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).

is described, which can be prepared without chemical alteration in uniform samples of chosen dimensions, and is chemically identical with the naturally occurring fibrin of clotted blood.

When purified preparations of fibrinogen and thrombin are allowed to react, fibrin clots of very different properties are formed depending upon the conditions of concentration, *p*H and ionic strength.[9] One type of clot is characterized by opacity, plasticity and remarkable ease of syneresis, which are considered to be associated with coarseness of structure. The ease of syneresis permits it to be compacted, under slight pressure, to form a strong, rubbery, opaque sheet, which has been called fibrin film.[10] This paper gives a description of the properties of fibrin film and their interpretation in terms of our previous discussion[9] of the structure of the clot from which it is derived.[11]

Materials

The fibrinogen used in these studies was obtained from Fraction I of human plasma.[14] Fibrin films were ordinarily prepared directly from this fraction, which contained 45 to 60% of fibrinogen. However, films with similar properties were also prepared from refractionated preparations[15] in which 90% or more of the protein was fibrinogen. The thrombin was obtained from Fraction III-2 of human plasma.[16,17] Concentrations of fibrinogen and thrombin were determined as previously described.[9] Both the fibrinogen and thrombin were stored dry *in vacuo*.

Preparation of Fibrin Film

Fraction I was dissolved in a sodium chloride–citrate buffer at a fibrinogen concentration of 5 g./liter, a *p*H of 6.2 to 6.4, and ionic strength 0.15. The other proteins present, principally β and γ globulins together with small amounts of α globulins and albumin, amounted to 3 to 4 g./liter.

A small volume of a solution of thrombin in 0.15 *M* sodium chloride was added to the Fraction I solution to bring the thrombin concentration to 0.3 to 0.5 unit/cc.[18] The mixture was thoroughly stirred, filtered through cheesecloth to remove any foam present, and poured into a rectangular tray, the depth of solution being ordinarily about 2 cm. The resulting clot became rigid and opaque in a few minutes, but the clotting reaction was allowed to proceed for one hour to ensure completion of the transformation of fibrinogen to fibrin. The clot was then pressed between layers of fine muslin under low pressure which was increased in stages from 5 to 25 g. per sq. cm. A solution containing salts and the proteins other than fibrin was expressed; the thickness of the clot was diminished to approximately one-fiftieth of its original value, while its area increased by only a few per cent.

The fibrin film formed in this way was produced by compaction of the clot in one dimension. Its size and shape were determined by the size and shape of the clot as projected on the plane of application of the compacting pressure. The film was rubbery and easily deformable. Upon release of stress, it returned to its original shape, although complete retraction was not instantaneous and under some conditions a permanent set was observed. It had the general characteristics of a cross-linked elastomer with delayed elastic return.

Almost all the proteins other than fibrinogen originally present were removed in the pressing, as shown by the fact that the weight of the film, dried at 100° after brief washing in distilled water, was only about 5% higher than the fibrinogen content of the original solution.[19] This small discrepancy, attributed to occlusion of other proteins, has been neglected, and the dry weight of the film may for most purposes be taken as equivalent to its fibrin content.

Films have been prepared with weights ranging from 0.1 to 60 mg. fibrin per sq. cm., varied by adjusting the depth and concentration of the fibrinogen solution before clotting. The corresponding range of thicknesses of the water-equilibrated film is 0.003 to 2 mm. Most of the films used in the studies reported here contained about 10 mg. per sq. cm.

Before measurements of physical properties were made, the films were stored for at least one day, and sometimes for as long as two months, either in a partly desiccated state at 5° or, after replacement of most of the water by glycerol, at room temperature.

Equilibria with Water and Glycerol

When immersed and equilibrated in water or 0.15 *M* sodium chloride, fibrin film prepared as described has a fibrin content of 30 ± 2% by weight. The water may be replaced by different mixtures of water and glycerol and the equilibrium swelling of the fibrin film in each mixture again determined. The fibrin content of the film is plotted against the percentage of glycerol in the swelling agent, or plasticizer, in Fig. 1. It is apparent that intermediate mixtures of glycerol and water swell the film more, and hence interact more strongly with the protein, than does pure water or a more concentrated glycerol solution.

Density.—The density of fibrin film equilibrated in water was measured by displacement of water in a pycnometer. Densities of other films, plasticized with less than the equilibrium amount of water, or with glycerol–water mixtures, were measured under mineral seal oil. In the case of glycerol–water mixtures, the composition of the plasticizer was estimated by heating a weighed strip of film at 100° for thirty minutes to remove the water, weighing, soaking in water

(9) J. D. Ferry and P. R. Morrison, THIS JOURNAL, **69**, 388 (1947).

(10) J. D. Ferry and P. R. Morrison, *J. Clin. Investigation*, **23**, 566 (1944).

(11) Fibrin film has been used in a variety of surgical applications,[12] and has been prepared under contract with the United States Navy from plasma fractions derived from blood collected by the American Red Cross. Its usefulness depends upon its mechanical properties as well as its faculty of being eventually absorbed after implantation in the tissues, in many cases with almost no irritative reaction. For most surgical applications, the film is treated by heat for sterilization, and its properties are somewhat different[13] from those of the untreated film described here.

(12) F. D. Ingraham, O. T. Bailey and C. J. Cobb, *J. Am. Med. Assoc.*, **128**, 1088 (1945); a bibliography is given by Ferry and Morrison, *Ind. Eng. Chem.*, **38**, 1217 (1946).

(13) J. D. Ferry, M. Singer, P. R. Morrison, J. D. Porsche and R. L. Kutz, THIS JOURNAL, **69**, 409 (1947).

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

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

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

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

(18) While the values of fibrinogen and thrombin concentrations, *p*H and ionic strength specified in this procedure can be varied somewhat, the films described in this paper were all prepared under the conditions given above.

(19) The fibrinogen content is taken as the amount of clottable protein recovered as fibrin by clotting under standard conditions where the yield of fibrin has been shown to be complete and the occlusion of other proteins, while not entirely precluded, is reduced to a minimum.[20]

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

one hour to remove the glycerol, drying at 100°, and weighing again.

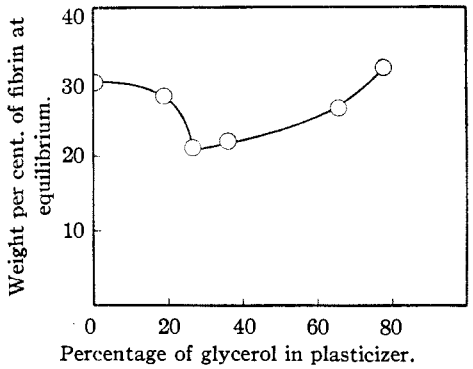

Fig. 1.—Equilibrium swelling of fibrin film in glycerol–water mixtures.

The densities are plotted against the percentage of fibrin in Fig. 2. Figures opposite the curves refer to the percentage of glycerol in the plasticizers. The values for the plasticizer mixtures alone were obtained from the "International Critical Tables." The dry fibrin, in this form, has a density of 1.304 ± 0.005.

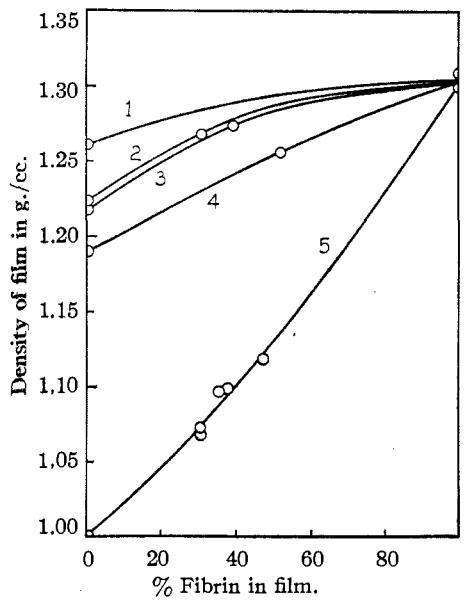

Fig. 2.—Densities of fibrin films at 25°, plotted against fibrin content. Plasticizers: 1, glycerol; 2, 86% glycerol; 3, 84% glycerol; 4, 73% glycerol; 5, water.

Compaction under Pressure.—When water-equilibrated film, containing 30% of fibrin, is pressed between moist layers of absorbent material such as fine muslin, the fibrin content is readily increased from 30 to 40% at a very low pressure (Fig. 3). To expel water beyond this point requires much higher pressures; however, at 30 kg. per sq. cm. a fibrin content of about 60% is attained. When the pressed film is immersed in water, it swells and the fibrin content returns nearly to its original equilibrium value (Fig. 4).

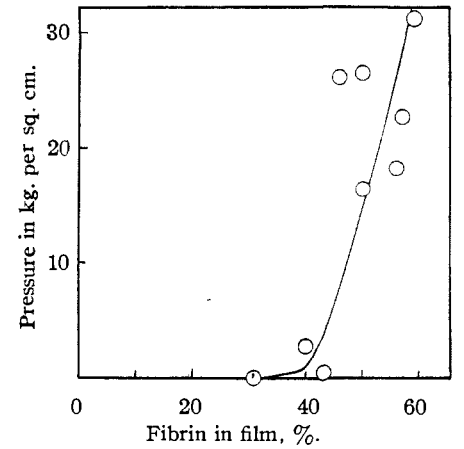

Fig. 3.—Compaction of fibrin film under pressure.

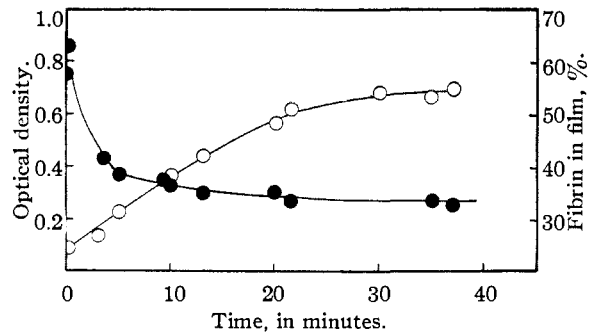

Fig. 4.—Imbibition of water by pressed fibrin film, with decrease in fibrin content (●) and rise in opacity (○).

Opacity

When water is removed from the highly opaque, water-equilibrated film, either by partial drying or by compaction under pressure as described above, the opacity decreases and the film becomes nearly transparent when a fibrin content of 60% is attained. As a measure of opacity, the transmission of the film to green light (Zeiss filter L-II) was measured in a Pulfrich photometer, and the optical density (negative logarithm of the fraction of light transmitted) was calculated. When the transparent pressed film is swollen in water, the opacity, like the fibrin content, returns to its original value (Fig. 4). The dependence of optical density on fibrin content was studied by adjusting the latter in three ways: (1) drying the film to 10% moisture (90% fibrin) and swelling it for brief periods; (2) pressing the film at high pressures and swelling it for brief periods (Fig. 4); (3) pressing the film at different pressures (Fig. 3). The optical densities of films subjected to these different treatments are plotted in Fig. 5 against the fibrin content, and are in good agreement.

Both pressing and drying decrease the volume of the film by diminishing its thickness, while the

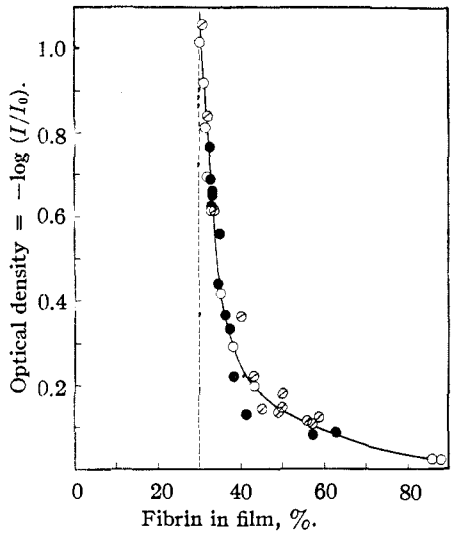

Fig. 5.—Optical densities of fibrin films plotted against fibrin content: O, dried and swollen; ●, pressed and swollen; ⊘, pressed at different pressures.

area remains essentially unchanged. Accordingly, Fig. 5 represents comparisons of the opacity of the same amount of fibrin per unit area, swollen to different extents. An alternative measure of the opacity is the optical density per unit thickness. This is plotted in Fig. 6, which represents comparisons of the opacity of different amounts of fibrin each swollen to unit thickness. In both plots, it is apparent that there is a much greater change in opacity with fibrin concentration below a concentration of 40% than above this value. This phenomenon, together with the similar effect in the curve for compacting pressure (Fig. 3), suggests that a structural transition occurs at 40% fibrin—an interpretation which is further supported by measurements of mechanical properties, described below.

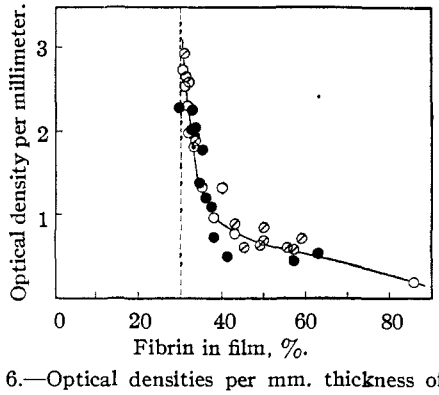

Fig. 6.—Optical densities per mm. thickness of fibrin films plotted against fibrin content: O, dried and swollen; ●, pressed and swollen; ⊘, pressed at different pressures.

When the aqueous plasticizer of fibrin film is replaced by glycerol–water mixtures, the fibrin content remaining approximately constant, the opacity is greatly diminished. The optical density per unit thickness of film, divided by the fibrin content, is plotted in Fig. 7 against the percentage of glycerol in the plasticizer. When the glycerol is washed out in water, the original opacity is regained.

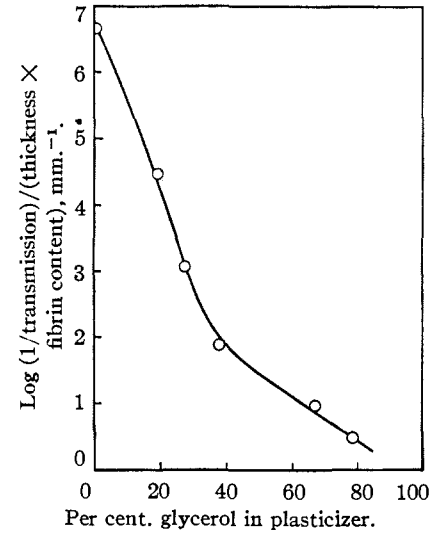

Fig. 7.—Optical densities of fibrin film per mm. thickness, divided by fibrin content, plotted against percentage of glycerol in the plasticizer.

As in the case of fibrin clots,[9] the opacity of fibrin film is due to scattering of light. The theory of light scattering by inhomogeneous structures[21] consisting of spherical elements (small compared with the wave length of light) with a refractive index n_1 immersed in a medium of refractive index n_2 specifies that the turbidity is proportional to $(n_1^2 - n_2^2)^2$. In an irregular solid structure, the scattering depends in a complicated way on the sizes and shapes of inhomogeneities, but if there are only two discrete values of refractive index the above factor still applies.[22] There are few data on refractive indices of solid proteins from which n_1 can be estimated; a value for the protein of rubber latex,[23] 1.519, may be cited to show the magnitude to be expected. The fact that it is much closer to the refractive index, n_2, of glycerol (1.473) than that of water (1.333) explains the effect shown in Fig. 7. It is impossible to analyze the data quantitatively, however, without knowledge of the degree of solvation of the fibrin elements responsible for the scattering.

The turbidity of the water-equilibrated fibrin film is still further increased by stretching; this must be due to rearrangement of the fibrin elements to form configurations which scatter light more strongly.

Porosity and Permeability

Further information concerning the structural

(21) *Cf.* W. Heller, *Phys. Rev.*, **68**, 5 (1945).
(22) P. Debye, personal communication.
(23) H. P. Stevens, *India-Rubber J.*, **105**, 329, 332 (1943).

discontinuities in fibrin film may be obtained from studies of the rate of flow of water through films under pressure and of ultrafiltration.

Films containing about 3 mg. of fibrin per sq. cm. were equilibrated in water and supported on Seitz type filter pads, and phosphate buffer of *p*H 6.2 and ionic strength 0.15 was forced through them at a pressure of 1 atmosphere. The rates of flow were of the order of 0.2 cc. per hour per sq. cm. of area. By assuming a model structure of cylindrical pores of circular cross-section perpendicular to the film surface, an average pore diameter can be calculated.[24] In the case of collodion membranes of high water content, there is some evidence that this procedure gives a reasonable estimate of the size of whatever openings actually exist.[25] Values calculated for fibrin film ranged from 45 to 100 Å.

In other experiments, solutions of hemoglobin and Fraction I from plasma were ultrafiltered through fibrin film, using the same experimental arrangement as in the rate of flow measurements. Hemoglobin passed through the film readily in almost undiminished concentration; the nonclottable proteins in Fraction I passed to a slight extent, and fibrinogen did not filter through at all. Since hemoglobin and the smaller proteins of plasma have diameters (on the basis of ellipsoids of revolution) of between 30 and 40 Å., it may be concluded that the openings in the film are at least of this order of magnitude. The failure of fibrinogen, which on the basis of an elongated ellipsoid has a diameter of about 35 Å., to pass through at a perceptible rate may be attributed to the small probability of the proper end-on orientation which would be necessary for penetration of such an elongated molecule (length 700 Å., contrasted with about 150 Å. for hemoglobin) into an opening not much larger than its smallest dimension, and to the probable tortuosity of the channels in the film.

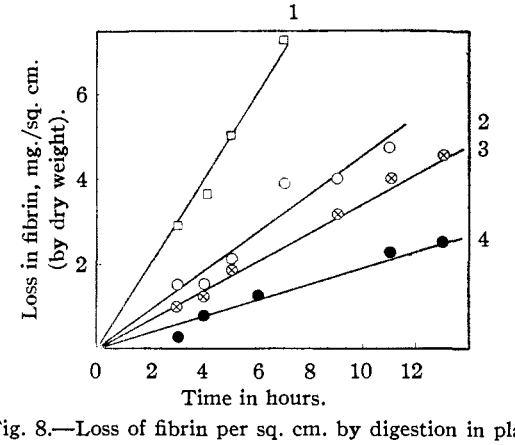

Fig. 8.—Loss of fibrin per sq. cm. by digestion in plasmin, plotted against time, at 25° in 0.15 *M* sodium chloride, *p*H 6.8; film area 2.0 ± 0.1 sq. cm.; initial film thickness: 1, 13 mg./sq. cm. of fibrin; 2, 3, 4, 6 mg./sq. cm. Concentration of plasmin in arbitrary units: 1 and 2, 20; 3, 10; 4, 5.

That the water-equilibrated film is permeable to small protein molecules is also indicated by the course of its digestion by plasmin, the proteolytic enzyme of plasma.[26] The weight of fibrin digested is proportional to the time (Fig. 8), and the rate of digestion increases with increasing concentration of enzyme. Moreover, the rate of fibrin destruction is roughly proportional to the thickness of the film (curves 1 and 2), indicating that the enzyme penetrates the structure and that digestion proceeds throughout the volume of film. If the film were impermeable and digestion limited to the surface, the rate of destruction should be independent of thickness.

Mechanical Properties

Stress-strain curves of strips of fibrin film were determined by measurements at room temperature with a scale and spring balance. The stress was calculated on the original cross-section, following the convention ordinarily used for rubberlike materials.

Cycle of Deformation.—Strips of film equilibrated with water were subjected to stepwise increases of load at equal time intervals until an elongation of about 100% was attained, and then the stress was reduced in the same manner. Stress-strain curves obtained in this way are plotted in Fig. 9 for several different rates of loading. There is marked hysteresis and some permanent set. Although the permanent set increases with decreasing rate of loading, there is comparatively little alteration in the hysteresis loop over a considerable range of loading rates. Similar results were obtained with film containing 31% of fibrin plasticized with 85% glycerol.

Creep and Creep Recovery.—Strips of film immersed in water were stretched at constant load and the changes in length were observed for one hour. The load was then removed and the changes in length were again followed. In every case, the phenomena of creep and creep recovery were observed (Fig. 10), accompanied by a small permanent set. It is of interest that the curves for creep and creep recovery do not have the same shape; the latter is much sharper. This shows that the Boltzmann superposition principle is not followed[27]; *i.e.*, the effects of loading and unloading are not additive.

Extension Curves.—In spite of hysteresis and creep, the stress-strain curve for extension gives a mechanical characterization of fibrin film which does not depend markedly upon the rate of loading in the range from 20 to 100 g./sq.mm./min. (Fig. 9). Curves of this sort were obtained,

(24) H. Bechhold, *Z. physik. Chem.*, **60**, 257 (1907).
(25) J. D. Ferry, *Chem. Rev.*, **18**, 373 (1936).
(26) L. R. Christensen and H. P. MacLeod, *J. Gen. Physiol.*, **28**, 559 (1945).
(27) L. Boltzmann, *Ann. Physik*, **7**, 624 (1876); H. Leaderman, "Elastic and Creep Properties of Filamentous Materials and other High Polymers," The Textile Foundation, Washington, 1943.

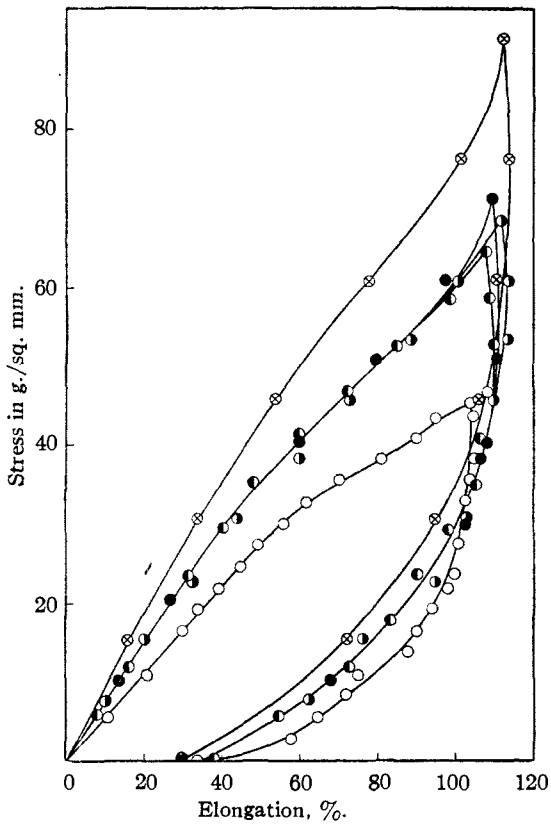

Fig. 9.—Load-elongation cycles of water-equilibrated fibrin film; loading rates in g./sq. mm./min.: ○, 5.4; ◐, 29; ◑, 38; ●, 97; ⊗, 112.

at a loading rate of about 50 g./sq.mm./min., for water-plasticized films of different fibrin contents. The fibrin content was adjusted by pressing or partial desiccation, and the measurements were made quickly before evaporation occurred. The results of several typical experiments are shown in Fig. 11. At the equilibrium fibrin content of about 30%, representing maximum swelling in water, the stress-strain curve is almost linear up to 100% elongation. The apparent modulus of elasticity, represented by the initial slope, is about 60 g./sq.mm. As the fibrin content is increased, the entire curve is shifted to higher stresses, and above a fibrin content of 40% it becomes convex to the stress axis, so that the initial stresses at low elongations are high.

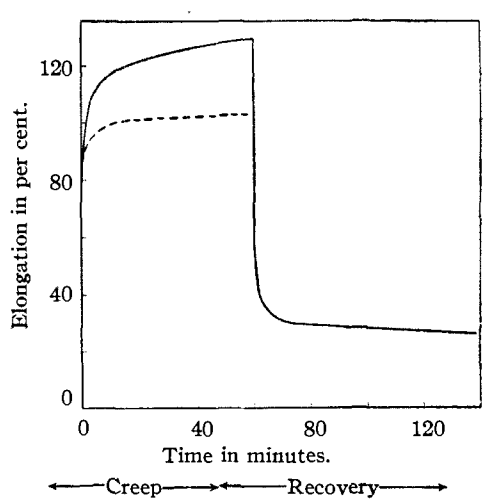

Fig. 10.—Creep and creep recovery of water-equilibrated fibrin film: stress, 60 g./sq. mm. The dashed curve, representing elongation during creep recovery subtracted from the value of elongation when load was removed, is given to facilitate comparison of creep and creep recovery.

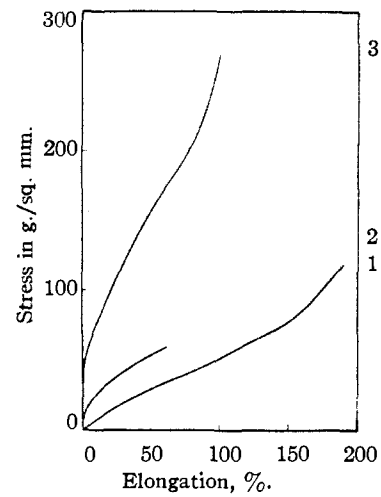

Fig. 11.—Stress-strain curves of water-plasticized fibrin film, fibrin contents: 1, 30%; 2, 44%; 3, 59%.

With glycerol–water mixtures as plasticizers, the same transition from a linear to convex stress-strain curve appears, but it occurs at a lower fibrin content, as indicated in Fig. 12. With 95% glycerol as plasticizer, the transition evidently occurs at a concentration below that of equilibrium swelling, since only convex stress–strain curves are observed.

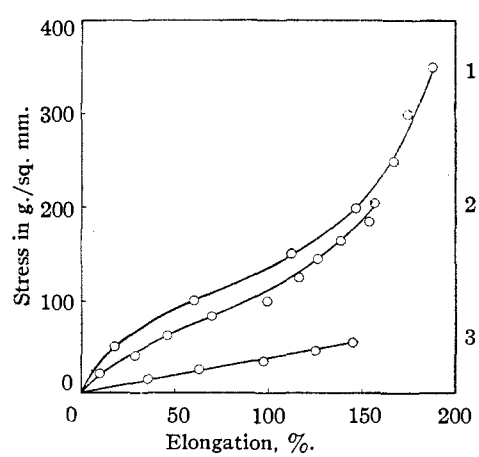

Fig. 12.—Stress–strain curves of fibrin film plasticized with 75% glycerol, fibrin contents: 1, 44%; 2, 33%; 3, 27%.

TABLE I

CALCULATION OF FROZEN-IN STRAIN FROM CHANGES IN AREA AND VOLUME

	Before treatment	After treatment: Acid swelling[a]	Thermal contraction[b]	KCNS 4*M*	Urea 6*M*
Appearance	Opaque	Transp.	Opaque	Transp.	Transp.
Fibrin content, %	28.5	12.4	27.6	21.3	18.0
Vol. fraction fibrin[d]	0.230	0.095	0.222	0.193	0.152
Prop. increase in volume (R_V)		2.42	1.04	1.19	1.51
Prop. increase in area (R_A)		0.701	0.369	0.517	0.676
Frozen-in strain ($\alpha = R_V^{1/3}/R_A^{1/2}$)		1.60	1.66	1.47	1.40
Fibrin content after washing, %				36	18
α calculated from shape after washing				1.37	1.35
Apparent modulus of elasticity,[c] g./mm.^{2}	67		18	23	25

[a] Swelling in 1 *M* acetic acid containing 0.05 *M* sodium chloride. [b] In water at 100° for one second. [c] Slope of stress-strain curve at a loading rate of about 50 g./sq. mm./min. [d] Assuming the volumes of fibrin and solvent are additive.

Tensile Strength and Elongation at Break.—Measurements of tensile strength and elongation at break are somewhat erratic, since the point of break may depend upon a chance flaw in the structure. Values obtained for water-equilibrated film have ranged from 10 to 15 × 10^6 dynes per sq. cm. for tensile strength calculated on initial cross-section, or 25 to 40 × 10^6 calculated on final cross-section; and from 100 to 200% for elongation at break. Values obtained with 75% glycerol as plasticizer are plotted against the fibrin content in Fig. 13. The tensile strength increases markedly above a concentration of 30% fibrin. The elongation at break goes through a maximum at about 40% fibrin.

Anistropy and Spontaneous Contraction

When a thin strip of film, plasticized with glycerol for transparency, is examined with a polarizing microscope in a direction parallel to the surface of the film, double refraction is observed. The principal vibration directions are parallel and perpendicular to the film surface, and the larger refractive index is associated with the former. In this respect the film behaves as though it contained fibrous elements aligned parallel to its surface, according to the Ambronn–Wiener theory of the double refraction of a fibrous structure.[28]

Further evidence of anisotropy is given by the spontaneous contraction which can be induced in fibrin film by a variety of treatments, resulting in a decrease in area and increase in thickness. For example, the film swells in 1 *M* acetic acid containing 0.05 *M* sodium chloride to somewhat more than twice its water-equilibrated volume. This swelling is anisotropic; the thickness increases while the area actually decreases slightly. The same phenomenon is observed in 4 *M* potassium thiocyanate and 6 *M* urea, with less volume increase, and upon dipping in water at 100° for one second with practically no volume increase. In all these treatments except the last, the film becomes quite transparent.

(28) E. G. Coker and L. N. G. Filon, "Photoelasticity," Cambridge University Press, Cambridge, 1931.

When both volume and shape change, the phenomenon may be formally represented as a uniform swelling superimposed on a shape change which consists of a contraction in area and increase in thickness. In such a representation, the anisotropy of the water-plasticized film is described by supposing that the water-plasticized film behaves as though it had been stretched uniformly in all directions in its own plane, with a consequent decrease in thickness; or compressed in thickness, with free expansion laterally. If it were strained thus, it ought to contract spontaneously, but it is presumably prevented from doing so by some restraint; there is a frozen-in strain. After treatment by heat or certain reagents, the restraint is released and lateral contraction occurs, in addition to the volume increase occasioned by the swelling.

If the frozen-in strain is denoted by α (ratio of stretched to unstretched length in any direction in the plane of the film), then the change in area upon relaxation (if no swelling occurred) should be given by the factor $1/\alpha^2$. If the volume increases upon swelling by a factor R_V, the uniform increase in area from this cause should be given by a factor of $R_V^{2/3}$. Hence the actual proportional change in area is $R_A = R_V^{2/3}/\alpha^2$, and thus $\alpha = R_V^{1/3}/R_A^{1/2}$.

Values of α calculated from changes in volume and area in the different treatments mentioned are given in Table I. Both heat and acid swelling result in a considerable release of frozen-in strain, and thiocyanate and urea somewhat less. After the latter reagents are removed by washing, the films remain transparent and retain their contracted shape; their apparent moduli of elasticity are considerably lower than that of the untreated film, as shown in the Table.

Discussion

The properties of fibrin film may be interpreted in the light of the structure previously postulated[9] for the coarse type of fibrin clot from which it is derived. It was assumed that the fibrinogen molecules are joined primarily by end to end linkages, forming chains about 35 Å. in diameter,

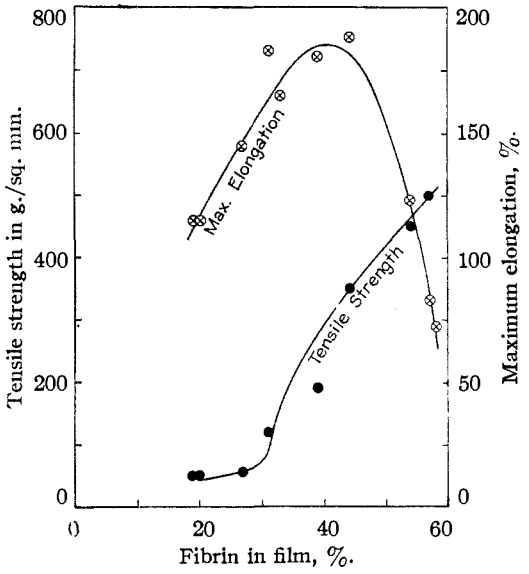

Fig. 13.—Tensile strength and elongation at break of fibrin film plasticized with 75% glycerol, plotted against fibrin content.

and that in the coarse clot the chains are aggregated side-by-side in bundles. The bundles are crosslinked in a coarse network; some of the crosslinks are considered to be primary chemical bonds, but many of them are thought to be secondary bonds, such as would be formed by intermittent lateral association of the chain bundles, as seen in the electron microscope.[29]

When the clot is compacted to form the film, fluid is evidently removed from the interstices between the chain bundles, and the latter are brought close together so that the network occupies only one-fiftieth its original volume. The ease with which this is accomplished indicates that the chains are fairly flexible and that any secondary bonds broken in the process are comparatively weak. New secondary bonds may be formed, of course, in the compacted state.

Interpretation of Anisotropy and Spontaneous Contraction.—When the fibrin network is formed in fairly dilute solution by clotting under the conditions described, the chain bundles are presumably oriented at random. When the clot is compacted to form a fibrin film, the bundles should be crumpled in such a way that the fibrinogen residues are preferentially oriented parallel to the plane of the film (Fig. 14). This agrees, of course, with the deduction from the sign of the birefringence of the film.

The entropy difference between the compacted, oriented structure and the unoriented structure which would have been formed if the compaction had been isotropic will cause a tendency for spontaneous rearrangement to the latter. In water-equilibrated fibrin film at room temperature, this rearrangement does not take place. Although the fibrin elements can easily rearrange in the plane of the film, as witnessed by the long-range elasticity, they apparently do not assume configurations which involve motions perpendicular to the plane or tilting of the fibrinogen residues at large angles with it. This constraint seems reasonable on purely steric grounds.

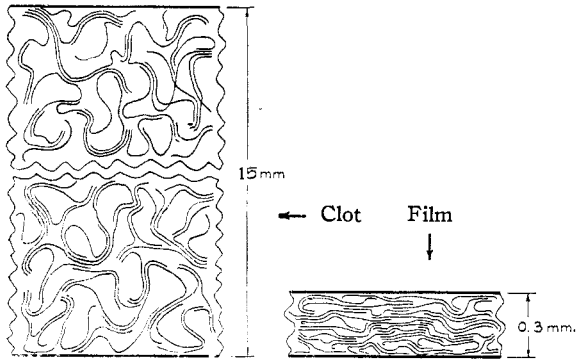

Fig. 14.—Schematic diagram of formation of fibrin film.

However, under the influence of acid, urea or potassium thiocyanate, at least partial rearrangement toward an isotropic structure occurs and the film contracts in its own plane. At the same time, the opacity irreversibly disappears (Table I). A possible explanation of this phenomenon is that the reagent destroys the side-by-side attractions between chains in a bundle and thus removes much of the constraint, permitting individual chains to assume random orientation (Fig. 15). The partial dissociation of protein structures by acid, thiocyanate, and urea is well known.[30] The original alignment is, of course, not regained after the reagent is removed, so that many crosslinks of the parallel association type are presumably permanently destroyed, accounting for the decrease in the modulus of elasticity.

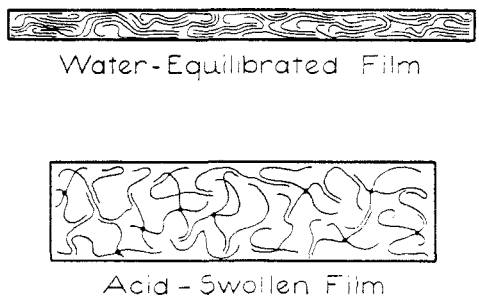

Fig. 15.—Schematic diagram of proposed mechanism of anisotropic swelling.

An alternative explanation of the spontaneous contraction is an intramolecular rearrangement

(29) C. Wolpers and H. Ruska, *Klin. Wochenschr.*, **18**, 1077, 1111 (1939).

(30) E. O. Kraemer, *J. Phys. Chem.*, **45**, 660 (1941); J. Steinhardt, *J. Biol. Chem.*, **123**, 543 (1938); T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford University Press, 1940, pp. 359-372, 407.

within the fibrinogen residues, such as has been postulated to explain the thermal contraction of collagen.[6a] Since most of the residues in the film are oriented parallel to the film plane, any contraction of the fibrinogen molecule itself would result in a change of shape of the sort observed. This mechanism might operate in the case of the contraction at 100°, which is not accompanied by disappearance of opacity.

Interpretation of the Properties of Water-Equilibrated Film.—The equilibrium swelling of the film represents a balance between the affinity of the fibrin for water and the entropy and energy changes associated with expansion of the structure. However, the thermodynamic equations for the swelling of polymer gels[31] do not apply to a highly polar system of this sort and it is not possible to calculate from swelling the concentration of cross-links in the structure. Nor is it possible to calculate from opacity measurements explicit dimensions of the inhomogeneities responsible for light scattering, which are without doubt the chain bundles and the water-filled interstices between them. The ultrafiltration results indicate that the interstices are of the order of 40 Å. or more in width.

The apparent modulus of elasticity of water-equilibrated film is about 6×10^6 dynes per sq. cm., which is the same order of magnitude as that of lightly vulcanized rubber,[32] and also that of elastin in the *ligamentum nuchae*.[7] This fact, together with the high elongations which can be achieved, suggests that the elasticity of fibrin film is at least partly due to the entropy of orientation of long flexible molecules, as in rubber.[31] The chains may be imperfectly flexible, however, so that their inherent stiffness contributes to the elasticity, as indicated by the fact that fibrin clots have considerably higher rigidities than can be accounted for by ordinary rubberlike elasticity.[9]

It is possible that an intramolecular transition, such as the α, β transition in keratin, may also be involved. However, Bailey, Astbury and Rudall[33] found that moderately stretched fibrin, like fibrinogen, gave an X-ray diagram similar to that of α-keratin. Only upon further stretching did a strong β pattern occur. This suggests that the deformation of water-equilibrated film, up to moderate elongations, is accompanied simply by the orientation of chain bundles.

The stress–strain curves in Fig. 9 and 11 do not permit evaluation of the portion of stress which is due to entropy of orientation; this must await studies of the effect of temperature on stress-strain equilibrium as carried out by Wöhlisch[7] on certain biological structures and by Bull[4c] on keratin. It is suggestive that the hysteresis loop of Fig. 9 resembles those obtained for keratin[4c] within an extension of 20%; except that here the extension is very much greater and the so-called "Hooke's law region" is absent. The hysteresis in keratin was attributed by Bull partly to viscous retardation and partly to the breaking and reforming of secondary bonds during extension and contraction. In the case of fibrin, the latter process might be represented by the pulling apart of chain bundles at the anastomoses which have been postulated, followed by the formation of new side-by-side associations between fibrin chains at high extensions where orientation facilitates the parallel alignment required.

Effect of Reduction of Water Content.—When the fibrin content of the water-plasticized film is increased from 30 to 40%, there is a marked change in opacity with very little change in mechanical properties and very little pressure required. It may be concluded that water is being removed from comparatively large interstices between the bundles of fibrin chains, so that the inhomogeneities responsible for most of the light scattering are eliminated, while the chain bundles do not approach so closely as to interfere much with each other.

When the fibrin content is increased above 40%, there is comparatively little further change in opacity, whereas the pressure required for compaction rises sharply and the stress–strain curve acquires a sharp curvature and high initial slope. Presumably this further removal of water necessitates crowding the fibrin chains together with expenditure of energy either by compression or deformation of the chains themselves or by rearrangement of secondary bonds. The compacted structure which results has a much higher initial modulus of elasticity, approaching that of keratin (about 10^8 dynes per sq. cm.). This may be attributed to a larger number of secondary bonds between the closely crowded chains, forming a more densely cross-linked structure. The easier stretch from 20 to 100% elongation (Curve 3, Fig. 11) may be explained by supposing that many of these additional secondary bonds are broken when the elongation exceeds 20%. Very similar interpretations of the stress–strain curves of keratin fibers have been made,[4b,c] although in the latter case the change in shape of the curve occurs in a much smaller range of elongation (5 to 20% instead of 20 to 100%).

The fact that the elongation at break goes through a maximum at a fibrin content of about 40% (Fig. 13) indicates that below this value the secondary bonds are so few that rupture occurs before maximum orientation is achieved, while above it they are too numerous to permit maximum orientation; *i.e.*, one system is too soft and the other too brittle.

The plasticizer content of the film at which the transition from a linear to a convex stress–strain curve occurs may be regarded as the

(31) P. J. Flory and J. Rehner, Jr., *J. Chem. Phys.*, **11**, 521 (1943).
(32) H. M. James and E. Guth, *ibid.*, **11**, 455 (1943).
(33) K. Bailey, W. T. Astbury and K. M. Rudall, *Nature*, **151**, 716 (1943).

minimum amount necessary to keep the fibrin bundles apart and prevent the formation of an excessive number of secondary crosslinks. It would follow that water is a better plasticizer, gram per gram, than glycerol. This agrees with the conclusions of Fuoss regarding the relationship of molecular size to plasticizing effectiveness, derived from studies of the electrical properties of polymer–plasticizer systems.[34]

Summary

1. Fibrin film is prepared by allowing human fibrinogen and thrombin to react under specified conditions and compacting the resulting clot under low pressure.

2. When equilibrated with water, fibrin film contains about 30% of protein. It is opaque, and its opacity increases upon stretching. Ultrafiltration experiments indicate that it has structural interstices at least 40 Å. in width. Its apparent modulus of elasticity, at a moderate rate of loading, is about 6×10^6 dynes per sq. cm. Its stress–strain curve shows marked hysteresis. Its tensile strength is 10 to 15×10^6 dynes per sq. cm., and its elongation at break is between 100 and 200%. Upon treatment with acid, boiling water, or certain other reagents, it contracts spontaneously in its own plane, increasing in thickness.

3. The fibrin content of the film can be reversibly increased to 60% by partial desiccation or compaction under pressure. As the fibrin content increases from 30 to 40%, there is a marked decrease in opacity, and but little change in mechanical properties. As it increases above 40%, much higher compacting pressures are necessary; there is but little further change in opacity, but marked change in mechanical properties. The initial modulus of elasticity becomes much higher, and the stress–strain curve acquires a pronounced curvature.

4. Substitution of glycerol for water as plasticizer in the film reduces the opacity greatly and lowers the value of the fibrin content at which the above-described transitions in physical properties occur.

5. These properties of fibrin film are discussed in relation to hypotheses concerning the structure of the film and the structure of the clot from which it is derived.

(34) D. J. Mead, R. L. Tichenor and R. M. Fuoss, THIS JOURNAL, **64**, 283 (1942).

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[CONTRIBUTION FROM THE DEPARTMENTS OF PHYSICAL CHEMISTRY AND ANATOMY, HARVARD MEDICAL SCHOOL, AND THE ARMOUR LABORATORIES]

Preparation and Properties of Serum and Plasma Proteins. X. Modification of the Physical and Chemical Properties of Fibrin Film by Heat Treatment[1,2]

BY JOHN D. FERRY, MARCUS SINGER, PETER R. MORRISON, JULES D. PORSCHE AND RUSSELL L. KUTZ

The properties of solid protein structures are markedly affected by heat treatment, especially in the presence of moisture. Effects which have been observed include the increased elongation of keratin fibers in steam or boiling water,[3] the spontaneous thermal contraction of collagen[4] and its hydrolysis to gelatin,[5] the "setting" of keratin by steaming,[6] and the orientation of artificial protein fibers achieved by drawing in steam.[7] The chemical changes produced by heat treatment may include intramolecular rearrangement, hydrolytic degradation and formation of new structural crosslinks.

(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 52 in the series "Studies on the 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) Woods, *Nature*, **132**, 709 (1933).

(4) Astbury, *Proc. Intern. Soc. Leather Trades' Chem.*, **24**, 69 (1940).

(5) Scatchard, Oncley, Williams and Brown, THIS JOURNAL, **66**, 1980 (1944).

(6) Astbury and Woods, *Phil. Trans. Roy. Soc.*, **A232**, 333 (1933).

(7) (a) Lundgren and O'Connell, *Ind. Eng. Chem.*, **36**, 370 (1944); (b) Astbury, *Nature*, **155**, 501 (1945).

Fibrin film is a protein structure derived from fibrinogen and thrombin and chemically identical with the natural fibrin of clotted blood.[8] It, too, is modified by heat treatment, and the nature of the change depends markedly upon the amount of moisture present. Among the properties which are altered are the imbibition of water, swelling in acid and alkali, mechanical properties, ease of digestion by enzymes, and affinity for dyes.

The properties of fibrin films which have been modified by heat in various ways are described here and compared with those of the untreated film,[8] and some conclusions are drawn concerning the probable physical and chemical changes which occur during the treatment.[9]

Materials and Methods

Fibrin film was prepared by clotting the fibrinogen of Fraction I of human plasma[10] with thrombin purified from

(8) Ferry and Morrison, THIS JOURNAL, **69**, 400 (1947).

(9) Many of these observations were made in the course of development of suitable methods for sterilizing fibrin film for surgical use, and in studying the stability of the film under various conditions. Fibrin film has been prepared under contract with the United States Navy from plasma fractions derived from blood collected by the American Red Cross.

(10) Cohn, Strong, Hughes, Mulford, Ashworth, Melin and Taylor, THIS JOURNAL, **68**, 459 (1946).