This increase in $2B'$ with increasing negative charge would also result from a very large proton polarization in COHb. We have not attempted to calculate any electrostatic effect since the calculations are much more complicated than in the cases considered above and particularly since they depend greatly upon the assumption as to the distribution of protons between the two proteins.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY, ITHACA, NEW YORK]

The Thermally-induced Transition in Fibrin[1,2]

BY GEORGE LOEB[3] AND HAROLD A. SCHERAGA

RECEIVED JUNE 1, 1961

The thermal transition in fibrin films and fibers was observed by following the changes in three properties as the temperature was changed: the length of the sample at zero force, the retractive force at constant length, and the optical birefringence. These properties were observed to undergo sharp changes at a well-defined temperature which depended on the diluent with which the sample was in equilibrium. The *p*H-dependence of the transition temperature was accounted for with the aid of a simplified model for the fibrin network. This model consisted of exactly similar, partially helical chains between cross-links. Each helix was assumed to be stabilized by two hydrogen bonds between ionizable side-chain groups, *viz.* one tyrosyl-carboxylate ion and one tyrosyl-histidine bond. The helical portions are disrupted during the transition. The *p*H-dependence of the transition temperature for the helix-random coil transformation was related to the ionization parameters of the side-chain groups. The values of the parameters chosen for the description of this model are consistent with theoretical expectations and with the results from experiments on proteins in aqueous salt and urea solutions.

Introduction

In recent years theories have been proposed to account for the stability of the native conformations of protein molecules and for the denaturation accompanying the loss of the stabilizing influences. The interactions of primary concern here are the hydrogen bonds between ionizable side-chain groups. Theoretical considerations[4] have shown how these interactions can account for the *p*H-dependence of reversible denaturation. The purpose of this paper is to provide experimental data for testing the applicability of the theory by determining the extent to which the thermal transition in the fibrous protein fibrin[5] can be described by a simple model and the theory cited above.[4] This study was carried on concurrently with similar studies of ribonuclease[6,7] and insulin.[6]

The point of view taken here is that the transition in fibrous proteins, observed as a shrinkage in the unconstrained fiber[5,8,9] or the development of a retractive force in a sample of fixed length, is similar to transformations observed in the case of other high polymers[10,11] in passing from a highly ordered microcrystalline form to a more random arrangement of chains in an amorphous form as the temperature of the sample is raised. The validity of this point of view in the case of collagen has been shown by recent work of Flory and coworkers.[12-17] Many other workers have made use of this point of view in experimental and theoretical studies of transitions in proteins and polypeptides.[11,18-28]

The related studies in this Laboratory on ribonuclease[6,7] and insulin[6] were carried out with proteins whose covalent structures are known[29-31] and whose properties may therefore be more easily interpreted in terms of molecular structure than in the case of collagen or fibrin. However, the prior treatment of these materials which allows them to be handled by the methods used here requires the introduction of cross-linkages which are foreign to the native structure, and it has not yet

(1) This work was supported by the Office of Naval Research (Contract Nonr-401(36)) and by a research grant (H-1662) from the National Heart Institute of the National Institutes of Health, U. S. Public Health Service.

(2) Presented, in part, at the Symposium on Blood Clotting, Division of Colloid Chemistry, 135th meeting of the American Chemical Society, Boston, Mass., April 1959.

(3) Union Carbide Fellow 1957-1958; American Viscose Corp. Summer Research Fellow, 1957; E. I. du Pont de Nemours Corp. Summer Research Fellow, 1958.

(4) H. A. Scheraga, *J. Phys. Chem.*, **64**, 1917 (1960).

(5) K. M. Rudall, Symposium Soc. Dyers & Colourists, Leeds, 1946, page 15; *Adv. Protein Chem.*, **7**, 253 (1952).

(6) A. Nakajima and H. A. Scheraga, *J. Am. Chem. Soc.*, **83**, 1575, 1585 (1961).

(7) J. Hermans, Jr., and H. A. Scheraga, *ibid.*, **83**, 3283, 3293 (1961).

(8) W. T. Astbury, *Proc. Intern. Wool Textile Research Conf., Australia*, **B**, 202 (1955).

(9) B. Low, in "The Proteins," Vol. IA, Ed. by H. Neurath and K. Bailey, Academic Press, Inc., New York, N. Y., 1953, page 235.

(10) P. J. Flory, *J. Am. Chem. Soc.*, **78**, 5222 (1956); *Science*, **124**, 53 (1956).

(11) L. Mandelkern, *Chem. Revs.*, **56**, 903 (1956); *Rubber Chem. and Technol.*, **32**, 1392 (1959).

(12) R. R. Garrett and P. J. Flory, *Nature*, **177**, 176 (1956).

(13) E. T. Dumitru, Ph.D. Thesis, Cornell University, Ithaca, N. Y., 1957.

(14) J. F. M. Oth and P. J. Flory, *J. Am. Chem. Soc.*, **80**, 1297 (1958).

(15) J. F. M. Oth, *Kolloid-Z.*, **162**, 124 (1959).

(16) P. J. Flory and E. Weaver, *J. Am. Chem. Soc.*, **82**, 4518 (1960).

(17) P. J. Flory and O. K. Spurr, Jr., *ibid.*, **83**, 1308 (1961).

(18) J. R. Colvin, *Arch. Biochem. Biophys.*, **46**, 385 (1953).

(19) J. A. Schellman, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **29**, 223, 230 (1955).

(20) T. L. Hill, *J. Polymer Sci.*, **23**, 549 (1957).

(21) J. T. Yang and P. Doty, *J. Am. Chem. Soc.*, **79**, 761 (1957).

(22) J. A. Schellman, *J. Phys. Chem.*, **62**, 1485 (1958).

(23) S. Bresler, *Discussions Faraday Soc.*, **25**, 158 (1958).

(24) B. H. Zimm and J. K. Bragg, *J. Chem. Phys.*, **28**, 1246 (1958); **31**, 5261 (1959).

(25) J. H. Gibbs and E. A. DiMarzio, *ibid.*, **29**, 1247 (1958); **30**, 271 (1959).

(26) S. A. Rice and A. Wada, Abstracts of the 134th meeting of the Amer. Chem. Soc., Chicago, Ill., p. 41 S, Sept. 1958; *J. Chem. Phys.*, **29**, 233 (1958).

(27) L. Peller, *J. Phys. Chem.*, **63**, 1194, 1199 (1959).

(28) L. Mandelkern, D. E. Roberts, A. F. Diorio and A. S. Posner, *J. Am. Chem. Soc.*, **81**, 4148 (1959).

(29) C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **235**, 633 (1960).

(30) D. H. Spackman, W. H. Stein and S. Moore, *ibid.*, **235**, 648 (1960).

(31) A. P. Ryle, F. Sanger, L. F. Smith and R. Kitai, *Biochem. J.*, **60**, 541 (1955).

been shown that these treatments do not in themselves deform the native conformation of the proteins studied.[32]

In the case of fibrin, we are more likely to be dealing with a conformation closely similar to that of a native globular protein for the following reason. The fibrin clot shows evidence of being formed of units which may be reversibly dissociated into globular units of conformation very closely similar to that of the native fibrinogen molecule[33] and, even when irreversibly clotted in the presence of calcium ion and serum factor, gives an X-ray diffraction pattern similar to that of other members of the class of fibrous proteins called the KMEF group.[8,9] This pattern has characteristics of the α-helix[34] which has been found to be present in crystals of the globular protein myoglobin.[35] It has been observed in this work that samples of the reversibly aggregated fibrin (fibrin s), made from highly purified preparations of fibrin in the absence of calcium ion, and those of the irreversibly clotted fibrin (fibrin i) give very similar behavior in the transition region when water, ethylene glycol or glycerol were used as diluent.[36] There is a very good probability, then, that the fibrin structure may be closely similar to that of many globular proteins of interest to the biochemist.

The work described here consisted of two parts. first, the determination of the transition temperature in fibrin as a function of *p*H and the concentration of denaturant, and, secondly, an attempt to ascertain whether the material was indeed in helical and random conformations below and above the transition temperature, respectively.

Experimental

Materials.—Armour Bovine Fraction I, lot No. S3904, was used for the preparation of samples of fibrin i. Several experiments were performed on clots (fibrin s) made from highly purified fibrinogen using the fractionation procedure of Laki[37] as modifed by Sturtevant, *et al.*[38] Eastman *p*-benzoquinone and reagent grade 37% formalin were used as cross-linking agents. USP urea was recrystallized twice by the method of Steinhardt,[39] with removal of alcohol at room temperature. All other materials were reagent grade. Some experiments were performed with reagent grade urea which was found to be free of cyanate according to the method of Werner, as recommended by Stark, *et al.*[40]

Films and Fibers.—Films were made by a procedure similar to that of Ferry and Morrison,[41] as follows. Eight grams of Armour Fraction I powder was dissolved in 100 ml. of a solution of 0.05 *M* KCl. This was diluted with an equal volume of 0.05 *M* $CaCl_2$, filtered through coarse filter paper and the *p*H was adjusted to 6.25 with a few drops of 0.1 *M* KOH or HCl. An equal volume of a solution of 8 mg. of Parke, Davis thrombin in 100 ml. of H_2O was added with stirring, the resultant foam was removed and the mixture was allowed to clot in a beaker of such size that the mixture formed a layer about 1 inch in thickness. After 1 hr. at room temperature, the clot was turned out onto a piece of muslin or filter paper, covered with another piece, bubbles of air were expressed, and the resulting sandwich was compressed between glass plates, making use of a series of increasing weights so that after 0.5 hr., 1 kilogram was used for a clot of 7 cm. diameter. After an additional hour, the now compacted film of fibrin i was cut into 3 mm. strips, thoroughly washed with 0.3 *M* KCl solution and stored in the refrigerator in 0.3 *M* KCl or distilled water until used.

Several experiments were performed on fibrin films (fibrin s) made from highly purified fibrinogen according to the modified Laki[37] procedure of Sturtevant, *et al.*,[38] and clotted in 0.15 *M* KCl solution in the same manner.

After washing and storage, the samples were allowed to equilibrate with an excess of the diluent solution, in which they were to be tested, for 48 hr. at 4°.

Several experiments were performed on fibers stabilized by cross-linking with *p*-benzoquinone. The studies of Mihalyi and Lorand[42] showed that the cross-linking of fibrinogen with quinone is effective at *p*H 8.5 and above. Accordingly, fibers were made by the following procedure. The clotting mixture was the same as that used for making films. It was chilled to 4° and, while cold, was drawn up into glass tubes of 5 mm. i.d. These tubes were allowed to stand at 25° for 90 minutes to allow clotting to occur. The clots were pushed out of the tubes with a rod of 5 mm. o.d. and allowed to fall into a wash solution of 0.3 *M* KCl to remove non-clotted protein. They were stretched with forceps approximately 50% of their length to orient the fibers and then washed in 5 or 6 portions of KCl. They were then immersed in a solution of 0.1 *M* phosphate buffer at *p*H 6 with 0.01 g. of *p*-benzoquinone per 100 ml. at 4° for 24 hr. to allow the quinone to diffuse into the samples. The *p*H of the solution then was changed to 8.7 at room temperature and tanning was allowed to proceed for the period of time desired. The fibers then were washed exhaustively with water and stored in water in the refrigerator. The fibers were held straight during tanning by clamps; care is needed to avoid kinking the tanned samples.

Several films were tanned in formalin by placing fibrin strips in formalin solutions at *p*H 7 for a given time and at a given formalin concentration, then washing with water.

Length Measurement.—Lengths were measured with either of two cathetometers, one purchased from Ole Dich, Copenhagen, Denmark, and the other from the Gaertner Scientific Corp., Chicago, Illinois. The sample was hung in a flat-sided glass cell surrounded by a flat-sided, glass water jacket which was kept at constant temperature with circulating water. A very small piece of chromel wire (or platinum wire at very low *p*H) was used as a weight to keep the sample hanging vertically. This was small enough so as not to exert enough force to affect the observed transition. The cell was filled with diluent and the length was measured as a function of temperature.

Force Measurement.—Forces were measured with an apparatus similar to that of Oth and Flory.[14] The transducer with which forces on the sample were converted to electrical signals was purchased from Statham Instrument Co., Hato Rey, Puerto Rico; model G-7A. This was coupled with a General Electric 8CE5 potentiometer recorder whose sensitivity could be varied in steps over the range from 0.2 to 500 mv. for full scale deflection of the 9.5 cm. chart. Oscillations of the recorder sensing element were eliminated by a 100 μfd. electrolytic capacitor across the input leads. The entire assembly was calibrated by hanging known weights from the transducer probe. The length of the samples could be measured simultaneously with the force, since the same flat-sided cell was used for the dynamometer as was described for the length measurement. The length of the sample could be changed during the course of an experiment by changing the distance between the clamps holding the sample by means of an adjusting screw. The upper clamp was hung directly from the transducer probe which was powered by three Mallory mercury bat-

(32) The possible disruption of the native structure by the introduction of cross-linkages does not invalidate the application of polymer theories or the deduction of thermodynamic parameters pertaining to the transitions observed in the other studies.[6] Without the support of parallel studies upon native conformations, however, the other studies might not apply to the native proteins.

(33) H. A. Scheraga and M. Laskowski, Jr., *Adv. Protein Chem.*, **12**, 1 (1957).

(34) R. B. Corey and L. Pauling, *Proc. Intern. Wool Textile Research Conf., Australia*, **B**, 249 (1955).

(35) J. C. Kendrew, *et al.*, *Nature*, **185**, 422 (1960).

(36) Fibrin i was used in the greater portion of this work because the reversibly clotted material is soluble in warm salt solutions and at extremes of *p*H.

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

(38) J. M. Sturtevant, M. Laskowski, Jr., T. H. Donnelly and H. A. Scheraga, *J. Am. Chem. Soc.*, **77**, 6168 (1955).

(39) J. Steinhardt, *J. Biol. Chem.*, **123**, 549 (1938).

(40) G. R. Stark, W. H. Stein and S. Moore, *ibid.*, **235**, 3177 (1960).

(41) J. D. Ferry and P. R. Morrison, *Ind. Eng. Chem.*, **38**, 1217 (1947).

(42) E. Mihalyi and L. Lorand, *Hung. Acta Physiol.*, **1**, 218 (1948).

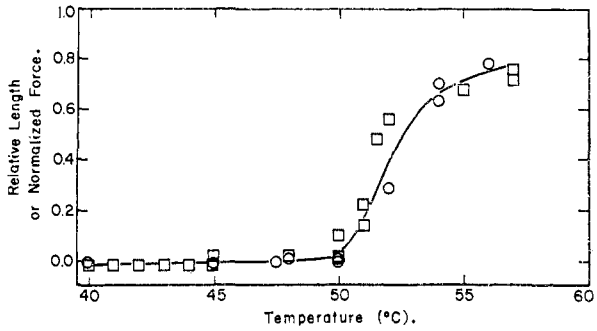

Fig. 1.—Comparison of length (□) and force (○) measurement techniques for untanned fibers (~1 mm. diameter) of fibrin i in water. Force ~0.3 g.; length ~2 cm. The change in length is negative, and the units are arbitrary.

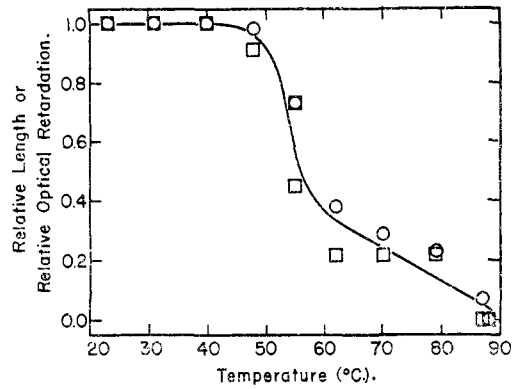

Fig. 2.—Length (○) and optical retardation (□) changes in the transition region for untanned fibers of fibrin i (similar to those of Fig. 1) in water. Original length ~0.5 cm.

teries, part #302478, and provision was made for displacing the zero of force signal by five chart widths. The sensitivity of the transducer was approximately 3×10^3 microvolts per volt of exciting voltage per ounce of applied force.

Birefringence Measurement.—Birefringence was measured with a Leitz petrographic microscope, using a Berek compensator and a Leitz 350° hot stage attachment for the microscope. Length measurements were made simultaneously by using a micrometer eyepiece.

***p*H Measurement.**—The *p*H of the diluent surrounding the samples was measured using a Beckman model G *p*H meter which was calibrated with Beckman reference buffers and a potassium acid phthalate solution (*p*H 4.01 for 0.05 *M* solution).

Results

Nature of the Transition.—Curves representing the temperature dependence of length, force, or birefringence showed a sharp transition in the neutral *p*H region; while the transition is not as sharp at extremes of *p*H, it is still quite apparent. The curves were obtained by maintaining the sample at a given temperature for 10 minutes then measuring the length and/or force and/or birefringence. The temperature was then increased by 2° and the procedure repeated. In several control experiments, the temperature was increased only after the property measured reached an apparently constant value. This, however, required extremely long periods of time. Since the dependence of the transition temperature on wide ranges of parameters was being measured, it was desired to perform many measurements upon samples

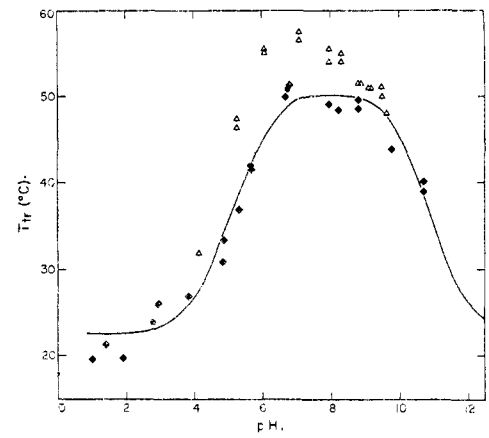

Fig. 3.—Dependence of transition temperature on pH, obtained with untanned films of fibrin i. △, ionic strength 0.15; ◆, ionic strength 3.0; ●, ionic strength 3.0, temperature raised after measured length was constant at each of these two *p*H values. Curve is theoretical (see text).

made at the same time. Also we did not wish to store the samples for extended periods, even in the refrigerator. Further, it is well known that thermal degradation, disulfide exchange, and possibly other undesirable side reactions occur when protein samples are held at elevated temperatures for long periods of time. It was felt that these considerations justified more rapid heating, especially since transition temperatures at both very low and neutral *p*H, measured in the long control runs, agreed very well with those obtained from the more rapid runs. The transition temperatures were derived from the more rapid heating experiments by taking the intersection of the heating curve below the transition range with the tangent to the linear portion of the heating curve in the transition region. In the longer "equilibrium" runs, the temperature of half-conversion was taken as the transition temperature. This half-conversion temperature was arrived at by extrapolating the linear high-temperature and low-temperature portions of the heating curves into the transition region. The fraction of material transformed at any temperature T was taken to be equal to $\frac{G - G_c}{G_a - G_c}$, where G_a is the value of the ordinate of the extrapolated high-temperature portion of the heating curve at the temperature T, G_c is the corresponding quantity for the extrapolated low-temperature form at temperature T, and G is the measured value of the property at temperature T. Typical heating curves, as determined from length, force and birefringence measurements, are shown in Figs. 1 and 2. The transition temperatures, T_{tr}, determined from the longer runs are shown as circles in Fig. 3, which shows the *p*H-dependence of the transition temperature at ionic strengths 0.15 and 3.0.

Effect of Tanning on the Transition Temperature.—It is expected that cross-links introduced into *crystalline* regions of a polymer network will raise the transition temperature.[10] In this work *p*-benzoquinone and formaldehyde were used as cross-linking agents. In the case of formaldehyde

the transition temperature could be raised up to 20° above that found in water. Quinone tannage, on the other hand, did *not* raise the transition temperature more than one degree, which is very close to the limit of experimental error. The differences may be understood on the basis of three points of view: first, that those residues which form cross-links with quinone are not found in the crystalline regions, while formalin may react with residues found in these regions. This is considered doubtful, as amino groups seem to be involved in both cases.[42,43] A second alternative is that the groups have different reactivity in amorphous and crystalline regions. A third, which seems most probable at present, is that the bulkier quinone cannot penetrate the crystalline regions of the sample, while formaldehyde can. The sharp dependence of permeability upon the size of the diffusing species seems to be indicated independently by the work of Harrison and Speakman, who showed this effect of the permeability in a homologous series of alcohols.[44] Similar effects were observed with insulin and ribonuclease.[6]

Reversibility.—Since the transitions will be considered from a thermodynamic point of view their reversibility is of interest. Several experiments were performed to obtain evidence for the reversibility of the transition. The transitions in the samples stabilized by quinone tanning were reversible, at least in the following sense: If the sample in 3 *M* KCl at *p*H 2.5 is heated from 4°, an increase in force will occur in the transition region, and at higher temperatures the slope of the force-temperature curve decreases to the value characteristic of the high-temperature form, the maximum slope being found in the transition region. If the sample is then cooled at constant length, the force decreases along the projection of the curve extrapolated from the high temperature region. If the temperature is then held at 4° for 3 days, the tension on the sample falls to a small amount. If the tension is reduced to zero by allowing the sample to contract *very slightly* and then relax for 2 days to come to equilibrium, a heating curve is obtained which shows an increase of force in the transition region. This starts at a lower temperature than the transition observed on the first heating, and the total force increase is larger than observed on the first heating. Perhaps this is due to (a) a less perfect arrangement within the crystallites when they reform and (b) a greater degree of crystallinity due to the long crystallization under stress. Samples have been taken around this cycle four times in some cases. This type of reversibility may not be true thermodynamic reversibility in the sense that *small* changes in external parameters do not lead to small reversals in the experimentally observable quantities; however, it has been observed that over reasonable time scales an appreciable amount of hysteresis is observed in re-forming crystallites in most polymeric systems.[11] We adopt the viewpoint that this is what is happening in the case of cross-linked fibrin also. The *untanned* samples showed similar indications of reversibility at high ionic strength after 2 weeks of standing at 4° but were not so definite in this respect as were the tanned samples.

Several experiments were performed upon fibrin s, made from fibrinogen purified by the modified Laki procedure.[37,38] The transitions in pure water, in glycerol, and ethylene glycol observed with these samples were not distinguishable from similar experiments performed on samples made with the Fraction I and calcium ion. Experiments in aqueous salt solution could not be compared due to the dissolution of the soluble fibrin at elevated temperatures when salt is present.

Structural Studies.—The model of the fibrin system to be considered here involves a transition from an ordered to a random phase. Efforts were made to evaluate the validity of this assumption by two approaches: X-ray diffraction and elasticity studies of the material above and below the transition region. The results of these experiments were not entirely satisfactory for the following reasons. The X-ray diffraction studies of fibers of fibrin i gave clear patterns on the dried material, which showed typical α and β patterns on untransformed and transformed material, respectively. We were interested, however, in patterns taken *in the presence of diluent.* Initial experiments on wet fibers, mentioned in footnote 33 of reference 6, appeared to confirm the conclusions deduced from patterns of dried fibers. However, subsequent experiments on other samples failed to provide unambiguous information. No clear-cut evidence was obtained with these swollen samples; the patterns were difficult to obtain in a clear form because of disorientation in the swollen films and fibers and also because of the scattering due to the large amount of diluent in the samples.

The results of the elasticity measurements were not conclusive insofar as the samples did not exhibit completely reversible stress-strain or stress-temperature behavior; that is, after stressing a sample it would not completely revert to its original length on releasing the tension, even when the sample was tanned. Also a cycling of temperature at constant length showed a lack of reversibility. Whether this is due to truly irreversible processes or simply to a long hysteresis is not known. We attempt a qualitative description of the results, recognizing this limitation.

According to the elastic equation for amorphous networks in equilibrium with a diluent phase[10,45]

$$f = BTL\left(1 - \frac{L_{i0}^3}{v_2 L^3}\right) \qquad (1)$$

where f is the retractive force developed in the amorphous elastometer, T is the absolute temperature, L is the length of the sample, L_{i0} is the length of the unstressed sample when not swollen with diluent, v_2 is the volume fraction of polymer at the length which generates the force f, B is an elasticity parameter which is defined by the nature of the network through the equation

$$B = k\nu(\langle\alpha\rangle_0/L_{i0})^2 \qquad (2)$$

(43) K. H. Gustavson, "The Chemistry of the Tanning Process," Academic Press, Inc., New York, N. Y., 1956.

(44) D. Harrison and J. B. Speakman, *Textile Research J.*, **28**, 1005 (1958).

(45) P. J. Flory, C. A. J. Hoeve and A. Ciferri, *J. Polymer Sci.*, **34**, 337 (1959).

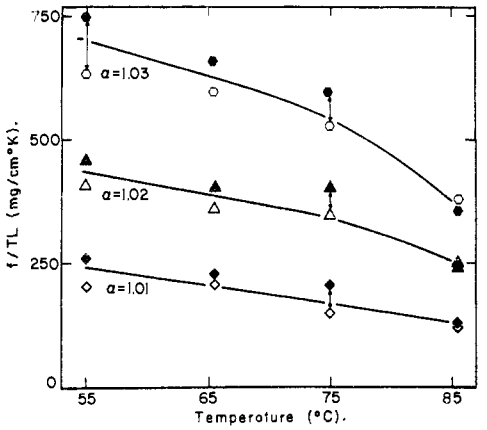

Fig. 4.—Elasticity of quinone-tanned fibers made from fibrin s (in water) at temperatures above the transition temperature; α is the extension ratio. Open and closed symbols indicate increasing and decreasing temperatures, respectively.

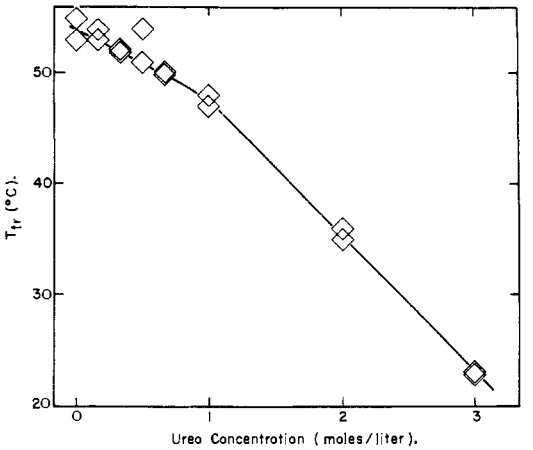

Fig. 5.—The effect of urea on the transition temperature for untanned films of fibrin i at pH 6.6, ionic strength 3.0.

where $<\alpha>_0 = (\overline{r_i^2}/\overline{r_0^2})^{1/2}v_2^{1/3}$, k is the Boltzmann constant, ν is the number of chains in the network, $\overline{r_i^2}$ is the mean square chain vector in the unstressed state at volume fraction v_2, $\overline{r_0^2}$ is the mean square chain vector in the configuration corresponding to the ideal case of a Gaussian distribution of chain configurations where interactions do not perturb the configuration (unconstrained free chains).

Correction for changes in volume fraction during deformation were estimated from the swelling of the sample as determined by measurement of the length (extrapolated to zero force) during the elasticity experiment, combined with the modulus of elasticity, as suggested by Flory,[10] and Flory and Spurr.[17]

A plot of f/TL *vs.* T (at constant value of L_{i0}^3/v_2L^3) should, then, be a straight line of zero slope if B were constant and the substance were an ideal elastomer. It was found, however, that a negative slope was always present.

We do not wish to present any quantitative statements based on these experiments, since the lack of complete reversibility may cause such numbers to be misleading. We do feel that there is an indication that the normal elastic behavior, as is found for example in the case of collagen or rubber, is not found in fibrin. A run on a tanned sample is illustrated in Fig. 4. The points were obtained in the order of increasing and decreasing temperature. The pronounced negative slope is clearly seen in spite of the large uncertainties in the experimental points.

This deviation from ideal behavior is in the direction of positive energy and entropy changes on elongation[45] *above the transition range.* This would seem to indicate either hindrances to free rotation in the amorphous polymer chains or the overcoming of other restraints upon the configuration of the chains. The deviations are in a direction *opposite* to those which would be observed if orientation by stretching induced interactions at these very small degrees of elongation. These results are not sufficiently conclusive, however, to rule out the postulated helix-random coil transition, which we continue to accept here.

pH-Dependence of the Transition Temperature.—The transition temperature is a function of pH. This dependence is shown by the points in Fig. 3. The curve drawn in the figure is a theoretical one constructed according to the theory outlined in the Discussion section and gives good agreement with the experimental data at ionic strength 3.0.

Effect of Urea.—The dependence of the transition temperature on urea at pH 6.6 and ionic strength 3.0 is shown in Fig. 5. The buffers used were phosphate at 0.05 M; the ionic strength was maintained with potassium chloride at 3.0.

Discussion

pH-Dependence of the Transition Temperature.—The purpose of this work was to determine whether the theory[4] that hydrogen bonding between polar side-chain residues stabilizes the native protein molecule can account for the pH dependence of the transition temperature. In order to eliminate electrostatic effects as much as possible, the work was done at extremely high ionic strength where ribonuclease showed reversibility and the absence of significant dependence of swelling on pH.[6] It must be borne in mind, however, that we have no guarantee that electrostatic effects have been completely eliminated by this procedure. In order to compare the experimental data with the theory of hydrogen bonding,[4] we have quite arbitrarily chosen a simple (and very probably oversimplified) model of the fibrin molecule on which to base our calculations. The model was arrived at from consideration of the amino acid composition of fibrinogen, which is the precursor of fibrin, shown in Table I.

If we make the assumption that all the chains of the molecule are of equal length (a chain is defined here as the section of polypeptide between covalent cross-links) and that all chains will be stabilized by the same type and number of hydrogen bonds, we can postulate that a unit of 10^5 grams of protein is a structure with 10 closed loops (corresponding to the 10 disulfide crosslinks), 20 chains, and about 40 residues per chain. This defines the covalent structure of our model.

TABLE I

AMINO ACID COMPOSITION OF FIBRINOGEN[46]

Residue	Number of residues in 10^5 g. of protein
Tyrosine	30
Histidine	17
Lysine	63
Aspartic and glutamic acids	~200
Half-cystine	19
Others	117
Total	846

The observed standard free energy of unfolding of such a system may be written[4] as

$$\Delta F_{\text{obsd.}}^0 = \Delta F_b^0 + \Delta F_H^0 \qquad (3)$$

where ΔF_b^0 is the standard free energy of unfolding the backbone if no hydrogen bond stabilization is present[19] and ΔF_H^0 is the contribution to the standard free energy of unfolding due to the possibility of hydrogen bond formation.[47] The free energy of mixing of solvent and unfolded form is absorbed in these parameters.

On the basis of our model and the work of Schellman,[19] we may estimate ΔF_b^0. We take the results, derived from optical rotation by Yang and Doty,[21] that the helical content of fibrinogen in its native state is approximately 35% of its amino acid residues. To simplify the treatment we keep the assumption that all chains are identical by allowing each chain to have random portions and also a helical portion equivalent to 35% of its residues. Then according to Schellman,[19] we may write

$$\Delta F_b^0 = (n - 4)\,\Delta H_{\text{res.}}^0 - T(n - 1)\,\Delta S_{\text{res.}}^0 - T(\Delta S_x^0/2) \qquad (4)$$

for each chain, where n is the number of amino acid residues in the helical part of the chain, $\Delta H_{\text{res.}}^0$ and $\Delta S_{\text{res.}}^0$ are the standard enthalpy and entropy changes, respectively, per residue, for the unfolding of an infinitely long helix to a random coil, and ΔS_x^0 is the molar entropy decrease of the random coil due to the introduction of cross-links in the *crystalline* form. This equation is derived on the basis that only two types of conformation are permitted to any chain: either 35% helical or else completely transformed to a non-helical form. The estimate of ΔS_x^0 according to Flory[10] is given by the equation

$$\Delta S_x^0 = -(3R\nu/4)[\ln n' + 3] \qquad (5)$$

where k is Boltzmann's constant, ν is the number of cross-linked helices and n' is the number of statistical elements[48] between cross-links. This treatment and an alternative one due to Kuhn, as used by Schellman,[19] lead to similar results for ΔS_x^0, namely, 10 ± 1 e.u. per mole of chains. We adopt this value of 10 e.u. in this discussion.

(46) G. R. Tristram, in "The Proteins," Vol. IA, Ed. by H. Neurath and K. Bailey, Academic Press, Inc., New York, N. Y., 1953, p. 215.

(47) M. Laskowski, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.*, **76**, 6305 (1954).

(48) We assume here that one amino acid residue forms one statistical unit (*i.e.*, $n' = n$), although it is possible that more than one residue is a better estimate due to restrictions on the bond angles and rotations in the peptide backbone. The result is not very sensitive to a factor of 2 in n/n' in any case.

The values of $\Delta H_{\text{res.}}^0$ and $\Delta S_{\text{res.}}^0$ adopted for use in this model may be approximated from theoretical considerations but the range of values obtained is so broad that in order to make any progress we must assign, on the basis of experimental studies, values which cause the transitions to fall in the temperature range commonly observed. The value of $\Delta H_{\text{res.}}^0$ adopted is 1500 cal./mole of hydrogen bonds on the basis of Schellman's analysis[19] of the thermodynamics of urea solutions. Adoption of this value forces a choice of $\Delta S_{\text{res.}}^0$ = 4 to 5 e.u. if reasonable estimates of the transition temperatures are to be forthcoming.[49]

We now turn to an estimate[47,50] of ΔF_H^0. If an i^{th} donor group DH, with dissociation constant K_1, and a j^{th} acceptor group A, with dissociation constant K_2, are held in the proper steric relationship to each other so as to be able to form a hydrogen bond DH. . .A, then the contribution of such a bond to the free energy of unfolding of that structure to any other conformation in which the bond is sterically not possible is given by

$$\Delta F_H^0 = -RT \ln (1 - x_{ij}) \qquad (6)$$

where x_{ij} is the fraction of such groups which actually are hydrogen bonded and is given by

$$x_{ij} = \frac{K_{ij}}{1 + K_{1j} + K_1/[H^+] + [H^+]/K_2} \qquad (7)$$

where K_{ij} is the equilibrium constant for forming a side-chain hydrogen bond.[47] Equation 6 may be obtained if we consider that the transition occurs in two steps. In the native protein, we postulate a specific structure which has the donor group DH in close proximity to the acceptor group A. If the groups are hydrogen bonded, then the bond must be destroyed in the transition process. If the reaction is reversible, we have an equilibrium between three forms of any chain: the native or crystalline form (form C), an intermediate form retaining the structure characteristic of the native but in which the side-chain hydrogen bonds are broken (form I), and the amorphous denatured form (form A). Since physical measurements of the kind used here do not distinguish between forms C and I, the observed reaction is

$$P_{(C+I)} \rightleftarrows P_{(A)} \qquad (8)$$

where P denotes the fraction of material in the form indicated by the subscript. Since x_{ij} is the fraction of groups which are hydrogen-bonded, and we postulate that a large number of identical chains are involved

$$\frac{P_{(C)}}{P_{(C+I)}} = x_{ij} \qquad (9)$$

(49) Since the transition temperature is given by

$$\Delta F_{\text{obsd.}}^0 = \Delta H_{\text{obsd.}}^0 - T\Delta S_{\text{obsd.}}^0 = 0$$

$$T_{\text{tr.}} = \frac{\Delta H_{\text{obsd.}}^0}{\Delta S_{\text{obsd.}}^0} \approx \frac{\Delta H_{\text{res.}}^0}{\Delta S_{\text{res.}}^0}$$

for moderate n, the relative error in $\Delta S_{\text{res.}}^0$ is almost equal to the relative error in $T_{\text{tr.}}$. For example, a 5% error in $\Delta S_{\text{res.}}^0$ (an accuracy which is impossible to achieve as far as a theoretical calculation is concerned at present) would lead to a 15° error in $T_{\text{tr.}}$, which is much too large. Therefore, in order to fix a base point for the calculation, it is necessary to assign an empirical value to $\Delta S_{\text{res.}}^0$ to agree with one point on the experimental curve of $T_{\text{tr.}}$ *vs.* *p*H, after the quantity ΔF_H^0 has been decided upon. The point chosen is at *p*H = 7 (see below).

(50) M. Laskowski, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.*, **78**, 5793 (1956).

i.e., x_{ij} is the fraction of untransformed material which is hydrogen-bonded. We define

$$K_0 = \frac{P_{(A)}}{P_{(I)}} \qquad (10)$$

as the intrinsic equilibrium constant for the backbone transition. Then[50]

$$K_{obsd.} = \frac{P_{(A)}}{P_{(C+I)}} = K_0(1 - x_{ij}) \qquad (11)$$

which shows that ΔF_H^0 is as given by eq. 6.

The model adopted involves 2 side-chain hydrogen bonds for each backbone chain, so the observed standard free energy of transition for one mole of chains is

$$\Delta F_{obsd.}^0 = \Delta F_b^0 - \Sigma RT \ln(1 - x_{ij}) \qquad (12)$$

where the sum contains two terms, corresponding to the two side-chain hydrogen bonds. The temperature at which $\Delta F_{obsd.}^0 = 0$ is taken as the transition temperature. The values of K_1, K_2 and K_{ij} at 300° and the values ΔH^0 used to calculate their changes with temperature are listed in Table II. The plot of transition temperature as a function of *p*H resulting from this model is shown in Fig. 3, together with the experimental points as observed. It will be noted that the fit is good for ionic strength 3.0 indicating that the model is adequate to explain the observed *p*H dependence within the *p*H range of 2.5 to 11 where charge effects are reduced by high salt concentration. The value of $\Delta S_{res.}^0$ required to match the calculated and experimental curves correctly[49] at *p*H 7 is 4.7 e.u.

TABLE II

PARAMETERS USED TO CHARACTERIZE SIDE-CHAIN HYDROGEN BONDS[a]

$K_{ij} = 4$; $\Delta H_{ij}^0 = -6$ kcal.

Bond type	pK_1 (300°K.)	pK_2 (300°K.)	ΔH_1^0 (kcal.)	ΔH_2^0
Tyrosyl-carboxylate ion	10.5	4.5	+6	0
Tyrosyl-histidine	10.5	6.5	+6	+7

[a] Justification of these values has been provided elsewhere.[47,51,52]

Sharpness of the Transition.—We now consider the sharpness of the transition curves. The sharpness is determined[19,53] by $\Delta H_{obsd.}^0/T_{tr.}^2$ of the transition and, according to our model, is

$$s = \left(\frac{d\alpha}{dT}\right)_{T_{tr.}} = \frac{d}{dT}[1 + \exp(\Delta F_{obsd.}^0/RT)]^{-1} = \frac{\Delta H_{obsd.}^0}{4RT_{tr.}^2} \qquad (13)$$

where $T_{tr.}$ is the transition temperature, $\Delta F_{obsd.}^0$ is the observed standard free energy of the transition, and α is the fraction of material which exists in the transformed form. The exact shape of the heating curve is determined by the mechanism of the transition, as has been shown in the various theoretical treatments.[22,24-27] In the case of fibrin, the transition at *p*H 7 occurs within less than 2°. Since this is very sharp, the fine details of the shape of the heating curve are not available. However, the sharpness is very much greater than that expected for a single independent helix of this chain length.[53] Therefore it appears that more than one helix cooperates in the transition. Assuming an all-or-none transition for each chain, we may calculate an approximate value of $\Delta H_{obsd.}^0$ of the unit involved in the transition. This allows an estimate of the number, $\mathfrak{N}$, of chains which unfold simultaneously in a reacting unit, to account for the sharpness of this transition *according to this model*. The $\Delta H_{obsd.}^0$ of the transition is according to our model

$$\Delta H_{obsd.}^0 = \Delta H_b^0 + \Delta H_H^0 \qquad (14)$$

where ΔH_b^0 is the contribution of the backbone, and ΔH_H^0 is the contribution of the side-chain hydrogen bonds. According to our model ΔH_b^0 for a single chain (with $n = 35\%$ of 40 or 14) is

$$\Delta H_b^0 = (n - 4)(\Delta H_{res.}^0) = 15 \text{ kcal./mole of chains} \qquad (15)$$

and we must calculate ΔH_H^0. This is given by[4,38]

$$\Delta H_H^0 = \Sigma x_{ij}\left[-\Delta H_{ij}^0 + \frac{(K_1/[H^+])\Delta H_1^0 - ([H^+]/K_2)\Delta H_2^0}{1 + K_1/[H^+] + [H^+]/K_2}\right] \qquad (16)$$

where the sum is taken over the two side-chain hydrogen bonds per chain. The enthalpy of unfolding per $\mathfrak{N}$ moles of chains is

$$\Delta H_{obsd.}^0 = \mathfrak{N}\{(n - 4)\Delta H_{res.}^0 + \Delta H_H^0\} \qquad (17)$$

It should be observed that the number of chains involved will *not* change the transition temperature unless the total $\Delta H_{obsd.}^0$ and/or $\Delta S_{obsd.}^0$ *per chain* are changed. This may be seen by comparing the expressions for the transition temperature and the sharpness. According to eq. 17 (and the first equation in ref. 49), it can be seen that $T_{tr.}$ is independent of $\mathfrak{N}$ since both $\Delta H_{obsd.}^0$ and $\Delta S_{obsd.}^0$ are proportional to $\mathfrak{N}$. These equations imply that the association of chains into groups does not change $\Delta H_{obsd.}^0/\mathfrak{N}$ or $\Delta S_{obsd.}^0/\mathfrak{N}$ very much. This need not cause much concern because the arbitrary choice of *p*H-independent parameters may be made to allow for this effect.

Since $T_{tr.}$ is independent of $\mathfrak{N}$, we can compute $\mathfrak{N}$ from eq. 13. Writing $s = (1/2)\text{deg.}^{-1}$ (since the transition occurs over 2° or less at *p*H 7), we find, for the model chosen, that $\mathfrak{N} = 18$, so that about 18 chains must be involved in a cooperative transition to account for the sharpness of the transition. The possibility that the interdependence between chains is responsible for sharpening of a helix–coil transition has been pointed out previously.[54,55] It may be seen that any interaction which increases $\Delta H_{obsd.}^0$ of the transition by increasing the total number of interactions which must be simultaneously broken as in the case of interdependence of a number of smaller units or by increasing the number of interactions per unit which are broken during the transition[53] will have a sharpening effect.

We may postulate that the chains are not completely independent because (a) hydrogen bonds or other secondary interactions may exist between

(51) G. I. Loeb and H. A. Scheraga, *J. Phys. Chem.*, **60**, 1633 (1956).

(52) H. A. Scheraga, *Ann. Rev. Phys. Chem.*, **10**, 191 (1959).

(53) H. A. Scheraga, R. A. Scott, G. I. Loeb, A. Nakajima and J. Hermans, Jr., *J. Phys. Chem.*, **65**, 699 (1961).

(54) B. H. Zimm, P. Doty and K. Iso, *Proc. Natl. Acad. Sci. U. S.*, **45**, 1601 (1959).

(55) P. J. Flory, *J. Polymer Sci.*, **49**, 105 (1961).

adjacent chains, and (b) because the cross-linkages force the chains to be interdependent.

The number 18 derived from the treatment above need not be interpreted as indicating that there are indeed well-defined groups of 18 chains going through the transition as a whole. There merely may be interactions such that any one chain may influence, and be influenced by, a large number of neighbors so that a change of conformation in one affects the others. The number 18 may be looked upon as defining a dimension of the "sphere of influence" about each chain without, as yet, specifying the extent of interdependence among any two specific chains in a region of sample. In fact, we might very well expect the degree of interdependence to be a smooth function of their spatial separation.

A similar calculation may be made at *p*H 2.8 where the effect of the side-chain hydrogen bonds is small. Here, the transition range is observed to be 5° and ΔH_H^0 less than 1 kcal. The number of chains which is indicated by this calculation is 9. The difference may be accounted for in two ways, *if we accept the model:* (1) Experimental error; in these runs, the length was followed at a given temperature until no further change was detected. The detection of changes is dependent upon the precision of the detection device; it is possible that a very slow change, which might continue for a long time, might go unnoticed and lead to a different slope for the heating curve. By the use of a large number of samples, each thermostated for long periods at small temperature intervals, this can be avoided. (2) The side-chain hydrogen bonds may be inter-, as well as intra-, chain. Thus, when the side-chain hydrogen bonds are broken by lowering *p*H, chains become more independent. This may occur in proteins in solution when adjacent chains in the same molecule may be involved.

The Effect of Urea.—The values of $\Delta H_{res.}^0$ and $\Delta S_{res.}^0$ chosen for this model are consistent with the results of both our and previous experiments on the effect of urea concentration upon the transition temperature. As originally proposed by Schellman,[19] we may indicate the effect of urea upon the polypeptide backbone. In order to do this, we must make use of the binding constant of urea to the polypeptide backbone. The value adopted by Schellman was based upon the association of urea into dimers in a bond which one would expect to be quite similar to the bond involved in the binding of urea to the polypeptide backbone. We must recognize the possibility that the binding constant may not be correct in the case of binding to polypeptides[56]; but on the other hand, the use of this value has led to fairly consistent estimates of $\Delta H_{res.}^0$ for the urea and peptide backbone bonds from experimental data when applied to ribonuclease,[6] insulin,[6] ovalbumin[19] and now fibrin. There should be less controversy about the value of ΔH^0 for binding chosen from the dimerization data if we accept that the major cause of deviation from ideality in urea solution is due to dimerization. The uncertainty in the binding constant K may therefore reflect an uncertainty in the entropy of binding of urea to the peptide backbone, which is an illustration of the generalization that enthalpy effects are generally more reliably extrapolated from one system to another if the bonds involved are similar than are entropy effects. This is reminiscent of the difficulty[49] in arriving at a value of $\Delta S_{res.}^0$. Taking the value of K obtained from the urea dimerization, we have in urea solution[19]

$$\Delta F_{obsd.} = \Delta F_b^0 - 2nRT \ln (1 + K\{U\}) \quad (18)$$

where $\{U\}$ is the urea concentration in moles/l. Schellman's value of $K = 0.041$ was modified to 0.025 to account for the fact[19] that 60% of the dimers have only one hydrogen bond. Since $\Delta F_{obsd.} = 0$ at $T_{tr.}$, we obtain

$$\Delta H_{res.}^0/T_{tr.} - \frac{1}{10}(\Delta S_x^0 + 13\ \Delta S_{res.}^0) = 2.8\ R \ln (1 + K\{U\}) \quad (19)$$

where the effect of urea upon the side-chain hydrogen bonds is neglected here for the present. We also neglect electrostatic effects since the ionic strength is high.

When a plot of log $(1 + K\{U\})$ *vs.* $1/T_{tr.}$ is constructed the slope gives $\Delta H_{res.}^0$ according to equation 19. We notice (Fig. 6) that at low urea concentrations the slope of the curve is much steeper than at concentrations above 1 *M*. This phenomenon has been noted several times, under different conditions of *p*H between 6 and 7.5, and at ionic strength 0.15 to 3.0. This steep slope may be explained in a number of ways, *e.g.*, in terms of an inverse transition following the formulation of Schellman[22] or as the effect of urea upon side-chain hydrogen bonds in our model. The slope above 1 *M*, however, gives a value of $\Delta H_{res.}^0$ of 1.65 ± 0.15 kcal., which is in very good agreement with previous estimates[6,19] and is consistent, within experimental error, with the value 1.5 kcal. chosen in the calculation of the *p*H dependence of the transition temperature. The intercept gives $\Delta S_{res.}^0 = 4.55 \pm 0.35$ e.u. Attempts to measure the effect of urea upon the transition temperature at low *p*H, where the effect of side-chain hydrogen

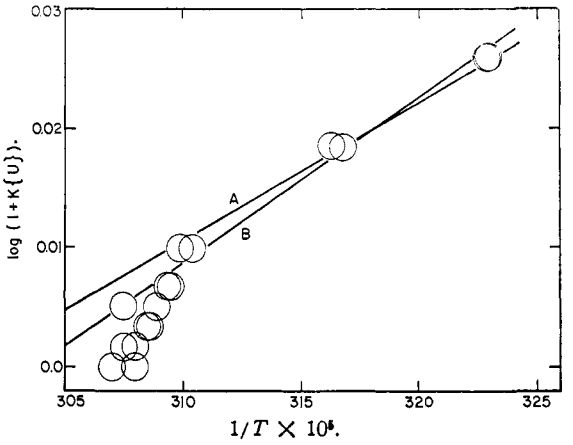

Fig. 6.—Treatment of data of Fig. 5 according to eqn. 19. The curves show the approximate limits of error. Curve A leads to $\Delta H_{res.}^0 = 1.5$ kcal., $\Delta H_{res.}^0 = 4.2$ e.u.; Curve B to $\Delta H_{res.}^0 = 1.8$ kcal., $\Delta S_{res.}^0 = 4.9$ e.u.

(56) I. M. Klotz and J. S. Franzen, *J. Am. Chem. Soc.*, **82**, 5241 (1960).

bonds is negligible, were not successful because of a large amount of scatter in the experimental results. If such measurements could be made, they might be a better confirmation of the value of $\Delta H_{res.}^0$ than measurements in the neutral *p*H region. The effect of decomposition of urea should also be minimized at low *p*H. However, the effect of charges on the protein might not be eliminated even at this high ionic strength and might be serious at low *p*H.

This study was undertaken concurrently with similar studies of insulin fibers and ribonuclease films[6] and the three should be considered as complementary. The case of tanned ribonuclease is especially interesting in view of its practically complete reversibility in diluents of high salt concentration and low *p*H. In fact, the reversibility of the transition previously noted in studies of ribonuclease solutions[7,57] was a strong factor in motivating a successful demonstration of reversibility of the transition in the cross-linked ribonuclease network. This in turn lends support to the postulate that, in the case of fibrin, the transition also may be truly thermodynamically reversible. That the transition is thermodynamically reversible has been shown to be probable, but was not proved, in this work.

The values of the significant parameters which result from the treatments are quite similar, and this encourages us to consider them, at least tentatively, to be generally applicable. The case of fibrin demonstrates their applicability to a system which was not previously subjected to the action of reagents which change the covalent structure of the molecule, as quinone or formaldehyde may.

(57) W. F. Harrington and J. A. Schellman, *Compt. rend. trav. lab. Carlsberg Ser. chim.*, **30**, 167 (1957).

These parameters fall into two classes. Those which are included in ΔF_H^0 are attributed to the ionization and hydrogen bonding of side-chain ionizable groups. Those which are included in ΔF_b^0, however, are based on rather arbitrary assumptions about protein structure, which may or may not be valid. The fact that our selection of the parameters $\Delta H_{res.}^0$ and $\Delta S_{res.}^0$, and a reasonable length for the "average" chain, leads to reasonable values of ΔF_b^0 may be fortuitous; it would, therefore, be misleading to take this as evidence for the α-helical conformation in this system. We have shown that the α-helical model, with reasonable values of the parameters, does give agreement with the experimental results; however, other models for the backbone structure, which have the same *p*H-independent values of ΔF_b^0 will also give satisfactory agreement. This qualification does *not* pertain to the significance of the good agreement for the *pH-dependent* aspect of the transition (Fig. 3).

The secondary structure of fibrin, as evidenced by the X-ray studies of several workers (see review by Low[9]) must undergo drastic changes during the transition. This lends support to the model used here in the sense that we may be confident that we are indeed observing changes in the secondary structure of the molecule, and not merely observing disorientation of the molecular units which have aggregated to form the elastomer and then have been forced into an oriented configuration by physical treatment.

Acknowledgment.—We are indebted to Drs. P. J. Flory, L. Mandelkern, A. Nakajima, and J. F. M. Oth for helpful discussions of this problem, and to Mrs. Miriam Taylor for aid in the experimental work.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF MINNESOTA, MINNEAPOLIS, MINNESOTA]

Studies of Rack Mechanisms in Heme-proteins. I. The Magnetic Susceptibility of Cytochrome c in Relation to Hydration

BY RUFUS LUMRY, AAGE SOLBAKKEN, JOHN SULLIVAN AND LLOYD H. REYERSON

RECEIVED JUNE 26, 1961

The magnetic susceptibilities of trypsinogen, oxidized cytochrome *c* (OCy) and reduced cytochrome *c* (RCy) have been determined as a function of the amount of water bound starting with the lyophilized material. The susceptibility change of trypsinogen is entirely due to bound water which maintains the susceptibility value of liquid water. The susceptibility of the protein does not change with the addition of bound water. Dry OCy and RCy have higher susceptibilities than hydrated or dissolved forms. This change is due to unpairing of spins in RCy and the same explanation is probably correct for OCy. The changes in electronic state of the iron ions is interpreted as being due to shifts from 1 to 3 unpaired electrons in OCy and from 0 to 2 unpaired electrons in RCy and attributed to changes in the fields due to imidazole ligands as their positions are altered by rearrangements in the protein conformation. This situation is interpreted as a rack mechanism in which the protein conformation controls in part the electronic state of the heme group and several possible consequences of this mechanism are presented. Cytochromes dried by lyophilization have different susceptibilities from those dried by water removal at 27° and the difference may result from the displacement of one or more imidazolium groups by water in the iron coördination shell.

The magnetic susceptibility of cytochrome *c* was first investigated in 1940 by Theorell[1] who used impure preparations of horse-heart and cow-heart cytochrome *c*. In studies at 22° he found a wide range of paramagnetic susceptibility values for ferricytochrome *c*, OCy, on varying the hydrogen ion concentration. In the neutral range of *p*H this compound yielded values from 2580 to 3340 $\times 10^{-6}$ c.g.s. units for χ. Van Vleck[2] and, more recently, Griffith[3] have pointed out the importance

(1) H. Theorell, *J. Am. Chem. Soc.*, **63**, 1820 (1941).

(2) J. H. Van Vleck, "The Theory of Electric and Magnetic Susceptibilities," Oxford, 1932.

(3) J. S. Griffith, *Biochem. et Biophys. Acta*, **28**, 439 (1958).