transported acids is also dependent, in part, on their own stability toward oxidation.

Particular study has heretofore been given to ascorbic acid.[14,15] In addition to being an active organic acid, ascorbic acid is also a powerful reducing agent. It can reduce quinone to quinol and can probably also form complexes with both.

The synergic action of phosphoric acid with tocopherol[6] must likewise be reconsidered; the apparent reduction and cyclization of tocoquinone to tocopherol by traces of phosphoric acid has never been satisfactorily visualized. The nature of the analogous complex in this case is also being investigated. With an increasing understanding of these exchange reactions various chemical and biochemical conceptions may be subject to revision.

The author is grateful to H. A. Mattill for suggesting this problem and for his help in the preparation of this paper.

(14) Calvin Golumbic and H. A. Mattill, THIS JOURNAL, **63**, 1279 (1941).

(15) V. P. Calkins and H. A. Mattill, *ibid.*, **66**, 239 (1944).

Summary

The antioxygenic action of quinone with phosphoric acid in delaying fat autoxidation has been investigated. From the experimental data a scheme has been proposed, which involves both adsorption and solution reactions and which is based upon an exchange mechanism. The scheme also explains the effectiveness of various organic acids and other synergists in the stabilization of vegetable oils and fats which contain phenolic inhibitors or their oxidation products.

IOWA CITY, IOWA RECEIVED AUGUST 27, 1946

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

Preparation and Properties of Serum and Plasma Proteins. VIII. The Conversion of Human Fibrinogen to Fibrin under Various Conditions[1,2]

BY JOHN D. FERRY AND PETER R. MORRISON

I.

The final step in the clotting of blood is considered to be the reaction between the proteins fibrinogen and thrombin to form a solid structure of fibrin. Many investigators have studied this process by clotting plasma, in which only about 4% of the protein is fibrinogen, and where the reaction may be complicated by the presence of other substances involved in the clotting complex (such as antithrombin), or by using fibrinogen and thrombin preparations of doubtful and unspecified purity.[3] Others have recognized the desirability of working with purified preparations of known fibrinogen content and thrombin activity, respectively.[3,4]

Various observers, even recently,[5] have questioned the enzymatic nature of thrombin. However, the fact that the amount of fibrin formed is independent of the amount of thrombin present, over a wide range,[6] and the ability of thrombin to convert over 10^5 times its own weight of fibrinogen to fibrin,[7] appear to be decisive evidence that the role of thrombin is catalytic. It has been suggested that the formation of the solid structure of fibrin does not involve any profound intramolecular changes in the fibrinogen (such as occur when globular proteins are denatured[9,10]) but merely the attachment of fibrinogen molecules together to form a network in which they retain their identity. The evidence for this view, although not yet conclusive, is, first, that the X-ray patterns of fibrin and fibrinogen are very similar,[11] and, second, that the products of partial digestion by a proteolytic enzyme of fibrin and of fibrinogen appear to be identical in solubility behavior, electrophoretic mobilities, and sedimentation constants.[12]

The conversion of fibrinogen to fibrin may thus be considered as a polymerization process, in which the "monomer" is itself a very large molecule. The polymer, fibrin, is of course usually crosslinked, since a rigid gel is formed even at

(1) This work has been carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

(2) This paper is Number 47 in the series "Studies on Plasma Proteins" from the Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

(3) For a critical review, see E. Chargaff, *Advances in Enzymol.*, **5**, 31 (1945); *cf.* E. Wöhlisch, *Ergeb. Physiol. exptl. Pharmakol.*, **43**, 174 (1940); A. J. Quick, "The Hemorrhagic Diseases and the Physiology of Hemostasis," C. C. Thomas Co., Baltimore, Md., 1942.

(4) (a) W. H. Seegers and H. P. Smith, *J. Biol. Chem.*, **140**, 677 (1941); (b) K. Laki, *Studies Inst. Med. Chem. Univ. Szeged*, **2**, 27 (1942).

(5) W. F. H. M. Mommaerts, *J. Gen. Physiol.*, **29**, 103, 113 (1945).

(6) P. R. Morrison, in preparation.

(7) For example, in one experiment[8] 1 unit of thrombin clotted 0.18 g. of fibrinogen. Estimating the maximum weight of thrombin present from the highest prothrombin activity thus far reported,[9] 1500 units per mg., we obtain a minimum weight ratio of fibrinogen to thrombin of 2×10^5.

(8) W. H. Seegers, E. C. Loomis and J. M. Vandenbelt, *Arch. Biochem.*, **6**, 85 (1945).

(9) W. T. Astbury and R. Lomax, *J. Chem. Soc.*, 846 (1935).

(10) H. Neurath, J. P. Greenstein, F. W. Putnam and J. A. Erickson, *Chem. Rev.*, **34**, 157 (1944).

(11) K. Bailey, W. T. Astbury and K. M. Rudall, *Nature*, **151**, 716 (1943).

(12) W. H. Seegers, M. Nieft and J. M. Vandenbelt, *Arch. Biochem.*, **7**, 15 (1945); C. G. Holmberg, *Arkiv. Kemi Mineral. Geol.*, **17A**, No. 28 (1944); G. Kegeles, W. H. Seegers and J. W. Williams, unpublished experiments.

high dilution. It is particularly striking that a solution containing as little as 0.02 to 0.04 g./liter of fibrinogen (a volume fraction of 3×10^{-5} or less) can form a rigid clot.[13]

The dimensions of the fibrinogen molecule, assuming as a model an elongated ellipsoid of revolution, have been calculated from measurements of double refraction of flow, viscosity, and osmotic pressure[14] to be about 35 × 700 Å.; the molecular weight is of the order of 300,000–500,000. The size of the monomer molecule is thus enormous compared to that of whatever chemical groups on its surface are responsible for intermolecular linkages during clotting.

Elucidation of the conversion process involves two virtually independent problems: the nature of the chemical bonds which link the fibrinogen units together; and the geometrical arrangement of the fibrinogen units in the fibrin structure. It has recently been suggested—although the evidence appears inconclusive—that at least some of the structural bonds are disulfide links.[16] In this paper we are primarily concerned with the second problem—the arrangement of the fibrinogen units, which largely determines the physical properties of the clot.

When purified preparations of fibrinogen and thrombin are allowed to react under controlled conditions, it is found that variables such as *p*H, ionic strength, and temperature affect not only the rate of the reaction (as judged, for example, by the "clotting time"), but also the properties of the clot formed.[17] We describe here the wide range of properties obtainable and the influence of various physical and chemical factors upon them. The descriptions include the opacity and modulus of rigidity, which can be measured quantitatively, and the friability, syneresis, and other mechanical properties, which are qualitatively or semi-quantitatively appraised. From these studies, some information may be derived concerning the structure of the clots and the mechanism of conversion of fibrinogen to fibrin.

II. Materials and Methods

The fibrinogen used in these studies was obtained from Fraction I of human plasma.[19] In some experiments this fraction, in which the protein is normally about 60% fibrinogen, was used without further purification. The preparations were dissolved in sodium citrate buffer at *p*H 6.3, clarified by repeated filtration, dried from the frozen state, and stored *in vacuo*. After this processing, the protein contained from 45 to 60% of fibrinogen. Its solutions were very stable to both spontaneous clotting and lysis. The additional protein in this material consists largely of β and γ globulins.[19] In other experiments, refractionated preparations[21] containing 80 to 90% of fibrinogen were employed. Stock solutions were made up in 0.2 or 0.25 *M* sodium chloride at a fibrinogen concentration of 20 to 40 g./liter and clarified by filtration through filter pads. Fibrinogen concentrations were determined as described below. Aliquots of stock solution were diluted with mixtures of sodium chloride and phosphate buffer solutions, together with small amounts of dilute sodium hydroxide or hydrochloric acid when necessary to adjust the *p*H to the required value.

The thrombin was obtained from Fraction III-2 of human plasma.[20,22] Its activity was 10 to 15 unit/mg. of protein (see below). Although this probably corresponds to a relatively low purity,[23] the preparations were free of plasmin (the proteolytic enzyme of plasma) and of lipoid components often found associated with thrombin. The material was stored as a dry powder and dissolved in 0.15 *M* sodium chloride to give clear stock solutions (concentration 1 to 100 unit/cc.).

Fibrinogen Assay.—Fibrinogen was determined gravimetrically after conversion to fibrin under standard conditions.[6] An aliquot of stock solution at *p*H 6.2 to 6.4 was diluted with 0.15 *M* sodium chloride to an approximate concentration of 1 g. of fibrinogen per liter. A small volume of thrombin solution was added to bring the thrombin concentration to 0.1 unit/cc. (see below), and the mixture was allowed to clot in a shallow vessel. After one hour, the clot was transferred to a cloth of fine texture and allowed to synerize under its own weight, the water, salt and proteins other than fibrinogen which were expressed being taken up by an absorbent pad. The thin film of moist fibrin which remained was washed in 0.15 *M* sodium chloride and in water, rolled up, dried at 100° and weighed.

The purity of each stock preparation was expressed as the ratio of fibrinogen to total protein, the latter determined by micro-Kjeldahl analysis, using a nitrogen factor[24] of 6.0.

Thrombin Assay.—The thrombin unit used in this Laboratory[22] is within the limits of experimental error identical with that employed by Seegers.[26] It is now defined in terms of a standard thrombin preparation and each new preparation is assayed against the standard at serial dilution. In practice it is found that one unit of thrombin is the amount which clots 1 cc. of fibrinogen (Fraction I) solution in a test-tube of 1 cm. i. d., in approximately 45 sec., the fibrinogen concentration being 10 g./liter, *p*H near 6.3, ionic strength 0.15 and temperature 25°.

These figures may vary slightly from one fibrinogen preparation to another, and also are somewhat dependent on the end-point chosen in measuring the clotting time. Disturbances due to such variations, however, are eliminated by the use of both the reference standard and the unknown thrombin on the same fibrinogen preparation at each test.

Clotting Time.—The clotting time of a given fibrinogen–thrombin mixture was taken as the time required for 5 cc.

(13) Measurements by Mr. V. Kimel in this Laboratory.

(14) These dimensions, which differ somewhat from previous estimates,[15] are based on recent measurements by J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. and Colloid Chem.*, in press; J. T. Edsall, J. F. Foster and H. Scheinberg, in preparation.

(15) E. J. Cohn, J. L. Oncley, L. E. Strong, W. L. Hughes, Jr., and S. H. Armstrong, Jr., *J. Clin. Investigation*, **23**, 417 (1944).

(16) (a) J. P. Baumberger, *Am. J. Physiol.*, **133**, P206 (1941); (b) R. N. Lyons, *Australian J. Expil. Biol. Med. Sci.*, **23**, 131 (1945).

(17) Many of these observations were made in connection with the development of fibrin products for surgical applications,[18] from the fractions of human plasma separated as described in other papers of this series.[19,20]

(18) J. D. Ferry and P. R. Morrison, *J. Clin. Investigation*, **23**, 566 (1944); *Ind. Eng. Chem.*, **38**, 1217 (1946).

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

(20) J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron and P. M. Gross, in preparation.

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

(22) J. T. Edsall and S. G. Miller, in preparation.

(23) Prothrombin has been prepared[8] with an activity of 1500 unit/mg.

(24) The nitrogen factor of fibrin is given by Brand, Kassell and Saidel[25] as 5.92; the nitrogen factor of Fraction I[19] is 6.1.

(25) E. Brand, B. Kassell and L. J. Saidel, *J. Clin. Investigation*, **23**, 437 (1944).

(26) W. H. Seegers, H. P. Smith, E. D. Warner and K. M. Brinkhous, *J. Biol. Chem.*, **123**, 751 (1938); E. D. Warner, K. M. Brinkhous and H. P. Smith, *Am. J. Physiol.*, **114**, 667 (1936).

of solution in a test-tube of 1.1 cm. i. d. to develop a characteristic rigidity. This end-point could be reproduced closely, and, although it depended upon subjective judgment, was satisfactory for the purposes of the present study.

Opacity.—Opacities of clots were determined with a Beckman quartz spectrophotometer. The clots were formed either in rectangular quartz cells 1.0 cm. thick or in selected test-tubes of 1.1 cm. i. d. The extinction coefficient was expressed as $\tau = (1/d) \log (I_0/I)$, where d is the light path in cm., I the transmitted intensity and I_0 the incident intensity.

A comparison of the extinction coefficients of a fibrinogen solution before and after clotting, plotted against the wave length, is shown in Fig. 1. The system contained 1.0 g./liter of fibrinogen (a preparation of 90% purity) and 0.15 M sodium chloride, at pH 6.85; it was clotted with 0.5 unit/cc. of thrombin. In the unclotted solution, the maximum at 2800 Å. is due to absorption by tryptophan and tyrosine residues, as characteristically observed in proteins[27]; the extinction above 3500 Å. is very small. In the clot, the much higher extinction at wave lengths above 3500 Å. is due to scattering, and is indicative of the changes in structure with which we are here concerned.

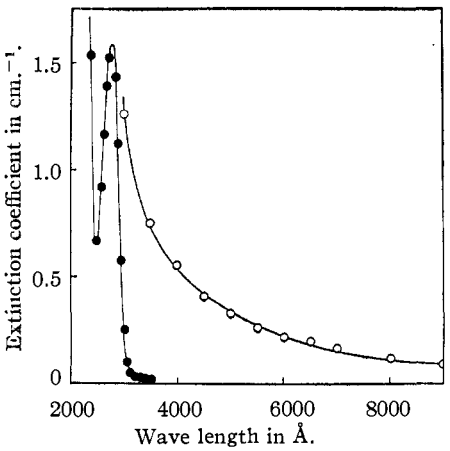

Fig. 1.—Extinction coefficients of a fibrinogen solution before (●) and after (○) clotting, plotted against wave length: fibrinogen concentration 1.0 g./liter, thrombin 0.5 unit/cc., NaCl 0.15 M, pH 6.85.

Opacity measurements were usually made at 6000 Å. Additional measurements at 3500 Å. are not reported directly but were employed to calculate ratios of scattering at 3500 Å. to that at 6000 Å. (see Fig. 18, below).

Rigidity.—Rigidities of clots were determined by measuring the velocity of propagation of transverse vibrations, using an optical method.[28] The clots were formed in rectangular glass cells, provided with clamps to hold the vibrator plates in place until rigidity had developed.[29] The modulus of rigidity is given by $G = V^2\rho$, where V is the velocity of wave propagation and ρ the density of the clot. The latter value was calculated from the density of the salt solution used as solvent (from the "International Critical Tables") and an assumed value of 0.75 for the partial specific volume of all protein present.

Friability.—A rough but characteristic measure of friability was obtained by pressing the clot with the finger. "Non-friable" clots compacted and expelled fluid without breaking; "slightly friable" clots expelled fluid but split under too great pressure; "friable" clots broke without expulsion of fluid; "very friable" clots crumbled at the slightest touch.

Syneresis.—A rough measure of the ease of syneresis, or compaction with loss of fluid, was obtained by placing a cylindrical clot about 1 cm. in diameter on filter paper and allowing it to compact under its own weight for three hours. The ratio of the volume before compaction to the final volume was taken as a syneresis index. Most of the clots described here did not undergo "retraction," or spontaneous syneresis within the vessel in which the clot is formed.

Mechanical Properties.—After syneresis, the ends of the clot were clamped between wooden jaws and it was stretched by applying a load which was increased in 5-g. steps until the clot broke. After each load increment, the length was measured, the entire load was removed for twenty seconds, and the length was measured again to determine permanent set. The tensile strength was taken as the breaking load divided by the final cross section at the point of rupture. The maximum elongation was taken as the increase in length at break expressed as percentage of the original length. The permanent set was taken as that produced at half the breaking load, expressed as percentage of the original length.

Measurements were made only on those clots described as "non-friable" or "slightly friable." The more friable preparations could not be clamped by the apparatus employed.

III. Course of the Conversion Reaction

The conversion of fibrinogen to fibrin may be followed by measuring various changes in the properties of the clotting system after thrombin is added. The most common measure of the rate of conversion is, of course, the clotting time, *i. e.*, the time required for the first appearance of fibrin or the development of a certain characteristic rigidity. The value of the latter depends upon the conditions of the test; when this involves the ability of the clot to support its own weight, the characteristic rigidity must be of the order of $g\rho l$ (where g is the acceleration due to gravity, ρ the density, and l a linear dimension of the order of the size of the sample), *i. e.*, 10^3 dynes/sq.cm. After "clotting," the rigidity continues to increase and eventually may attain a much higher value; its change with time can be measured. The increase in opacity during the process of conversion may also be followed.[30] Finally, the amount of fibrin formed at any point may be determined by rapid compaction of the clot as described above in the procedure for gravimetric assay, expressing the unconverted fibrinogen, and weighing the fibrin after washing and drying. This process, however, can be carried out only in a limited range of concentration and pH.

The recoverable fibrin and the increase in opacity, both expressed as percentages of the final values attained (in about three hours), for a clotting system containing 1 g./liter of fibrinogen and 0.1 unit/cc. of thrombin at pH 6.3 and ionic strength 0.15, are plotted against the time in Fig.

(27) C. Dhéré, "Recherches Spectrographiques," Fribourg, 1909; W. Stenström and M. Reinhard, *J. Biol. Chem.*, **66**, 819 (1925); J. L. Crammer and A. Neuberger, *Biochem. J.*, **37**, 302 (1943).

(28) J. D. Ferry, *Rev. Sci. Instruments*, **12**, 79 (1941); THIS JOURNAL, **64**, 1323 (1942).

(29) We are much indebted to Dr. S. H. Armstrong, Jr., for the design of these clamps.

(30) K. Klinke and G. Elias, *Z. ges. exper. Med.*, **77**, 717 (1931); K. K. Nygaard, "Hemorrhagic Diseases. Photo-electric Study of Blood Coagulability," C. V. Mosby Co., St. Louis, 1941; H. F. Deutsch, *J. Clin. Investigation*, **25**, 37 (1946).

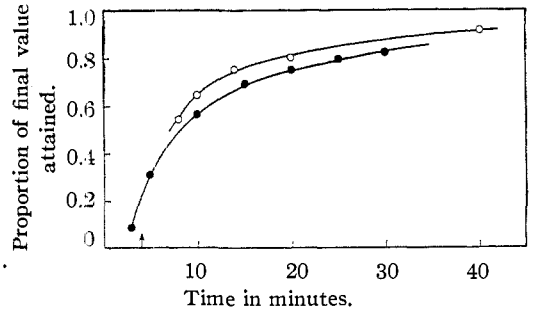

Fig. 2.—Recoverable fibrin (O) and opacity increase (●) plotted against time, during clotting of solution with fibrinogen concentration 1 g./liter, thrombin 0.1 unit/cc., ionic strength 0.15, *p*H 6.3. Arrow denotes clotting time.

2. The rigidity and increase in opacity are similarly plotted in Fig. 3 for a system containing 16 g./liter of fibrinogen and 1.0 unit/cc. of thrombin at *p*H 6.33 and ionic strength 0.3. The conventional clotting times are also indicated. The figures show that the opacity starts to increase before the moment of "clotting" is reached. As the conversion proceeds, the amount of recoverable fibrin is the first of the three variables to reach its maximum value. The opacity continues to increase and approaches its maximum value later, while the rigidity increases more slowly still.

These changes are readily understood when clotting is considered as a three-dimensional polymerization. The clotting time corresponds to the "gel point" described by Flory,[31] which occurs long before the gel fraction becomes unity (*i. e.*, the recoverable fibrin reaches its maximum value). The succeeding changes in opacity and rigidity show that, even after all the protein is bound to the network, the reaction proceeds to form additional crosslinks and structures which scatter light more strongly.

Kinetic Studies of the Conversion Reaction.—Measurements of opacity and rigidity are not at present suited for studies of the kinetics of conversion of fibrinogen to fibrin, because relations of these quantities to the degree of partial conversion have not been established. The only direct measurement is the gravimetric estimation of fibrin. Studies of this sort in very dilute systems where the reaction proceeds slowly indicate that the reaction is roughly first order with respect to both fibrinogen and thrombin.[32] Experiments by Laki[4b] in which the clotting process was halted at intervals by the addition of potassium permanganate led to similar conclusions.

However, these results cannot be interpreted in terms of the reaction of individual groups on the fibrinogen molecule without knowledge of the geometry of the polymerization. We shall not attempt to deal further with kinetics at present,

(31) P. J. Flory, THIS JOURNAL, **63**, 3083, 3091, 3096 (1941).

(32) P. R. Morrison, V. Kimel, J. D. Ferry and J. T. Edsall, unpublished experiments.

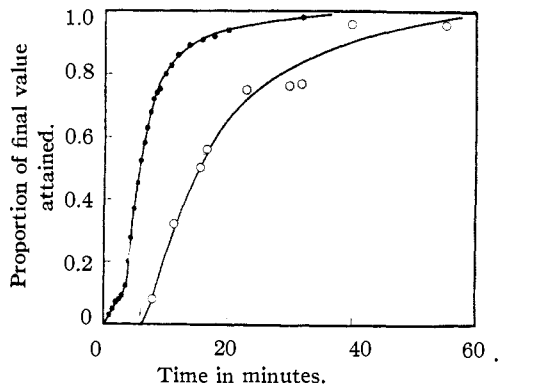

Fig. 3.—Rigidity (O) and opacity increase (●), plotted against time, during clotting of solution with fibrinogen concentration 16 g./liter, thrombin 1.0 unit/cc., ionic strength 0.3, *p*H 6.33. Arrow denotes clotting time.

and shall consider only the properties of clots in which the conversion of fibrinogen to fibrin has reached completion.

IV. Properties of Clots: Effect of Fibrinogen and Thrombin Concentrations and *p*H

Stock solutions of Fraction I, in which 46% of the protein was fibrinogen, were diluted to give samples ranging in concentration from 1.5 to 23.9 g./liter of fibrinogen

TABLE I

OPACITIES AND RIGIDITIES OF FIBRIN CLOTS

Ionic strength 0.30 (0.25 sodium chloride + 0.05 phosphate), thrombin 0.2 unit/cc.

Fib. concn. g./liter	*p*H	Clotting time, min.	Opacities Soln. τ_0	Opacities Clot τ	G $\times 10^{-4}$ dyne cm.$^{-2}$
1.5	6.82	4.00	0.01	0.30	
3.0	6.77	4.25	.02	.24	0.42
6.0	6.74	7.00	.04	.23	1.24
12.0	6.68	9.25	.06	.21	2.56
23.9	6.64	13.50	.09	.30	10.78

TABLE II

OPACITIES AND RIGIDITIES OF FIBRIN CLOTS

Ionic strength 0.30 (0.25 sodium chloride + 0.05 phosphate), thrombin 1.0 unit/cc.

Fib. concn. g./liter	*p*H	Clotting time, min.	Opacities Soln. τ_0	Opacities Clot τ	G $\times 10^{-4}$ dyne cm.$^{-2}$
1.5	6.31	1.42	0.01	0.50	0.18
	6.80	1.50	.01	.09	.16
	7.21	1.25		.06	
3.0	6.29	1.58	.02	.82	
	6.77	1.42	.02	.15	.37
	7.12	1.33		.12	.46
6.0	6.26	2.50	.04	1.03	
	6.72	1.83	.03	0.32	1.65
	7.03	1.67		.16	1.31
12.0	6.23	6.00	.06	.68	4.39
	6.64	2.92	.06	.26	3.24
	6.94	2.00		.15	3.50
23.9	6.18	8.50	.09	1.42	
	6.57	5.83	.09	0.39	12.58
	6.81	4.00		.19	18.02

TABLE III

OPACITIES AND RIGIDITIES OF FIBRIN CLOTS

Ionic strength 0.30 (0.25 sodium chloride + 0.05 phosphate), thrombin 4.0 unit/cc.

Fib. concn. g./liter	pH	Clotting time. min.	Opacities Soln. τ_0	Opacities Clot τ	G $\times 10^{-4}$ dyne cm.$^{-2}$
1.5	6.36	0.67		0 16	0.10
	6.80	.67	0.01	.10	.09
	7.21	.42		.04	.08
2.9	6.35	.75		.25	.31
	6.73	.48	.02	.12	.25
	7.17	.33		.08	.25
5.8	6.32	.83		.30	
	6.78	.58	.02	.14	.89
	7.13	.42		.09	.45
11.7	6.31	1.17		.34	3.00
	6.75	0.75	.05	.16	2.40
	7.05	.67		.12	2.40
23.4	6.30	1.50		.42	13.90
	6.71	1.33	.07	.18	11.59
	6.96	0.83		.15	10.40
	7.42	1.00		.17	7.44

and in pH from 6.18 to 7.21, the ionic strength being kept constant at 0.30 (0.25 sodium chloride + 0.05 phosphate buffer). Small volumes of thrombin solution were added to bring its concentration to 0.2, 1.0 and 4.0 unit/cc. The clotting times were noted, and after three to four hours (for the systems most dilute in thrombin, eighteen hours) the opacities and rigidities of the resulting clots were measured. The results are given in Tables I, II and III.

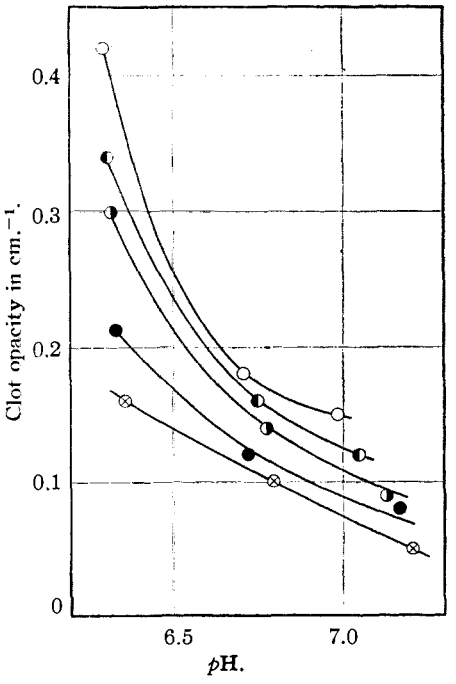

Fig. 4.—Opacities of fibrin clots plotted against pH, at thrombin concentration 4 unit/cc. and ionic strength 0.30; fibrinogen concentrations in g./liter: ⊗, 1.5; ●, 2.9; ◑, 5.8; ◐, 11.7; ○, 23.4.

Opacity.—The opacity, or extinction coefficient, τ, varied from 0.05 to 1.6; in appearance the clots ranged from near transparency to milky turbidity. The extinction of the unclotted solution,[33] τ_0, which is also given in the tables, occasionally amounted to about one-third the opacity of the clot, in concentrated, nearly transparent systems; in dilute, more opaque systems, it was very much smaller than that of the clot.

The opacity, τ, from Table III, for 4 unit/cc. of thrombin, is plotted against the pH in Fig. 4 for several different concentrations of fibrinogen. The opacity falls sharply with increasing pH. The effect of fibrinogen concentration is remarkably slight over a range of sixteen-fold; this means that the opacity per unit fibrinogen concentration falls off with increasing concentration, as shown in Fig. 5.

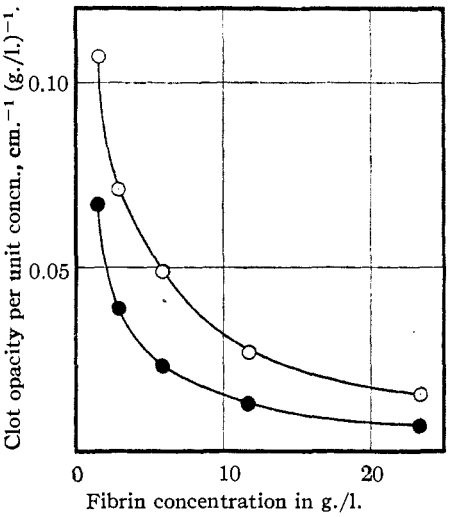

Fig. 5.—Opacity per unit fibrin concentration, plotted against fibrin concentration in g./liter; ○, pH 6.35; ●, pH 6.8.

Rigidity.—The rigidity values from Table III, for a thrombin concentration of 4 unit/cc., are plotted in Fig. 6. They decrease somewhat with increasing pH, and increase enormously with increasing fibrinogen concentration. The logarithm of the rigidity, at pH 6.8, interpolated from Fig. 6, is plotted against the logarithm of fibrinogen concentration in Fig. 7, together with values similarly interpolated at different thrombin concentrations. The slopes of the lines, which are the same within experimental error, show that the rigidity varies approximately at the 1.57 power of the fibrinogen concentration. The effect of thrombin concentration is relatively slight.

Qualitative Studies.—Several series of clots were prepared from samples of purified fibrinogen of 75 to 85% purity. The ionic strength of the stock solutions, which contained 20 g./liter of fibrinogen, was 0.30 (0.20 sodium chloride + 0.10 phosphate buffer), and dilutions were made with 0.30 *M* sodium chloride. The friability, syneresis, tensile strength, maximum elongation and permanent set (at half the breaking load) of each clot were recorded. These results are presented in Fig. 8 as contour graphs in which fibrinogen concentration is plotted against pH, at a thrombin concentration of 0.5 unit/cc. The results at thrombin concentrations of 0.02 and 10 unit/cc. were quite similar, except as noted in the text below.

(33) The extinction of the unclotted solution is probably due to light scattering, not only by individual fibrinogen molecules, but also by traces of aggregated material. To distinguish between these sources of scattering, it is necessary to study the angular dependence of scattered light intensity and also the effects of exhaustive procedures for clarification, which were not attempted here.

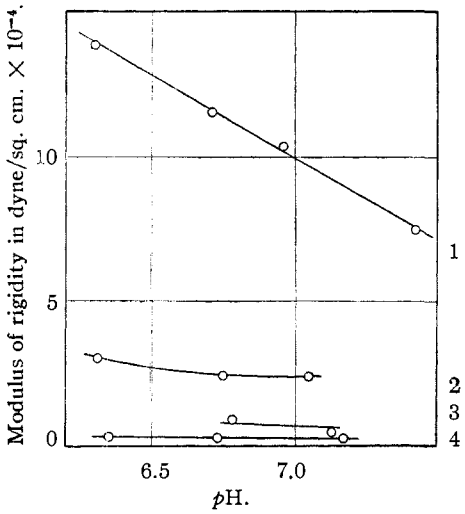

Fig. 6.—Rigidities of fibrin clots plotted against *p*H, at thrombin concentration 4 unit/cc. and ionic strength 0.30; fibrinogen concentrations in g./liter: 1, 23.4; **2**, 11.7; 3, 5.8; 4, 2.9.

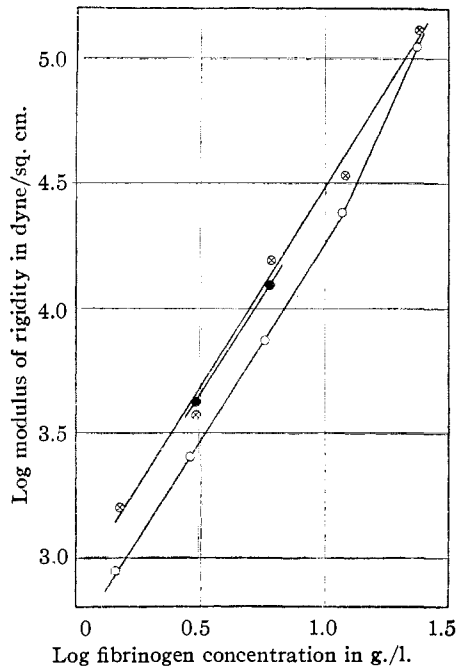

Fig. 7.—Logarithm of rigidity at *p*H 6.8 plotted against logarithm of fibrinogen concentration; thrombin concentrations in unit/cc.: ●, 0.2; ⊗, 1.0; O, 4.0.

Friability.—The friability increases with increasing *p*H, and does not vary much with fibrinogen concentration. When the thrombin concentration is very low, however, the friability increases with fibrinogen concentration.

Syneresis.—The tendency to syneresis decreases with increasing *p*H and also with increasing fibrinogen concentration. When the thrombin concentration is very low, the contours shown become convex to the coördinate axes, so that syneresis occurs in dilute solution even at a high *p*H. It is this effect which permits fibrinogen assays to be carried out by the method described above.[6]

Mechanical Properties.—The tensile strength and maximum elongation of the synerized clots decrease with

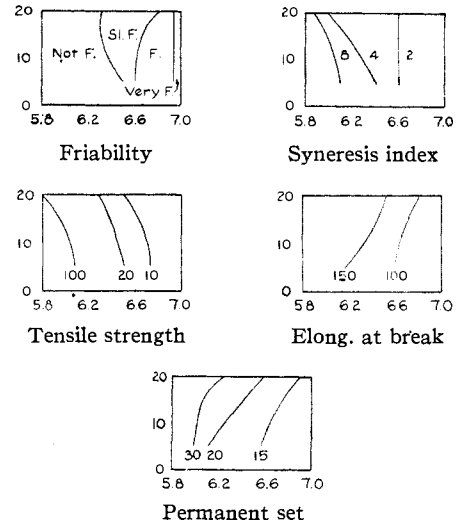

Fig. 8.—Friability, syneresis, tensile strength, elongation at break, and permanent set of fibrin clots, as functions of *p*H and fibrin concentration.

increasing *p*H. The permanent set decreases with increasing *p*H.

Classification of Clot Types.—With respect to most of the properties described, the different clots may be classified as varying between two extreme types, which, in anticipation of the conclusions to be reached below concerning their structures, are termed fine and coarse.[34] The fine clot is transparent and very friable, and does not synerize. Its tensile strength and maximum elongation are low because of its friability; its permanent set is low, for within the limits of deformation it is almost perfectly elastic. The coarse clot is very opaque and non-friable, and synerizes very readily. It has a higher tensile strength and elongation, but also a high permanent set.

Of the variables considered in this section, *p*H has by far the greatest influence upon the properties of a fibrin clot; increasing *p*H shifts them toward those of the fine type,[35] and the complete transition from coarse to fine is achieved in the *p*H range from 6.2 to 7.2. The influence of fibrinogen and thrombin concentrations is less marked. Increasing fibrinogen concentration diminishes the opacity per unit concentration and diminishes the tendency to syneresis; the shift is toward fineness. Increasing thrombin concentration (except in the range of very dilute thrombin) diminishes the opacity somewhat, representing a shift toward fineness.

V. Properties of Clots: Effect of Ionic Strength, Temperature and Added Reagents

Further studies were made to determine the influence of the ionic strength and temperature during clotting upon the properties of the clots formed. In addition, it was found that the addition of small amounts of certain polyhydroxyl compounds, such as glycerol, had marked effects, and these were explored.

Since the opacity is perhaps the most striking difference between coarse and fine clots, and can be measured quantitatively, it was used as the principal index of clot character in these experiments.

Opacity.—Stock solutions of Fraction I were diluted to a concentration of 5.0 g./liter of fibrinogen, the ionic

(34) These characteristic types of clots were previously described as Type A and Type B,[18] respectively.

(35) Howell, *Am. J. Physiol.*, **35**, 143 (1914), noted that when oxalated plasma was made alkaline before adding calcium the clot formed was transparent and structureless in the ultramicroscope. Laki (ref.) employed transparent clots at *p*H 7.2 for optical studies.

strength being varied as specified below. The solutions were clotted with thrombin at a concentration of 1.0 unit/cc. After sixteen hours, the opacities of the resulting clots were measured.

The opacity, τ, is plotted against the ionic strength, at *p*H 6.3, in Fig. 9. In sodium chloride, as the ionic strength is increased from 0.15 to 0.5, the opacity falls and passes through a minimum. With calcium chloride, the fall in opacity is much more marked.

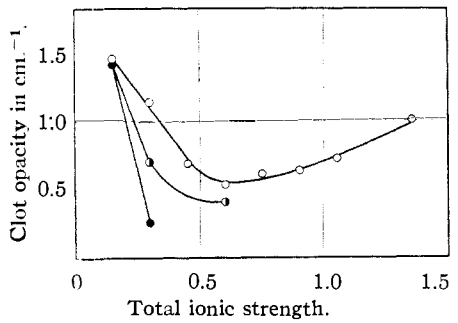

Fig. 9.—Opacities of fibrin clots plotted against ionic strength, at fibrin concentration 5 g./liter, thrombin concentration 1 unit/cc., and *p*H 6.3: O, sodium chloride; ◑, 0.15 *M* calcium chloride + sodium chloride; ●, calcium chloride.

The opacity is plotted against the temperature of clotting in Fig. 10, for an ionic strength of 0.15. At *p*H 6.3, the effect is slight. At *p*H 6.8, the opacity decreases with increasing temperature, thus accentuating the difference between the two *p*H values.

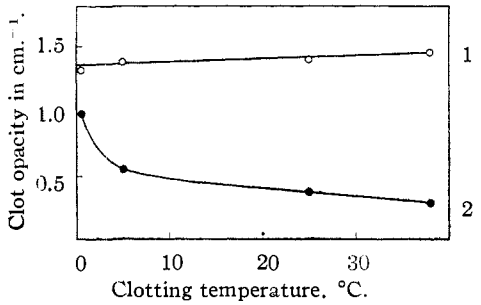

Fig. 10.—Opacities of fibrin clots plotted against temperature, at fibrin concentration 5 g./liter, thrombin concentration 1 unit/cc., and ionic strength 0.15: O, *p*H 6.3; ●, *p*H 6.8.

In Fig. 11, the opacity is plotted against the mole fraction of glycerol added before clotting. At *p*H 6.3, where the opacity in the absence of glycerol is high, a marked drop is produced by the presence of as little as 0.02 mole fraction of glycerol.[36]

Several other polyhydroxyl compounds produce this effect. The opacity is plotted in Fig. 12 against the mole fraction of glucose, glycerol, propylene glycol and ethylene glycol added before clotting. The effectiveness of these compounds in decreasing opacity decreases in the order named.

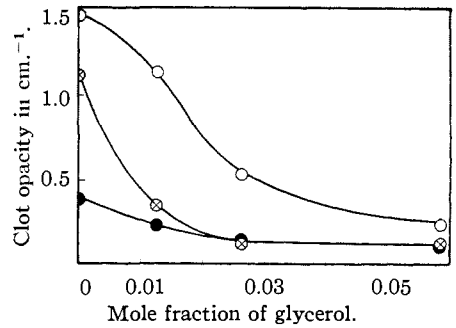

Fig. 11.—Effect of glycerol on clot opacity: O, *p*H 6.3, ionic strength 0.15; ⊗, *p*H 6.3, ionic strength 0.30; ●, *p*H 6.8, ionic strength 0.15.

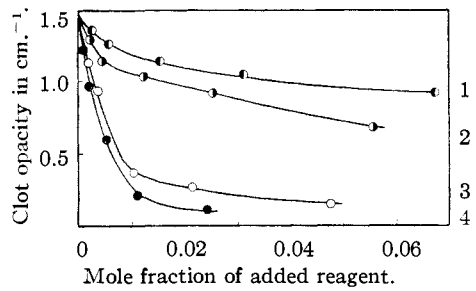

Fig. 12.—Effect of polyhydroxyl compounds on clot opacity—fibrin 5 g./liter; thrombin 1 unit/cc.; *p*H 6.3; ionic strength 0.15: 1, ethylene glycol; 2. propylene glycol; 3, glycerol; 4, glucose.

However, a polyhydroxyl compound of much higher molecular weight—starch—has the opposite effect. In Fig. 13, the opacity is plotted against the concentration of starch.[38] At *p*H 6.8, the opacity is markedly increased by the presence of starch. At *p*H 6.3, the presence of 0.059 mole fraction of glycerol lowers the opacity from 1.6 to 0.5, as shown above. However, the further addition of

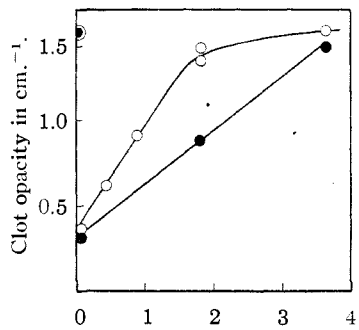

Fig. 13.—Effect of starch on clot opacity—fibrin 5 g./liter; thrombin 1 unit/cc.; ionic strength 0.15: O, *p*H 6.7; ⊙, *p*H 6.3; ●, *p*H 6.3 with 0.059 mole fraction of glycerol.

(36) Since the light scattering depends upon the difference in refractive index between the protein and the surrounding medium, the addition of large amounts of glycerol would be expected to reduce opacity by raising the refractive index of the medium and correspondingly diminishing the refractive index difference. However, the magnitude of this effect, which is considered in the following paper,[37] would be negligible for the small proportions of glycerol employed in the studies reported here; thus, the opacity decrease is due to changes not in refractive index, but in structure, as shown also by the effect of glycerol on friability and syneresis.

(37) J. D. Ferry and P. R. Morrison, THIS JOURNAL, **69**, 409 (1947).

(38) Soluble starch (J. T. Baker) was triturated in cold water, dissolved in hot water at a concentration of 5%, and centrifuged to remove a small amount of insoluble material.

starch progressively increases the opacity and nullifies the effect of the glycerol.

Other Properties.—Examination of the clots described above, as well as those formed in other experiments with purified fibrinogen (*cf.* Fig. 8), showed the effect of the variables considered in this section upon the friability and syneresis.

Increasing the ionic strength from 0.15 to 0.45 (sodium chloride) increased the friability and diminished the syneresis index. At *p*H 6.2, changing the temperature of clotting had very little effect on the properties of the clot. Addition of glycerol before clotting in amounts up to a volume fraction of 0.1 increased the friability and decreased the syneresis index markedly.

Description in Terms of Clot Types.—The above results may be summarized in the statement that increasing ionic strength (up to 0.5), increasing temperature (at the upper end of the *p*H range), and increasing concentration of glycerol and other polyhydroxyl compounds of low molecular weight shift the clot properties in the direction of fineness; whereas increasing starch concentration shifts them in the direction of coarseness.

VI. Clotting Times

The experimental variables which have been studied influence the clotting time as well as the properties of the final clot.

Thrombin Concentration.—The reciprocal clotting time is plotted against thrombin concentration for several different fibrinogen concentrations (*p*H 6.64 to 6.81) in Fig. 14. The curvature shows that the clotting time is not quite inversely proportional to the thrombin concentration.

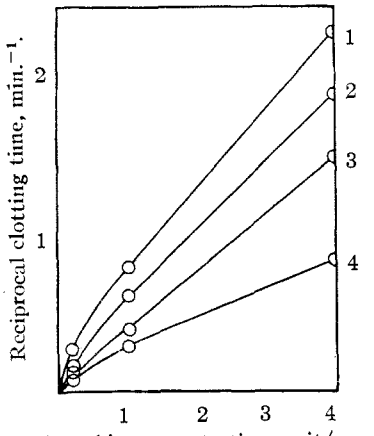

Fig. 14.—Reciprocal clotting time plotted against thrombin concentration, at ionic strength 0.30 and *p*H 6.64 to 6.81—fibrinogen concentrations in g./liter: 1, 3; 2, 6; 3, 12; 4, 24.

Fibrinogen Concentration.—The clotting times for 1 unit/cc. of thrombin, from Table II, are plotted against the *p*H in Fig. 15 for several different concentrations of fibrinogen. When the fibrinogen concentration is high the clotting time decreases with increasing *p*H. It increases with increasing fibrinogen concentration in the range covered here. At higher dilutions, however, where the conversion is first order with respect to fibrinogen as described above, the clotting time decreases with increasing fibrinogen concentration. The transition between the two ranges is shown in Fig. 16, where the clotting time of a preparation containing 74% of fibrinogen is plotted against fibrinogen concentration at a *p*H of 7.17 and ionic strength 0.15, at two different thrombin concentrations. The minimum shown has been noted by several investigators.[39]

(39) E. Wöhlisch, W. Diebold and O. Kiderlen, *Arch. ges. Physiol.*, **237**, 599 (1936).

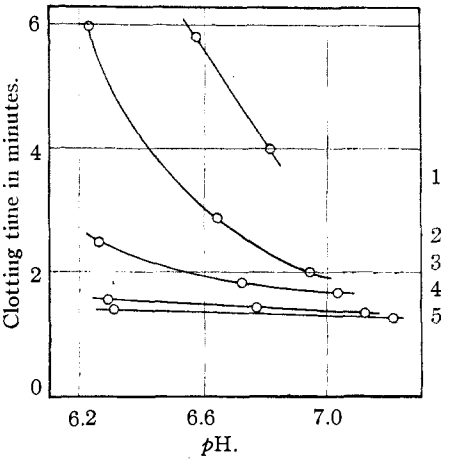

Fig. 15.—Clotting time plotted against *p*H, at ionic strength 0.30 and thrombin 1 unit/cc.—fibrinogen concentrations in g./liter: 1, 24; 2, 12; 3, 6; 4, 3; 5, 1.5.

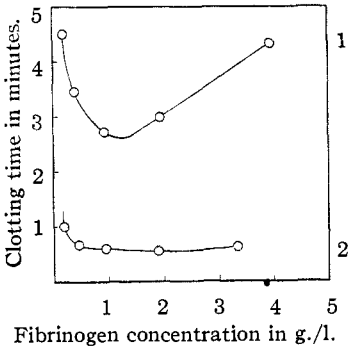

Fig. 16.—Clotting time plotted against fibrinogen concentration, at ionic strength 0.15 and *p*H 7.17—thrombin concentrations in unit/cc.: 1, 0.1; 2, 1.0.

Ionic Strength.—Increase in ionic strength prolongs the clotting time, as shown in Fig. 17 for a solution of Fraction I in sodium citrate, *p*H 6.3, at a fibrinogen concentration of 5 g./liter and various concentrations of thrombin.

Glycerol.—The addition of glycerol also markedly increases the clotting time, as shown in Table IV.

TABLE IV

EFFECT OF GLYCEROL ON CLOTTING TIME

Fibrinogen (80% purity) 20 g./liter, thrombin 0.5 unit/cc., ionic strength 0.30

Glycerol, mole fraction	0	0.013	0.026	0.059
Clotting time, min., *p*H 6.36	4	7	9	14
Clotting time, min., *p*H 7.06	1.7	3	6	8

VII. Discussion

The observed differences in the properties of clots formed under various conditions presumably depend upon the manner in which the fibrinogen molecules are linked together during polymerization.

Interpretation of Opacity Measurements.—In dilute solutions of spherical, isotropic particles, small compared with the wave length of light, the light scattering, of which the opacity is a measure, varies inversely with the fourth

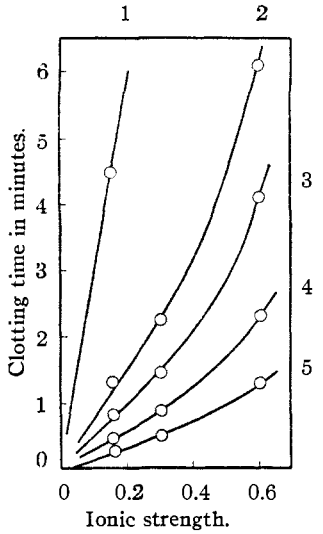

Fig. 17.—Clotting time plotted against ionic strength, for fibrinogen concn. 5 g./liter and *p*H 6.3—thrombin concns. in unit/cc.: 1, 0.1; 2, 0.5; 3, 1.0; 4, 2.0; 5, 5.0.

power of the wave length and is proportional to the molecular weight.[40] When the particle size is larger, the scattering increases with less than the inverse fourth power of the wave length and less than the first power of the molecular weight and becomes dependent upon particle shape.[41,42] In a solid structure, such as a fibrin clot, it is impossible to calculate explicitly the size and shape of structural units from opacity measurements, but the magnitude of opacity and the deviation from proportionality to $1/\lambda^4$ may both be taken as measures of the size of inhomogeneities of refractive index within the structure.

In our studies, the ratio of opacities at 3500 and 6000 Å. has been taken as a measure of the dependence of scattering upon wave length. Proportionality to $1/\lambda^4$ gives 8.6 for this ratio. Observed values for unclotted fibrinogen have ranged from 8.6 to 10. Observed values for clotted fibrin ranged from 7.4 for clots of the fine type (whose opacities were only slightly greater than that of an unclotted solution) to as low as 1.3 for clots of the coarse type (whose opacities were very much greater). Thus, both the magnitude of opacity and its dependence upon λ indicate that in the clots described as "fine" the structural inhomogeneities are but little larger than individual fibrinogen molecules, whereas in "coarse" clots they are very much larger.

When the opacity per unit concentration of fibrin, τ/c, at 6000 Å., is plotted against the 3500/6000 ratio, the data of Figs. 9, 10, 11 and 13, and of Tables II and III, all fall on a single curve, provided the fibrin concentration (*i.e.*, original fibrinogen concentration) is at least 5 g./liter and the thrombin concentration at least 1 unit/cc. (Fig. 18). This single function, for systems with different values of *p*H and ionic strength as well as those containing glycerol or starch, is further indication that the effects of these various environmental factors can be described by a single variable, as implied in the gradation from fine to coarse. However, when the fibrinogen concentration is very low, the relation shown in Fig. 18 does not hold; points lying to the right of the curve are observed.[43]

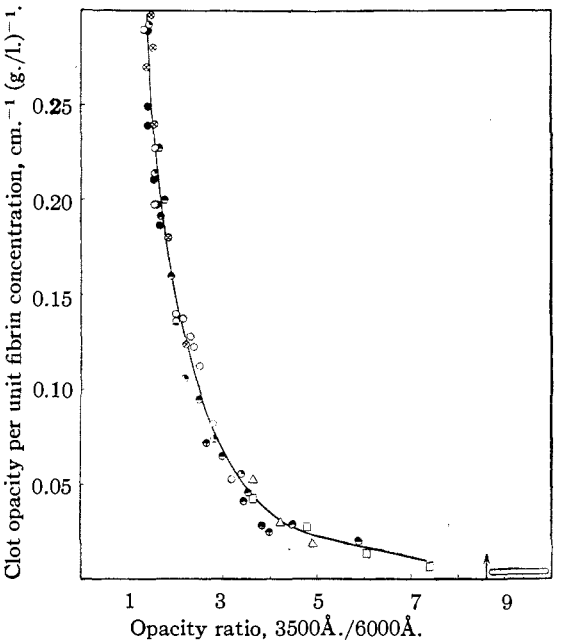

Fig. 18.—Opacity per unit concentration of fibrin, at 6000 Å., plotted against the ratio of opacities at 3500 and 6000 Å. The arrow shows the theoretical ratio for small particles ($\tau\lambda^4$ = constant) and the area, the range of values found for solutions of fibrinogen.

If the empirical relation of Fig. 18 holds for a system in which the fibrinogen is partially converted to fibrin, then measurements of the 3500/6000 ratio during clotting can serve to evaluate the change in τ/c during clotting, and, from the latter quantity and τ itself, the increase in fibrin concentration with time may be calculated. This is illustrated in Fig. 19 for a solution of Fraction I (46% fibrinogen). The calculated curve of relative fibrin concentration agrees fairly well with two points given by gravimetric determination.

Characterization of Clot Types.—The appropriateness of the terms "coarse" and "fine" in characterizing the two extreme types of fibrin clot is thus shown by deductions from opacity measurements. It is also supported by the other properties observed. The friability of the fine clot is consistent with the presence of delicate

(40) P. Debye, *J. Appl. Phys.*, **15**, 338 (1944).
(41) R. S. Stein and P. Doty, THIS JOURNAL, **68**, 159 (1946).
(42) W. Heller, personal communication.
(43) At *p*H 6.3 and a thrombin concn. of 0.1 unit/cc., increasing the fibrinogen concn. from 0.5 to 1.5 g./liter increased the 3500/6000 ratio from 1.4 to 3.4 while the opacity per unit concn. at 6000 Å. remained constant at about 0.66 cm.$^{-1}$ (g./l.)$^{-1}$.

structural elements easily broken by concentration of stress,[44] and the ease of syneresis of the coarse clot is consistent with the presence of large interstices from which fluid is readily expressed. Also, studies of the sieving out of protein molecules of various sizes when fluid is expressed from clots, to be reported elsewhere,[6] indicate that the interstices of the fine clot are much smaller than those of the coarse type.

Intermolecular Forces during Clotting.—A generalization may now be made concerning the conditions which determine clot structure. The conditions which favor decreasing coarseness are those which would be expected to diminish attractive forces and the tendency to aggregation. The isoelectric point of fibrinogen[12] is near 5.5; increasing the *p*H from 6.2 to 7.2 increases the net charge considerably,[12] and hence the mutual electrostatic energy; any tendency to aggregation should be thereby diminished. The decreased activity coefficient of the single protein molecule relative to that of an aggregate implied in this statement is of course reflected in the generally observed increase in protein solubility with net charge.[45] Likewise, solubility studies on globulins[45] such as fibrinogen show that increasing the ionic strength causes the activity coefficient to fall sharply and pass through a minimum; the tendency to aggregation should be similarly affected. The profound influence of *p*H and ionic strength in shifting the properties of the clot toward fineness parallels this exactly.

It may be inferred that glycerol and glucose, which also increase the fineness of clot structure, lower the activity coefficient of fibrinogen by some strong specific interaction; it would be desirable to investigate this by thermodynamic studies. Starch evidently has the opposite effect, leading to aggregation. Interaction of fibrinogen with these compounds probably involves their hydroxyl groups; the compounds of lower molecular weight may tend to solvate the protein, whereas the starch may serve as a bridge between protein molecules. The analogy with the contrasting behavior of monovalent and polyvalent antigens in antigen–antibody reactions[46] is suggestive.

Comparison with Gelation in other Systems.—A relation between the coarseness of gel structure and intermolecular forces of attraction which act during formation of a gel has been observed in other systems. Thus, in collodion membranes the size of the structural interstices can be varied from about 10 to about 10,000 Å., and the coarseness depends primarily upon the proportions of solvent and non-solvent in a solution which is concentrated by evaporation.[47] The higher the proportion of non-solvent, the more the nitrocellulose tends to aggregate before its concentration becomes high enough for gelation, and the coarser is the resulting gel. The extreme types of structure obtained, termed by Elford[48] ultragel and microgel, correspond to the fine and coarse clots of fibrin. Similar effects have been noted in solutions of other cellulose derivatives[49] and of Vinylite.[50]

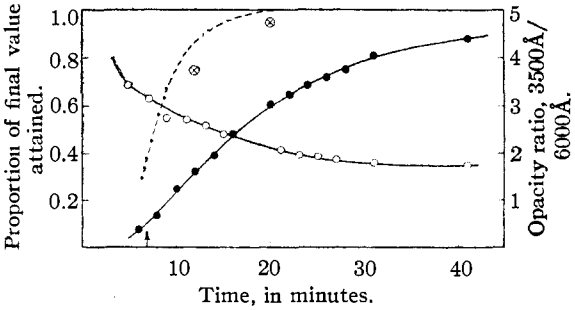

Fig. 19.—Changes in opacity (●), ratio of opacities at 3500 and 6000 Å. (○), and recoverable fibrin (⊗) during clotting of a solution with fibrinogen solution 6.0 g./l., thrombin concentration 0.2 unit/cc., ionic strength 0.30 and *p*H 6.3. The dashed curve represents the quotient of τ to τ/c interpolated from Fig. 18.

Relation of Clot Properties to Rate of Conversion.—Without attempting to formulate the kinetics of the reaction between fibrinogen molecules, one may be fairly sure that the rate constant includes a factor which depends upon mutual interaction energy; this factor can often be expressed as the ratio of the activity coefficient of a complex to the product of activity coefficients of the reactants.[51,52] Accordingly, changing environmental conditions to diminish intermolecular attraction should not only shift clot properties toward those of the fine type but also decrease the rate of conversion. If the extent of reaction at the clotting point is always the same, the "clotting time" should be correspondingly prolonged.

This correlation between prolonged clotting time and fineness of structure is shown in the effect of increasing ionic strength, which in the range from 0.15 to 0.5 decreases opacity (Fig. 9) and increases clotting time (Fig. 17). Similarly, increasing fibrinogen concentration above about 3 g./liter decreases the opacity per unit concentration (Fig. 5) and increases the clotting time (Figs. 15, 16). Also, glycerol decreases the opacity (Fig. 11) and increases the clotting time (Table IV).

(44) *Cf.* H. Mark in E. Ott, "Cellulose and Cellulose Derivatives," Interscience Publishers, New York, N. Y., 1943, p. 1039.

(45) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, Chapter 24.

(46) L. Pauling, D. H. Campbell and D. Pressman, *Physiol. Rev.*, **23**, 203 (1943).

(47) W. J. Elford, P. Grabar and J. D. Ferry, *Brit. J. Exp. Path.*, **16**, 583 (1935); P. Grabar, *Cold Spring Harbor Symp. Quant. Biol.*, **6**, 252 (1938).

(48) W. J. Elford, *Proc. Roy. Soc.* (London), **106B**, 216 (1930).

(49) H. M. Spurlin, A. F. Martin and H. G. Tennent, *J. Polymer Sci.*, **1**, 63 (1946).

(50) A. K. Doolittle, *Ind. Eng. Chem.*, **36**, 239 (1944).

(51) G. Scatchard, *Chem. Rev.*, **10**, 229 (1932).

(52) S. Glasstone, K. J. Laidler and H. Eyring, "The Theory of Rate Processes," McGraw-Hill Book Co., New York, N. Y., 1941.

On the other hand, the changes in clotting time caused by varying the thrombin concentration and the temperature do not depend primarily on interaction between fibrinogen molecules; they are due, of course, to the effects on the reaction rate of a change in concentration of catalyst and the Arrhenius factor, respectively. Accordingly, the above correlation between prolonged clotting time and fineness of structure is not observed. On the contrary, increasing thrombin concentration and temperature *diminish* the clotting time and shift clot properties toward fineness. This effect probably depends on the ratio of the reaction rate to the translational and rotational diffusion rates of fibrinogen molecules. When the ratio is high, aggregation during clotting will tend to be suppressed; just as in the crystallization of a solid, a rapid reaction produces fine crystals and a slow reaction coarse crystals.

The effect of *p*H on clotting time, however, is opposite to that expected from these considerations. Increasing *p*H increases the fineness of clot structure, and since this is attributed to a change in fibrinogen interaction, concomitant prolongation of the clotting time would be expected; instead it is shortened (Fig. 15). This anomaly might be interpreted as due to a change in the number of reactive groups per molecule with *p*H, but further work will be necessary to clarify it.

VIII. The Structure of Fibrin

In very dilute solution, the mode of junction of fibrinogen units in forming a clot is primarily end-to-end. This is indicated by the fact that a volume fraction of as little as 3×10^{-5} suffices for gelation,[13] which means that the ratio of length to diameter of the network strands is very great. A rough lower limit for this ratio may be calculated by assuming a regular lattice structure for the network, with fully-extended cylindrical strands between crosslinks. For a diamond lattice (four strands meeting at a crosslink) the value is 190; for a simple cubic lattice (six strands meeting at a crosslink) it is 280. Other structures, for example involving three strands meeting at a crosslink (trifunctional branching[31]), might be assumed, but the order of magnitude of the ratio would probably be unaffected. In any case, the strands are undoubtedly far from fully extended, so that the actual value of the ratio is greater than 190 to 280. Since the fibrinogen molecule has an axial ratio[14] of about 20, the number of fibrinogen units joined end-to-end in an average network strand must be greater than 10 to 14, to permit gelation at such high dilution.[53] It is probable, therefore, that the groups involved in polymerization of fibrinogen are located primarily on the ends of the molecule. The crosslinking bonds may, however, be on either the ends or the sides.

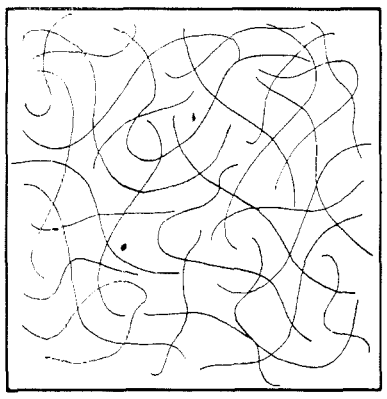

Fig. 20.—Schematic illustration of proposed structure of the fine clot.

The Fine Clot.—In the fine clot, the low opacity and the small deviation from Rayleigh scattering indicate that the structural unit is small; it may be assumed to be a single chain of fibrinogen molecules, joined end to end. The low permanent set indicates that the crosslinks are fairly strong; the fact that fibrin is insoluble in reagents such as urea and potassium thiocyanate, which often dissociate protein structures,[54,55] is evidence that at least some of the crosslinks are primary chemical bonds. Secondary bonds would not be expected in any case, since the conditions under which the fine clots are formed are unfavorable to aggregation (see above).

A schematic illustration of the proposed structure of the fine clot is given in Fig. 20.

It is of interest to test the applicability of the theory of rubberlike elasticity[56-58] in interpreting the rigidity of the fine-structure clot. The theory has been developed for a network of long molecules with many bonds about which free rotation can occur, so that transitions among many different configurations take place readily. The postulated fibrin network may differ in two respects from this ideal model: the chains may be extended more nearly taut between crosslinks, and, if 35 Å. in diameter, they may be imperfectly flexible, with an inherent stiffness. Nevertheless, the properties of the fine clot are similar

(53) Another mechanism for providing a strand with an axial ratio of several hundred would be the opening of a single fibrinogen molecule to form an extended polypeptide chain, as observed in the denaturation of corpuscular proteins.[9-11] This appears unlikely, not only because of the evidence already cited,[11-12] but also because of the stability of the fine-structure clot. Denatured corpuscular proteins are generally so hydrophobic that they aggregate and form compact precipitates unless held in solution by urea, strong acid or alkali, or detergents. By contrast, a fine-structure fibrin clot is well-dispersed at *p*H 7; it is rigid, it is true, owing to the three-dimensional network, but the above discussion shows that the strands have no tendency to roll up and the opacity studies show that there is no great tendency to lateral aggregation.

(54) E. O. Kraemer, *J. Phys. Chem.*, **45**, 660 (1841).

(55) J. Steinhardt, *J. Biol. Chem.*, **123**, 543 (1938).

(56) F. T. Wall, *J. Chem. Phys.*, **10**, 132, 485 (1942); **11**, 527 (1943).

(57) H. M. James and E. Guth, *ibid.*, **11**, 455 (1943).

(58) P. J. Flory and J. Rehner, Jr., *ibid.*, **11**, 512 (1943).

to those of a swollen gel of vulcanized rubber; and it is known that a polymer such as cellulose acetate, which has considerable molecular stiffness,[41] can manifest rubberlike elasticity to some extent.[59]

The theory of rubber-like elasticity[56] specifies for a tetrahedral model[58] the number of crosslinks per cc., n, as $G/2kT$, where G is the modulus of rigidity.[60] In a clot containing 10 g./liter of fibrin, $G = 2$ to 3×10^4 dynes/sq.cm. (Fig. 7); thus $n = 2.5$ to 3.7×10^{17} cc.$^{-1}$. Since at this concentration the number of fibrinogen units per cc. is 13 to 20×10^{15} (depending on the value taken for the molecular weight), about 20 crosslinks per fibrinogen unit would be required to account for the observed rigidity on the basis of ordinary rubber-like elasticity. But it is geometrically impossible to have such a densely crosslinked structure in so dilute a system; for a diamond lattice model, cylindrical, fully-extended chains at this concentration must have a ratio of length to diameter of at least 9 in order to form a continuous solid structure. Since this figure is approximately one-half the axial ratio of the fibrinogen molecule, the number of crosslinks cannot exceed one or two per fibrinogen unit, and is probably much smaller because the chains are not fully extended. The rigidity of the clot is thus much higher than can be accounted for by the mechanism of ordinary rubberlike elasticity. It presumably involves some other mechanism, perhaps the inherent stiffness of the chains.

The Coarse Clot.—High opacity and departure thereof from Rayleigh's law indicate that the coarse type of clot has much larger structural units. It may be assumed that they are not single chains of fibrinogen molecules but bundles of such chains. Since the conditions under which coarse clots are formed favor intermolecular attraction, it is reasonable to expect aggregation of this sort. It is confirmed by the observation of fibrils in the electron microscope,[61] ranging in diameter from 200 Å. (corresponding to a bundle of about twenty fibrinogen chains) to very much higher values. The larger fibrils are visible in the ordinary microscope.[62]

Fewer primary crosslinks would be expected in this structure, since many potential combining groups would be hidden within chain bundles. However, the modulus of rigidity is if anything slightly higher for a coarse than for a fine clot of the same fibrin concentration. This may be partly due to a greater inherent stiffness of the coarser chain bundles, and partly to presence of secondary crosslinks which make up for the loss of primary crosslinks. Some of the secondary links may be produced by bifurcations of chain bundles and intermittent lateral association of bundles; the electron microscope shows examples where two fibrils converge, are joined for some distance, and then diverge again.[61] The existence of weak secondary bonds is indicated by the characteristic properties of the coarse clot: the plasticity and permanent set, the lack of friability, and the ease of syneresis, in which fluid is removed from the relatively coarse interstices in the structure and the chain bundles are compacted together.

A schematic illustration of the proposed structure of the coarse clot is given in Fig. 21. This picture is further developed in the following paper,[37] in which elastic films derived from coarse clots are described.

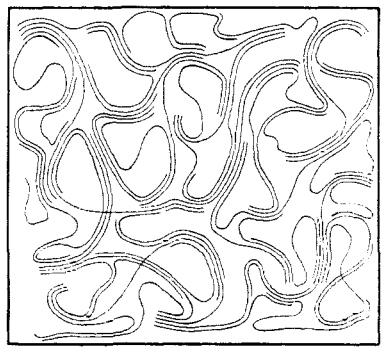

Fig. 21.—Schematic illustration of proposed structure of the coarse clot.

Further information concerning the number of combining groups on the fibrinogen molecule, and their location on its surface, as well as their chemical identity, may be forthcoming from

(59) J. G. McNally and S. E. Sheppard, *J. Phys. Chem.*, **35**, 100 (1931).

(60) The modulus of elasticity, E, is $3kT\nu/V$, where ν/V is the number of chains per unit volume; in the tetrahedral model, the number of crosslinks is half the number of chains; and $G = E/3$, since Poisson's ratio = ½. Thus $n = G/2kT$.

(61) C. Wolpers and H. Ruska, *Klin. Wochenschr.*, **18**, 1077, 1111 (1939). These observations were made on plasma after delaying the normal clotting process by addition of heparin. It is hoped that electron microscope studies may be made on clots formed from purified fibrinogen and thrombin.

NOTE ADDED IN PROOF.—Electron micrographs of clots formed from bovine Fraction I and thrombin have been recently made by Drs. C. V. Z. Hawn and K. R. Porter (personal communication). At *p*H 6.8 the observed structure was similar to that found by Wolpers and Ruska, with fiber widths of 1000 to 3000 Å. In these specimens staining revealed a striking longitudinal periodicity of about 200 Å which points to a very precise lining up of the individual molecules in forming fibers. As expected, at the higher *p*H of 8.0, the structure was much finer, approaching the width of one or two fibrinogen molecules.

(62) When examined under the microscope at a magnification of 900 diameters, clots made from Fraction I at *p*H 6.0 appeared as three-dimensional networks. At a fibrinogen concentration of 1.0 g./l., as the thrombin concentration was increased from 0.02 to 0.5 unit/cc., the width of the fibrils decreased from 1 to 2μ until they could barely be seen. The size of the meshes similarly decreased from 20 to 30μ to much smaller values. This corresponds to the observations of others[63] that with rapid clotting much smaller fibers are formed. At *p*H 7.0 no structure has been seen with any values of fibrinogen or thrombin concentration we have employed. When clotting was initiated by grains of thrombin, much larger fibers were formed. This situation may be more analogous to that in blood or plasma where clotting appears to progress from the platelets.

(63) W. H. Howell, *Am. J. Physiol.*, **35**, 143 (1914); L. M. Tocantins, *ibid.*, **114**, 709 (1936).

careful organic chemical studies on the one hand, and from a detailed investigation of the kinetics of fibrin formation and application of the theories of crosslinking on the other.

Summary

1. Solutions of human fibrinogen, ranging in purity from 46 to 90%, were clotted with human thrombin, and the effects of fibrinogen concentration, thrombin concentration, *p*H, ionic strength, temperature and the addition of certain polyhydroxyl compounds upon the clotting time, opacity, rigidity, friability, syneresis and mechanical properties of the resulting clots were determined.

2. After addition of thrombin, the opacity begins to increase; later, the solution "clots" or becomes rigid; the amount of recoverable fibrin, the opacity, and the rigidity subsequently increase, and attain their final values in the order named. This behavior is interpreted as a three-dimensional polymerization.

3. The fibrin clots formed under different conditions may be classified as ranging in properties between two extreme types: the fine clot, which is transparent, elastic, friable and non-synerizing, with a low elongation at break and low permanent set; and the coarse clot, which is opaque, plastic and non-friable, synerizes very readily, and has a high elongation at break and high permanent set.

4. The opacity per unit fibrin concentration decreases with increasing fibrin concentration in both types of clots. The variation of opacity with wave length deviates only slightly from Rayleigh's law in the fine clot, but deviates markedly in the coarse clot.

5. The modulus of rigidity of the fine clot varies with approximately the 1.6 power of concentration. It is higher than can be accounted for by the theory of ordinary rubber-like elasticity.

6. The properties characteristic of the fine clot are favored by increasing fibrinogen concentration, increasing *p*H, increasing ionic strength (from 0.15 to 0.5), increasing temperature (at *p*H 6.8), and the addition of glycerol and other polyhydroxyl compounds of low molecular weight. The properties characteristic of the coarse clot are favored by the addition of soluble starch.

7. Under the conditions which favor formation of the fine clot, the forces of attraction between fibrinogen molecules are lowered and the tendency to aggregation is diminished. These conditions are usually associated with longer clotting times. However, when the clotting time is prolonged without altering the interaction between fibrinogen molecules, the properties of the clot are shifted toward coarseness.

8. The proposed structure of the fine clot is a network of chains, consisting of fibrinogen molecules joined end to end, crosslinked at least partly by primary chemical bonds. The proposed structure of the coarse clot is a network of bundles of such chains, crosslinked largely by secondary bonds and by lateral association.

BOSTON, MASS. RECEIVED JULY 22, 1946

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

Preparation and Properties of Serum and Plasma Proteins. IX. Human Fibrin in the Form of an Elastic Film[1,2]

BY JOHN D. FERRY AND PETER R. MORRISON

The physical and chemical properties of solid protein systems and their relation to structure are of basic importance in physiology; and they present many interesting problems in macromolecular chemistry. The natural structures of keratin have been extensively studied with respect to X-ray analysis,[3] mechanical properties,[4] and combination with acids and bases[5]; and collagen[6] and elastin[7] have also been investigated in their natural forms (tendon and elastic ligament, respectively).

Other studies of solid proteins have been made with artificial structures—fibers and films—prepared from denatured proteins.[8] These systems have the advantage that they can be prepared with reproducible and uniform dimensions, but because of the drastic chemical treatment involved in their formation they may be quite different chemically from their native protein precursors and from the natural structures of biological interest.

In this paper, a solid protein structure of fibrin

(1) This work has been carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

(2) This paper is Number 48 in the series "Studies on Plasma Proteins" from the Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

(3) W. T. Astbury, *J. Chem. Soc.*, 337 (1942); *Chem. Ind.*, **40**, 491 (1941).

(4) (a) W. T. Astbury and H. J. Woods, *Phil. Trans. Roy Soc.*, **A232**, 333 (1933); (b) M. Harris, L. R. Mizell and L. Fourt, *Ind. Eng. Chem.*, **34**, 833 (1942); (c) H. B. Bull, THIS JOURNAL, **66**, 1253 (1944); *ibid.*, **67**, 533 (1945).

(5) J. Steinhardt, *Ann. N. Y. Acad. Sci.*, **41**, 287 (1941).

(6) (a) W. T. Astbury, *Intern. Soc. Leather Trades Chem.*, **24**, 69 (1940); (b) R. S. Bear, THIS JOURNAL, **66**, 1297 (1944).

(7) E. Wöhlisch, *Arch. ges. Physiol.*, **246**, 469 (1942); *Kolloid Z.*, **104**, 14 (1943).

(8) H. P. Lundgren and R. A. O'Connell, *Ind. Eng. Chem.*, **36**, 370 (1944); W. T. Astbury, *Nature*, **155**, 501 (1945).