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The Conversion of Fibrinogen to Fibrin. VIII. Sedimentation and Viscosity Studies on Clotting Systems Inhibited by Urea and on Solutions of Fibrin in Urea[1,2]

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The sedimentation constant and intrinsic viscosity of fibrinogen in solutions of urea at concentrations up to 3.5 *M* (*p*H 6.3 and 7.5, ionic strength 0.15) are similar to the corresponding values in the absence of urea. When fibrinogen reacts with thrombin in the presence of 1.0 *M* urea at *p*H 6.3 or 2.35 *M* urea at *p*H 7.5, no clot is formed but a new component appears in the ultracentrifuge with a sedimentation constant extrapolated to infinite dilution of about 24 *S*. At the same time the viscosity increases and becomes non-Newtonian; the apparent viscosity depends on velocity gradient at gradients as low as 10 sec.$^{-1}$, as measured by a falling sphere technique with glass spheres about 0.1 mm. in diameter. These systems are very similar to those previously studied in which hexamethylene glycol rather than urea is employed as the inhibitor. The sedimentation constant and intrinsic viscosity of fibrin dissolved in 3.5 *M* urea are similar to the corresponding values for fibrinogen, indicating that the fragments resemble fibrinogen in size and shape. When a solution of fibrin in 3.5 *M* urea at *p*H 7.5 is diluted to reduce the urea concentration to 2.35 *M*, at constant *p*H and ionic strength, a new component again appears in the ultracentrifuge with a sedimentation constant extrapolated to infinite dilution of about 24 *S*. At the same time the viscosity increases and becomes non-Newtonian. Similar results are obtained by replacing the 3.5 *M* urea with 0.5 *M* hexamethylene glycol. The fast component in sedimentation and the high non-Newtonian viscosity are attributed to an intermediate fibrinogen polymer with twice the cross-section area of fibrinogen and a range of lengths of the order of ten times that of fibrinogen. It can evidently be formed either directly from fibrinogen by the action of thrombin or from the fibrinogen-shaped fragments obtained by dissolving fibrin in concentrated urea.

Urea, like hexamethylene glycol (and a number of other substances), is a reversible inhibitor of the conversion of fibrinogen to fibrin; at a concentration of about 0.5 *M* (at *p*H 6.3, ionic strength 0.45), it prevents clotting, but does not permanently damage either fibrinogen or thrombin.[3] In fibrinogen–thrombin systems inhibited by hexamethylene glycol, although clotting does not occur, there is nevertheless a partial, arrested polymerization of the fibrinogen which is revealed by sedimentation and viscosity measurements.[4] Similar sedimentation and viscosity studies on urea-inhibited systems are reported in the present paper.

In addition, urea, unlike hexamethylene glycol, can be employed to attack the problem of the structure of fibrin from the opposite direction, since in concentrated solution it dissolves clots.[5-7] Mihályi[6] showed that the properties of solutions of fibrin in urea depended on the urea concentration and *p*H, some having low specific viscosities of the order of those of fibrinogen solutions, while others had much higher viscosities and appeared to be thixotropic. He did not, however, measure the dependence of viscosity on concentration or on velocity gradient. The latter properties, together with the sedimentation behavior of solutions of fibrin in urea, have been investigated in the present study; striking similarities between what may be termed partly depolymerized fibrin and partly polymerized fibrinogen have been found, as briefly reported in a recent note.[8]

(1) This is Paper 10 of a series on "The Formation of Fibrin and the Coagulation of Blood" from the University of Wisconsin, supported in part by research grants from the National Institutes of Health, Public Health Service. This work was supported also by a grant from Eli Lilly and Company, and by the Research Committee of the Graduate School of the University of Wisconsin from funds supplied by the Wisconsin Alumni Research Foundation.

(2) Based in part on the Ph.D. Thesis of Paul Ehrlich (present address: National Bureau of Standards, Washington, D. C.).

(3) S. Shulman, *Arch. Biochem.*, **30**, 353 (1951).

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

(5) K. Laki and L. Lóránd, *Science*, **108**, 280 (1948).

(6) E. Mihályi, *Acta Chem. Scand.*, **4**, 344 (1950).

(7) Dissolution of fibrin has been observed in this Laboratory also in solutions of guanidine hydrochloride and histamine dihydrochloride.

(8) S. Shulman, P. Ehrlich and J. D. Ferry, THIS JOURNAL, **73**, 1388 (1951).

Materials and Methods.—Bovine fibrinogen (Fraction I, Preparation 129-18, protein 70–73% clottable) and thrombin (Preparation C-173B, 4.7 units per mg.) were obtained from Armour and Company. For some experiments, the Fraction I was refractionated by alcohol precipitation as described in Paper III of this series,[4] to give a preparation with 89–91% clottable protein, hereafter referred to as "refractionated." From the report of Lóránd[9] and others that 3 to 4% of the mass of fibrinogen is split off during conversion to fibrin, it would follow that about 93% of the protein in this preparation was fibrinogen. The urea was a commercial product of reagent grade.

Stock fibrinogen solutions were prepared as previously described,[4] being dialyzed either against 0.45 *M* sodium chloride or against a buffer containing sodium phosphate of ionic strength 0.05 with the remainder of the specified ionic strength made up by sodium chloride. Fibrinogen assays were made by the method of Morrison.[10] Solutions containing urea were made up by mixing solutions of stock fibrinogen, concentrated urea, concentrated buffer, and salt to give the desired concentrations of the various components. In kinetic studies of inhibited systems, the reaction time was reckoned from the introduction of the thrombin, which was always added last.

To prepare a solution of fibrin, a clot (of the opaque type) was first formed in cellophane tubing, at *p*H 6.3, ionic strength 0.45, fibrinogen 10 to 15 g./l., and thrombin 1 unit/ml. The clotting time under these conditions[3] is about 10 min. The cellophane tube, tied at both ends, was kept in a stoppered test-tube (containing a few drops of salt solution) for about 4 hr. to permit the conversion to fibrin to approach completion.[11] It was then immersed in a large volume (about 50 times the clot volume) of a 3.5 *M* urea solution of ionic strength 0.15 (0.10 sodium chloride and 0.05 phosphate buffer). The high urea concentration required for dissolution was thus achieved by dialysis rather than addition of solid urea, as used by Mihályi[6]; it is desirable to avoid even temporary urea concentrations above 3.5 *M*, because above 4 *M* denaturation is apparent[12] (see below). The *p*H at which the dialysis and resulting clot dissolution were carried out was 6.3 in some experiments and 7.5 in others; the temperature was usually that of the room (25 ± 1°) but sometimes 3°. The time of dialysis was 12 to 20 hr., with constant stirring to hasten equilibration. Since the volume of the cellophane tube changed slightly during dialysis, the protein concentration in the final solution was somewhat different from that of the original clot; it was calculated with adequate accuracy by opening the tube, removing the solution as completely as possible, weighing the solution, and calculating the volume from the density of the solvent. Dissolution was complete except for occasional

(9) L. Lóránd, *Nature*, **167**, 992 (1951); K. Laki, personal communication.

(10) P. R. Morrison, THIS JOURNAL, **69**, 2723 (1947).

(11) J. D. Perry and P. R. Morrison, *ibid.*, **69**, 388 (1947).

(12) E. Mihályi, *Acta Chem. Scand.*, **4**, 317 (1950).

traces of fibers or gel fragments, which amounted to less than 1% of the total volume; these were removed by filtration through a stainless steel screen to give the stock fibrin solution.

Dilutions of a stock fibrinogen or fibrin solution were made by adding a solvent which contained the same concentrations of urea, salt and buffer and had the same *p*H as the stock. The viscosity and density of this solvent were used in calculations of relative viscosities, corrected sedimentation constants, etc. of the protein solutions.

Viscosity measurements were made in Ostwald viscosimeters, in which the maximum velocity gradient[13a] (*i.e.*, the gradient at the capillary wall) for water at 25° was 2100 sec.$^{-1}$ as calculated from the formula[13b] $G_m = hg\rho r/2\eta l$, where h is the average distance between levels, g the acceleration of gravity, ρ and η the density and viscosity of the solution, and r and l the radius and length of the capillary. The velocity gradients for various solutions studied were calculated from the observed flow times. When possible, intrinsic viscosities were obtained by extrapolating plots of η_{sp}/c against c to zero c, where η_{sp} is the specific viscosity and c the concentration of fibrinogen (or fibrin) in g. per deciliter. The concentration used was that of fibrinogen or fibrin, respectively, the small contribution of other protein to the viscosity being ignored. Viscosity measurements were also made with a special falling sphere technique which is described in detail in the following section.

Centrifugation experiments were made both with the Svedberg oil turbine ultracentrifuge, Department of Chemistry (through the kindness of Professor J. W. Williams), and the Spinco electrically driven ultracentrifuge, Department of Physiological Chemistry (through the kindness of Professor P. P. Cohen). The rotor speeds were 50.4 × 10^3 and 59.6 × 10^3 revolutions per minute, respectively. Sedimentation constants were calculated from the slopes of plots of the logarithm of the distance from the axis of rotation to the center of the schlieren peak against the time corrected for viscosity changes in the solvent due to changes in temperature. The partial specific volume of the protein was taken as 0.75. Measurements were made at several fibrinogen (or fibrin) concentrations from 1 to 7 g./l. and extrapolated to infinite dilution.

Technique of Falling Sphere Viscosity Measurements.—Because some of the solutions studied showed non-Newtonian flow, it became of interest to measure viscosities at lower velocity gradients than those provided in the Ostwald viscosimeters. Reduction of the gradient by decreasing the capillary bore was undesirable because of possible significant changes in capillary radius due to adsorption of denatured protein as well as danger of plugging by traces of foreign material. An entirely different method was therefore sought.

The falling sphere method is ordinarily applied to highly viscous systems such as honey or concentrated polymer solutions, and is not considered suitable for liquids like water whose viscosities are in the centipoise range. Nevertheless, by using very small glass spheres, of the order of 0.1 mm. in diameter, it was found that such viscosities could be successfully measured within a precision of a few per cent. The maximum velocity gradient was much smaller than in the Ostwald viscosimeters, and it could be varied by using spheres of different sizes. This technique also has the advantage that the body of the liquid is less disturbed than in either the Ostwald or Couette type of apparatus, and there is less opportunity for surface denaturation in protein solutions since the ratio of glass area to volume of solution is relatively small.

Success of the method depended on the availability of miniature glass beads accurately spherical in shape, which are used by the Minnesota Mining and Manufacturing Company in self-reflecting highway signs. The spheres were selected and their diameters were measured individually, several at a time, under a microscope with a micrometer ocular. A single measured sphere was picked up by a steel needle moistened with glycerol and introduced into the top of a column of solution; its velocity of fall was then timed. The solution was contained in a selected Pyrex tube 8 mm. in diameter and 75 cm. long. Several of these tubes were mounted in a thermostat, with provision for adjusting their axes accurately vertical, and illuminated by a bright fluorescent lamp from behind. The descent of the sphere could be followed with the unaided eye, even for a diameter as small as 0.1 mm.

According to Stokes' law, for a Newtonian liquid the velocity of fall should be proportional to the square of the sphere diameter for spheres of different sizes in the same liquid. (The effect of the container wall, referred to below, would introduce a small correction but would be nearly constant.) Tests in water and sucrose solution showed this to be true provided that the sphere diameter did not exceed a critical value. From the work of Arnold,[14] it would be expected that for spheres above a certain critical diameter inertia effects would cause the velocity to be less than that given by Stokes' law; according to his experience, deviations should appear when the diameter (in cm.) exceeds $2.8[\eta^2/g(\rho_1 - \rho_2)\rho_2]^{1/3}$. (Here η is the viscosity of the liquid, g the acceleration of gravity, and ρ_1 and ρ_2 the densities of the sphere and the liquid, respectively.) For a viscosity of 1 centipoise with densities of 2.5 and 1.0, respectively, the critical diameter is calculated to be 0.11 mm., and our measurements confirmed this value.

The density of the spheres was stated by the manufacturer to be in the neighborhood of 2.5; correct results were obtained for liquids of known viscosity by using the value 2.54. On one microscope employed, the diameters could be measured directly with the micrometer ocular; on another, it was necessary to apply a constant correction (attributed to a diffraction or reflection error at the edge of the sphere) which never exceeded 4%, to obtain consistent results on a series of liquids of known viscosity. With these adjustments, viscosities were calculated by the Ladenburg formula

$$\eta = gd^2(\rho_1 - \rho_2)t/18s(1 + 2.4d/D)$$

where t is the time required to traverse distance s, and d and D are the diameters of the sphere and tube, respectively.

Representative results are shown in Fig. 1 for two sucrose solutions and for one inhibited clotting system in 2.35 *M* urea at *p*H 7.5. Apparent viscosities measured with different sizes of spheres are plotted against the maximum velocity gradient calculated by the formula $G_m = gd(\rho_1 - \rho_2)/6\eta$. The data for the sucrose solutions are in close agreement with the viscosities determined by the Ostwald viscosimeter, represented by horizontal lines. The clotting solution, whose high viscosity permits a greater range of sphere sizes without exceeding the critical limit, shows a distinct decrease of the apparent viscosity from 8.5 to 6.2 centipoise as the velocity gradient is increased from 30 to 140 sec.$^{-1}$. At a still higher value of G_m = 593 sec.$^{-1}$, obtained in the Ostwald viscosimeter, the apparent viscosity of this solution was lower still, being only 3.6 centipoise.

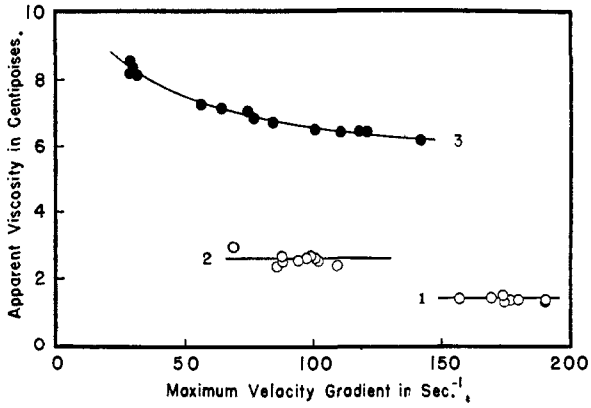

Fig. 1.—Viscosity at 25° determined by falling sphere method, plotted against maximum velocity gradient: 1 and 2, sucrose solutions (160 and 320 g./l., respectively); 3, inhibited clotting system in 2.35 *M* urea, *p*H 7.5, ionic strength 0.15, fibrinogen concentration 7 g./l., thrombin 1 unit/ml., reaction time 42 to 43 hr. at 25°.

(13) (a) The maximum velocity gradient is used rather than the more usual average velocity gradient (which for a capillary viscosimeter is smaller by a factor of 3/4) for purposes of comparison with falling sphere viscosity measurements, in which there is no obvious definition for the average gradient. (b) H. Lamb, "Hydrodynamics," Dover Publications, New York, 1945.

(14) H. D. Arnold, *Phil. Mag.*, [6] **22**, 755 (1911).

Properties of Fibrinogen in Urea Solutions.—The behavior of fibrinogen without thrombin in the presence of urea was first investigated by viscosity and sedimentation measurements as well as by qualitative tests of denaturation. Table I

TABLE I

SEDIMENTATION AND VISCOSITY MEASUREMENTS ON FIBRINOGEN (FRACTION I), WITH AND WITHOUT UREA, AT IONIC STRENGTH 0.15

Expt. no.	Urea concn., M	pH	$[\eta]$ (g./100 ml.)$^{-1}$	$d(\eta_{sp}c)/dc$, (g./100 ml.)$^{-2}$	$S_{20,w}$ svedb. extrap.	UC[a]
8	0	6.3	0.31	0.14		
9	0	6.3	.25	.12		
10	0	6.3	.32	.16		
18	0	6.3			7.9	Sp
20[b]	0	6.3	.30	.04		
11	0	7.5	.32	.24		
12	2.35	7.5	.34	.09		
13	2.35	7.5	.36	.11		
14	3.0	6.3	.36	.08		
15	3.5	6.3	.39	.06		
16	3.5	6.3	.38	.06	9.0	OT
17	3.5	6.3	.34	.05		
19	3.5	6.3			8.0	Sp

[a] OT, Svedberg oil turbine; Sp, Spinco ultracentrifuge. [b] Refractionated fibrinogen, prepared by precipitation at 0° at ionic strength 0.08; measurements at ionic strength 0.45.

summarizes values of the intrinsic viscosity, $[\eta]$, the slope of the plot of reduced specific viscosity against concentration from which the intrinsic viscosity is obtained, and the sedimentation constant reduced to water at 20° and extrapolated to zero protein concentration. All these measurements (except one viscosity experiment) were made at ionic strength 0.15 (0.10 sodium chloride and 0.05 phosphate). The pH values and urea concentrations were chosen to correspond to those used for inhibition and dissolution experiments as described below. These measurements were made on Fraction I rather than refractionated material. However, previous studies[4] have shown no perceptible difference in sedimentation constant or intrinsic viscosity between these two preparations.

The intrinsic viscosities in the absence of urea are in reasonable agreement (except for one value) with the figure of 0.34 previously reported for both Fraction I and refractionated material at ionic strength 0.45, and the values in 2.35 M urea at pH 7.5 and in 3.5 M urea at pH 6.3 are only a little higher.[15] The sedimentation constant in the latter solvent also agrees with the value in the absence of urea and with the previously reported figure[4] of 8.4 S. It may be concluded that the fibrinogen has not suffered any marked changes in size or shape in these solvents.[16]

(15) Table I shows that the slopes of the intrinsic viscosity extrapolations vary considerably with solvent composition; this may be explained qualitatively by differences in intermolecular interaction, probably largely electrostatic. The slope at ionic strength 0.45 is much less than that at 0.15, indicating that the latter salt concentration is insufficient to suppress electrostatic effects; and at ionic strength 0.15 the slope is greater at pH 7.5 than at 6.3, as might be expected from the higher net charge on the protein. Urea evidently diminishes the slope at both pH values.

(16) The sedimentation constant measured in the oil turbine ultracentrifuge appears to be somewhat higher than that in the Spinco, here and also in Table II. A similar discrepancy has been noted by others.[17a,b] The differences are not large enough to affect any conclusions drawn in the present study, however.

Further evidence that denaturation of fibrinogen did not occur in 2.35 M or even 3.5 M urea at pH 7.5 was obtained from tests in which fibrinogen solutions in this solvent were kept for 16 hr. at room temperature and then the urea was removed by dialysis (at constant pH and ionic strength); there was no precipitation nor obvious change in viscosity. When similar tests were made in 3.5 M urea at pH 6.3, coagulation occurred upon removal of urea at ionic strength 0.15 but not at ionic strength 0.45, it may be concluded that some irreversible changes take place in this solvent, even though the size and shape of the fibrinogen are not significantly altered. These results are in qualitative accord with the studies of Mihályi,[12] from which it would be expected that in 3.5 M urea at pH 7.5 fibrinogen should be stable for times of the order of several hours, while denaturation would occur more rapidly at pH 6.3. The rate of denaturation decreases very rapidly with decreasing urea concentration, so denaturation introduces no complications at the lower levels of urea employed in the inhibition experiments described in the following section.

Inhibited Clotting Systems.—Since the effectiveness of urea as an inhibitor decreases with increasing pH in the neutral zone at ionic strength 0.15, the inhibited polymerization reaction with thrombin proceeds at comparable rates in 1.0 M urea at pH 6.3 and in 2.35 M urea at pH 7.5. The reaction was followed at 25° in these two solvents by sedimentation and viscosity measurements, just as in previous experiments with hexamethylene glycol as the inhibitor,[4] and with closely similar results.

After reaction for one day, the sedimentation diagram revealed two peaks; an example is shown in Fig. 2. The sedimentation constants of these two components, extrapolated to infinite dilution, are given in Table II, together with the values obtained in hexamethylene glycol-inhibited systems.[4]

TABLE II

SEDIMENTATION CONSTANTS, EXTRAPOLATED TO INFINITE DILUTION, OF SLOW AND FAST COMPONENTS OF INHIBITED CLOTTING SYSTEMS (FRACTION I; TIME OF REACTION 1 TO 2 DAYS)

Inhibitor	Concentration, M	pH	Ionic strength	Concentration Fibrinogen, g./l.	Concentration Thrombin unit/ml.	$S_{20,w}$, slow extrap.	$S_{20,w}$, fast extrap.	UC[a]
Urea	1.0	6.3	0.15	4.0	1.0	7.8	24	Sp
Urea	2.35	7.5	.15	7.0	1.0	7.8	22	Sp
Hexamethylene glycol[4]	0.36–0.41	6.3	.45	3.7	0.7	8.5	24.5	OT

[a] OT, Svedberg oil turbine; Sp, Spinco ultracentrifuge.

It is evident that the same species are present in all three systems, and that the slow component has the same sedimentation constant as unaltered

(17) (a) G. Kegeles and F. J. Gutter, THIS JOURNAL, 73, 3770 (1951); (b) J. F. Taylor, private communication.

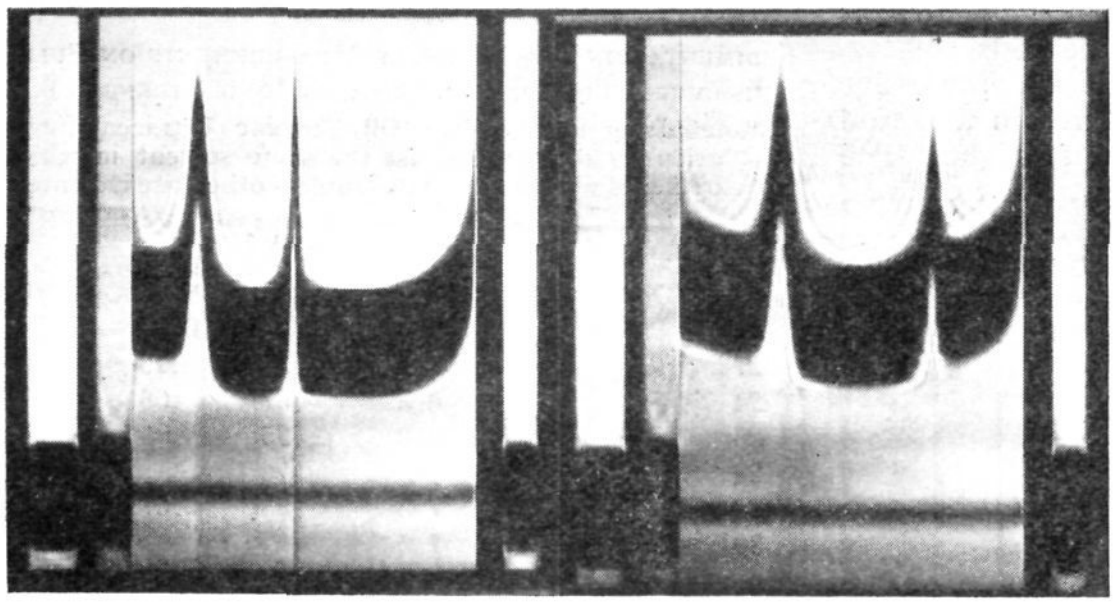

Fig. 2.—Sedimentation diagram of a urea-inhibited clotting system (with Fraction I); fibrinogen 4 g./l., thrombin 1.0 unit/cc., *p*H 6.3, ionic strength 0.15, urea 1.0 *M*, reaction time 25 hr.; sedimented in Spinco ultracentrifuge at 59,600 r.p.m.; photographs at 20 and 32 min., respectively, after attaining top speed.

fibrinogen. The experiments were not extensive enough to demonstrate with urea, as with hexamethylene glycol, that the sedimentation constants remained unchanged with progressive reaction nor to follow the changes in peak areas with time. After two days, the area corresponding to the fast component was about 30% of the total, and this proportion did not change with dilution from 4 to 2 g./l. Below 2 g./l., the areas could not be estimated. The slow component is attributed to unaltered fibrinogen (or a chemically altered molecule with the same gross size and shape), and the fast component is attributed to an intermediate polymer.

The viscosities of inhibited clotting mixtures increased progressively with time, as previously observed with hexamethylene glycol. Instead of appearing to approach a constant value, however, the viscosity passed through a maximum and slowly declined. In the urea-inhibited mixtures, the falling sphere method was used to follow changes in viscosity with time, instead of the capillary method,[4] because spheres could be conveniently dropped at intervals through a single sample of solution with negligible disturbance of the system. In such a series, spheres were selected with as nearly the same diameters as possible, to avoid introducing another variable. Plots of viscosity against time are shown in Fig. 3; they indicate that the polymerization reaction is somewhat slower at 2.35 *M* urea, *p*H 7.5, than at 1.0 *M* urea, *p*H 6.3; and that the reaction rate depends on the concentration of fibrinogen present. The ordinate is designated *apparent* viscosity because each value is characteristic of the velocity gradient prevailing in that determination. Because of the non-Newtonian character of these solutions, as illustrated in Fig. 1, no quantitative conclusions concerning the rate of polymerization can be drawn from such viscosity measurements at single velocity gradients, nor from the corresponding measurements in hexamethylene glycol reported previously.[4]

The falling sphere method served also to study the dependence of apparent viscosity on velocity gradient by dropping spheres of different sizes through an inhibited clotting mixture within a short time interval. When combined with an Ostwald viscosity measurement, this provided a considerable range of velocity gradients. The data plotted as curve 3 of Fig. 1 refer to a system in 2.35 *M* urea, *p*H 7.5 (*cf.* curve 2, Fig. 3) at reaction times between 42 and 43 hr., when the properties of the solution are changing only slightly with time. The drop in apparent viscosity with increasing velocity gradient from 30 to 140 sec.$^{-1}$ is not surprising in view of flow birefringence measurements on comparable inhibited systems in hexamethylene glycol, reported in Paper VI of this series.[18] The above range of velocity gradients corresponds to values of the parameter $G\eta_0/T$, which determines flow orientation, between 0.001 and 0.05; and flow birefringence reveals progressive orientation of elongated particles occurring in exactly this range. Such orientation, of course, causes a progressive fall in apparent viscosity.[19] Unfortunately, the data do not permit a safe extrapolation of the apparent viscosity to zero velocity gradient, and there is no adequate theory for quantitative interpretation of the values at finite velocity gradients to estimate the shape of the elongated aggregates present. Nevertheless, the observation of non-Newtonian flow is a valuable

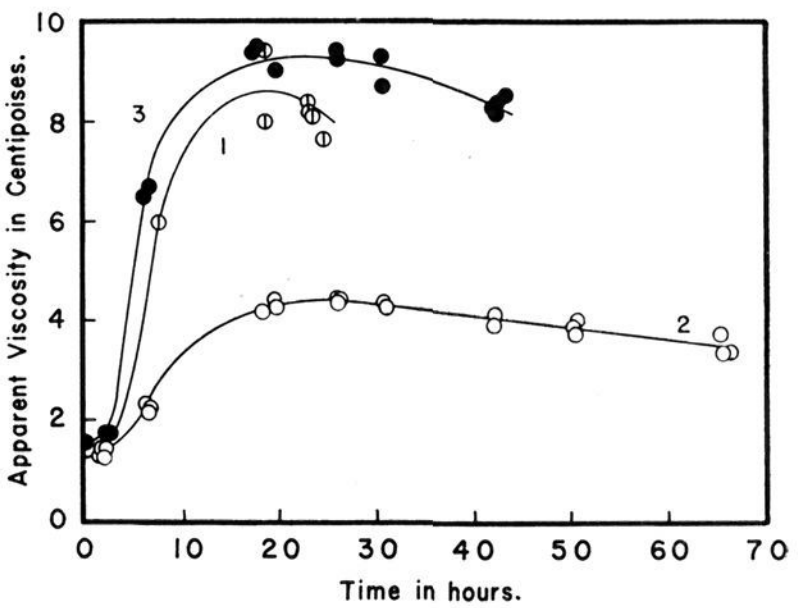

Fig. 3.—Increase in viscosity at 25° with time in urea-inhibited clotting systems, at ionic strength 0.15, thrombin concentration 1 unit/cc.: curve 1, urea 1.0 *M*, *p*H 6.3, fibrinogen 4 g./l.; curve 2, urea 2.35 *M*, *p*H 7.5, fibrinogen 4 g./l.; curve 3, urea 2.35 *M*, *p*H 7.5, fibrinogen 7 g./l. Measurements by the falling ball method with spheres corresponding to maximum velocity gradients (G_m) of 250 to 290 sec.$^{-1}$ for water.

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

(19) J. R. Robinson, *Proc. Roy. Soc.* (*London*), **A170**, 519 (1939).

confirmation of the presence of such aggregates, as deduced from other experimental methods.[20]

Viscosity measurements were made on progressive dilutions of stock inhibited mixtures in a period when the change with time was slight. The diluent contained the same concentration of urea, of course, as the stock. Results from both methods are shown in Fig. 4, where the reduced specific viscosity is plotted against the concentration of (original) fibrinogen. The value from the sphere

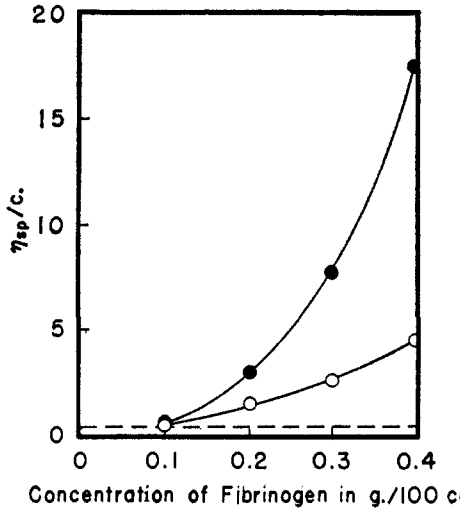

Fig. 4.—Reduced specific viscosity at 25° plotted against concentration for urea-inhibited clotting system (*p*H 6.3, ionic strength 0.15, urea 1.0 *M*, original fibrinogen concentration 4 g./l., thrombin 1 unit/cc.). Reaction time before first dilution, 24 hr.: ●, falling sphere method (G_m = 270 for water); O, capillary method (G_m = 2100 for water). The dashed line represents the behavior of fibrinogen.

method is always higher than that from the Ostwald, so non-Newtonian flow is always present, but the difference diminishes with dilution and both values appear to approach an intrinsic viscosity not far from that of fibrinogen (whose corresponding plot, on this scale, appears as a nearly horizontal line). This indicates, as in the hexamethylene glycol experiments,[4] that the elongated aggregates responsible for the high viscosity dissociate with dilution. It must be recognized, however, that the drop in η_{sp}/c is exaggerated by the non-Newtonian character of these solutions. The use of a single Ostwald viscosimeter, or the use of spheres of nearly constant diameter in the falling sphere technique, corresponds to conditions of constant average shearing stress; with dilution, the average (and maximum) velocity gradient increases; so that even in the absence of dissociation there would be an increased non-Newtonian deviation which would cause a drop in the apparent reduced specific viscosity. These two effects can be separated only by recourse to other experimental methods, such as light scattering.

Solutions of Fully Depolymerized Fibrin.—Sedimentation and viscosity data for solutions of fibrin in 3.5 *M* urea are summarized in Table III. The intrinsic viscosity and the sedimentation con-

(20) Unpublished viscosity studies on clotting systems inhibited by hexamethylene glycol have shown exactly similar behavior.

TABLE III

SEDIMENTATION AND VISCOSITY MEASUREMENTS ON FULLY DEPOLYMERIZED FIBRIN IN UREA, AT IONIC STRENGTH 0.15

Sedimentation in Svedberg Oil Turbine Ultracentrifuge; dissolution by dialysis against the same solvent in which measurements were made at 25°, unless otherwise indicated.

Expt. No.	F'gen[a]	Urea concn., *M*	*p*H	$[\eta]$ (g./100 ml.)$^{-1}$	$d(\eta_{sp}/c)/dc$, (g./100 ml.)$^{-2}$	$s_{20,w}$ extrap.
26	Rf	2.0[b]	6.3[b]	0.34	0.07	
27	Rf	3.0[b]	7.4[b]	.34	.10	
24	FrI	3.5	6.3	.37	.06	
25	Rf	3.5	6.3	.35	.06	
29	Rf	3.5	6.3			9.8
28	FrI	3.5	7.5	.35	.37	
30	FrI	3.5[c]	7.5			8.8

[a] FrI, Fraction I; Rf, refractionated. [b] Dissolved at *p*H 6.3, urea concentration 3.5 *M*, and brought to the indicated *p*H and urea concentration by a second dialysis. [c] Dissolved at 3°.

stant extrapolated to infinite dilution, both at *p*H 6.3 and at 7.5, are very close to the values for the original fibrinogen, indicating that the fragments formed by the dissolution process have the same gross size and shape as fibrinogen molecules. It has been reported by Steiner and Laki[21] that light scattering measurements on fibrin dissolved in 6 *M* urea at *p*H 8.4 provide the same molecular weight as that of fibrinogen in the same solvent, in agreement with the above conclusion. (Of course, the fragments cannot be identical with fibrinogen if peptides have been split off during clotting,[9] but the loss of 3 or 4% of the molecule in this way would hardly affect the gross size and shape as measured by any of these methods.) It is for this reason that the solutions described in Table III are denoted "fully depolymerized fibrin." As shown in the table, the urea concentration can be reduced after dissolution to 2.0 *M* at *p*H 6.3 or 3.0 *M* at *p*H 7.4 without altering the intrinsic viscosity of the fibrin fragments. Further reduction of the urea concentration, however, causes marked viscosity increases, as observed by Mihályi[6]; such systems are denoted "partly depolymerized fibrin" and are described in the following section.

The data of Table III indicate that denaturation—*i.e.*, marked structural changes in the fibrinogen units—has not occurred under the conditions of dissolution. Further evidence was provided by the fact that the viscosity of a solution of fully depolymerized fibrin in 3.5 *M* urea remained constant for at least 48 hours at room temperature; while a similar solution in 4 *M* urea, above the level used in the experiments reported in Table III, showed an increased turbidity and viscosity which increased with time, indicating denaturative changes. Dialysis of solutions of fibrin in 3.5 *M* urea against buffered salt solutions to remove the urea has repeatedly given gels which closely resemble the original fibrin (*cf.* Mihályi[6]); these gels, observed in the electron microscope, have the characteristic network structure of fibrin.[22]

(21) R. F. Steiner and K. Laki, THIS JOURNAL, 73, 882 (1951); *Arch. Biochem. Biophys.*, 34, 24 (1951).

(22) Electron microscope observations were made in collaboration with Dr. P. Kaesberg, of the Department of Biometry and Physics and Department of Soils.

Solutions of Partly Depolymerized Fibrin.—Solutions of fully depolymerized fibrin (prepared from Fraction I), containing 7 g. of fibrin per l. in 3.5 *M* urea at *p*H 7.5, were diluted with equal volumes of 1.2 *M* urea at constant salt and *p*H to reduce the urea concentration to 2.35 *M*, the level employed in inhibited clotting systems at this *p*H. The apparent viscosity rose sharply within an hour and then remained fairly stable (though decreasing slightly) for a number of hours; an example of measurements in the Ostwald viscosimeter is shown in Fig. 5. Measurements with the falling sphere

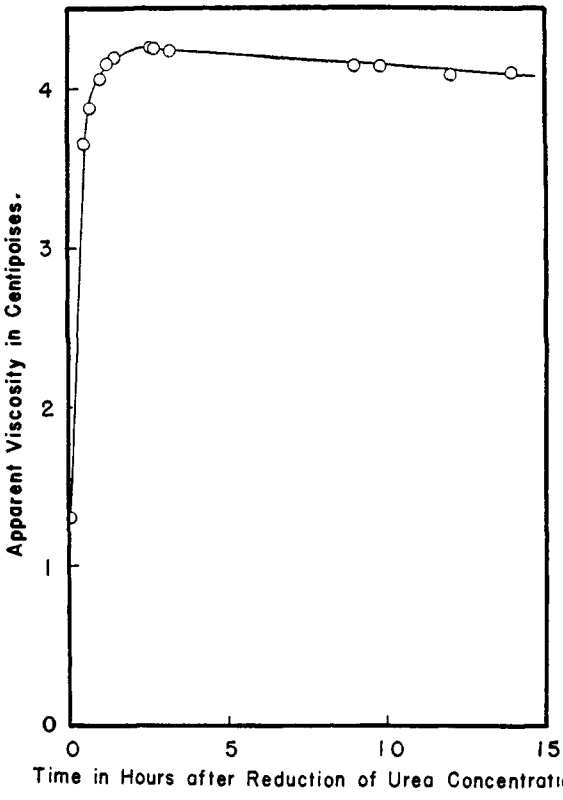

Fig. 5.—Apparent viscosity at 25° measured by capillary viscosimeter (G_m = 2100 for water) for dissolved fibrin, 6.8 g./l., *p*H 7.5, ionic strength 0.15, plotted against the time after reduction of urea concentration from 3.5 to 2.35 *M*.

technique at different velocity gradients, performed after the maximum viscosity had been attained and subsequent changes with time were slight, showed that such solutions were markedly non-Newtonian. One set of data at 20 and 25° is depicted in Fig. 6, where a corresponding curve for an inhibited clotting system (curve 3 of Fig. 1) is included for comparison. When diluted with solvent, these fibrin solutions suffered a marked drop in viscosity, just like the inhibited clotting systems; Fig. 7 shows reduced specific viscosity plotted against concentration for one series of dilutions together with single values obtained in several different experiments, and the curve closely resembles the corresponding one in Fig. 4.

The viscosity behavior of these fibrin solutions is clearly very similar to that of inhibited clotting systems in which arrested polymerization has occurred at the same urea concentration and *p*H.

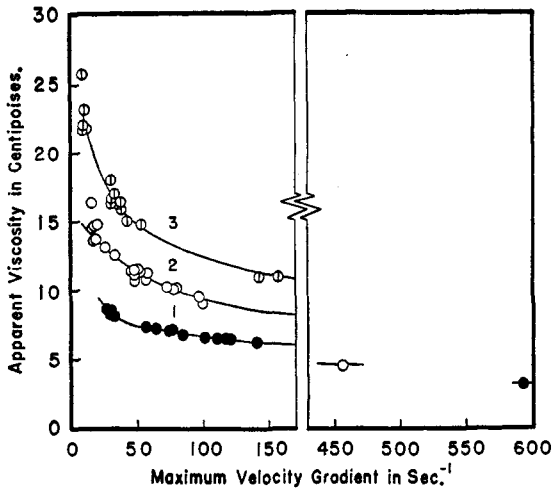

Fig. 6.—Dependence of apparent viscosity on maximum velocity gradient in solutions of partly depolymerized fibrin: curve 1, curve for inhibited clotting system reproduced from Fig. 1; curve 2, fibrin (7 g./l.) in 2.35 *M* urea, *p*H 7.5, ionic strength 0.15, at 25°; curve 3, the latter system at 20°. The two points at high velocity gradient are capillary measurements.

This indicates that reduction of the urea concentration has permitted the fibrinogen-shaped fragments formed by clot dissolution to aggregate partially, giving the same intermediate polymer which is formed directly from fibrinogen in inhibited clotting systems. Confirmation of this conclusion is provided by sedimentation measurements; the solutions in 2.35 *M* urea exhibit two peaks in the ultracentrifuge; and the sedimentation constants of the two components, which are plotted against concentration in Fig. 8, extrapolate to values of 9 and 24 Svedberg units, identical with

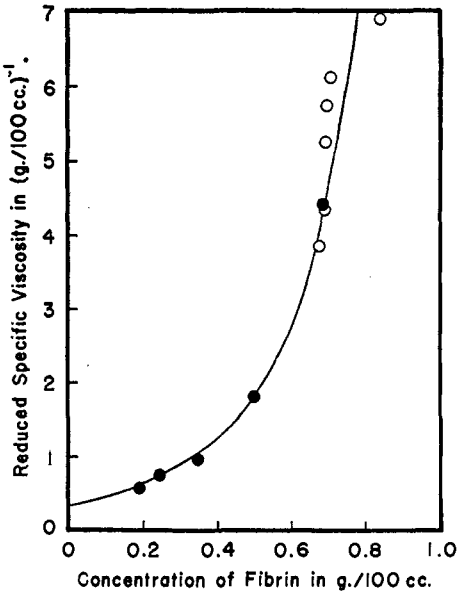

Fig. 7.—Reduced specific viscosity at 25° (measured by capillary viscosimeter, G_m = 2100 for water) of dissolved fibrin in 2.35 *M* urea, *p*H 7.5, ionic strength 0.15, plotted against protein concentration: ●, dilutions from the system referred to in Fig. 5; ○, different systems at their viscosity maxima.

those of the clotting mixtures inhibited by either urea (Table II) or hexamethylene glycol.[4] It is for this reason that the term "partly depolymerized fibrin" is employed.

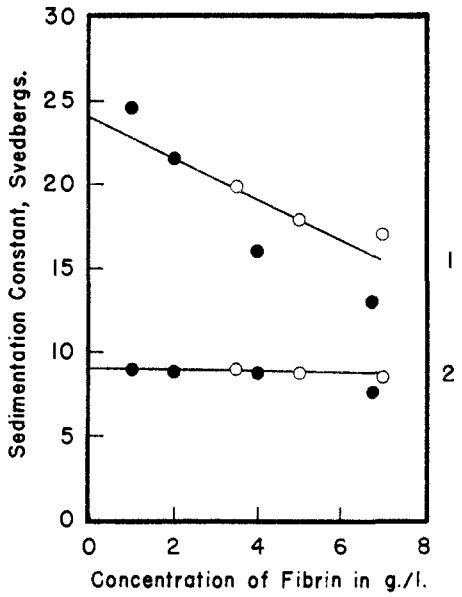

Fig. 8.—Sedimentation constants of fast (1) and slow (2) components in solutions of fibrin (from Fraction I) in 2.35 *M* urea, *p*H 7.5, ionic strength 0.15, measured in the Svedberg oil turbine ultracentrifge and reduced to water at 20°: open circles, experiment 38; black circles, experiment 39.

In the experiments of Fig. 8, the relative area of the fast peak in the ultracentrifuge was again about 30% of the total, and did not change with dilution down to a protein concentration of 2 g./l. Below this concentration the areas could not be measured.

Two additional experiments were performed to test the reversibility of interconversion between the fully depolymerized fibrin fragments and the intermediate polymer represented by the fast component in the ultracentrifuge. In the first, an inhibited clotting system in 2.35 *M* urea at *p*H 7.5 was allowed to react 140 hr., with formation of the intermediate polymer. The urea concentration was then increased to 3.5 *M* by dialysis; the ultracentrifuge diagram subsequently showed a single peak, with a sedimentation constant of 7 *S* at a finite concentration of 7. g./l. Thus, as expected, urea at a concentration capable of dissolving fibrin can dissociate the intermediate polymer into fragments with the same sedimentation constant as fibrinogen. In the second experiment, fibrin was dissolved in 3.5 *M* urea and then this solvent was replaced by 0.5 *M* hexamethylene glycol at *p*H 6.2 and ionic strength 0.45, by dialysis against a large volume of the latter solution. This is the hexamethylene glycol system used in inhibited clotting studies.[4,18] The fibrin remained in solution but instead of the single centrifuge peak characteristic of fibrin in 3.5 *M* urea it exhibited two peaks whose sedimentation constants at the finite concentration of 3 g./l. were 7 and 19 *S*, respectively. At the same time, there was a large increase in viscosity. Thus the glycol solvent medium which permits the intermediate polymer to be formed directly from fibrinogen by the action of thrombin also permits it to be formed from fibrin which has been depolymerized by urea.

Discussion

Since the intermediate identified by an extrapolated sedimentation constant of about 24 *S* has now been obtained in four different ways, it becomes increasingly important to determine its size and shape and other properties. The appearance of the above component in the ultracentrifuge is always accompanied by a high, non-Newtonian viscosity. It was originally suggested[4] that this viscosity was not due to the intermediate itself (Φ), but to a small proportion of extremely elongated material (Φ_n) formed by end-to-end aggregation of Φ. The amount of Φ_n was supposed to be too small to detect in a sedimentation diagram. The sedimentation constant of 24 *S* for Φ was interpreted as representing a hexamer of fibrinogen with twice the length and three times the cross-section area.

In the light of more recent work, however, this picture is not tenable. In the present viscosity measurements at low velocity gradients, values of η_{sp}/c higher than 10 have been obtained, where c represents the total concentration of fibrinogen plus all polymers. Neglecting corrections for hydrodynamic interaction, which would decrease this, and for extrapolation to zero velocity gradient, which would increase it, we can estimate roughly the length of an elongated particle which would give this over-all value for the solution if its concentration was too small to detect in the ultracentrifuge—say 2% of the total protein. Using the formula of Simha,[23] we find the unlikely length of 100,000 Å. Actually, flow birefringence measurements, both with[18] and without[24] added glycerol, show that most of the lengths are in the general neighborhood of 4000 Å., and that a substantial proportion of the total protein (instead of a very small fraction of it) is long enough to orient at low velocity gradients.

For these reasons and also evidence from light scattering experiments which will be reported subsequently,[25] the concept of two stages of polymerization, Φ and Φ_n, is replaced by the simpler concept of a single intermediate polymer Φ, which is itself sufficiently elongated to account for the high viscosity and the flow birefringence. The latter measurements indicate lengths in the neighborhood of six to ten times that of fibrinogen, and in this range the sedimentation constant corresponds fairly well to a side-by-side degree of polymerization of twofold. For example, on the basis of an elongated ellipsoid of revolution with twice the cross-section area of fibrinogen, the sedimentation constant calculated from the equations of Perrin[23] would be 23 for a sixfold end-to-end polymerization and 25 for a tenfold end-to-end polymerization. A solution in which about a third of the fibrinogen was converted to such polymers would have a

(23) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids, and Peptides," Reinhold Publishing Corp., Inc., New York, N. Y., 1943.

(24) J. D. Ferry, S. Shulman and J. F. Foster, *Arch. Biochem. Biophys.*, submitted.

(25) S. Katz, K. Gutfreund and S. Shulman, unpublished experiments.

zero-gradient value of η_{sp}/c less than, but of the same order of magnitude as, the observed values at moderate concentrations at the lowest velocity gradients achieved. The length of such polymers would be sufficient to account qualitatively for the non-Newtonian viscosities observed.

The drop in apparent η_{sp}/c with dilution is then attributed to dissociation of the intermediate Φ itself, but as mentioned above the drop is exaggerated by the non-Newtonian character of the flow. A certain amount of dissociation is not inconsistent with the persistence of the fast peak in the ultracentrifuge when systems containing the intermediate polymer are diluted, since the value of the sedimentation constant is not very sensitive to length until the end-to-end degree of polymerization falls below five. The effect of dilution is being further investigated by light scattering.[25]

Thus the properties of the intermediate fibrinogen polymer, Φ, identified in urea and hexamethylene glycol solutions are consistent with a cross-section area double that of fibrinogen and a range of lengths of the order of ten times that of fibrinogen. When it is formed from fibrinogen and thrombin, its immediate precursor may well be a chemically altered fibrinogen such as the molecule identified in acidic clotting systems by Laki,[26] which may be symbolized F′. When it is formed from urea-dissolved fibrin, its immediate precursor is a fibrinogen-shaped fragment, symbolized F*, which Laki[26] has already surmised as possibly identical with F′. Probably F′ (and F*) has a molecular weight smaller than that of fibrinogen by 3 to 4%, having undergone the loss of one or more peptide units totalling this amount.[9] The number and location of these peptide units are no doubt reflected in the detailed geometry of formation of Φ. The observed transformations which interrelate these substances are summarized in Fig. 9.

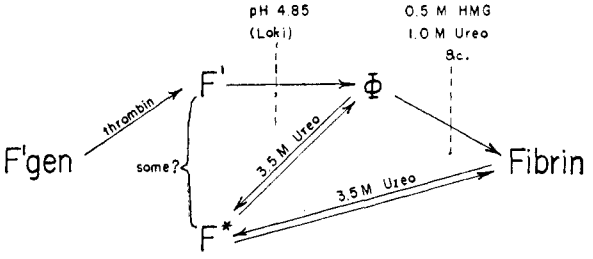

Fig. 9.—Relations among fibrinogen, fibrin and the intermediates Φ, F′ and F*.

Acknowledgments.—We are much indebted to the Minnesota Mining and Manufacturing Company for furnishing the glass spheres used in falling sphere viscosity measurements, and to Mr. W. Van Valkenburg and Mr. K. Gutfreund for help in some of the experiments.

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

[FROM THE DEPARTMENT OF CHEMISTRY, INDIANA UNIVERSITY, AND THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, UNIVERSITY OF ISTANBUL]

The Reaction of Native and Denatured Ovalbumin with Congo Red[1]

BY FELIX HAUROWITZ, FRANK DIMOIA AND SHEVKET TEKMAN

Heat-treated ovalbumin and serum albumin combine with congo red to form complexes which remain red at *p*H 2, while mixtures of congo red with the unheated proteins have a purplish-blue color at *p*H 2. Since the absorption spectra of congo red in acid solutions of unheated and heat-treated albumins differ considerably, the kinetics of denaturation can be examined spectrophotometrically. The number R of red dye molecules bound per protein molecule is roughly proportional to c, the concentration of the blue dye molecules. After complete denaturation of the native proteins by heat, R increases by four in ovalbumin, and by two in serum albumin. Ovalbumin exposed to temperatures of 65 or 70° combines with less congo red than completely denatured ovalbumin exposed to 75°. The denaturation of ovalbumin by heat follows first-order kinetics with respect to protein concentration; the reaction order with respect to time is about 6.0 at 65°, 4.2 at 70° and 3.4 at 75°. It is concluded that the cleavage of hydrogen bonds and/or salt bridges, which presumably obeys first-order kinetics, is followed by the irreversible formation of new *intra*molecular cross-links between the peptide chains, and that this second phase of denaturation is a reaction of high order with respect to time.

Introduction

The color reactions of denatured proteins with nitroprusside,[2] phosphomolybdate[3,4] and diazobenzenesulfonate[4] are more intensive than those given by the same proteins in the native state. The low reactivity of the native proteins has been attributed to the inaccessibility of some of the groups in the closely folded peptide chains[5] and/or to their combination with other groups by hydrogen bonding.[6]

The present investigation was undertaken in order to find whether denaturation involves a similar increase in reactive cationic groups. Such an increase would alter profoundly the physical-chemical behavior of soluble proteins and particularly their electrokinetic potential.[7] Electrometric titration cannot give any information in this respect because all of the ionic groups of a protein are accessible to the small hydrogen ions. Previous investigations[8] had indicated that denaturation of proteins in-

(1) This work was supported by research grants from the U. S. Public Health Service, National Institutes of Health, and from the Graduate School of Indiana University. Some of the material was presented by Frank DiMoia in partial fulfillment of the requirements for a degree of Doctor of Philosophy at Indiana University. Earlier results were reported at the Meeting of the American Chemical Society in New York, September, 1947 (Abstracts, p. 29 C).

(2) A. C. Mirsky and M. L. Anson, *J. Physiol.*, **19**, 427 (1936).

(3) M. L. Anson, *ibid.*, **24**, 399 (1941).

(4) F. Haurowitz and S. Tekman, *Biochim. Biophys. Acta*, **1**, 484 (1947).

(5) F. Haurowitz, *Kolloid-Z.*, **71**, 198 (1935).

(6) M. Levy and A. E. Benaglia, *J. Biol. Chem.*, **186**, 829 (1950).

(7) H. B. Bull, *J. Phys. Chem.*, **39**, 577 (1935).

(8) F. Haurowitz, *Kolloid-Z.*, **74**, 208 (1936).