60	.27	53.5	.065– .066	21.3–23.0	310–1230	2.15	580	1.10	725	
13	SHI-232	75	.35	53.5	.062– .066	22.0–23.7	415–1230	1.62	640	1.49	660	
14	SHI-232	75	.35	53.5	.115– .117	7.5– 8.2	555–1740	1.22	700	1.42	660	
15	SHI-232 (Repeatedly dried and filtered)	75	1.02	53.5	.066	22.0–22.4	208–1250	0.45	970	0.95	750	0.15
16	LHI-346 Purified	75	1.0	53.5	.061– .063	24.0–24.6	300–1150	.69	840	1.07	730	.26
17	LHI-346 Purified	75	0.51	53.5	.059– .060	24.9–25.4	285–1100	.77	790	1.24	695	.23
18	Non-clottable protein of Fr. I	0	ca. 1.0	53.5	.068– .071	20.0–20.7	450–1290	2.02	595	2.15	580	
19	Prep. 1	0	ca. 1.0	53.5	.115– .118	7.6– 8.1	550–1770	1.69	630	2.30	570	

[a] The following symbols are employed in this table: G, velocity gradient; η, viscosity; Θ, rotary diffusion constant; Δ, phase retardation (see text); c, concentration in g. protein/100 cc.; subscript zero in heading of last column denotes limiting value as $G\eta \rightarrow 0$. [b] "% Clot" denotes the percentage of the protein present which was clottable with thrombin, in each preparation.

solvent in poise). The effect of concentration is not negligible and is in the expected direction, the lowest χ values for any given value of $G\eta$ being found in the most concentrated protein solutions. However, the curves found for protein concentrations of 0.12 and 0.25% lie very close together; whereas those at 0.50% show distinct evidence of molecular interaction. The net result of the interaction in this, as in other systems previously studied, is to cause an apparently greater degree of molecular orientation than is found at very low concentrations, where the molecules are free to orient independently. It is interesting to note that the magnitude of the concentration effect is of the same order as that previously observed in zein solutions,[8] although the latter were about ten

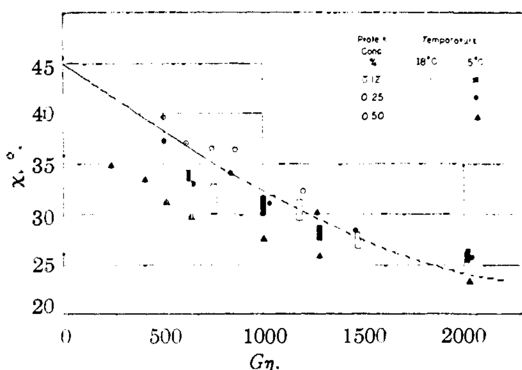

Fig. 1.—Double refraction of flow: purified fibrinogen. Results are shown for three different protein concentrations and two different temperatures, in glycerol-water (54% glycerol). The curve is drawn for a prolate ellipsoidal molecule 700 Å. long, of axial ratio 18 to 1.

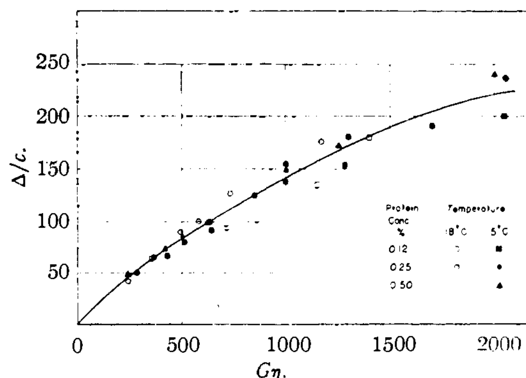

Fig. 2.—Double refraction of flow: purified fibrinogen. Phase differences divided by concentration, for the same solutions shown in Fig. 1.

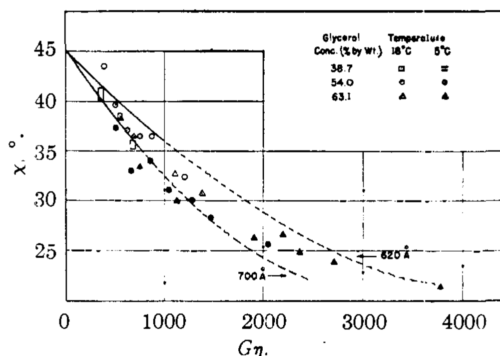

Fig. 3.—Purified fibrinogen measurements at three glycerol concentrations. Theoretical curves for ellipsoidal molecules 620 and 700 Å. long are shown for comparison with experimental points.

times as concentrated as the fibrinogen solutions studied here. The greater length of the fibrinogen molecules would be expected to give rise to interaction effects at much lower concentrations than in the case of the smaller zein molecules.[11a]

In Fig. 2, the function Δ/c is plotted, where Δ is the observed phase retardation, and c is the concentration in grams protein per 100 cc. solution. The double refraction, $n_e - n_0$, is related to Δ by the equation $n_e - n_0 = 4.24 \times 10^{-8}\Delta$. The phase difference Δ, divided by $G\eta c$, at low concentrations is approximately 0.18 for fibrinogen (see Fig. 2 and Table I); whereas in zein[8] it is approximately 0.007. This great difference is due primarily to the much greater length of the fibrinogen molecule and the consequently much slower rotary Brownian movement of the major axis. Thus the degree of orientation which can be achieved in a given solvent, at a given velocity gradient, is much greater for fibrinogen than for zein because the tendency to disorientation is far less; and the observed double refraction, at a given value of $G\eta$, is a much larger fraction of the maximum possible double refraction attainable at complete orientation. This maximum is a function of the inherent optical anisotropy of the protein molecule, and of the difference in refractive index between the molecule and the solvent. Further discussion on this point will be given in a later paper of this series.

Effect of Variation in Solvent Composition.—In Fig. 3 are plotted the results of a series of measurements on the same fibrinogen preparation, the protein concentration being substantially constant (0.25 to 0.32%) while the glycerol concentration was varied from 38.7 to 63.1%. When χ is plotted against $G\eta$ the measurements in all these solvents lie relatively close together, as would be expected if the size and shape of the fibrinogen molecules are independent of solvent composition. The only serious deviation is found in measurements made at the lowest glycerol concentration and at low temperature (Experiment 7, Table I; data not shown in Fig. 3). It should be noted, however, that in this particular run, the protein solution became rather cloudy and measurements were unusually difficult.

Temperature Dependence.—Measurements in two temperature ranges, one near 18° and the other near 5°, are recorded in Figs. 1, 2 and 3. In each case, measurements made at the higher temperature are denoted by open symbols, and those made at the lower temperature by shaded symbols. The temperature effect is, in general, relatively slight, except for the case of the solution in 38.7% glycerol, which has already been discussed above. The small effect of temperature that can be noted is in the expected direction; that is, χ values at a given value of $G\eta$ are, in general, slightly lower at the lower temperature. A very small effect in this direction would be expected simply from the decrease of rotary Brownian movement with decreasing temperature.[12] There may also be a tendency toward aggregation of the fibrinogen molecules at lower temperatures, but this would certainly appear, from the experimental evidence, to be slight, in the solvents studied here.

Calculation of Molecular Dimensions.—The principal use of double refraction of flow measurements today is to determine rotary diffusion constants of asymmetric molecules, and thence to estimate molecular dimensions. The observed extinction angle, χ, is a unique function of the parameter $\alpha = G/\Theta$, where G is the velocity gradient and Θ the rotary diffusion constant. Since G is readily determined from the experimental conditions, Θ is immediately given if α is known. The relation between χ and α is known explicitly only for χ values between 45 and about 36°, corresponding to a relatively small degree of molecular orientation. In the study of zein,[8] it was found that most of the measured χ values lay in this range, and the Θ values and molecular lengths could be deduced directly. Fibrinogen is two or three times as long a molecule as zein, and, although it was studied in less viscous solvents, it is so much more readily oriented that most of the observed χ values lay below 35°. The general

(11a) In Figs. 1, 2 and 3 the value of η is the viscosity of the solvent. A calculation was also made employing the viscosity of the solution instead of that of the solvent. From the data of Oncley, Scatchard and Brown[6] the relative viscosity of a 0.5% solution is approximately 1.13, and that of a 0.12% solution is approximately 1.03. Thus the relative viscosities of the two solutions differ by only 10%. (It is assumed that the increase in relative viscosity due to fibrinogen is the same in glycerol–water as in aqueous salt solution.) On the other hand, the initial slope of the curves in Fig. 1, when χ is plotted as a function of $G\eta$, is approximately 1.9 times as great for the most concentrated as for the least concentrated solution. Hence, the larger part of the observed effect of fibrinogen concentration would still remain apparent, even if the viscosity of the solution had been employed in our calculations, instead of that of the solvent.

(12) Besides the direct effect of temperature variation, which is proportional to T, there is, of course, a secondary and much larger effect on rotary Brownian movement due to the increase in viscosity of the solvent with decrease in temperature. The latter effect, however, is already accounted for in the data as presented, since the χ and Δ values are plotted against $G\eta$, and not simply against G.

differential equations for orientation in the three dimensional case[13,14] have not been solved so as to give numerical relations between χ and α in this range.

The most reliable estimates of molecular length, as given in Table I, are, therefore, those derived from measurements at relatively low velocity gradients. We have, however, made an attempt to evaluate the relation between χ and α at higher gradients by a semi-empirical method. As an approach to the problem, we have tentatively assumed that the true orientation distribution function in the three dimensional system may be approximated, when the degree of orientation is very large, by the distribution function for orientation in two dimensions, for which an exact relation has been given by Boeder[15] for all values of α. Specifically, we have taken Boeder's two-dimensional value of χ as valid for α values above 20, and have drawn a smooth interpolation curve connecting this portion of the curve with the region of low α values ($\alpha \leqq 2$), for which the corresponding values of χ are accurately known in the three dimensional case from the equation

$$2\chi = \tan^{-1} 6/\alpha.$$

Some points on the resulting curve are given in Table II, and Boeder's values for the two-dimensional case are given for comparison. The values of rotary diffusion constant and of molecular

TABLE II

VALUES OF χ (IN DEGREES) CORRESPONDING TO VARIOUS α VALUES

α	χ semi-empirical plot[a]	χ two dimensional
0	45	45
0.5	42.6	41.5
1.0	40.2	38.25
1.5	37.8	..
2.0	35.8	32.2
3.5	30.3	25.9
5.0	26.2	22.0
7.5	21.0	17.5
10.0	17.1	15.0
12.0	15.0	13.9
15.0	12.9	12.6
20.0	11.2	11.2

[a] The values listed under χ (semi-empirical plot) for $\alpha \leqq 2$ are exact values derived from the solution of the true three-dimensional equation. Other χ values in this column, listed as corresponding to α values greater than 2, are semi-empirical (see text).

length, calculated from χ values below 35°, are thus semi-empirical and should be given far less weight than those obtained from χ values above 35°. The validity of the underlying assumptions involved in this treatment is still by no means established. However, it is of interest to note that the values of length calculated from the data on the semi-empirical curve agree closely with those calculated for χ values above 35°, where the theory is well established.

The numerical calculations of molecular length from the rotary diffusion constants may be conveniently made as follows. Assuming the molecule to be a prolate ellipsoid of revolution with an axial ratio greater than 5 to 1, the fundamental equations of Perrin and others[16] lead to the relation

$$\Theta = \frac{3kT}{16\pi\eta a^3}\left(2 \ln \frac{2a}{b} - 1\right) \equiv \frac{3kT}{16\pi\eta a^3} Q \qquad (1)$$

The symbol Q is employed to denote the quantity in parenthesis in equation (1). The molecular length $l = 2a$, where a is the major semi-axis. It should be noted that the rotary diffusion constant Θ varies with temperature and viscosity, but the quantity $\eta\Theta/T$ should be independent of the particular solvent and temperature chosen for study, if the dimensions of the molecule remain unchanged.[17] In terms of the molecular length l and of $\eta\Theta/T$ equation (1) can be rewritten in the form

$$l^3 = 1.5 \frac{kQ}{\pi}\left(\frac{\eta\Theta}{T}\right)^{-1} = 0.659 \times 10^{-16}\, Q \left(\frac{\mu\Theta}{T}\right)^{-1} \qquad (2)$$

In order to calculate values of l from the Θ values estimated from double refraction of flow, it is necessary to choose a suitable value of the axial ratio, which in turn determines the function Q. In the case of fibrinogen, we have based this choice on viscosity measurements reported by Oncley, Scatchard and Brown,[6] which give a value of approximately 0.25 for the intrinsic viscosity H_0. An axial ratio of approximately 18 would be in satisfactory accord with this value of H_0, and the corresponding value of Q is 6.16. Inserting this value in equation (2), and taking the cube root of both sides of the equation, we obtain l (in Å.) = $740\,(\eta\Theta/T)^{-1/3}$. From this equation, the values of length given in Table I have been calculated from the observed values of $\eta\Theta/T$.

In estimating the most probable value of length for the molecule of human fibrinogen from these data, we must consider critically the errors that may affect the data. These errors fall into three principal classes.

1. Errors due to false light reflected from the walls of the cylinders and leading to distorted values of χ and Δ. These errors are most serious at low velocity gradient and low protein concentration (see discussion in references (7) and (8)).

2. Errors due to thermal gradients arising from the heat generated during flow. This heat generation is proportional to $G^2\eta$. Such disturbances are therefore most likely to occur at high viscosity and high velocity gradient. For two different systems, in which the value of $G\eta$ is the same, these disturbances are most serious in the solvent of lower viscosity.

3. Deviations due to molecular interaction, leading generally to a higher degree of orientation than would be obtained for a system of molecules far enough apart to orient independently.

(13) A. Peterlin, *Z. Physik*, **111**, 232 (1938); A. Peterlin and H. A. Stuart, *ibid.*, **112**, 1, 129 (1939).

(14) O. Snellman and Y. Björnstahl, *Kolloid-Beihefte*, **52**, 403 (1941).

(15) P. Boeder, *Z. Physik*, **75**, 258 (1932).

(16) For a summary of these relations, see J. T. Edsall, *Advances in Colloid Sci.*, **1**, 269 (1942); *cf.* E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, Chapter 21.

(17) The function $\eta\Theta/T$ has already been employed in describing the measurements on zein.[8]

TABLE III

REPORTED PHYSICAL CONSTANTS OF SOME HUMAN FIBRINOGEN PREPARATIONS AND CALCULATED VALUES FOR CERTAIN ELLIPSOIDAL MODELS[b]

	Calculated values						
	Model 1	Model 2	Model 3	Model 4	Model 5	Exptl. values	Ref. no. for exptl. data
Sedimentation[a] constant, $s_{20,w}$ (Svedberg units)	11	9.5	12.8	11	8.5	8.5–9	19,6
Diffusion constant, $D_{20,w}$ ($\times 10^7$ cm.²/sec.)	2.05	1.8	1.95	1.7	1.55	1.1–1.3	19
Partial specific volume, $\bar{v}$	(0.72)	(0.72)	(0.72)	(0.72)	(0.72)	0.72	18
Viscosity coefficient, H_0	.23	.316	.23	.316	.25	0.25	6
Frictional ratio, f/f_0	1.9	2.13	1.9	2.13	2.37	..	
Molecular weight from osmotic pressure ($\times 10^{-3}$)	460	460	570	570	500	580	6
Molecular weight from s and D ($\times 10^{-3}$)						670	19
Rotary diffusion constant, $\Theta_{20,w}$, for rotation about minor axis (sec.$^{-1}$ $\times 10^{-3}$)	33	25	28	19	50	35	This paper
Relaxation time for rotation of major axis, $\tau_{20,w}$ ($\times 10^5$ sec.)	1.5	2.0	1.8	2.7	1.0	1.4	This paper

[a] The studies of Oncley[6] indicate, besides the principal component of purified human fibrinogen (Fr. I-2), two minor components which sediment more rapidly, one with $s_{20,w}$ approximately equal to 12 S, and the other with $s_{20,w}$ = 20 S approximately. These components together make up a few per cent. of the protein in Fraction I-2. [b] Dimensions of the assumed models are as follows: Model 1, length 700 Å.; cross section 39 Å., hydration = 0; Model 2, same as Model 1, but with hydration 0.3 g./g. protein; Model 3, length 750 Å.; cross section 41.5 Å.; hydration = 0; Model 4, dimensions same as Model 3; hydration as in Model 2. Models 1-4 inclusive are prolate ellipsoids of revolution. All molecular weights and dimensions are given for the anhydrous molecules. Model 5: Oblate ellipsoid of revolution; diameter ($2b$) 343 Å.; thickness at center ($2a$) 9.8 Å.; hydration as in models 2 and 4.

Errors of Class 2 are difficult to evaluate; their presence is generally indicated by a blurring of the field and a distortion of the image of the gap, due to thermal gradients, which disturb the flow. Errors of Class 1 are most serious at low protein concentrations; those of Class 3, at high protein concentrations.

The two highest values in Table I for the calculated length of the fibrinogen in Fraction I-2, Run 167 appear to be unreliable, that in Experiment No. 5 because of the relatively high protein concentration, that in Experiment No. 7 because of the cloudiness of the solution and the difficulty of making accurate measurements. If these two values are rejected, the average of the other seven values listed for this preparation leads to an estimated molecular length of approximately 700 Å. Assuming a prolate ellipsoid of revolution with an axial ratio of 18 to 1, the value of $\eta\Theta/T$ should be 1.18. In water, at 20°, the rotary diffusion constant Θ_b, for rotation around the minor (b) axis, is thus 3.5×10^4 sec^{-1}. The corresponding relaxation time,[16] τ_a, for the rotary motion of the a axis, is 1.4×10^{-5} sec.

Critical Comparison with Other Data on the Properties of Fibrinogen.—Oncley, Scatchard and Brown[6] have determined the specific viscosity, the sedimentation constant, and the molecular weight from osmotic pressure, for the same fibrinogen preparations studied by us. Armstrong, Budka, Morrison and Hasson[18] have determined the partial specific volume. Holmberg,[19] using human fibrinogen prepared by an elaborate salting out procedure, determined sedimentation and diffusion constants.[20] All of these data are listed in Table III, together with corresponding calculated values for four prolate ellipsoidal models. In all these models an axial ratio of 18 to 1 has been assumed. The length has been varied from 700 to 750 Å., and the hydration from zero to 0.3 g. of water per gram of protein.[21] No one model reproduces all the reported experimental data very closely. The best fit appears to be obtained with the model of molecular weight 460,000 and a length of 700 Å., assuming a hydration value somewhere between 0 and 0.3. Axial ratios significantly higher or lower than 18 would be difficult to reconcile with the experimental data; higher values would give unduly high intrinsic viscosities and relaxation times; lower values would give unduly high sedimentation constants. None of these models yields a diffusion constant very close to that reported by Holmberg,[19] and any prolate ellipsoidal model adjusted to fit his diffusion constant gives values for the other physical quantities which are not readily reconciled with the experimental data. It should be added that Holmberg himself emphasizes the fact that his preparation was not strictly homogeneous, and claims no high accuracy for his diffusion constant. His sedimentation constant, however, is in excellent agreement with that determined by Oncley on the same preparations studied by us.

(18) S. H. Armstrong, Jr., M. J. E. Budka, K. C. Morrison and M. Hasson, THIS JOURNAL, **69**, 1747 (1947). See Table III, footnote.

(19) C. G. Holmberg, *Arkiv. Kemi. Mineral. Geol.*, **17A**, No. 28 (1944).

(20) Holmberg assumed a partial specific volume of 0.75 in calculating a molecular weight from these data. Using the measured specific volume,[18] we have revised Holmberg's molecular weight to 670,000, the value given in Table III.

(21) In evaluating the physical constants for these and other molecular models that were considered, we have found the nomograms of J. Wyman, Jr., and E. N. Ingalls, *J. Biol. Chem.*, **147**, 297 (1943), an almost indispensable aid to rapid calculation. The hydration factor of 0.3 g. H_2O/g. of protein corresponds to h = 0.42 in the notation of Wyman and Ingalls.

Nanninga[22] has studied bovine fibrinogen obtained by salting out with ammonium sulfate. He obtained a molecular weight of 441,000 by osmotic pressure, and a value of $H_0 = 0.24$, indicating an axial ratio (prolate ellipsoid) near 20 to 1. From this he calculated $2a = 725$ Å., thus deducing a model remarkably close to that deduced for human fibrinogen from the data considered in the present paper.

As noted in Table III, our purified fibrinogen is not homogeneous in the ultracentrifuge, but contains small amounts of two components which sediment much more rapidly than the main component. These may represent aggregates of the principal protein component, the properties of which have been treated in the preceding discussion. If so, the aggregation is probably side by side, rather than end to end, since a very elongated aggregate would not be expected to give much higher sedimentation constants than the individual molecules of shorter length. Moreover, the presence of elongated aggregates would be readily revealed by the double refraction of flow measurements, since such aggregates would be very easily oriented at low velocity gradients; hence the calculated values for molecular length at low gradients would be considerably greater than those derived from measurements at the higher gradients.[23] Actually the calculated lengths at high and low gradients are very nearly the same.

In the preceding discussion, we have placed some reliance on the relative uniformity of the calculated lengths of the fibrinogen molecules for measurements at low and at high velocity gradients (see Table I). However, this conclusion depends upon the validity of the functional relation between χ and α, shown in Table II, and is therefore subject to all the uncertainties present in that relationship. In general, there is a slight downward trend of calculated molecular length with increasing velocity gradient. However, this is small and it contrasts very sharply with the extreme heterogeneity in length in such protein preparations as those of myosin, in which the observed χ values obtained by von Muralt and Edsall[24] may be interpreted in the light of Sadron's[23] theory of double refraction of flow in polydispersed systems. Similar results have been obtained by us[25] on gelatin solutions, in which the high degree of polydispersity is reflected in curves of a very different sort from those here reported for fibrinogen. If considerable polydispersity were present in the fibrinogen solutions, the observed curve for χ as a function of $G\eta$ should flatten out markedly at high values of $G\eta$, and become nearly horizontal. The fact that no such behavior is observed is a strong indication that these preparations of fibrinogen are relatively uniform with respect to molecular length.

(22) L. Nanninga, *Arch. neerland physiol.*, **28**, 241 (1946).

(23) C. Sadron, *J. Phys. Radium*, [7] **9**, 381 (1938).

(24) A. L. von Muralt and J. T. Edsall, *J. Biol. Chem.*, **89**, 315, 351 (1930).

(25) I. F. Foster and J. T. Edsall, unpublished work.

All of the discussion hitherto has presupposed that the fibrinogen molecule is rod-shaped, and from the appearance of the needle shaped aggregates which are formed when fibrinogen is converted into fibrin, it is difficult to picture the molecule otherwise. However, it must be pointed out that a model consisting of a disk-shaped oblate ellipsoid (Model 5, Table III) reproduces all the experimental data at least as well as any of the prolate ellipsoidal molecules. This disk-shaped model, however, is only 9.8 Å. thick at the center, which is about the thickness of a protein monolayer. Physically, such a model appears highly dubious. However, it is conceivable that the fibrinogen molecule might be built of a peptide chain coiled around itself in a single plane in such a manner as to form a flat disk. The initial step in the action of thrombin on fibrinogen would then presumably be the loosening of the attachments holding the different portions of the disk together, so that the molecule could uncoil into a long thread-like structure. Whether such a model could possibly correspond to physical reality is a question that must remain for further experimental study.

Studies on Other Fractions Related to Fibrinogen.—In addition to Fraction I–2, several other less pure preparations of Fraction I of human plasma were studied, and the results are tabulated in Experiments 10–17 inclusive, of Table I. The molecular lengths of the principal components in these preparations appear to be essentially the same as those in the purified fibrinogen Fraction I-2. Since fibrinogen is the dominant component in all these preparations, these studies alone can give little indication of the molecular dimensions of the non-clottable proteins in Fraction I. However, one preparation was studied in which all the fibrinogen had been removed by clotting with thrombin, the residual nonclottable protein being taken into solution. The results on this preparation are recorded in Experiments 18 and 19 of Table I. They indicate that these non-clottable components, or at least a portion of them are, like fibrinogen, very elongated molecules, their length being of the order of 600 Å., only slightly less than the value of 700, estimated for fibrinogen itself. The nature of the elongated component of this non-clottable fraction is now being further investigated.

Summary

1. Double refraction of flow measurements have been made on purified human fibrinogen, using glycerol–water–salt mixtures as solvents. Other closely associated protein components in Fraction I of human plasma were also studied.

2. The results indicate, for purified fibrinogen, a rotary diffusion constant ($\Theta_{20,w}$) of approximately 35,000 sec.$^{-1}$.

3. From these and other related physico-chemical data, it is concluded that human fibrinogen can be approximately described as a prolate ellip-

soid 700 Å. long, with an axial ratio of 18 to 1. A disk-shaped (oblate) ellipsoidal model is also compatible with all the observed data, but appears inherently less probable.

4. Some of the non-clottable protein of Fraction I also consists of elongated molecules of the order of 600 Å. long.

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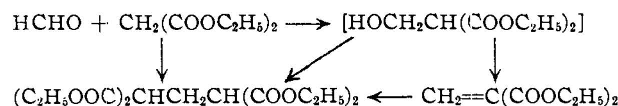
[CONTRIBUTION FROM THE STERLING-WINTHROP RESEARCH INSTITUTE]

A New Synthesis of *dl*-Serine[1]

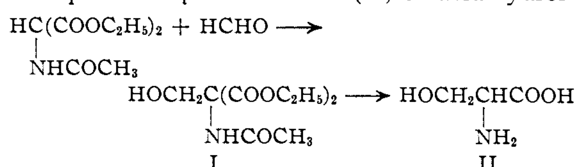
BY JOHN A. KING[2]

The amino acid *dl*-serine has been synthesized by four general methods: (1) a Strecker type of synthesis on glycolaldehyde[3] or ethoxyacetaldehyde[4,5,6]; (2) a Claisen-type formylation of ethyl hippurate with subsequent reduction and hydrolysis[7,8]; (3) an alkylation of the sodium enolate of ethyl phthalimidomalonate with chloromethyl ether followed by hydrolysis[9,10]; and (4) from acrylic esters by conversion to the α-bromo-β-alkoxy (or -hydroxy) esters followed by saponification, amination and hydrolysis.[11-14] None of these methods is especially convenient, the starting materials for some of them are difficult, laborious or expensive to prepare, and the over-all conversion to pure serine is not particularly good by any of them. The relative inaccessibility of serine prompted a search for a convenient and economical synthesis that would give a good yield of product without the use of high-pressure amination or drastic acid hydrolysis of an ether linkage. The present paper reports such a synthesis.

In the Knoevenagel condensation of formaldehyde with ethyl malonate in the presence of catalytic amounts of diethylamine the product iso-

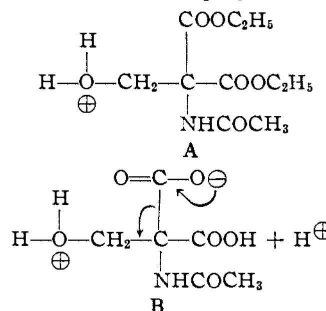


lated is the bis compound, ethyl α,α'-dicarbethoxyglutarate,[15] but when a trace of caustic soda is used as the condensation catalyst it has been claimed[16] that the bimolecular condensation product, methylolmalonic ester, is the initial reaction product. Apparently, however, this aldol-like primary condensation product has never been isolated from the reaction because of its tendency to undergo dehydration to methylene malonic ester or further condensation to the bis compound. Should formaldehyde similarly condense with the now readily available ethyl acetamidomalonate[17,18,19] the product, ethyl α-acetamido-α-carbethoxy-β-hydroxypropionate (I), would be incapable of intramolecular dehydration and might be expected to produce serine (II) on acid hydrol-



ysis. Formaldehyde and ethyl acetamidomalonate were found to condense to give a quantitative yield of I. However, concentrated hydrochloric acid hydrolysis of I caused complete destruction of the molecule and gave no serine; the nitrogen came out as ammonium chloride and the rest of the molecule was converted to pyruvic acid. The same results were obtained with 1 N hydrochloric acid.

This behavior appears more rational after consideration of the electronic structure of the molecule. The oxygen of the hydroxyl group in the molecule is inherently nucleophilic and, in the presence of strong mineral acid, is susceptible to proton attack, forming the oxonium ion A. Under the reaction conditions employed the ester link-



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(3) Fischer and Leuchs, *Ber.*, **35**, 3788 (1902).

(4) Leuchs and Geiger, *ibid.*, **39**, 2644 (1906).

(5) Dunn, Redemann and Smith, *J. Biol. Chem.*, **104**, 511 (1934).

(6) Redemann and Icke, *J. Org. Chem.*, **8**, 159 (1943).

(7) Erlenmeyer, *Ber.*, **35**, 3769 (1902).

(8) Erlenmeyer and Stoop, *Ann.*, **337**, 236 (1904).

(9) Mitra, *J. Indian Chem. Soc.*, **7**, 799 (1930).

(10) Maeda, Terumi and Suzuki, *Bull. Inst. Phys. Chem. Research* (Tokyo), **17**, 267 (1938); *C. A.*, **34**, 6931 (1940).

(11) Schiltz and Carter, *J. Biol. Chem.*, **116**, 793 (1936).

(12) Carter and West, *Organic Syntheses*, **20**, 81 (1940).

(13) Wood and du Vigneaud, *J. Biol. Chem.*, **134**, 413 (1940).

(14) Mattocks and Hartung, *ibid.*, **165**, 501 (1946).

(15) Knoevenagel, *Ber.*, **27**, 2346 (1894).

(16) D'Alelio, U. S. Patent 2,330,033; *cf.* Welch, *J. Chem. Soc.*, 257 (1930), who believes that the primary reaction product is monomethylolmalonic ester when amine catalysts are used.

(17) Locquin, *Bull. soc. chim.*, [4] **49**, 42 (1931).

(18) Snyder and Smith, THIS JOURNAL, **66**, 350 (1944).

(19) Albertson, Archer and Suter, *ibid.*, **66**, 500 (1944); **67**, 36 (1945).