Debye–Hückel equation. We tried to use a similar computation for our solvents water–dioxane, using dielectric constants of 65 and 45, respectively, for 20% and 40% dioxane. The charge was considered as maximum since the titration curves[7] indicate that almost the maximum positive charge is reached at *p*H 3 in 20% dioxane and at *p*H 3.5 in 40% dioxane. The number of bound anions was taken similar to the value in aqueous solutions, *i.e.*, one anion per two chemical units.[9] Slight differences in the number of bound anions would not introduce significant differences in the energies of repulsion.

On this basis we find in 20% dioxane and in 40% dioxane, respectively, 2,250 and 3,200 cal. for the change in electrostatic free energy resulting from the association of two molecular units, that we call ΔF^e. If we let ΔF^a be the term for the attractive forces involved in the process, we can write for the total free energy change of association ΔF

$$\Delta F = \Delta F^a + \Delta F^e \qquad (1)$$

In water, we found an equilibrium constant of 500 for the association of two units. Then $\Delta F = -3{,}600$ cal. ΔF^e is calculated as 1,800 cal. and from equation 1, $\Delta F^a = -5{,}400$ cal. If the attractive forces in the association process were the same in water and in the mixed solvents, then

in 20% dioxane, $\Delta F = -5400 + 2250 = -3150$ cal.

in 40% dioxane, $\Delta F = -5400 + 3200 = -2200$ cal.

and the corresponding equilibrium constants would be 220 and 44, respectively. However, an inspection of the sedimentation curves on Fig. 1 indicates that the association of the molecular units is extremely weak in 20% dioxane and practically nonexistent in 40% dioxane. Consequently, the equilibrium constants calculated above are much too high, their true values must be inferior to unity. For obtaining such figures, the absolute value of ΔF^a in equation 1 must be much lower than 5,400.

The lowering of the absolute value of the attractive term in dioxane solutions may be explained by the following considerations. This term results from two kinds of forces: (1) the attractions between insulin units; (2) the attractions between insulin and solvent molecules. During the association process, there is a negative free energy change resulting from the first forces and a positive free energy change resulting from the decreased solvation of the associated particles. From the considerations already developed, it is evident that the attractions between insulin and solvent molecules are much higher in dioxane–water than in pure water. Consequently, the total attractive term ΔF^a will be higher in dioxane–water than in water, and its absolute value will be smaller.

From this discussion, we see that the weak association of insulin units in dioxane solutions results not only from the lower dielectric constant of the medium but also for an important part, from the interactions between insulin particles and solvent molecules. A similar situation was encountered in the study of the binding of organic ions by proteins in water–dioxane mixtures.[10]

This work was supported by grants from the Lilly Research Laboratories, Indianapolis, and the "Centre National de Biologie physicochimique."

(9) E. Fredericq, *Bull. soc. chim. Belges*, **65**, 960 (1956).

(10) E. Fredericq, *ibid.*, **64**, 639 (1955).

LIÉGE, BELGIUM

[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

Molecular Stability and Sub-structure of Bovine Fibrinogen

BY JOHN E. FITZGERALD,[1] NATHANIEL S. SCHNEIDER[2] AND DAVID F. WAUGH[3]

RECEIVED AUGUST 2, 1956

As indicated by determination of molecular weight (light scattering) and sedimentation constant, fibrinogen retains its characteristic size and shape up to *p*H 10.8 and independently of protein concentration and ionic strength. Above *p*H 12.8 the molecule decomposes and yields one A fragment of $M = 230{,}000$ and $S = 5.5$ (at 0.1% protein) and approximately eight B fragments of $S = 1.77$, $D = 12.4 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ (at 1% protein) and thus $M = 14{,}500$. The dimensions calculated for the anhydrous A fragment are 724 × 27 Å. (length and diameter of prolate ellipsoid assuming 0.3 g. of water per gram protein) and 600 × 30 Å. (assuming 0.75 g. of water per gram protein). If the anhydrous B fragment carries 0.3 g. of water per gram protein, it has dimensions of 67 × 22 Å. Combinations of fragments would readily yield the nodose structures seen in electron micrographs. At values intermediate between *p*H 10.8 and 12.8 the fibrinogen molecule may undergo a number of rapid changes in frictional resistance without a change in molecular weight, the maximum change in S recorded here being from $S^0_{20} = 8.1$ to $S^0_{20} = 5.5$ (*p*H 12.2). The clear increase in frictional resistance is due to molecular expansion and is attributed to a partial liberation of the B fragments rather than to an isotropic swelling. At *p*H 12.2 there is a slow denaturation such that after 60 min. at 4° a rapid return to *p*H 7.7 finds 90% of the protein insoluble. Below *p*H 12.2 denaturation proceeds too slowly and above irreversible changes appear. After denaturation a maximum recovery of solubility and clottability at *p*H 7.7 may be obtained by incubation for 4–6 hours at intermediate *p*H values, the most effective being *p*H 10.82 at room temperature. After such treatment, 90% of the original protein is soluble and 70% of the soluble protein is clottable. The sizable fraction of protein which is soluble but non-clottable has a molecular weight and sedimentation coefficient typical of fibrinogen and thus resembles fibrinogen in size and shape. A rapid return from *p*H 12.2 to 7.7 thus stabilizes structural changes leading to insolubility while at *p*H ~10.8 energy barriers are such as to allow a gradual rearrangement to a more native family of structures. From the foregoing it is clear that fibrinogen does not dissociate under conditions where it is altered by thrombin and thus the complexity of the single critical contact between thrombin and fibrinogen, predicted from kinetic data, may be determined when the necessary associated chemical events are known.

(1) Fellow American Cancer Society. A part of this work was reported in J. E. Fitzgerald, Ph.D. Thesis, Department of Biology, Massachusetts Institute of Technology, May, 1955.

(2) Public Health Service Fellow of the National Heart Institute.

(3) For supporting this research, the authors are indebted to the Medical Research and Development Board, Office of the Surgeon General, Department of the Army, Washington, D. C. (Contract No. DA-49-007-MD-198).

The process of activation produces a modified fibrinogen which will polymerize, the end result being the formation of the fibril network characteristic of the clot. Studies of the kinetics of activation of fibrinogen over a wide range in thrombin concentration, fibrinogen concentration, ionic strength,[4] *p*H and temperature[5] show that a single critical contact only is required for activation.

However, studies of the *over-all* clotting process reveal certain chemical changes which, in a preliminary way, suggest that there may be three or four thrombin sensitive sites per fibrinogen molecule. These studies examine the appearance of acidic peptides (Lorand,[6] Bettleheim and Bailey[7]), of end groups (ref. 7, Lorand and Middlebrook[8]), of hexose (St. Zara and Bagdy[9] and Laki[10]), and examine the number of critical sites inhibited by formaldehyde.[11]

At the time our studies were indicated, there were suggestions that fibrinogen may be constructed of three or four sub-units. Thus, Caspary and Kekwick report that human fibrinogen dissociates,[12] and in electron micrographs fibrinogen appears as nodose filaments of variable length,[13] the average filament containing about four "beads." From the foregoing there emerged the possibility that thrombin splits an acidic peptide and/or a polysaccharide from each of three or four sub-units of fibrinogen.

If it be assumed that a majority of the chemical events just enumerated are required for activation, the single critical contact specified by kinetic data suggests that the mechanism of activation is more complicated than previously inferred. Either the critical contact may consist of a sequence of connected events in which, for example, four peptides appear, or there occurs before the activation process a dissociation of fibrinogen into sub-units. Clearly, the process of activation might require the splitting of only one peptide and/or one saccharide unit, the others following but not being connected with activation.

The possibility that fibrinogen dissociates before or during activation is considered here. We have examined mainly conditions of protein concentration, *p*H, ionic strength, and temperature under which fibrinogen will retain its physical integrity and clottability and similar conditions which result in expansion (swelling) and finally fragmentation.

Materials and Methods

Materials.—The fibrinogen was prepared essentially according to the method of Laki[10] from fraction I of bovine plasma prepared by the Armour Laboratories. The cold insoluble globulins were first removed and fibrinogen was precipitated by adding saturated $(NH_4)_2SO_4$ to 23% of saturation. The protein at 16 mg./ml. was dialyzed against 0.3 *M* KCl to remove all $(NH_4)_2SO_4$. Aliquots of 2 ml. were rapidly frozen in lusteroid centrifuge tubes and stored in the deep freeze. In one preparation, to facilitate low ionic strength studies, a solution of fibrinogen in 0.3 *M* KCl was dialyzed against 0.01 *M* KCl adjusted to *p*H 10 (NH_4OH) to keep the fibrinogen in solution.

The weight concentration of protein was determined by heating 2-ml. samples to constant weight at 110° and subtracting the weight of KCl present in the solvent. An average value of 1.54 was obtained for the slope of the plot of optical density at λ 280 mμ, against concentration in mg. per ml. The determination of clottable protein was carried out in a standard *p*H 7 buffer at a fibrinogen concentration of about 1 mg. per ml. and a thrombin concentration of 0.10 unit per ml. The optical density of the supernatant was determined at four hours clotting time, after compaction and removal of fibrin and the value checked at about 12 and 16 hours. Preparations ranged from 92 to 95% clottable.

The most generally used solvent, referred to as standard phosphate buffer, had an ionic strength of 0.15 of which 0.05 was due to phosphate and 0.10 due to KCl.[4] High *p*H solutions were generally made up with KOH and were unbuffered except as noted in the text. Precautions were taken to minimize *p*H alterations using closed vessels or keeping solutions in an atmosphere of nitrogen. Where possible, *p*H values were checked before and after experiments.

Methods.—Light scattering measurements were made with the Brice–Speiser light scattering photometer using the semimicro hemicylindrical cell designed by P. M. Doty.[14] Measurements were made of the 90° scattering and the dissymmetry ratio at λ 4358 Å. The protein refractive index increment was taken as $dn/dc = 0.197$ (see ref.[17]).

Solutions were clarified by centrifugation for 45 to 90 minutes in the #40 rotor of the Spinco preparative centrifuge using the standard dural caps with a baked glyptal coating for moderate *p*H solutions and tapered nylon plugs for high *p*H solutions.

Ultracentrifuge runs were made in the model E Spinco Ultracentrifuge equipped with Schlieren cylindrical lens optics. For most of the work the 12 mm. 4° Kel-F center piece was used but in examining low molecular weight materials a Kegeles type of synthetic boundary cell[15] constructed from a 2° centerpiece was used. The rotor temperature was measured at intervals during the run using the radiation method described by Waugh and Yphantis.[16]

The Region of Stability

Accurate characterization of fibrinogen has been impeded by the ease with which the protein "denatures" and forms soluble aggregates. Recently, examinations of purified fibrinogen by Katz, *et al.*,[17] and Shulman[18] have resulted in a downward revision of molecular weight from 500,000 to 340,000. The light scattering molecular weight of the fibrinogen used here when dissolved in standard phosphate buffer is 382,000 with a dissymmetry of 1.13, both values being higher than those previously reported.[17,18] However, the sedimentation coefficient of 7.6 determined at 0.1% protein is in reasonable agreement with the value of ~7.7 determined by Shulman[18] and the common extrapolated value evident in Fig. 1 ($S^0_{20} = 8.1$) lies 3.8% above the extrapolated value of Shulman, as is expected from the difference in techniques used in determining rotor temperature.[16] Examination of

(4) D. F. Waugh and B. J. Livingstone, *J. Phys. Chem.*, **55**, 1206 (1951).

(5) D. F. Waugh and M. J. Patch, to be published.

(6) L. Lorand, *Biochem. J.*, **52**, 200 (1952).

(7) F. R. Bettleheim and K. Bailey, *Biochem. Biophys. Acta*, **9**, 578 (1952).

(8) L. Lorand and W. R. Middlebrook, *Biochem. J.*, **52**, 196 (1952).

(9) St. Zara and D. Bagdy, *Biochem. Biophys. Acta.*, **11**, 313 (1953).

(10) K. Laki, "Blood Clotting and Allied Problems," J. E. Flynn, editor, Trans. Fourth Conf. New York, Josiah Macy, Jr., Foundation, 1951, p. 217.

(11) D. F. Waugh and B. J. Livingstone, *J. Phys. Colloid Chem.*, **55**, 3 (1951).

(12) E. A. Caspary and R. A. Kekwick, *Biochem. J.*, **56**, XXXV (1954).

(13) C. E. Hall, *J. Biol. Chem.*, **179**, 857 (1949); B. W. Siegel, J. P. Merman and H. A. Scheraga, *Biochem. Biophys. Acta*, **11**, 329 (1953).

(14) Manufactured by Pyrocell Mfg. Co., 207–211 East 84th Street, New York 28, N. Y.

(15) G. Kegeles, THIS JOURNAL, **74**, 5531 (1952).

(16) D. F. Waugh and D. A. Yphantis, *Rev. Sci. Instr.*, **23**, 609 (1952).

(17) S. Katz, K. Gutfreund, S. Shulman and J. D. Ferry, THIS JOURNAL, **74**, 5706 (1952).

(18) S. Shulman, *ibid.*, **75**, 5846 (1953).

the sample in the artificial boundary cell of Pickels, *et al.*,[19] revealed a shoulder on the fast side of the peak, which is probably responsible for the high molecular weight determined by light scattering. Assuming that the 5% non-clottable protein constitutes the observed contaminant, its molecular weight calculated from the light scattering result for fibrinogen would be about 1×10^6. This value will be used where it becomes necessary to take account of the presence of the high molecular weight component.

Were dissociation of fibrinogen to occur, as indicated in the introduction, we would expect a reversible equilibrium in which the sub-unit state would be favored by dilution and by the introduction of a higher net charge. However, dissociation is significant in the activation mechanism only if the protein retains its clottability. Since solutions do not clot above *p*H 10.5, test samples have been returned to *p*H 7 and ionic strength 0.15, and clotting has been examined within a range of thrombin and fibrinogen concentrations previously studied in this Laboratory.

Electron microscope work carried out by others[13] and by one of us (J.E.F.) suggested the possibility that dissociation occurred at protein concentrations of 30 γ per ml. Accordingly determinations of the molecular weight of fibrinogen at 30 to 90 γ per ml. were carried out at room temperature by light scattering. The molecular weight so obtained, 382,000, is consistent with those obtained at higher concentrations using a variety of conditions. In addition, light scattering measurements were carried out on six samples at a protein concentration of 10 γ per ml. The individual results were in good agreement and the average value falls directly on the curve obtained by extrapolation from higher concentrations. The results are summarized in the first line of Table I, which gives the solvent, *p*H and ionic strength in addition to the pertinent light scattering data.

Molecular weight determinations were also made at room temperature at *p*H 9.2 and *p*H 9.75 (lines 2 and 3 of Table I). There is no evidence of a decreased molecular weight.

TABLE I

LIGHT SCATTERING MOLECULAR WEIGHT OF BOVINE FIBRINOGEN

Solvent	*p*H	μ	Concn. range, g. $\times 10^{+5}$ per ml.	Z	$M \times 10^{-5}$
Phosphate buffer	7.0	0.15	1.0 to 9.1	1.13	382
Borate buffer	9.2	.15	2.4 to 13.7	1.13	376
Glycine buffer	9.75	.30	16.7 to 76.7	1.13	376
Phosphate buffer "urea treated fibrinogen"	7.0	.15	3.4 to 15.4	1.14	372
4×10^{-4} Versene in phosphate buffer	7.0	.15	6.1 to 15.3	1.14	379
Dialysis *vs.* NH_4OH *p*H 9.5, 48 hr.	9.5	0	3.0 to 8.0	1.14	468

Sedimentation coefficients have also been obtained at room temperature for several *p*H values, as shown in Fig. 1. Up to the highest *p*H value used in this series, *p*H 10.8, there is no significant change in the extrapolated value of S, although as the *p*H increases, the negative slope of the S *vs.* c plot also increases. Such a result is expected since electrostatic particle interaction increases with increasing net charge. The lowest concentration examined in the series, 0.025% at *p*H 9.1, is near the useful limit of the ultracentrifuge and the lower reliability of this value may affect the extrapolation. The extrapolated sedimentation coefficient, $S^0_{20} = 8.25$, suggests that the molecules retain their original molecular weights.

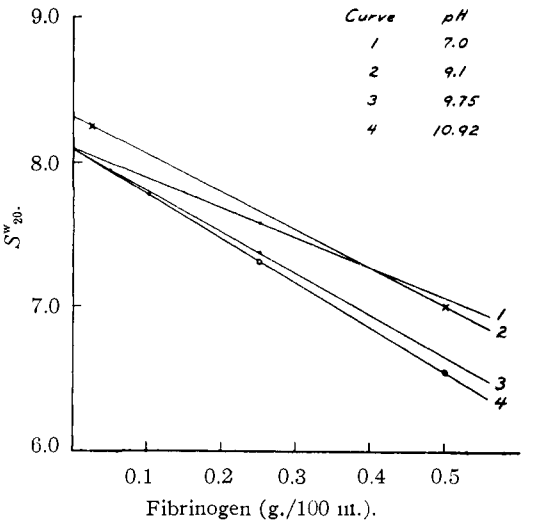


Fig. 1.—Effect of *p*H on the concentration dependence of sedimentation of fibrinogen, $\mu = 0.30$.

Fibrinogen preparations may contain a serum factor whose presence, along with calcium, makes clots insoluble in urea. Such a factor might also be involved in stabilizing against dissociation. This possibility was examined by employing a technique described by Shulman[20] which effectively destroys clot stabilizing properties through the action of 2 M urea for 2 hours at *p*H 7.0 (4°). The results of an examination of urea treated fibrinogen in standard phosphate buffer is given in line 4 of Table I. It is apparent that this treatment has not affected molecular weight.

Finally, neither treatment with 10^{-4} Versene,[21] (line 5 of Table I), an agent which sequesters divalent cations, nor dialysis for 48 hours against NH_4OH at *p*H 9.5 to remove added salt (line 6) will effect a downward displacement of molecular weight, even at protein concentrations below 100 γ per ml.

Non-clotting Activation of Fibrinogen by Thrombin.—The possibility that dissociation takes place as a consequence of activation has been examined in a preliminary fashion. Fibrinogen solutions, dialyzed against ammonium hydroxide at *p*H 9.0–11.0, will not clot on the addition of thrombin. However, activation has been effected, for on neutralizing or increasing the ionic strength, clots form immediately. The amount of clot increases with time of incubation with thrombin.

Light scattering studies were carried out on a

(19) E. G. Pickels, W. F. Harrington and H. K. Schachman, *Proc. Natl. Acad. Sci., U. S.*, **38**, 943 (1952).

(20) S. Shulman, *Nature*, **171**, 606 (1953).

(21) Ethylenediaminetetraacetic acid made by Bersworth Company, Framingham, Mass.

0.045% solution of fibrinogen dialyzed against ammonium hydroxide at *p*H 10.4 (to remove all added salt). No change in scattering was observed over a period of 36 hours following the addition of thrombin to give 0.08 NIH unit at *p*H 10.4 per ml. in the clarified solution in the light scattering cell. At *p*H 9.0, however, under similar conditions, there was a slow aggregation, partially reversible by increasing the *p*H to *p*H 10.4, which leveled off in 5 hours at about 9 × the scattering of fibrinogen (Fig. 3).

Region of Instability

Above a *p*H of about 11.5 there is an obvious change in the properties of fibrinogen including its configuration, molecular stability and clottability. While the system is complex in the range from approximately *p*H 11.6 to *p*H 12.8, above *p*H 12.8 there is an essential simplification and we shall, therefore, consider first the behavior of fibrinogen at a *p*H close to 13.

Fibrinogen at *p*H 13.—Two light scattering examinations were made on fibrinogen samples at *p*H 13 (25°), from one to three hours after preparation of the samples. The first examination in 0.1 *M* NaOH with 0.25 *M* added KCl gave a molecular weight of 242,000 and a dissymmetry of 1.06. Thus, at this *p*H there is an over-all decrease of almost 40% in molecular weight. A time study of the decrease in light scattering intensity was made by mixing a clarified solution containing 0.1% fibrinogen and 2 *M* KCl with an equal volume of *p*H 13 solvent directly in the light scattering cell. It was found that there was an initial sharp decrease in scattering which appeared to level off after about 40 minutes to a final value less than 70% of the original scattering. The system was not completely stable at *p*H 12.85, for aggregates appeared after 8 hours.

A fibrinogen sample at high *p*H examined in the ultracentrifuge exhibited two peaks: a slow moving, rapidly diffusing peak which contained about 20% of the protein and a faster, sharp and symmetrical main peak. Sedimentation measurements performed at *p*H 12.85 and $\mu = 0.3$, on 0.5 and 0.1% fibrinogen solutions gave coefficients of 4.03 and 5.08, respectively, for the fast peak. These extrapolate at zero concentration to $S^0_{20} = 5.4$. The slow moving peak of the 0.5% solution had a coefficient of $S_{20} = 1.8$. The peak was not sufficiently well defined in the 0.1% solution to permit its measurement. For convenience the material of the fast $S^0_{20} = 5.4$ peak will be referred to as the A component and that of the slow $S_{20} = 1.8$ peak as the B component. The B component was found to be insensitive to ionic strength over the range 0.1 to 0.5, but as the ionic strength was decreased the A component showed signs of polymerizing, for a "fast" shoulder appeared on the corresponding peak.

Information to be presented in subsequent sections suggested that the B component would remain stable at lower *p*H values while the A component would aggregate. This formed the basis of a fractionation procedure. The fibrinogen is exposed at room temperature for one hour to *p*H 12.9 as a 0.6% solution containing 0.3 *M* KCl. The *p*H of the solution is reduced to about *p*H 11 by the addition of 1 *M* HCl and the solution is then dialyzed overnight at 4° against a large excess of standard phosphate buffer. The highly opalescent solution which results is centrifuged for 2 hours at 30,000 r.p.m. to remove the aggregated A component. The supernatant, now clear, is dialyzed overnight against several changes of distilled water to remove all salt and is finally lyophilized.

Sedimentation and diffusion coefficients were determined from runs carried out on this material in an artificial boundary forming cell[15] at a 1% protein concentration in standard phosphate buffer. All but a small fraction of the protein was in a single peak which appeared to be symmetric throughout the run. A sedimentation coefficient of 1.77 *S* was obtained. Certain difficulties encountered in determining diffusion coefficients lay in a base line distortion which was considered to be due to the known small amount of high molecular weight contaminant. The peak was therefore bisected by a line parallel to the meniscus and only the half peak on the meniscus side was used. The calculated peak areas were reasonably constant and small zero times, less than 300 seconds, were obtained. The diffusion coefficient obtained is 12.4×10^{-7} cm.^{2} sec.$^{-1}$. A second run, less subject to the difficulties outlined above, was made by centrifuging at 60,000 r.p.m. for a few minutes to remove the contaminant after which the speed was reduced and kept at 20,000 r.p.m. The diffusion coefficient obtained from the initial period of this run is 14.6×10^{-7} cm.^{2} sec.$^{-1}$, only 10% higher than the previous value. A check on the limits of placing the base line indicates that the error from this operation is less than 15%. Using the sedimentation and average diffusion constants at the single concentration, a molecular weight of 14,500 is calculated for the B component.

From the areas of the peaks ($A = 0.8$, $B = 0.2$), the molecular weight of the B component, the concentration and molecular weight of the contaminant and the weight average molecular weight of the mixture (light scattering), the molecular weight of the A component may be estimated as $M \sim 230{,}000$. Apparently each fibrinogen molecule yields one fragment of $M \sim 230{,}000$ and approximately eight fragments of $M \sim 14{,}500$.

Fibrinogen at *p*H 11.6–12.8.—In this *p*H range the system is complicated by aggregation. The composition of the solution at a given *p*H, temperature and ionic strength is dependent upon time for apparently only the B component is relatively stable. We conclude from the experiments to be described that the first alteration to take place when fibrinogen is brought to a *p*H in excess of *p*H 11.0 is a change in shape and hydration without a change in molecular weight. This we term expanded fibrinogen. There follows, under appropriate conditions, a fragmentation into the A and B components, the latter showing a tendency to accumulate and the former (A) a tendency to aggregate. Aggregation is retarded by working at low ionic strengths and fragmentation proceeds slowly at low temperatures.

Changes in combined hydration and shape with-

out changes in size may be demonstrated at *p*H 12.4 and at ionic strengths below 0.1. At room temperature, scattering was studied as a function of time giving the results shown in Fig. 2. Here the ratio of scattering intensity at time t to the initial intensity (both at 90°) is plotted against time for representative fibrinogen solutions. It is seen that a ratio of unity is obtained if t is small. The molecular weights at small values of t were determined by working rapidly. Two runs gave values of 374,000 and 354,000 which are near that for the fibrinogen used (377,000). Both runs showed a dissymmetry of 1.14 and slopes of reciprocal scattering *versus* concentration similar to those obtained for fibrinogen. Ultracentrifuge runs were performed at room temperature at protein concentrations of 0.25 and 0.134% at *p*H 12 and $\mu \sim 0.07$. Full speed was reached within 20 minutes of *p*H adjustment. Single peaks having sedimentation coefficients of $S = 4.7 \pm 0.2$ and $S = 4.8 \pm 0.3$, respectively, were observed.

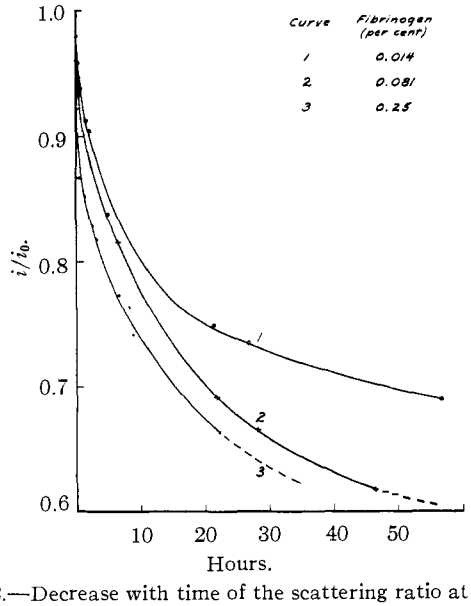


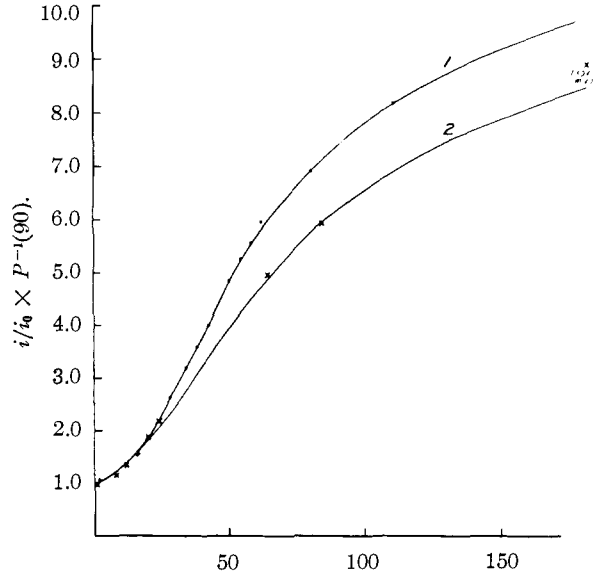
Fig. 2.—Decrease with time of the scattering ratio at 90° of fibrinogen at *p*H 12.0 and $\mu = 0.064$.

In view of the fact that the reversibility studies to be described were carried out at 4°, sedimentation diagrams were obtained at this temperature. Single peaks were observed at *p*H 12.2, the ionic strengths, protein concentrations and sedimentation constants being: $\mu = 0.12$, $c = 0.54\%$, $S = 4.47$; $\mu = 0.09$, $c = 0.33\%$,[22] $S = 4.97$; and $\mu = 0.03$, $c = 0.04\%$, $S = 5.34$. These extrapolate to $S = 5.5 \pm 0.16$ at $c = 0$. Under these conditions the reversibility of the system was examined with the result that, within the time period required to perform ultracentrifuge examination, extensive reversibility with respect to sedimentation coefficient and solubility could be demonstrated (see below). There can be no doubt that fibrinogen is capable of undergoing a reversible change in frictional resistance without a change in molecular weight.

(22) The total protein concentration in the cell was 0.5%. Gel was found in the base of the cell after the run. The concentration given is that of the supernatant.

The curves of Fig. 2 show that the expanded state, at room temperature and $\mu = 0.064$, is followed by a fragmentation since the scattering ratio decreases within about 50 hours to values between 0.6 and 0.7. Beyond this time aggregation takes place. Over the time periods covered, the dissymmetry values decreased slightly, the maximum change being from 1.19 to 1.09 for 0.25% protein. Sedimentation analyses were carried out at 24 hours on the same solutions as used to obtain the scattering data of Fig. 2, curves 2 and 3 (0.08 and 0.25% protein). The symmetrical main peaks led to a sedimentation constant of $S^0_{20} = 5.5$. The sedimentation pattern of the 0.25% protein solution exhibited a much smaller, rapidly diffusing peak of $S \sim 2$. Thus, fragmentation is found both by light scattering and by ultracentrifugation.

At *p*H values intermediate between 11 and 12.8, aggregation is favored by increasing the ionic strength above 0.1. The system becomes quite sensitive to precise levels of these variables. Solutions of fibrinogen at *p*H 12, 25° and $\mu = 0.3$ illustrate a situation in which aggregation is rapid. In Fig. 3, which shows data obtained at a



Min. after adjustment to *p*H 12 (curve 1) or after thrombin (curve 2).

Fig. 3.—Formation of aggregates in solutions of fibrinogen: curve 1, *p*H 12.0, $\mu = 0.30$; curve 2, *p*H 9.0, $\mu \sim 0$, thrombin activated.

protein concentration of 0.035%, even from small times onward there is a gradual increase in scattering which appears to reach a limiting average molecular weight about 10 times that of the starting fibrinogen. However, aggregation continues, for within 24 hours visible aggregates appear. Fibrinogen concentrations of 0.5% (*p*H 12.0, 25° and $\mu = 0.3$) have been examined in the ultracentrifuge at 53 and 330 minutes after preparation. At 53 minutes two peaks of equal area are clearly seen. These have coefficients of $S = 6.3 \pm 0.3$ and $S \sim 9.4$, the latter having a "fast" shoulder. There is also present a small peak of roughly $S \sim 2$. At 330 minutes the $S \sim 9$ peak has gained at the expense

of the $S \sim 6.3$ peak and the slow $S \sim 2$ peak is now well defined.

Reversibility

The "expanded" molecule represents a physical situation in which a return toward normal might occur. Conditions which produce expansion with slow fragmentation or aggregation have, therefore, been used. Preliminary experiments at once indicated that, at 25°, loss of solubility is too rapid, even at *p*H values in the vicinity of *p*H 11, to be attractive. Studies were performed at 4°, μ = 0.035 (KCl–KOH), and *p*H values near *p*H 12. At intervals, aliquots of the alkaline fibrinogen were returned to *p*H 7.7 by the addition of 1 ml. of potassium phosphate of *p*H $\sim$ 7 and μ = 1.0. This return was accomplished either as a single rapid neutralization to determine the extent of alteration at the high *p*H or by adjusting to an intermediate *p*H and then, after a time interval, to *p*H 7.7.

The Optimal *p*H for Denaturation.—The fibrinogen concentration was held at 1.0 mg. per ml. throughout. At various times an aliquot of 4 ml. was brought to *p*H 7.7 in a single step, centrifuged at room temperature and the protein concentration in the supernatant determined. The series of *p*H values of 11.95, 12.06, 12.15, 12.29 and 12.5 was used. These *p*H values are those in the solutions at 25°, rather than at the temperature of the reaction vessel, which was 4°. Denaturation at *p*H 11.95 and 12.06 was considered to proceed too slowly to be of use, since only a 50% loss of solubility was recorded after 60 minutes. At *p*H 12.15, solubility dropped, quite uniformly in several experiments, to 10% of the initial value in 60 minutes and 5% in 120 minutes. There was no tendency for the supernatant optical density to increase after this time. At *p*H 12.19 and *p*H 12.5, while the loss of solubility was rapid, the solubility reached a minimum at about 30 and 10 minutes, respectively, and thereafter increased. This was particularly true of the sample at *p*H 12.5 and is taken to indicate the progress of irreversible changes. The optimum *p*H for denaturation was taken as *p*H 12.15.

Recovery of Solubility and Clottability.—Fibrinogen solutions containing 1.0 mg. of protein per ml. were allowed to stand for 60 minutes at *p*H 12.15, the other conditions being 4° and μ = 0.035. After 60 minutes, volumes sufficiently large so as to furnish a number of aliquots were returned, by adding potassium phosphate solution, to *p*H values of 10.71, 10.82, 10.98, 11.03 and 11.27, these now being the *p*H values at the incubation temperature of the solutions, namely, 25°. Aliquots of each intermediate solution were withdrawn at successive times, returned to *p*H 7.7 and precipitates removed after 1 hour. Solubility was recovered in each case, the recovery being complete within 4 to 6 hours. The most effective *p*H was *p*H 10.82 and the soluble protein, after incubation for 24 hours in the intermediate buffer at 25°, was 90% of the original. The other intermediate *p*H values and corresponding recoveries of solubility were *p*H 10.71, 70%; *p*H 10.98, 78%; *p*H 11.03, 45% and *p*H 11.27, 25%. The *p*H at which recovery is allowed to take place is thus seen to be critical, the optimum value being close to *p*H 10.8. Temperatures below 25° have been used. At 4° the rate of recovery is reduced but apparently not the extent.

Clottability was determined from supernatant optical densities after removal of clots (0.1 NIH unit thrombin per ml.; incubation time 1 hr.). The clot thus obtained was a granular or stringy precipitate and not a gel, an end result which is consistent with observations made with native fibrinogen at low concentrations.[23]

Clottability also returned to a major extent and as rapidly as solubility. Of the protein soluble at *p*H 7.7 an average of 70% was clottable, the range being from 60 to 79% with no clear differences between samples held in intermediate buffers for different lengths of time. A sizable fraction of the protein returned from an insoluble to a soluble form is thus non-clottable. The sedimentation coefficient of this soluble but non-clottable "fibrinogen" is close to that of native fibrinogen, as will be seen.

Sedimentation Behavior during Recovery.—Analytical runs were carried out on alkaline, intermediate and returned solutions. The alkaline solution, containing 1.04 mg. of protein per ml., was examined at 5°, full speed being attained 27 minutes after adjustment to *p*H 12.2 (μ = 0.045). A single, sharp, symmetrical peak was observed whose sedimentation coefficient was 5.34 *S*, a value in agreement with that given previously.

After 24 hours at the intermediate *p*H of 10.87 and 4°, 81% of the protein was soluble when the solution was returned to *p*H 7.7 and 25°. An aliquot of the intermediate solution, which contained about 0.92 mg. of protein per ml., was warmed to 25° and examined immediately in the ultracentrifuge. A single peak having a "fast" shoulder was observed. The sedimentation coefficient varied with centrifugation time from about S = 6 to 7, the best average value being $S \sim 6.5$. In view of the absence of fragmentation and the extent of reversibility under these conditions, it is clear that the decrease in sedimentation coefficient from $\sim$ 7.9 *S* to $\sim$ 6.5 *S* is due to molecular expansion.

The returned solution at *p*H 7.7 was first clarified of its insoluble material by centrifugation, the supernatant at this stage containing 0.67 mg. of protein per ml. At room temperature, a single peak having a sedimentation coefficient of S = 8.01 was observed. It is clear from the clottability of this material that a sizable fraction of these molecules, which have the same sedimentation coefficient as fibrinogen, will no longer react with thrombin.

Discussion

Expansion and Fragmentation.—At high *p*H fibrinogen yields two characteristic fragments. These may be described, approximately, as molecules having, respectively, M = 230,000 and S = 5.5 (A) and M = 14,500 and S = 1.77 (B). Each fibrinogen molecule yields one of the former and about eight of the latter. The A fragments show a pronounced tendency to aggregate on lowering

(23) D. F. Waugh and B. J. Livingstone, *Science*, **113**, 121 (1951)

the *p*H or increasing the ionic strength while the B fragments are relatively stable.

The best approximation to the configuration of native fibrinogen from sedimentation and diffusion[18] is an ellipsoid of revolution having a major axis of 860 Å. and a minor axis of 29 Å. A combination of partial specific volume and viscosity suggests corresponding axes of 640 and 34 Å. Shulman has suggested that the discrepancy might be resolved if the molecule had the characteristics of a nodular rod of about 600 × 50 Å. We may assume that the A fragment, since it accounts for about 70% of the fibrinogen molecule, represents a central rod to which the eight B fragments are attached thus yielding a nodose structure. We would expect the length of this rod to be close to that of the fibrinogen molecule. If the A fragment, at *p*H 13, carries 0.3 or 0.75 g. of water per gram protein, the corresponding axial ratios and lengths will be 27 and 724 Å. or 20 and 600 Å. The anhydrous B fragment has dimensions of 67 × 22 Å. (length and diameter) if it carries 0.3 g. of water per g. protein. The properties of the fragments at *p*H 13 suggest that they are not particularly unfolded since waters of less than 0.7 g. per protein are adequate in both cases and 0.3 g. water per g. protein would be acceptable.

Our experiments show that fibrinogen may undergo a change in frictional resistance without a change in molecular weight so that a sedimentation constant of $S = 5.5$ may be observed. Under other conditions, such as in the intermediate solutions at *p*H 10.87 (see Sedimentation Behavior during Recovery), the sedimentation coefficient of the expanded molecule appears to be $S \sim 6.5$. In order to alter $S = 8.1$ into $S = 5.5$ at constant M, the frictional resistance must increase by a factor of 1.47. Assuming an ellipsoidal shape, maintenance of a constant axial ratio would require about a fivefold increase in kinetic water. However, the fact that fragmentation follows expansion prompts a preliminary analysis on the assumption that the expansion of fibrinogen is due to the partial liberation of the B fragments. If each fragment contributes to the expanded molecule its individual frictional resistance the total frictional resistance would be

$$F = F_A + 8F_B \qquad (1)$$

where the molar frictional resistance, F, is readily obtained from M, S and $\bar{V}$ (= 0.72[24]). The frictional resistance of the equivalent anhydrous sphere in a solvent having a viscosity of 0.01 poise is

$$F_0 = 0.7514 \times 10^{15} M^{1/3} \qquad (2)$$

Table II lists certain values.

TABLE II

FRICTIONAL RESISTANCE PER ORIGINAL FIBRINOGEN MOLECULE

Condition	F (× 10^{+17})
Fibrinogen, F_0	0.53
Fibrinogen, F	1.20
Fragments, F_0	1.93
Fragments, F	3.00

(24) H. A. Scheraga, W. R. Carroll, L. F. Nims, E. Sutton, J. K. Backus and J. M. Saunders, *J. Polymer Sci.*, **14**, 427 (1954).

It is apparent that the process of expansion could lead at least to a total increase in frictional resistance by a factor of 2.5 and thus to a decrease in sedimentation coefficient from 8.1 to 3.2. On this basis, the maximum observed decrease to 5.5 S can be accounted for by a partial loosening.

Correlations of the studies reported here with those of Schauenstein and Hochenegger[25] and Klingenberg and Schauenstein[26] are instructive. These authors have examined fragmentation and the changes in ultraviolet absorption which take place in alkaline solutions. They report that above a *p*H of 12.7, or in the presence of cupraethylenediamine, fibrinogen and fibrin yield an insoluble fraction I amounting to 76% of fibrinogen (A fragment) and a soluble fraction II obtained in a yield of about 15% (B fragment). Fraction I was found to contain all of the tyrosine of fibrinogen and fraction II probably all of the arginine. In native fibrinogen, up to a *p*H of 11.6, only about 50% of the tyrosine groups appear to be free to ionize. Between a *p*H of 11.6 and 12.8 there is a gradual appearance of those which have been blocked. From the localization of groups, the *p*H range in which blocked phenolic groups are freed, and the *p*H at which fragmentation takes place, it is suggested[25] that a hydrogen bond between tyrosine and arginine side chains is responsible for bonding the fragments in fibrinogen.

No changes in absorption are noted below *p*H 11.6 although we have observed some expansion at *p*H 11.75. At *p*H 12.2, on the other hand, where expansion is extensive but fragmentation essentially absent at 5° and slow at 25° (see Fig. 2), about one-half of the blocked tyrosines are free.[25] Such observations suggest that expansion requires at least the breaking of an appropriate hydrogen bond. We are also in agreement that fragmentation and ultraviolet absorption changes are rapid and complete at *p*H 12.85 suggesting, as noted, that the tyrosine group is further involved in fragment integration.

The A fragment may not be a covalently bonded structure. We have found that at *p*H 12.85 in the presence of 6 M urea fibrinogen clearly decomposes into smaller units whose size is about $M \sim 40{,}000$. Quite frequently particles of varying length but considerably smaller than that of fibrinogen or the A fragment are observed in electron micrographs.[13] In view of the considerable stability of fibrinogen, we must conclude that the fragments seen in electron micrographs arise through a process which splits the original molecules along cleavage planes which may well be those demonstrated at high *p*H and in the presence of urea.[27] At least one cause of fragmentation may be strong local electrostatic repulsion due to a non-uniform distribution of charge, as is indicated to be present by the splitting out of acidic peptides during activation. The removal of

(25) E. Schauenstein and M. Hochenegger, *Z. Naturforsch.*, **8b**, 473 (1953).

(26) H. G. Klingenberg and E. Schauenstein, *ibid.*, **8b**, 473 (1953).

(27) Dr. C. E. Hall, Dept. of Bio., Mass. Inst. of Tech., has recently carried out studies of fibrinogen, using the electron microscope, which show that the smaller particles previously observed are products of the preparative procedures.

water would decrease the dielectric constant and increase repulsive forces correspondingly.

Reversibility of the Expanded States.—The existence of a group of soluble molecules which have the same sedimentation coefficient as fibrinogen but which are not activated by thrombin suggests that only small structural changes are needed to block activation. Were expansion to be uniform, the individual side chain and main chain segments must suffer considerable displacements in space with respect to each other. It seems unlikely that even a small fraction of the total population could return to a form both soluble and susceptible to thrombin activation. On the other hand the view that the fibrinogen molecule consists of a central core to which is attached smaller fragments is entirely acceptable for the mechanism of expansion suggested above does not require that the components themselves change radically their shape or kinetic water but only that the B units alter the mode of their attachment.

It is of importance that the intermediate *p*H leading to a maximum recovery lies at the juncture of the *p*H range where the fibrinogen molecule is entirely stable and the *p*H range where demonstrable expansion takes place. Evidently a rapid return from *p*H 12.2 to *p*H 7.7 stabilizes those structural changes which are responsible for insolubility, while at *p*H $\sim$ 10.8 the energy barriers between neighboring structures are sufficiently reduced so that a gradual return to the family of native structures is possible.

Molecular Stability and Activation.—Evidence in favor of the view that thrombin must act on the intact fibrinogen molecule is strong and may be summarized as follows: first, fragmentation does not take place at least up to *p*H 11.4 where the molecules carry a high net charge (more than 286 excess negative charges per molecule). It is to be noted that the *p*H maximum for the rate constant associated with activation is *p*H 7.6 and the rate constant falls rapidly on either side of this value.[5] The possibility that dissociation will occur at *p*H values in the range of *p*H 6 to *p*H 9, where each molecule carries a maximum of about 40–50 excess negative charges, seems remote since an attractive energy which can lead to stability at *p*H 11.4 will exert an effect which increases exponentially with the decrease in net charge. Second, in all probability the cleavage planes revealed by fragmentation at high *p*H are the weakest portions of the molecule and if dissociation occurred at all it would give essentially these fragments as subunits. Fragmentation has been found to be irreversible for there is no tendency for the fragments to reassociate to form molecules similar to fibrinogen. Indeed, the B fragment shows little tendency to aggregate while the A fragment shows a strong tendency to form either A aggregates or an A precipitate.[28] Third, as we have seen only slight structural changes are required to produce inactivation toward thrombin. Fourth, the inherent stability of the activated molecule is indicated not only by the absence of a molecular weight or sedimentation coefficient change on activation at higher *p*H values but also in the results of several physical investigations on the products obtained when fibrin is dissolved in urea, 1 *M* KBr at *p*H 5.3, etc. (see Ferry[29]). The products so obtained have the size and shape of fibrinogen, differing from fibrinogen only in electrophoretic mobility.

Recently, Caspary and Kekwick[12] have given evidence for the dissociation of human fibrinogen under normal conditions at low concentrations. We have also examined human fibrinogen under similar experimental conditions and have found no evidence for dissociation down to concentrations of 0.01%.

From the above it is evident that activation must involve the intact fibrinogen molecule and thus we might expect the critical contact required for activation to be complex. The complexity may be considerably less than that indicated at present by the chemical changes, for the chemical events associated with activation and those taking place after activation have not yet been separated unambiguously.

CAMBRIDGE, MASS.

(28) Reversibility after fragmentation has been reported.[25,26] At the *p*H values used, however, it is unlikely that splitting had occurred.

(29) J. D. Ferry, *Physiol. Rev.*, **34**, 753 (1954).