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).

Fraction III-2,[11,12] and compacting the resulting clot, as described in the previous paper.[8] The thickness of most films corresponded to about 14 mg. of fibrin per sq. cm.

Films were placed in glass tubes for heat treatment, and were usually rolled loosely together with parchment paper to prevent sticking of adjacent layers. Steam treatment was carried out in an autoclave.

The following procedures were used in studying the properties of the films after heat treatment.

Water-Equilibrated Fibrin Content.—The film was equilibrated in distilled water, blotted, quickly weighed, heated at 100° for twenty-four hours, and reweighed. In some experiments, equilibration with water vapor at different relative humidities was carried out over saturated solutions of $BaCl_2 \cdot 2H_2O$, $Mg(NO_3)_2 \cdot 6H_2O$, and $Ca(NO_3)_2 \cdot 4H_2O$.

Opacity.—The transmission of film to green light (Zeiss filter L-II) was measured in a Pulfrich photometer, and the optical density was calculated as the negative logarithm of the fraction of light transmitted.

Swelling in Acid and Alkali.—The swelling agents used were very dilute hydrochloric acid and sodium hydroxide, and 1 *M* acetic acid and ammonium hydroxide. Water-equilibrated film was blotted, weighed, immersed in the acid or alkali for twenty-four hours, and again blotted and weighed. The swelling index was taken as the ratio of the weight of acid-swollen film to the weight of fibrin (calculated from the initial wet weight and the equilibrium fibrin content previously determined).

Mechanical Properties.—Strips of water-equilibrated film about 1 sq. mm. in cross-section were stretched with a spring balance and scale, at a loading rate of about 50 g./min. The stress was calculated on the initial cross-section (as calculated from the initial length, the weight and the density of water-equilibrated fibrin[8]).

Affinity for Dyes.—The affinities of the fibrin for a basic dye, methylene blue, and an acidic dye, Orange G, were measured by equilibrating film in dilute dye solutions and determining the optical density of the dye bound.[13]

The compositions of the solutions were as follows: methylene blue, 1.0×10^{-5} *M*, in 0.02 *M* phosphate buffer at *p*H 7.0; Orange G, 2.0×10^{-5} *M*, in 0.01 *M* phosphate buffer at *p*H 6.5. Pieces of film 2 × 2 cm. were suspended for forty-eight hours at 25° in a large excess of dye solution (6 to 8 pieces in 1 liter), which was stirred constantly and replaced with fresh solution after the first twenty-four hours.

The optical densities of the moist, stained films were determined with a Pulfrich photometer, using a green filter (Zeiss L-II) for methylene blue and a blue filter (Zeiss L-III) for Orange G. Correction was made for the optical density of the undyed fibrin. Calculation showed that the contribution to optical density from dye solution imbibed by the film was negligible. Other tests, in which the uptake of dye by film from a small volume of solution was determined by difference, showed that the optical density of the stained film was proportional to the amount of dye bound per sq. cm. of fibrin.[14] The dye bound per unit weight of fibrin was thus proportional to the optical density divided by the film weight in mg. per sq. cm. This latter ratio, found to be constant for films of different thicknesses which had received the same treatment,[14] is used in reporting all data.

Digestion by Trypsin.—A 1% solution of commercial trypsin (Cenco) in *M*/15 phosphate buffer at *p*H 8.0 was centrifuged and clarified by filtration. A piece of film about 1 × 1 cm. was immersed in 5 cc. of trypsin solution with 1 cc. of 0.1% Merthiolate at 37°. The end-point of digestion was taken as the time when gentle agitation broke the film into small fragments.

(11) Oncley, Melin, Richert, Cameron and Gross, in preparation.

(12) Edsall and Miller, in preparation.

(13) A study of the affinities of modified and unmodified fibrin film for acid and basic dyes under various conditions of *p*H, ionic strength, and concentration will be reported elsewhere.[14] In the experiments described here, these conditions were invariant.

(14) Singer and Morrison, in preparation.

Results of Heat Treatment

The effects of heat on fibrin film with moisture contents ranging from the maximum of 70% to zero will first be briefly described.

Treatment of Film with Water at 100°.—When water-equilibrated fibrin film, containing about 30% fibrin, is treated for 1 sec. with boiling water or steam, irreversible contraction takes place in the plane of the film[8]; the opacity persists, even after drying, and the tensile strength drops to a small fraction of its original value. The consistency of the treated material resembles that of boiled egg white. It was found, however, that this gross deterioration of mechanical properties could be avoided if the water content of the fibrin was first reduced. When film was pressed or partly desiccated to a fibrin content of 60% and then treated for 1 sec. with boiling water or steam, a somewhat smaller contraction took place (α, the "frozen-in strain factor," was 1.3 as compared with 1.66 for water-equilibrated film[8]); the product remained transparent, imbibed less water than before (equilibrium fibrin content of 50%), and retained its tensile strength.

Treatment of Partially Desiccated Film in Saturated Steam.—Samples of fibrin film were desiccated to various extents and exposed to saturated steam at different temperatures and for varying periods of time.[15]

The exchange of water between film and vapor phase was followed in one series (at 109°) by removing samples at intervals during the treatment. When the moisture content of the film was initially below 20%, it rose in steam; when initially above 50%, it fell. After ten minutes in saturated steam, the moisture content became essentially constant. The final values lay between 20 and 25% when the initial values were less than 20% (Table I).

TABLE I

DEPENDENCE OF MOISTURE CONTENT IN SATURATED STEAM (109°, 5 LB./SQ. IN.) UPON INITIAL MOISTURE CONTENT

Initial moisture content, %	3	10	19	50	70[a]
Moisture content after steaming 25 min., %	20	24[b]	25[b]	36[b]	66

[a] Normal moisture content—undesiccated. [b] The same value was obtained in saturated steam at 121° (15 lb./sq. in.).

In several experiments, films desiccated to an initial moisture content of about 20% were exposed to saturated steam at temperatures ranging from 100 to 130° for times up to eight hours. The films were then reimmersed in water, and the water-equilibrated fibrin content, tensile strength, and elongation at break were determined. The results of one series at 121° (15 lb./sq. in.) are given in Table II. In the first few minutes of steaming, a change occurs which results in much

(15) We are much indebted to Dr. E. A. Bering, Jr., for conducting many of these experiments.

less swelling in water (as shown by the higher fibrin content) and *increases* the tensile strength.

TABLE II

CHANGES IN PROPERTIES OF FIBRIN FILM IN STEAM AT 121° (15 LB./SQ. IN.)

Time	Color	Water-equilibrated: Fibrin content, %	Tensile strength, g./sq. mm.	Elong. at break, %
0	White	*ca.* 30	160–220	210–260
5 min.	Colorless	57	520	270
20 min.	Colorless	57	480	180
40 min.	Colorless	51	520	270
1 hr.	Colorless	53	350	270
2 hrs.	Straw	52	250	220
4 hrs.	Brown	45	100	120
7 hrs.	Dark brown	24	Very weak	

Further steaming changes the properties relatively little until, as the steaming is prolonged beyond forty minutes, there is gradual deterioration of the protein structure, as evidenced by loss of tensile strength. The water-equilibrated fibrin content also decreases, representing greater swelling and indicating a loosening of the structure, and eventually the maximum elongation falls off as the film becomes too weak to be stretched to its normal elongation. In addition, there is increasing discoloration.

Results at other temperatures were quite similar, the changes occurring more rapidly the higher the temperature.

Treatment of Dry Film.—To explore the effect of heat upon fibrin film in the absence of moisture, films were desiccated at room temperature to reduce the moisture content below 50% and the remaining water was removed, after chilling in a bath of Dry Ice and alcohol, by evacuation. The dry films were sealed in tubes *in vacuo* and heated at 170° for one and one-half hours.

After treatment, the tubes contained ammonia and hydrogen sulfide. The films, upon reimmersion in water, were transparent and yellow in color. The water-equilibrated fibrin content was 58%; tensile strength, 200 g./sq. mm.; elongation at break, 60%.

Properties of Steam-Treated Fibrin Film

Of the various modified films described above, that obtained by exposure of fibrin film with an initial moisture content of 20% to steam at 121° for twenty minutes, henceforth described as "steam-treated film," was selected for further study.[16] A comparison of its properties with those of unmodified film follows.

Equilibrium with Water.—When partly desiccated fibrin film is immersed in water, it rapidly imbibes liquid and swells to its previous equilibrium volume (Ref. 8, Fig. 4). For unmodified film containing 10% of moisture (90% fibrin), the initial rate of uptake is about 10 mg./sq. cm./min., and the final fibrin content attained is 30%. For steam-treated film, the corresponding values are 2 mg./sq.cm./min. and 57%. The lower affinity of the steam-treated film for water is also shown by equilibration with water vapor at different relative humidities[15] (Fig. 1).

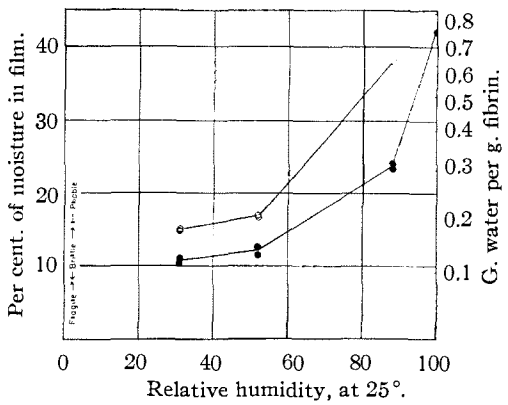

Fig. 1.—Equilibration of fibrin film with water vapor at 25°: ○, unmodified film; ●, steam-treated film.

Opacity.—Water-equilibrated steam-treated film is quite transparent; the optical density per mm. ranges from 0.1 to 0.2, as compared with about 3.0 for the very opaque unmodified film. However, when unmodified film (equilibrium fibrin content 30%) is pressed or desiccated to raise the fibrin content to the equilibrium value of steam-treated film (57%), the opacity falls to about 0.5. Thus the normal opacity difference is largely attributable to the difference in fibrin content.

Permeability.—Unlike unmodified film, the steam-treated material is quite impermeable to hemoglobin. It is readily permeable, however, to smaller molecules, including sodium chloride, glucose, diglycine, and α-aminobutyric acid.

Swelling in Acid and Alkali.—Whereas unmodified film swells to a weight of eight times its fibrin content in hydrochloric acid at *p*H 3 or sodium hydroxide at *p*H 8, the swelling index of steam-treated film changes very little from the value characteristic of water (Fig. 2).[17] Further-

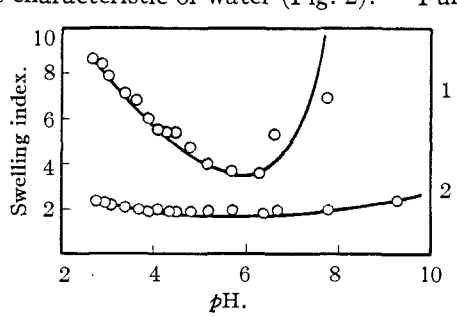

Fig. 2.—Swelling of fibrin film in dilute hydrochloric acid and sodium hydroxide: 1, unmodified film; 2, steam-treated film.

(16) This treatment effectively sterilizes fibrin film, as shown by bacteriological tests, and the films which have been prepared under contract with the United States Navy have been processed in this way.

(17) A more detailed description of the swelling of fibrin film and its relation to the titration curve will be reported elsewhere.

more, the swelling is isotropic, no contraction in the plane of the film being observed; α, the "frozen-in strain factor," is therefore 1.00 as contrasted with 1.60 for unmodified film.[8]

Mechanical Properties.—A load-elongation curve for water-equilibrated steam-treated fibrin film is shown in Fig. 3. Curves for unmodified film, both water-equilibrated (fibrin content 29%) and partly desiccated (fibrin content 59%) are given for comparison. When the two films are compared at equal fibrin contents, their mechanical properties are quite similar.

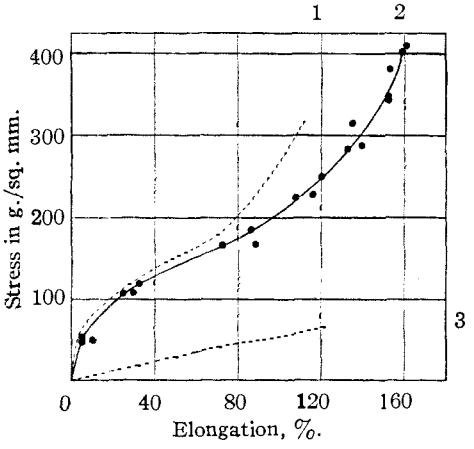

Fig. 3.—Stress-strain curves of fibrin film: 1, unmodified film, 59% fibrin; 2, steam-treated film, 57% fibrin (water-equilibrated); 3, unmodified film, 29% fibrin (water-equilibrated).

Affinity for Dyes.—The optical density divided by film weight in mg./sq.cm., for steam-treated film stained by the dilute dye solutions whose compositions are given above, is 0.044 for Orange G and 0.025 for methylene blue. The corresponding optical density values for unmodified film[18] in the two dyes are 0.012 and 0.006 $(mg./sq.cm.)^{-1}$, showing that the heat treatment increases the binding of both. The changes during the course of the treatment are shown in Fig. 4. The initial contact with steam produces an enormous increase in Orange G affinity, which subsequently gradually decreases; the methylene blue affinity increases throughout the treatment.

The values for optical density may be converted to moles of dye bound per g. protein by using the appropriate factors, whose determination is described elsewhere.[14] The results in moles $\times 10^{-5}$ per g. are, for untreated film, 0.10 of Orange G and 0.15 of methylene blue; and for steam-treated film 0.39 of Orange G and 0.64 of methylene blue. These are, of course, very much less than the expected maximum acid and base binding capacities, which, on the basis of amino acid analyses,[19]

(18) The unmodified film must be immersed in glycerol to reduce light scattering[8] before the optical density of bound dye is determined.

(19) Brand, Kassell and Saidel, *J. Clin. Investigation*, **23**, 437 (1944).

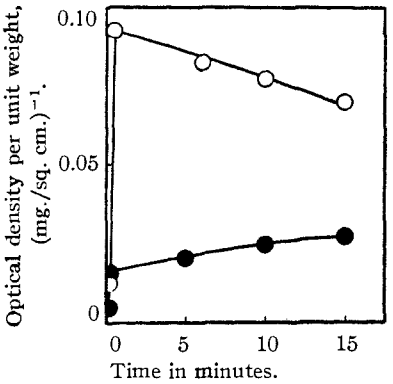

Fig. 4.—Changes in affinity of fibrin film for dyes during exposure to steam: O, orange G; ●, methylene blue.

should be at least 63×10^{-5} and 192×10^{-5} respectively.[20] Lowering the *p*H increases the binding of Orange G enormously, while raising the *p*H correspondingly increases that of methylene blue.[14] From the work of Fraenkel-Conrat and Cooper[21] it would be expected that below *p*H 2 the Orange G binding would represent total acid binding capacity, and above *p*H 11 the methylene blue binding would represent total base binding capacity.

Digestion by Enzymes.—The digestion time of steam-treated film by trypsin under the conditions described above is twenty-six hours, as compared with about half an hour for unmodified film. The digestion of steam-treated film by pepsin is similarly much slower than that of unmodified film.

Interpretation of Properties.—When unmodified film is pressed or desiccated to a fibrin content of 60% or more, the fibrin chains are thought to be crowded together, with the formation of additional secondary cross-links, as evidenced by the mechanical properties.[8] Such bonds are readily broken by the introduction of water, as shown by imbibition accompanied by the return of opacity as the voids between chains reopen.

In steam-treated film, which has been heated at a fibrin content of 75%, some of these secondary bonds have evidently been replaced by cross-links of a more permanent nature, since the film no longer swells to its former equilibrium value in water, and the swelling in acid and alkali is also greatly diminished. The transparency and mechanical properties of steam-treated film, equilibrated in water, correspond to its relatively high fibrin content of about 57%. The denseness of the structure, which does not possess the wide voids of water-equilibrated unmodified film, also explains its impermeability to hemoglobin.

The increased binding of dyes may be due both to chemical reactions involving the combining

(20) The figure for maximum acid-binding capacity does not include the contribution of lysine, and is undoubtedly far too low. The value has been determined by the authors by direct titration of swollen fibrin as 122×10^{-5} mole/g.

(21) Fraenkel-Conrat and Cooper, *J. Biol. Chem.*, **154**, 239 (1944).

TABLE III

CHANGES IN THE PROPERTIES OF STEAM-TREATED FIBRIN FILM AT MODERATE TEMPERATURES

Moisture content 21%

Temp., °C.	Time in days	Fibrin content, water-swollen	Tensile strength, g./sq. mm.	Elong. at break, %	Digest. time, hr.[a]	Swelling index HAc[b]	Swelling index NH_4OH[b]	Optical density[c] stained M. b.[d]	Optical density[c] stained O. G.[e]
	0[f]	57	500	230	26	3.8	7.9	0.025	0.044
	54	55	430	180	(60)	3.0	15.2	.036	.010
50	104	54	420	180	27	2.6		.047	.010
	198	51.5	270	160	16	2.3	27.2	.067	.004
	40	53	380	190	22	2.6	9.1	.052	.006
	68	52	300	160	23	2.5		.065	
57	95	51	360	170	27	2.2		.083	.006
	157	51	230	160	19	2.4	20.0	.088	.008
	198	47	260	120	16	2.1	17.8	.127	.009
	18	50	320	180	21	2.2	11.8	.094	.007
70	31	48	230	180	18	2.1	14.3	.119	.010
	43	47	230	120	9	2.2	10.0	.145	.010

[a] In 1% commercial trypsin, in $M/15$ phosphate buffer at pH 8.0. [b] 1 molar. [c] Optical density divided by weight of film in mg. per sq. cm. [d] Methylene blue, 1.0×10^{-5} M, in 0.02 M phosphate buffer, pH 7.0. [e] Orange G, 2.0×10^{-5} M, in 0.01 M phosphate buffer, pH 6.5. [f] Average of values of twenty representative preparations.

groups and to molecular rearrangements providing greater accessibility. The sudden changes upon initial exposure to steam may result from the molecular rearrangements associated with the lateral contraction in the plane of the film which occurs at the same time. The subsequent changes are probably due to chemical alteration. They may be enhanced by prolonged heat treatment, as shown in the following section.

Prolonged Treatment at Moderate Temperatures

The alterations observed when steam treatment of fibrin film is prolonged (Table II)—discoloration, loss of tensile strength, and increased imbibition of water—also take place more slowly at lower temperatures, and are accompanied by changes in the swelling in acid and alkali and the affinity for dyes. The course of these changes was studied in the presence of various amounts of moisture.

Films were treated with steam at 121° for twenty minutes and dried to different moisture contents by very brief evacuation of their containers; air was then readmitted and the containers were sealed. After heating at various temperatures from 25 to 121° for different intervals of time, the containers were opened, the films were immersed in water, and their properties were measured.

Changes in physical and chemical properties eventually became apparent; they were more rapid the higher the temperature and the higher the moisture content. Examples are shown in Table III for film containing 21% moisture. The equilibrium fibrin content in water fell, representing increased imbibition; the tensile strength and elongation at break decreased; the digestion time in trypsin was diminished; the swelling index in acetic acid decreased, while that in ammonium hydroxide increased. The affinity of the film for methylene blue increased markedly, while that for Orange G decreased. In addition, a brown discoloration of the film developed.

Correlations among Different Properties.—By far the most sensitive index of changes in the film noted was its affinity for methylene blue. This property is also the most reproducible and hence most suited to quantitative measurement. With a view to using the methylene blue binding alone as an index of alterations, its correlation with the other properties was tested at various temperatures. The methylene blue optical density is plotted in Fig. 5 against Orange G optical density, in Fig. 6 against the water-swollen fibrin content, in Fig. 7 against the swelling index in 1 M acetic acid, and in Fig. 8 against the tensile strength. In every case the points at all temperatures fall reasonably close to a single curve. (The values for tensile strength are rather scattered, because this is an inherently more erratic measurement than the others.) It may be concluded that the changes taking place in fibrin film at different temperatures are qualitatively sim-

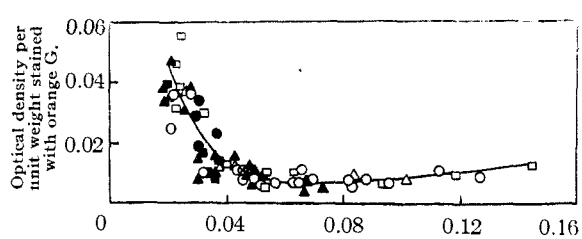

Fig. 5.—Optical density divided by film weight in mg./cm.^{2} for films stained with methylene blue, plotted against the same quantity for orange G: ●, room temperature; ■, 37°; ▲, 50°; ○, 57°; □, 70°; △, 100°; ⊗, 120°.

ilar, differing only in rate, and that the affinity for methylene blue may serve as a single measurement to represent the extent of alteration.

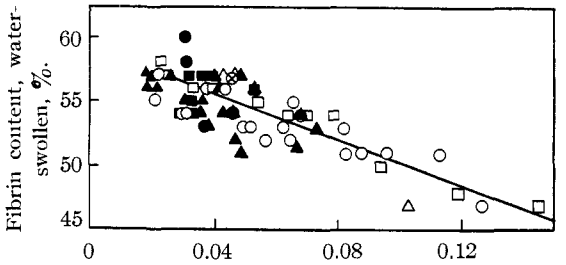

Fig. 6.—Optical density divided by film weight in mg./cm.² for films stained with methylene blue, plotted against water-swollen fibrin content. Key to temperatures same as in Fig. 5.

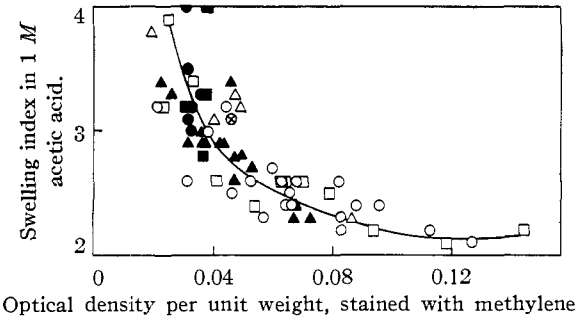

Fig. 7.—Optical density divided by film weight in mg./cm.² for films stained with methylene blue, plotted against swelling index in 1 *M* acetic acid. Key to temperatures same as in Fig. 5.

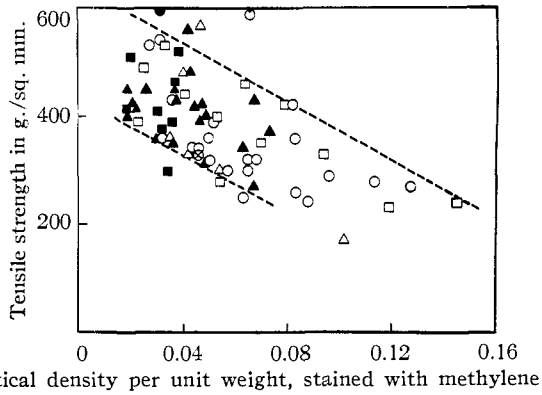

Fig. 8.—Optical density divided by film weight in mg./cm.² for films stained with methylene blue, plotted against tensile strength. Key to temperatures same as in Fig. 5.

Rate of Change of Methylene Blue Optical Density as a Function of Temperature and Moisture Content.—The methylene blue optical density is plotted against time in Fig. 9 for several temperatures, at a moisture content of 21%. The curves are not linear; the change is more rapid at first and then proceeds more slowly. The rate of change increases markedly with increasing temperature. Similar plots are given for different moisture contents at 57° in Fig. 10 and at 70° in Fig. 11. The rate of change of methylene blue binding also increases with moisture content.

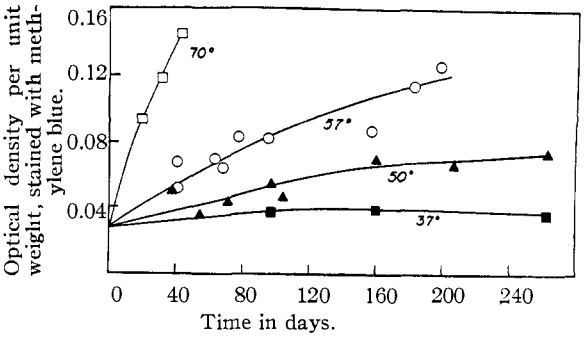

Fig. 9.—Increase with time of optical density divided by film weight in mg./cm.² for films stained with methylene blue; moisture content 21%.

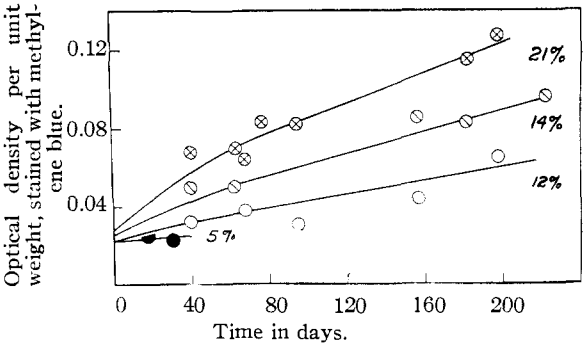

Fig. 10.—Increase with time of optical density divided by film weight in mg./cm.² for films stained with methylene blue; temperature 57°; moisture content 21% (⊗), 14% (⊘), 12% (○), 5% (●).

The Arrhenius equation may be applied by plotting, against the reciprocal absolute temperature, the logarithm of the time required for a given extent of alteration. The latter has been chosen as an increase in methylene blue optical density per mg./sq.cm. of fibrin of 0.03, which corresponds to the point where the decreases in water-equilibrated fibrin content (Fig. 6) and tensile strength (Fig. 8) are clearly distinguishable over the normal scatter of these measurements. The plot is given in Fig. 12, with two straight lines representing contours at moisture contents of about 12 and 21%, respectively. The slopes are identical and correspond to an activation energy of about 24 kcal.

Discussion

The changes in physical properties produced by heat treatment of fibrin film can be explained in

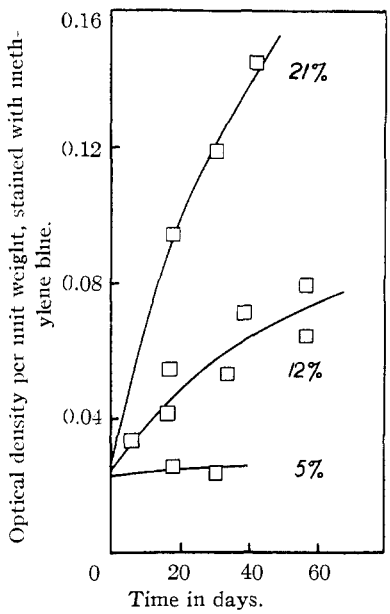

Fig. 11.—Increase with time of optical density divided by film weight in mg./cm.^{2} for films stained with methylene blue: temperature 70°; figures indicate moisture contents.

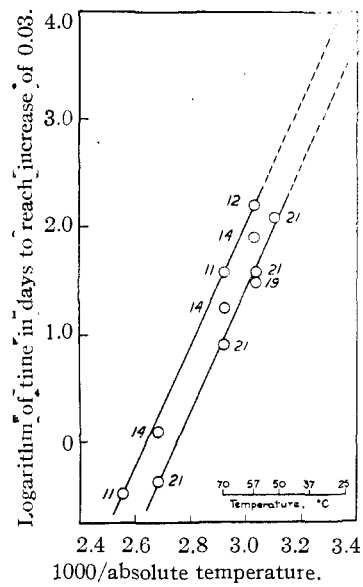

Fig. 12.—Time required for an increase of 0.03 in methylene blue optical density, plottedl ogarithmically against $1000/T$. Figures opposite points denote moisture contents.

terms of two concurrent processes: breaking of bonds and formation of new cross-links.

The occurrence of both breakdown and cross-linking reactions in the treatment of other high molecular weight systems by heat and light has been noted,[22] and in the case of rubber their respective effects on the mechanical properties have been isolated and measured separately.[23] The balance between cross-linking and breakdown depends primarily on the dilution of the system; higher dilution favors breakdown because the latter is a process of lower order.[22] This rule should apply generally regardless of the nature of the chemical reactions involved in degradation and cross-linking.

In brief treatment at 100° at a moisture content of 70%, degradation predominates, as shown by loss of tensile strength. On the other hand, in treatment at 121° for twenty minutes, at a moisture content of 25% (a much more concentrated system) cross-linking predominates, as shown by the discussion above of the properties of steam-treated film. At 170° in the absence of moisture (a still more concentrated system) the extent of cross-linking is even greater, as shown by the low elongation of the product.

When steam-treated film is subjected to additional prolonged heating at moderate temperatures, degradation predominates at moisture contents greater than 10%, as shown by loss of tensile strength and increased imbibition of water.

(22) Spence and Ferry, THIS JOURNAL, **59**, 1648 (1937); Taylor and Tobolsky, *ibid.*, **67**, 2063 (1945).

(23) Tobolsky and Andrews, *J. Chem. Phys.*, **13**, 3 (1945).

Nature of the Degradation Process.—The changes in acid and alkali swelling and in dye affinity which accompany degradation at moderate temperatures must involve charged groups. The degradation itself—*i.e.*, breaking of bonds in the crosslinked network—would tend to cause increased swelling not only in water, as observed, but also in acid and alkali. In 1 *M* acetic acid, however, the observed swelling is less, indicating that there is a smaller net positive charge present at *p*H 2.4 and that the consequent decrease in electrostatic forces overbalances the effect of network destruction. The increased swelling in alkali may be due to a larger net negative charge at *p*H 11.6 as well as destruction of the network.

A decreased net positive charge at *p*H 2.4 might result either from destruction of basic groups or creation of acidic groups. Either process would shift the isoelectric point of the protein in the acid direction, and would cause[24] a marked increase in basic dye binding and marked decrease in acid dye binding near neutrality, in agreement with the changes observed. The deduction of a decrease in net positive charge at acid reactions is also supported by studies of dye affinity in the corresponding *p*H range, reported elsewhere.[14]

One possible degradation reaction is the breaking of peptide bonds. However, peptide hydrolysis would create both carboxyl and amino groups, resulting in an *increased* net positive charge at *p*H 2.4, in disagreement with the deduction from acid swelling. It is possible that the shift in

(24) Sheppard, Houck and Dittmar, *J. Phys. Chem.*, **46**, 158 (1942).

charge is determined by reactions unrelated to structural changes, such as hydrolysis of amide groups to yield free carboxyl. Further study will be required to identify the nature of the degradation process.

Nature of the Cross-linking Process.—The cross-linking process may involve any of the reactions which have been postulated in the setting of keratin fibers, *e. g.*, the combination of amino groups with carboxyl groups or with cysteine residues.[25] The evidence of Lundgren[26] that condensation between side-chain amino and carboxyl groups of proteins occurs at 165° in the absence of moisture is suggestive in interpreting the cross-linking of dry fibrin film at 170°.

Summary

1. Fibrin film modified by exposure, at a moisture content of 20%, to steam at 121°, has a higher water-equilibrated fibrin content, a correspondingly lower opacity and higher tensile strength, lower swelling in acid and alkali, lower permeability, greater resistance to enzymatic digestion, and higher affinity for dyes, than unmodified film. The changes in physical properties may be attributed to cross-linking.

2. When the treatment is carried out at a moisture content of 70%, structural breakdown predominates over cross-linking.

3. Prolonged heating of steam-treated fibrin film at moderate temperatures results in increased water imbibition, decreased tensile strength, decreased resistance to tryptic digestion, decreased swelling in acid, increased swelling in alkali, decreased affinity for acid dye, and increased affinity for basic dye. The rate of change increases with the moisture content and also with the temperature, corresponding to an activation energy of 24 kcal.

4. These changes indicate structural breakdown accompanied by either destruction of basic groups or creation of acidic groups.

(25) Speakman and Hirst, *J. Soc. Dyers Colourists*, **59**, 124 (1943).
(26) Lundgren, personal communication.

BOSTON, MASS.
CHICAGO, ILL.

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Preparation and Properties of Serum and Plasma Proteins. XI. Quantitative Interpretation of Electrophoretic Schlieren Diagrams of Normal Human Plasma Proteins[1,2]

BY S. H. ARMSTRONG, JR.,[3] M. J. E. BUDKA AND K. C. MORRISON

Introduction

In a little over a decade since the introduction of the electrophoresis apparatus of Tiselius,[4] a considerable body of measurements of the distribution of electrophoretic components in normal human plasma has appeared. The earlier studies carried out in the main at *p*H values between 7.0 and 8.0, and at ionic strengths between 0.1 and 0.2, yielded values in good agreement.

Observations, both in this and in other laboratories,[5] had begun to give evidence, at the time of the initiation of the program of large-scale plasma fractionation, for systematic variations in the apparent distribution of the electrophoretic components of the plasma proteins as a function of conditions of electrophoresis in neutral and alkaline *p*H range. Because electrophoretic analyses assumed increasing importance both in the control of purity and yield of the products of plasma fractionation[6,7] and in the characterization of pathological plasma proteins, a systematic study of certain of the variables influencing apparent distributions has been carried out. In this study, findings on artificial mixtures of known composition made from purified proteins of known electrophoretic characteristics have been used to aid in the interpretation of data obtained under parallel conditions of electrophoresis on the more complex natural mixtures presented by plasma and its fractions. The results have been considered in the light of recent theoretical treatments.

To convert schlieren diagram data (given in terms of ratio of refractive index increment of a given component to total refractive index increment of the mixture) to a basis of weight of dried protein or of protein nitrogen, conversion factors have been calculated from independent measurements of refractive index increments of whole plasma and certain of its fractions.[8] In that valid use of refractive index increments requires the

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

(2) This is Number 56 in the series "Studies on Plasma Proteins" from Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

(3) Welch Fellow in Internal Medicine of the National Research Council.

(4) A. Tiselius, *Trans. Faraday Soc.*, **33**, 524 (1937).
(5) L. G. Longsworth, *Chem. Rev.*, **30**, 323 (1942).
(6) E. J. Cohn, J. L. Oncley, L. E. Strong, W. L. Hughes, Jr., and S. H. Armstrong, Jr., *J. Clin. Investigation*, **23**, 417 (1944).
(7) 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).
(8) Details of refractive index increment measurements will be presented in a separate communication in this series.