than half that found by the first method and a ridiculous a^0. Also, it is not possible to find a single J that will make the theoretical and experimental curves coincide over any range of concentration. Permutations and combinations of the above do not give anything much better in the way of a fit. A viscosity correction does not give enough aid and the simple association correction would push the Λ' curve up instead of down. At the present time, we can only hope that further work on this type salt and particularly mixed solvent work will help our interpretive problem.

To illustrate the difficulties involved in adding these "corrective" terms to the original limiting law, Table IV gives the relative size of the terms in the CuBDS and LaNTS extended equations at a few concentrations. To say that we are straining the theory in these cases is certainly an understatement. Approximations of no import for a 1–1 may become very important for a 3–3.

We hope to shed more light on these unsettled questions by the extensive experimental program we have in progress. At present we are exploring both the low-field and high-field conductance of the $BDS^=$ salts of the other stable divalent ions as well as other lanthanide $NTS^=$ salts. We also have prepared salts of benzenetrisulfonic acid and 4,4′-diphenyldisulfonic acid and are doing preliminary work with them. In addition, we are collaborating with workers at other universities in the exploration of the activity coefficients and transference numbers of these salts. It is hoped that this paper will stimulate more interest in the properties of this intriguing new class of strong electrolytes. Finally, one must say that the success of the extended Fuoss–Onsager theory in treating these high charge type salts is a striking demonstration of the essential correctness of the theory.

TABLE IV

Salt	$C \times 10^4$	Λ^0	$SC^{1/2}$	$EC \log C$	JC	Λ
CuBDS	1	114.54	4.52	− 1.29	0.84	109.57
	10	114.54	14.25	− 9.70	8.35	98.94
	50	114.54	31.95	− 37.20	41.75	87.14
LaNTS	1	140.5	14.1	− 23.5	15.8	118.7
	10	140.5	44.5	−176.3	158.0	77.7
	50	140.5	99.6	−676.6	790.0	154.3

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

Thermodynamic Study of Shrinkage and of Phase Equilibrium under Stress in Films Made from Ribonuclease[1,2]

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Ribonuclease films were used for studies of shrinkage and phase equilibrium under stress. The films were prepared by coagulating ribonuclease with formaldehyde, compacting the clot to a thin film, partially cross-linking with *p*-benzoquinone, stretching in hot urea solution and further cross-linking to obtain a partially oriented, partially crystalline material. The film is considered to consist of two regions, *i.e.*, amorphous zones which crystallize on stretching and separate zones which contain crystallites in equilibrium with the surrounding amorphous zone. With these films, investigations were carried out on the dependence of the helix-random coil transition temperature on the *p*H of the medium, using measurements of retractive force. The change in force with temperature was reversible in the presence of 2 *M* KCl at low *p*H where no side-chain hydrogen bonding is present. The *p*H-dependence of the transition temperature was adequately accounted for by means of a reasonable model and a recent theory of reversible protein denaturation, in which side-chain hydrogen bonds play a dominant role. The heat and entropy change per peptide residue, accompanying the helix-random coil transition, were obtained from similar experiments on the dependence of the transition temperature on the concentration of urea. The crystalline unit in the assumed model consisted of two helical portions of 12 amino acid residues each, connected at both ends by cross-links. Force-temperature curves as a function of relative length, as well as data on swelling and its dependence on ionic strength, also were obtained, the data being subjected to a thermodynamic analysis from the point of view of phase equilibrium under stress. It was thus possible to obtain estimates of the contributions of the heat of solution, heat of rupture of a backbone hydrogen bond and the heat change accompanying the change of composition of the fiber induced by a change in its length. The model and the results are compatible with known structural features of ribonuclease and the elastic behavior of other fibrous proteins.

Introduction

The effect of side-chain hydrogen bonds on the elastic properties of protein fibers and on the configurations of proteins in solution recently has been treated theoretically[4] on the basis of earlier work on elastic mechanisms in proteins[5] and on the stability of side-chain hydrogen bonds.[6] The purpose of this paper is to provide experimental evidence for the earlier theoretical considerations[4] and to obtain further information about the role of side-chain hydrogen bonds in the mechanism of shrinkage. Ribonuclease was chosen for this study since the amino acid sequence[7] and disulfide bridge positions[8] are known, and some information is

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(2) Presented before the Division of Biological Chemistry at the 138th Meeting of the American Chemical Society, New York, N. Y., September, 1960.

(3) Visiting Research Fellow (1958–1960) from the Department of Textile Chemistry, Kyoto University, Kyoto, Japan.

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available about its secondary and tertiary structure.[9] Films were prepared from ribonuclease and cross-linked with *p*-benzoquinone. The chain configuration in such films need not be the same as in native ribonuclease.

The point of view adopted in this work is that of Flory,[5] *viz.*, that the shrinkage of fibrous proteins involves a crystalline-to-amorphous phase transition, the soundness of this approach having been amply demonstrated.[10-19] It is further assumed here that the crystalline form is an α-helix and the amorphous form is a random coil so that the process may be regarded as a helix-to-random coil transition. Several workers have considered this transition (denaturation), and the effect of temperature, *p*H, and concentration of chemical denaturants thereon, from both a thermodynamical and statistical mechanical point of view.[20-26] It should be noted that the theory is essentially the same, independent of whether the transitions occur in singly dispersed molecules in dilute solution or in the (swollen) solid phase of a fiber.

Experimental

Preparation of Films.—Armour bovine ribonuclease (Lot 647-213) was used for preparing films. Stock solutions of ribonuclease were made by dissolving *ca.* 100 mg. of the protein in 1.5 ml. of buffer which was 0.1 *M* in phosphate and 0.43 *M* in NaCl (*p*H 9.5).[27] The formaldehyde clotting reagent was prepared by diluting a 40% solution with *p*H 9.5, 0.1 *M* phosphate buffer to a concentration of 2.5 g./100 ml.

To clot the ribonuclease, 1.5 ml. of the dilute formaldehyde solution was added to an equal volume of the protein solution with rapid stirring. Just before gelation began, the solution was poured onto a glass plate in an area surrounded by a glass cylinder of 3.5 cm. diameter. Clotting was allowed to proceed for 20 min. on the glass plate, after which the clot was transferred to a piece of muslin on a glass plate, covered with another piece of muslin and a glass plate and compressed with a weight of 700 g. for 1 hr. The resulting film then was washed with water.

In order to partially cross-link the film, it was immersed in a 1% solution of *p*-benzoquinone (buffered with *p*H 9.5 phosphate) at room temperature for 1 hr. and then washed with water. The cross-linked film was cut into strips of 1 mm. width and stored in water at 4°. Films prepared in this manner probably have their chains preferentially oriented in the plane of the film.

In order to obtain an oriented film, the strips were stretched 30% of their length in a 4 *M* urea solution[28] at 65° on a metal stretching frame and then re-immersed in the buffered *p*-benzoquinone solution at room temperature for 1 hr. under stress to fix the oriented configuration with more cross-links. After cross-linking, the stress relaxed in the stretched state, indicating that the oriented configuration was maintained in the absence of external stress. The oriented films were washed with water and stored in distilled water at 4°. Such films were stable for at least a month, using their unchanged force-temperature characteristics as a criterion of stability.

The cross-linking with formaldehyde and *p*-benzoquinone probably involves the basic amino acid residues, primarily lysine ϵ-amino groups,[30-32] of which there are 10 in ribonuclease.[7] The cross-linking reactions between amino groups and formaldehyde and *p*-benzoquinone, respectively, have been discussed by Mihalyi and Lorand.[32] Under the conditions used here, the degree of cross-linking with formaldehyde is very low.[32] Furthermore, when such cross-links are introduced into collagen, they tend to disappear upon storage in water.[18] Therefore, after the initial preparation of the film, *p*-benzoquinone was used to obtain more permanent cross-links. Since *p*-benzoquinone darkens on standing at *p*H 9.5 due to polymerization, fresh solutions of this reagent always were used.

Force Measurement.—The dynamometer for the measurement of force was essentially the same as that of Oth and Flory[13]; the sample length was 1 to 2 cm., and the sample was immersed in a buffer whose *p*H and temperature could be controlled during the measurement of the force. The strain gauge was a model G7A-1-2500 (Statham Instrument Co., Puerto Rico) with a capacity of 1 ounce (28 g.) and maximum excitation voltage of 36 volts. The input voltage was obtained from 3 Mallory mercury batteries of 9 volts each, arranged in series with a variable resistor to control the input voltage. The sensitivity of the gauge was 3354 microvolts per input volt per ounce. The output voltage was fed into a General Electric model 8CE5CP19A recording potentiometer having a sensitivity adjustable from 0.2 to 500 millivolts full-scale in 11 steps and two chart speeds of 6 in./hr. and 6 in./min., respectively. The system was calibrated by hanging known weights on the strain gauge and noting the deflection of the recorder pen. In experiments of long duration, the calibration was carried out before and after the run.

The lengths of the samples were measured simultaneously with the force by means of a cathetometer with a precision of ± 0.001 cm.

Results and Discussion

State of Film.—If a film is heated to induce a helix-random coil transition, the length will decrease at constant force, or the force exerted by the film will increase at constant length. If the transition is a reversible one, then a thermodynamic analysis can be carried out. In such an analysis it is helpful if one can demonstrate experimentally that the initial and final states are indeed helical and randomly coiled, respectively. For such purpose, X-ray diffraction photographs were taken of ribonuclease films at 50% extension *in the presence of buffer*. However, the photographs were not of

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(27) The *p*H was chosen as 9.5 to obtain a suitable reaction rate in the subsequent clotting with formaldehyde. At higher *p*H (*e.g.* *p*H 11) the clotting reaction was too fast, being almost instantaneous; at lower *p*H (*e.g.* *p*H 5.8) the reaction was too slow (about 24 hr.); at *p*H 9.5 gelation occurred in about 2 min. These observations are in accord with the notion that formaldehyde reacts with uncharged amino groups. The NaCl was added to reduce electrostatic repulsions between protein molecules during gelation.
(28) Urea at 65° was used to unfold the molecule since the denaturation of ribonuclease with urea is reversible.[29]
(29) W. F. Harrington and J. A. Schellman, *Compt. rend. trav. lab. Carlsberg, Ser. Chim.*, **30**, 21 (1956).
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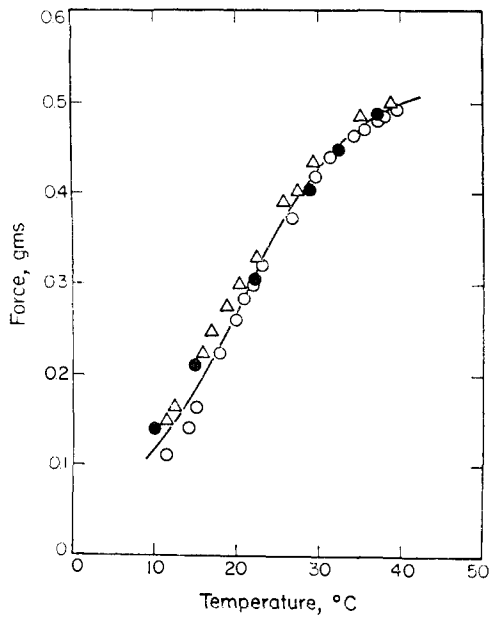

Fig. 1.—Change in contractile force with temperature in a buffer solution at *p*H 2.35 containing 2 *M* KCl: O, ascending; ●, descending temperature in the first run; △, ascending temperature in the second run; T_{tr} = 23.5°.

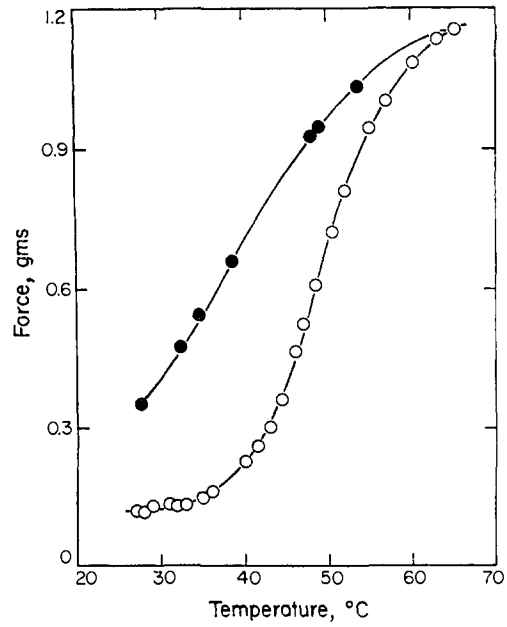

Fig. 2.—Change in contractile force with temperature in a buffer solution at *p*H 6.53 containing 2 *M* KCl: O, ascending; ●, descending temperature; T_{tr} = 49°.

sufficient clarity, because of the low degree of extension and high degree of swelling, to enable us to decide whether oriented crystallites were present in the film at low temperature. Higher extensions could not be induced because the films would then rupture. We shall, therefore, have to *assume* that the sample in its initial state contains (partially oriented) crystalline regions distributed among amorphous regions.[33] This assumption is compatible with the results of studies of dilute solutions of ribonuclease in water,[29,36,37] from which the presence of α-helices is inferred.[9,29,36,37] Similarly, it will be assumed that the chains are in the randomly coiled configuration above the transition temperature.

Force-Temperature Behavior.—Since our primary concern here is to assess the influence of side-chain hydrogen bonding on the helix-random coil transition[4] (and, therefore, on the elastic properties), 2 *M* KCl was used to minimize electrostatic contributions to the free energy of the transformation. As will be shown below, the degree of swelling is essentially independent of *p*H in 2 *M* KCl.

The first set of experiments was designed to examine the reversibility of the heat-induced transition. The sample was mounted between two clamps in a buffer at a constant temperature until equilibrium was attained and the length adjusted to take up the slack at zero force at that temperature. The temperature then was raised at a rate of about 1°C./10 min. After denaturation the sample was cooled slowly to the starting temperature and then reheated at the original rate. Fig. 1 shows the relation between the contractile force, in a buffer solution at *p*H 2.35 containing 2 *M* KCl, and temperature at constant length. The behavior in a buffer solution at *p*H 6.53 containing 2 *M* KCl is shown in Fig. 2. The transformation is reversible at *p*H 2.35 but hysteresis is observed at *p*H 6.53. As will be discussed below, there is a maximum degree of side-chain hydrogen bonding at *p*H 6.53 but essentially none at *p*H 2.35. It seems that the helix can re-form readily at *p*H 2.35. At *p*H 6.53, where the recrystallization requires the formation of the side-chain hydrogen bonds as well as the backbone ones, the process is slower, giving rise to the observed hysteresis. In dilute solution, the heat-induced denaturation is reversible[29,37] in the *p*H range of 1 to 7. Presumably, the constraints in the crystalline cross-linked fiber are somewhat different from those in the native molecule in dilute solution.

The transition temperature, T_{tr}, is taken as the inflection point obtained in a run of *increasing* temperature starting from essentially zero force.

Model for Elastic Behavior.—According to a recently proposed model for native ribonuclease in water,[9] about 60% of the molecule is helical and divided into several short helical portions. Since our films were obtained by cross-linking with formaldehyde and *p*-benzoquinone, it is likely that the introduction of cross-links reduces the helical content. We shall therefore assume that the crystalline portions are representable by units of the type shown in Fig. 3. The assumption of a higher degree of crystallinity of the same type would not alter the

(33) We have been more successful in demonstrating crystallinity by X-ray diffraction with other fibers (fibrin[34] and insulin[35]) in buffer with or without stretching. If a fibrin fiber, made in an analogous way as the ribonuclease film, is extended about 300%, an α-keratin X-ray pattern is obtained, even in buffer; with the insulin fiber we observed a transformation from a non-oriented crystalline pattern to an amorphous one as the temperature was increased in the presence of buffer. However, the degree of swelling of fibrin and insulin fibers was less than that of the ribonuclease film. If the degree of swelling is large, it is very difficult to detect crystallinity by X-ray diffraction.

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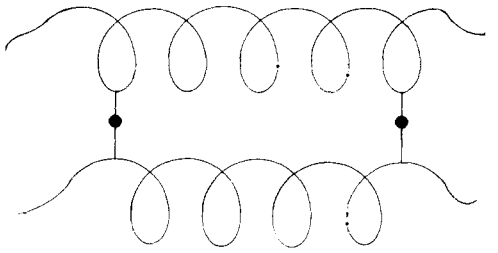

Fig. 3.—A model for the assumed helical portion of ribonuclease in the cross-linked film.

thermodynamic argument. This crystalline unit is composed of 2 α-helices, each containing 12 residues and cross-linked at both ends as shown. The remainder is considered to be in the amorphous region.

The free energy change, ΔF^0_{obsd}, for converting the helical portions of the unit to random coils may be computed from the equation of Schellman,[20] applied to the backbone helix, and the equation of Flory[5] for the effect of the cross-links. For the present, we are considering the behavior at very low pH where no side-chain hydrogen bonds are formed.

$$\Delta F^0_{obsd} = 2\{(n-4)\Delta H^0_{res} - (n-1)T\Delta S^0_{res}\} - T\Delta S^0_x \quad (1)$$

where n is the number of amino acid residues in the chain, and ΔH^0_{res} and ΔS^0_{res} are the enthalpy and entropy changes, respectively, per residue, for the unfolding of an infinitely long helix to an unstressed, *i.e.*, isotropic, random coil. As in a previous theory,[6] the free energy of mixing of amorphous polymer with diluent has been omitted. This omission could reflect itself in somewhat different values for ΔH^0_{res} and ΔS^0_{res} in eq. 1. Since our measurements were made at *very low* force, we can assume the random coil to be isotropic. The quantity ΔS^0_x is the molar entropy decrease of the random coil due to the introduction of cross-links in the *crystalline* form and is given by the expression[5]

$$\Delta S^0_x = -(3R\nu/4)[\ln n' + 3] \quad (2)$$

where ν is the number of cross-linked helices and n' is the number of statistical elements (assumed here equal to n) between cross-links. In our use of eq. 2 it is assumed that it is valid for short chains. With $n = 12$ and $\nu = 2$, eq. 1 becomes

$$\Delta F^0_{obsd.} = 16\Delta H^0_{res} - 22T\Delta S^0_{res} + 16.5T \quad (3)$$

Before ΔF^0_{obsd} can be evaluated it is necessary to know the magnitude of ΔH^0_{res} and ΔS^0_{res}. These values can be obtained from measurements of the transition temperature in the presence of urea.[20] Such experiments were carried out at pH 2.63 in the presence of 2 M KCl to eliminate side-chain hydrogen bonding and charge effects, respectively. According to Schellman,[20] the free energy of combination, ΔF^0_{comb}, of the extended chain with urea is given by eq. 4 (valid for $p > 20$).

$$\Delta F^0_{comb} = -2pRT\ln(1+Kc) \quad (4)$$

where p is the number of amino acid residues which may react with urea when the helix is unfolded and is equal to 24 in our model. The quantity K is the equilibrium constant for the binding of a urea molecule to a C=O or N–H group of a peptide unit, and

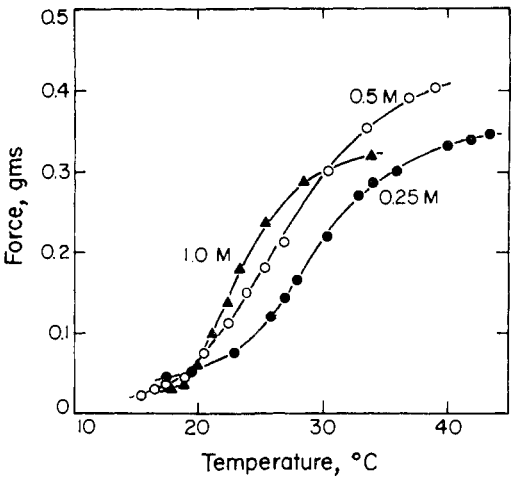

Fig. 4.—Typical force-temperature curves in urea solution (pH 2.63, 2 M KCl); the urea concentration is indicated on the curves.

c is the concentration of urea in moles/liter. The value of K for the association of urea monomers to dimers was found[38] to be 0.041 at 25°. However, this value cannot be used in eq. 4 since the value 0.041 applies to urea dimers which are mixtures of two forms containing one and two hydrogen bonds, respectively. However, from Schellman's treatment of the heat of association of urea,[38] it can be estimated that 60% of the dimers have only one hydrogen bond. We shall, therefore, take K as 60% of 0.041 or 0.025. Since c is in the range of 0 to 1, in the experiments to be described, $Kc < 1$ and eqn. 4 may be approximated by

$$\Delta F^0_{comb} = -48RTKc \quad (5)$$

The temperature dependence of K may be obtained from

$$K/K_{25} = \exp\left[-\frac{\Delta H_u}{R}(1/T - 1/T_{25})\right] \quad (6)$$

where ΔH_u is the heat of formation of the complex between urea and the peptide CO or NH group.

If the value of ΔF^0_{comb} of eq. 5 is added to the right hand side of eq. 3 and ΔF^0_{obsd} set equal to zero at the transition temperature, we obtain

$$\frac{1}{T_{tr}} = \frac{11\Delta S^0_{res} - 8.25}{8\Delta H^0_{res}} + \frac{3RcK_{25}}{\Delta H^0_{res}} e^{+\Delta H_u/RT_{25}} e^{-\Delta H_u/RT_{tr}} \quad (7)$$

Thus, if K is known as a function of temperature, a plot of $1/T_{tr}$ against $ce^{-\Delta H_u/RT_{tr}}$ should give a straight line, the slope and intercept of which will give ΔH^0_{res} and ΔS^0_{res}. Figure 4 shows some typical measurements of the force-temperature behavior in urea. The transition temperatures are plotted, according to eqn. 7, for $\Delta H_u = -3$, -6 and -9 kcal./mole, respectively, in Fig. 5. From the initial slopes of these curves we obtain $\Delta H^0_{res} =$ 1800, 1750, and 1700 cal./mole for $\Delta H_u = -3$, -6 and -9 kcal./mole, respectively. These values are in good agreement with estimates of Schellman.[20]

(38) J. A. Schellman, *Compt. rend. trav. lab. Carlsberg, Ser. Chim.*, **29**, 223 (1955).

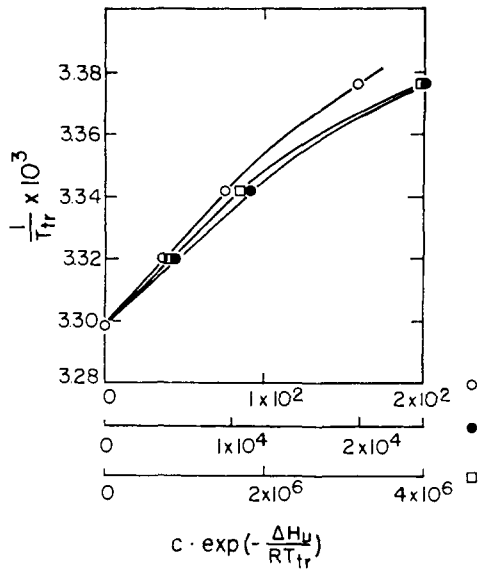

Fig. 5.—Relation between $1/T_{tr}$ and urea concentration, in a buffer solution of pH 2.63 containing 2 M KCl; the symbols O, ●, and □ correspond to $\Delta H_u = -3, -6, -9$ kcal./mole, respectively.

In a previous paper[39] it has been shown that

$$|\Delta H^0_{ii}| < |\Delta H_u| \quad (8)$$

where ΔH^0_{ij} is the heat of formation of a side-chain hydrogen bond between an i^{th} donor and j^{th} acceptor. Since we are taking[6] $\Delta H^0_{ij} = -6$ kcal./mole for the side-chain hydrogen bond, we shall take $\Delta H^0_{res} = 1700$ cal./mole (corresponding to $\Delta H_u = -9$ kcal./mole). Since the value of ΔH^0_{res} is not very sensitive to the choice of ΔH_u, we are not committing any significant error in our essentially arbitrary choice of ΔH_u. Therefore, no significance should be attached to the value $\Delta H_u = -9$ kcal./mole selected here. By the above criterion, it could have been zero without affecting our calculated value of ΔH^0_{res} very much. With the value of $\Delta H^0_{res} = 1700$ cal./mole, we obtain $\Delta S^0_{res} = 4.83$ e.u. from the intercept of the appropriate curve in Fig. 5. These values of ΔH^0_{res} and ΔS^0_{res} may be substituted in eq. 3. However, before doing so, we shall first consider the effect of side-chain hydrogen bonding in the next section.

pH Dependence of T_{tr}.—Equation 3 does not yet contain the pH-dependent contribution to ΔF^0_{obsd}. However, if the pH is varied, then ΔF^0_{obsd} (and consequently T_{tr}) depends on pH.[4] This pH dependence has been treated[4] by introducing an additive, pH-dependent term, ΔF^0_H, to the right hand side of eq. 3; the term ΔF^0_H arises from side-chain hydrogen bonds between ionizable groups.[6] For heterologous, single hydrogen bonds

$$\Delta F^0_H = -RT\Sigma \ln (1 - x_{ij}) \quad (9)$$

$$x_{ij} = \frac{K_{ij}}{1 + K_{ij} + K_1/[H^+] + [H^+]/K_2} \quad (10)$$

where K_{ij} is the equilibrium constant[6] for the formation of a side-chain hydrogen bond between an i^{th} donor and j^{th} acceptor, x_{ij} is the fraction of the molecules having this hydrogen bond, K_1 and

(39) M. Laskowski, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.*, **83**, 266 (1961).

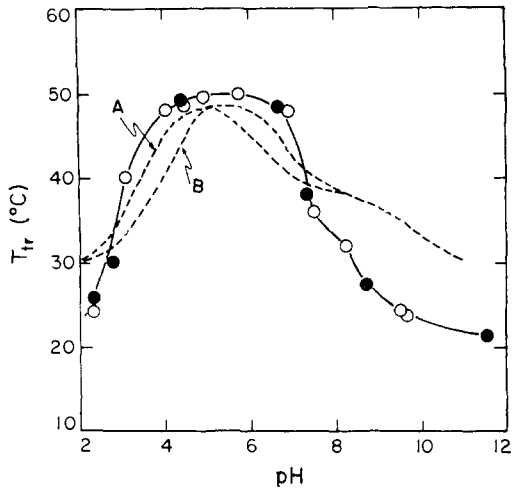

Fig. 6.—Experimental (solid line) and calculated (broken lines) results for pH dependence of transition temperature, T_{tr}. Experimental values in 0.07 M buffer solutions, O; in the same buffer with added 2 M KCl, ●. Calculated curves were obtained with the numerical values listed in Table I, assuming 1 tyrosyl-carboxylate and 1 histidyl-carboxylate hydrogen bond.

K_2 are the ionization constants of the donor and acceptor groups, respectively, in the *absence* of the hydrogen bond, and $[H^+]$ is the hydrogen ion activity. In using these equations the temperature dependence of K_1, K_2 and K_{ij} must be taken into account. With the aid of eq. 9, eq. 3 becomes

$$\Delta F^0_{obsd} = 16\Delta H^0_{res} - 22T\Delta S^0_{res} + 16.5T - RT\Sigma \ln (1 - x_{ij}) \quad (11)$$

where the sum is taken over all the side-chain hydrogen bonds between the helices illustrated in Fig. 3. The transition temperature is obtained as a function of pH from eqn. 11 by setting $\Delta F^0_{obsd.}$ equal to zero. Details of such calculations have been described previously.[4] As in the case of eq. 1, the omission of the mixing term should be noted. However, it will be shown below that the degree of swelling of ribonuclease films in 2M KCl is independent of pH. Therefore, this mixing term will not contribute to the pH-dependence of $\Delta F^0_{obsd.}$

Experimental data for the pH-dependence of T_{tr} are shown in Fig. 6. These were obtained from force-temperature measurements at various pH values. It should be noted that the same experimental curve (solid line in Fig. 6) is obtained both in the presence and absence of 2 M KCl. The two dashed curves in Fig. 6 are theoretical ones, calculated from eq. 11, using the values of ΔH^0_{res} and ΔS^0_{res} obtained in the previous section from experiments in urea solutions and assuming two side-chain hydrogen bonds per helical unit given in Fig. 3 to be contributing to the sum in eqn. 11. The two side-chain bonds are assumed to be 1 tyrosyl-carboxylate ion and 1 hystidyl-carboxylate ion hydrogen bond with parameters given in Table I. These parameters were chosen on the basis of previously published data.[6,40] The slightly higher value of $K_{ij} = 10$ was used to take into account an increased stability due to electrostatic interactions.

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

Lysyl groups were not considered as donors since they are used up in the cross-linking reaction. This is demonstrated by the fact that the *p*H of the water around a film (isoionic) is 6.5, as compared with that of native ribonuclease in dilute solution (*p*H 9.6). The theoretical curves fit the experimental data fairly well, especially in the region of maximum stability and at low *p*H. The discrepancy at high *p*H may be due to electrostatic interactions which were neglected here or, of course, to inadequacies of the model.

Effect of Swelling.—Since measurements of shrinkage and elasticity are usually carried out in the presence of solvent, it is necessary to consider the effect of *p*H, temperature and force on the degree of swelling. Since our experiments on the *p*H-dependence of T_{tr} were carried out at a force as close to zero as possible, we shall examine here the effects of swelling at zero force.

TABLE I

ASSUMED VALUES AT 25° FOR MODEL CALCULATION[a]

	Tyrosyl-carboxylate Curve A	Tyrosyl-carboxylate Curve B	Histidyl-carboxylate Curve A	Histidyl-carboxylate Curve B
K_1 (donor)	$10^{-9.5}$	$10^{-9.5}$	$10^{-6.5}$	$10^{-6.0}$
K_2 (acceptor)	$10^{-4.0}$	$10^{-4.5}$	$10^{-4.0}$	$10^{-4.5}$
ΔH_1^0, kcal./mole	6		7	
ΔH_2^0, kcal./mole	0		0	
K_{ij}	4		10	
ΔH^0_{ij}, kcal./mole	−6		−6	

[a] ΔH^0_{res} = 1700 cal./mole. ΔS^0_{res} = 4.83 e.u.

The degree of swelling, Q, was computed as the ratio of the weight of the wet film, W, to that of the dry film, W_0. The films were allowed to come to equilibrium with solvent for 3 days at constant temperature. The solvent on the surface of the film was removed with a piece of filter paper. The swollen film was weighed and then washed with water, dried *in vacuo* at 100° and then re-weighed. Table II shows the data obtained. The weight ratio, Q, is related to the volume ratio, q, by

$$q = \frac{\bar{v}\rho_1 + (Q - 1)}{\bar{v}\rho_1} \qquad (12)$$

where $\bar{v}$ is the partial specific volume of the protein and ρ_1 is the density of the solvent.

TABLE II

THE CHANGES OF THE DEGREE OF SWELLING WITH *p*H AND TEMPERATURE AT DIFFERENT IONIC STRENGTH[a]

(A) In 0.07 *M* buffer solution

$Q = W/W_0$

*p*H	2.35	3.09	4.93	5.87	6.91	7.48	8.35	9.53
30°	10.77	5.03	4.43	4.65	6.39	7.21	7.79	8.34
45°	9.96	4.80	4.23	4.36	6.21	6.90	8.00	8.59
60°	9.73	4.34	3.92	4.13	6.00	7.17	8.22	8.84

(B) In 0.07 *M* buffer solution + 2 *M* KCl

$Q = W/W_0$

*p*H	2.35	2.84	4.44	6.53	7.36	8.73
30°	4.44	4.35	4.68	4.56	4.71	4.98
45°	4.15	4.10	4.25	4.34	4.28	4.80
60°	3.71	3.85	3.64	4.10	3.80	4.57

[a] Some of these data apply to mixed crystalline and amorphous phases, some to completely amorphous material, depending on the transition temperature at the given *p*H and ionic strength.

Above the transition temperature, the swollen phase can be treated as an amorphous network. At swelling equilibrium equation (13) holds for the amorphous, isotropic state, neglecting any non-random cross-linking which may occur in *that part* of the amorphous network which arose from melted crystallites.[41]

$$-[\ln(1 - v_2) + v_2 + \chi_1 v_2^2] = \left(\frac{V_1}{\bar{v}M_c}\right)\left(v_2^{1/3} - \frac{v_2}{2}\right) \qquad (13)$$

where the volume fraction of protein in the swollen phase, v_2, is related to q (or Q) by the equation

$$v_2 = \frac{1}{q} \qquad (14)$$

V_1 is the molar volume of the solvent, χ_1 is a free energy parameter for the polymer-solvent interaction and M_c is the molecular weight per cross-linked unit.

The differential heat of dilution, $\Delta\bar{H}_1$, is given by the expression[42]

$$\Delta\bar{H}_1 = RT\kappa_1 v_2^2 \qquad (15)$$

where κ_1 is the heat parameter for the polymer-solvent pair and is related to χ_1 by the equation[18,43]

$$\kappa_1 = -T\frac{\partial\chi_1}{\partial T} \qquad (16)$$

Therefore, the integral heat of solution per mole of peptide units, ΔH_{sol}, is obtained by integrating eq. 15 from $n_1 = 0$ to n_1 at constant polymer volume, $\bar{v}$ and M_{unit}; this leads to eq. 17.

$$\Delta H_{sol} = \frac{\bar{v}}{V_1} M_{unit} RT\kappa_1(1 - v_2) \qquad (17)$$

where M_{unit} is the molecular weight per peptide unit (*i.e.* ~ 100).

We are justified in applying the above theory to our swelling data at *p*H 2.5 since T_{tr} is lower than 30° at this *p*H, *i.e.*, the protein is in the randomly coiled condition. Since v_2 is known from q (eq. 14) (q being obtainable from the Q-data of Table II using eq. 12) and V_1 is known, it is possible to compute χ_1 from eq. 13 if M_c is known. Assuming that the randomly coiled units have 20–30 residues between cross-links, the value of M_c would be 2000–3000. The χ_1-values, calculated from the *p*H 2.84 data of Table IIB are plotted against temperature in Fig. 7 (see also Table III). The resulting values of 0.4–0.5 seem reasonable.[44] The values of κ_1, computed from the slopes in Fig. 7, and ΔH_{sol} from eq. 17, are shown in Table III. The values of ΔH_{sol} will be used in the discussion in the next section.

The *p*H-dependence of the degree of swelling, Q, at two different ionic strengths and temperatures is plotted in Fig. 8. The degree of swelling is independent of *p*H in the presence of 2 *M* KCl but markedly dependent on *p*H at low and high *p*H in the absence of the KCl. Even in the absence of KCl, the degree of swelling is independent of *p*H in the intermediate *p*H range where the crystalline form is most stable (see Fig. 6). The presence of 2

(41) P. J. Flory, "Principles of Polymer Chemistry," Cornell University Press, Ithaca, N. Y., 1953, p. 579.

(42) Ref. 41, p. 523.

(43) Ref. 41, p. 510.

(44) Spurr[18] obtained 0.59 for collagen in 3 *M* KCNS solution at 60°.

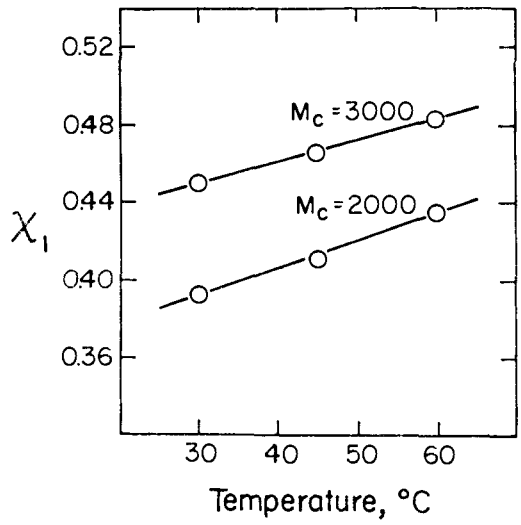

Fig. 7.—χ_1 calculated from swelling data, assuming M_c equal to 2,000 and 3,000, respectively, plotted against temperature; *p*H 2.84, 0.07 *M* buffer solution containing 2 *M* KCl.

M KCl seems to suppress electrostatic effects and render Q independent of *p*H. Therefore, we may conclude that our discussion of the *p*H-dependence of T_{tr} is not influenced by *p*H-dependent swelling. This adds validity to our assumption that side-chain hydrogen bonding plays an important role in the stabilization of the crystalline form.

TABLE III

κ_1 AND ΔH_{sol} OBTAINED FROM SWELLING DATA, AT *p*H 2.84 IN 0.07 *M* BUFFER CONTAINING 2 *M* KCl

		30°	45°	60°
$\bar{v}$ (assumed)		0.7	0.7	0.7
ρ_1 (measured)		1.08619	1.07929	1.07237
V_1		18.0787	18.1774	18.3068
χ_1	M_c = 2000	0.392	0.411	0.435
	M_c = 3000	.450	.466	.484
κ_1	M_c = 2000	− .42	− .44	− .47
	M_c = 3000	− .36	− .37	− .40
ΔH_{sol} (cal.)	M_c = 2000	−843	−910	−995
	M_c = 3000	−723	−765	−847

Thermodynamics of Phase Equilibrium under Stress.—When a protein film, composed of amorphous and crystalline phases, is immersed in a diluent, phase equilibrium can be established.[5] We shall assume that solvent can mix with the amorphous regions; we shall further assume that the constraints of the system prevent the crystalline phase from swelling but that the solvent can make contact with the side-chains so that the latter can equilibrate with hydrogen ions of the solvent, *i.e.*, the pure crystalline phase is defined by the *p*H and melts to an amorphous phase which is in equilibrium with diluent. We shall thus describe the phase equilibria as

$$\text{crystalline phase} \rightleftarrows \text{amorphous phase} \rightleftarrows \text{diluent phase} \quad (18)$$

When a uniform force, f, is applied along the axis of the film strip, these equilibria will be affected.[5] As pointed out by Flory,[5] Katchalsky,[45] Prins and Hermans[46] and Kuhn,[47] the film should be considered as an open system since the number of moles of diluent in the amorphous protein phase may change with a change in length due to the presence of the surrounding diluent phase.[48] According to Katchalsky,[45] equation (19) applies to such an open system, where the diluent contains only one component

$$f - T\left(\frac{\partial f}{\partial T}\right)_{P,L} = \left(\frac{\partial H}{\partial L}\right)_{P,T,n_1,n_2} + \left(\frac{\partial n_1}{\partial L}\right)_{P,T,n_2}\Delta\bar{H}_1 \quad (19)$$

where T is the absolute temperature, P the pressure, L the length of the sample, H the enthalpy, $\Delta\bar{H}_1$ the partial molar heat of dilution and n_1 and n_2 are the number of moles of diluent and protein, respectively. Now, representing the changes in enthalpy and length in bringing the sample from the crystalline to the amorphous state in a closed system by ΔH and ΔI, respectively, we obtain the equation at the transition temperature, T_{tr}

$$f - T_{tr}\left(\frac{\partial f}{\partial T}\right)_{P,L} = \left(\frac{\Delta H}{\Delta L}\right)_{P,T_{tr},n_1,n_2} + \left(\frac{\partial n_1}{\partial L}\right)_{P,T_{tr},n_2}\Delta\bar{H}_1 \quad (20)$$

If we apply this relation at *p*H 2.73, where the contributions of the side-chain hydrogen bonds vanish, we may write ΔH of eq. 20 as

$$\frac{M_{unit}}{W_0}\Delta H = \Delta H_{sol} + \Delta H^0_{res} \quad (21)$$

where ΔH_{sol} is given by eq. 17, and the other terms have been defined. The second term on the right hand side of eq. 20 may be expressed in terms of the temperature dependence of q and L, since

$$n_1 = \frac{W_0\bar{v}}{V_1}(q - 1) \quad (22)$$

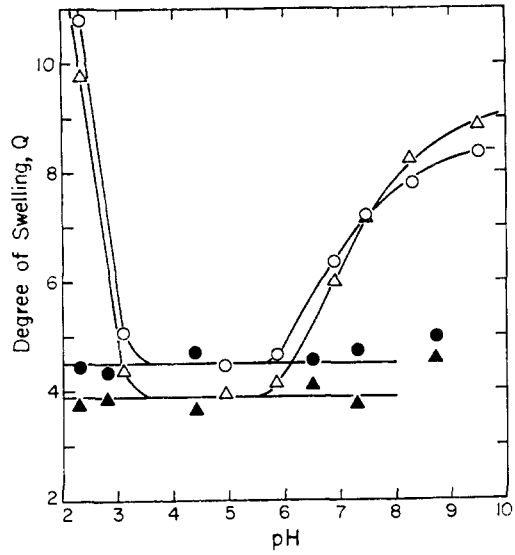

Fig. 8.—*p*H dependence of degree of swelling at two different ionic strengths: 0.07 *M* buffer solution, 30°, ○; 60°, △; 0.07 *M* buffer solution + 2 *M* KCl, 30°, ●; 60°, ▲.

(45) A. Katchalsky, Conference on Contractibility, Mellon Institute, Pittsburgh, Pa., Jan. 27–30, 1960, p. 22.

(46) W. Prins and J. J. Hermans, *ibid.*, p. 26.

(47) W. Kuhn, *ibid.*, p. 14.

(48) We are indebted to Drs. P. J. Flory, L. Mandelkern, C. A. Hoeve and A. Katchalsky for helpful discussions of this problem.

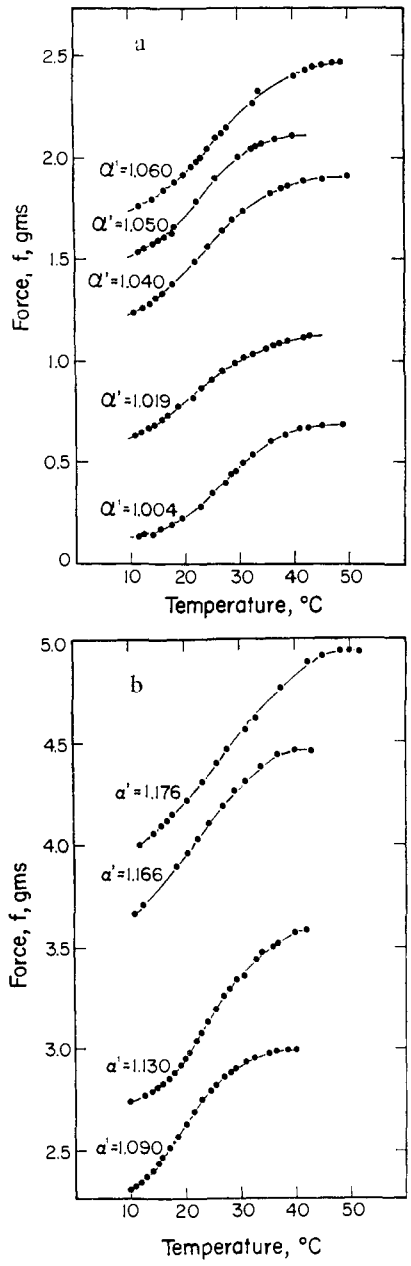

Fig. 9.—Temperature dependence of contractile force, f, at different relative lengths, α', in 0.07 M buffer solutions containing 2 M KCl; pH = 2.73; L_0 = 1.603 cm. at 11°.

and, at constant temperature

$$dn_1 = \frac{W_0\bar{v}}{V_1}\,dq$$

Using the values of $\bar{v}$ and V_1 at T_{tr}, we obtain eq. 23 from eq. 20, 21 and 22.

$$\frac{M_{unit}}{W_0}\left\{f - T_{tr}\left(\frac{\partial f}{\partial T}\right)_{P,L}\right\} = \left(\frac{\Delta H_{sol}}{\Delta L}\right)_{P,T_{tr},n_1,n_2} + \left(\frac{\Delta H^0_{res}}{\Delta L}\right)_{P,T_{tr},n_1,n_2} - \frac{M_{unit}\bar{v}}{V_1}\left(\frac{\partial q}{\partial T}\right)_{L,P,n_2}\left(\frac{\partial L}{\partial T}\right)^{-1}_{P,n_1,n_2}\Delta\bar{H}_1 \quad (23)$$

The left hand side of eq. 23 is obtainable from experiment and we can deduce therefrom information about the enthalpy terms on the right hand side of eq. 23. It should be noted that the correction term (last term on the right hand side of eq. 23) is only an approximation since our diluent contains more than one component, and we have introduced an inconsistency by keeping L and $\overline{r_0^2}$ independent of temperature in our treatment of swelling.

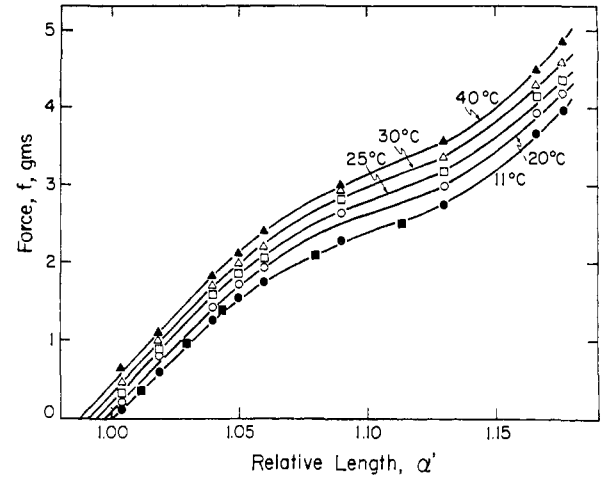

Fig. 10.—Relation between force, f, and relative length, α', in 0.07 M buffer solutions containing 2 M KCl. Results shown by (■) were obtained directly from force-relative length measurements at 11°. Other results were obtained from Fig. 9: pH = 2.73; L_0 = 1.603 cm. at 11°; cross sectional area of dried sample = 0.0023 cm.[2]

Fig. 9 shows some experimental results of force-temperature measurements in pH 2.73 buffer containing 2 M KCl at different relative length, α', where α' is the ratio of the length, L_f, at temperature T under the force f, to the length, L_0, at a given temperature (here 11°) under zero force, *i.e.*, $\alpha' = L_f/L_0$. These data were obtained directly by heating the sample at fixed length. The data of Fig. 9 are re-plotted in Fig. 10 as f *vs.* α' at several temperatures. Also shown in Fig. 10 (solid squares) are the results of a direct set of measurements of the stress-strain curve (*i.e.* f *vs.* α') at 11°. The direct measurements agree with those obtained from Fig. 9, demonstrating again the reversibility[49] of the equilibria involved.

Some data characterizing the curves of Fig. 9 are shown in Table IV. It can be seen that T_{tr} is independent of α' up to 15% extension; however, at higher extensions T_{tr} is inclined to increase, probably due to a change in the state of the amorphous material above the transition temperature (*i.e.*, the

(49) Fig. 11 is another demonstration of the reversibility at constant temperature at pH 2.79, 2 M KCl. The length of the sample at zero force at 11° was L_0 = 1.1246 cm. The sample was extended to L = 1.1377 cm. and brought to equilibrium at this temperature for about 3 hr. This was taken as "zero time" in Fig. 11. The relative length at this stage was 1.0116. The force remained constant at about 0.42 g. for 1.25 hr.; after obtaining point no. 1, the sample was extended 0.005 cm. and the force determined (points no. 2 and 3). The force decayed toward no. 3. After obtaining point no. 3 the length was reduced to its original value and the force determined (points no. 4, 5 and subsequent ones at 0.42 g.). After 2 hr. at equilibrium, point no. 6 was obtained and then the sample shortened 0.010 cm. It can be seen that the force then rises (points no. 7 and 8) to its equilibrium value. After obtaining point no. 8, the length was increased to its original value and points no. 9, 10, etc., were obtained. The decrease in force after points no. 2 and 9 in Fig. 11 is considered to be due to a tendency to crystallize and the increase in force after points no. 4 and 7 to a tendency to melt.

TABLE IV

TRANSITION TEMPERATURE AT DIFFERENT RELATIVE LENGTHS α', IN BUFFER SOLUTIONS OF pH 2.73 CONTAINING 2 M KCl

α'	1.004	1.019	1.040	1.060	1.130	1.176
T_{tr}, °C.	25	25	25	25	25	28
f, g., at T_{tr}	0.35	0.91	1.57	2.05	3.18	5.80
ΔL, cm.[a]	−0.023	−0.026	−0.031	−0.035	−0.043	−0.061
$(\partial L/\partial T)_f \times 10^3$ at T_{tr}[b]	−1.0	−1.3	−1.5	−1.7	−2.1	−3.2

[a] This is the total change in L in the transition at constant force (see Fig. 12). [b] From Fig. 12.

transition temperature for the crystalline-amorphous transformation increases as the tension on the amorphous form increases due to a decrease in the entropy of the amorphous form on stretching).

Fig. 10 shows the relation between f and α'. Within a relatively small range of low α' values, the retractive force f was proportional to α'. After the initial linear region the slopes of the curves decrease and then increase again at high α'. In an ideal homogeneous fibril,[10] melting should take place at a sharply defined temperature. Starting from a completely amorphous state at $f = 0$, the force f should first increase linearly with α' until the critical force for crystallization is reached; then it should be constant and independent of α', since there is coexistence of amorphous and crystalline regions, until it is totally crystalline; then f should increase sharply. However, if the sample contains partially crystalline regions in the initial stages at $f = 0$ in an ideal fibril, f should be zero until the fibril is totally crystalline and increase sharply with α'. The curves shown in Fig. 10 are not ideal but correspond to a schematic curve which Flory[10] gave for an inhomogeneous fibril. Presumably the whole curve at a temperature below the transition point ($\sim$ 25°) in Fig. 10 corresponds to the coexistence situation of amorphous and crystalline phases. That is, with increasing α' crystallization is taking place while there is coexistence of both phases. Flory[10] pointed out that such diffuse melting may be due to variations in chemical structure along the fiber axis or to non-uniformities in the cross-sectional area. The last rather steep slope portion of the curve in Fig. 10 is presumed to be due to the stress-strain behavior of a material rich in crystalline regions.

The data of Fig. 10 may also be considered in the following way. A vertical line (*i.e.*, increase in temperature at constant α') corresponds to an increase in f due to melting at any particular value of α'. An isothermal increase in α' leads to an increase in f due to an increasing amount of crystallization. Because of the diffuseness cited above, the coexistence region is not as sharply defined as it would be in the case of an ideal fibril. Above some critical temperature (greater than 40°), the system would be totally amorphous at all α'.

The data of Fig. 10 are re-plotted in Fig. 12 as L *vs.* T at constant f. From these curves it can also be seen that the low-temperature, partially crystalline material undergoes a helix-random coil transition around 25°. Further, we can obtain ΔL at constant force from these curves as the difference in L at high and low temperatures. The slope at T_{tr} gives $(\partial L/\partial T)_f$. $\Delta\bar{H}_1$ can be calculated from eq. 15 and is about −10 cal./mole for $\kappa_1 = -0.44$ at 45°; the measured value of $\partial g/\partial T$ is about −0.02, both quantities at zero force. Since q was not obtained as a function of L, we cannot evaluate the third term on the right hand side of eq. 23. However, we can make a rough estimate using the

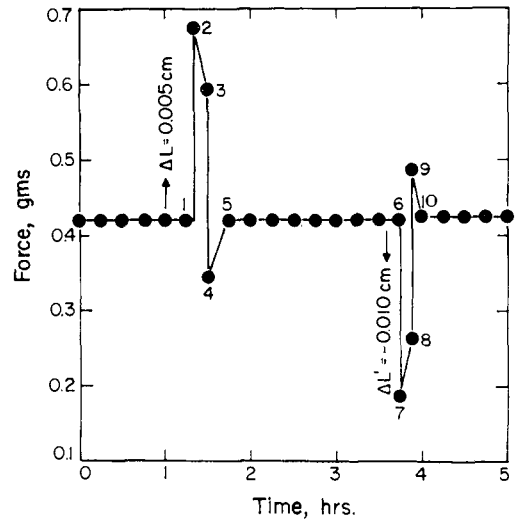

Fig. 11.—Establishment of equilibrium force at 11° in a buffer solution of pH 2.79 containing 2 M KCl, L = 1.1377 cm.

values obtained from the f *vs.* L function in place of the q *vs.* L function. From the values at zero force in the f *vs.* L curve we obtain[50]

$$-\frac{M_{unit}\bar{v}}{V_1}\left(\frac{\partial g}{\partial T}\right)_{L,P,n_2}\left(\frac{\partial L}{\partial T}\right)^{-1}_{P,f,n_2}\Delta\bar{H}_1\,\Delta L = -16 \text{ to } -18 \text{ cal./peptide unit} \quad (24)$$

Comparing this value to that of $\Delta H_{sol} = -765$ to −910 cal./peptide unit (Table III), we see that the contribution of the term in eq. 24 is about 2% of the first term on the right hand side of eqn. 23. While we have thus justified its neglect here, it is still important that future experiments be carried out to determine $(\partial n_1/\partial L)_{P,T,n_2}$, as Katchalsky has emphasized.[45,48]

If we neglect the third term on the right hand side of eqn. 23, we can evaluate the second term since we know the first term and also the left hand side of the equation. In Fig. 13 $[f - T(\partial f/\partial T)_{P,L}]$ is plotted against temperature using numerical values obtained from Fig. 9. First of all, it should be

(50) As can be seen from Table II, the degree of swelling is large and makes a significant contribution to f *in this system*. We have assumed that the change in force with length is due to a change in swelling at the transition temperature. Since ΔL is small (see Table IV) we have used the value of $(\partial q/\partial T)_{P,n_2}$ at $f = 0$ for $(\partial q/\partial T)_{L,P,n_2}$. The term $\left(\frac{\partial L}{\partial T}\right)_{P,n_1,n_2}$ of eq. 23 can be replaced by $(\partial L/\partial T)_{P,q,n_2}$ at T_{tr}, *i.e.*, constancy of n_1 and n_2 implies constancy of q. Again, attributing the change in force with length to a change in swelling at T_{tr}, $(\partial L/\partial T)_{P,q,n_2}$ was replaced by $(\partial L/\partial T)_{P,f,n_2}$.

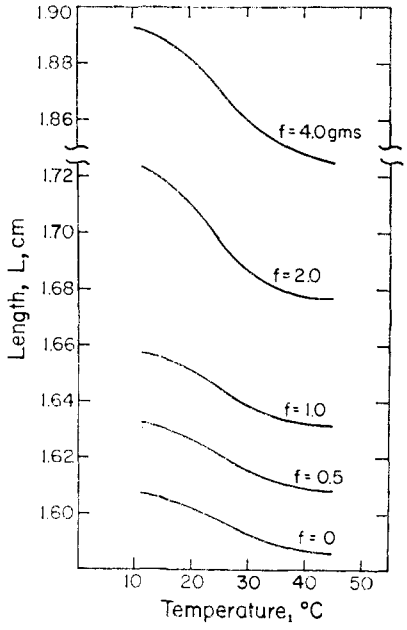

Fig. 12.—Change in length with temperature at constant force; data from Fig. 10.

noticed that these curves are independent of α'. The minimum occurs at the transition temperature. Neglecting the second term on the right hand side of eq. 20, we can obtain ΔH at the transition temperature from the minimum value of the ordinate in Fig. 13. Using ΔH_{sol} from Table III, we can then calculate ΔH^0_{res} from eq. 21. These values are listed in Table V. It can be seen that ΔH (M_{unit}/W_0) is essentially zero. Therefore, $\Delta H_{sol} \sim -\Delta H^0_{res}$ if we neglect the third term of eq. 23; *i.e.*, the contribution of the heat of solution is just compensating the contribution of the heat of dissociation of the hydrogen bond. Thus the calculated values of ΔH^0_{res} are +765 to +910 cal./peptide unit (about 40–50% of the value of 1700 obtained from the urea experiments). This means that the degree of crystallinity of the film is about 40–50%.

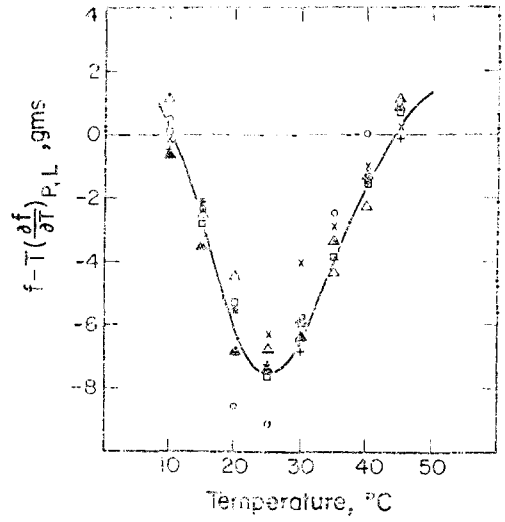

Fig. 13.—Relation between $[f - T(\partial f/\partial T)_{P,L}]$ and temperature; data from Fig. 9: $\alpha' = 1.004$ +, $\alpha' = 1.019$ ×, $\alpha' = 1.040$, ▲; $\alpha' = 1.060$ □, $\alpha' = 1.130$ O, $\alpha' = 1.176$, △.

In conclusion, the helical units shown in Fig. 3 are distributed in a partially oriented fashion in the film, occupying about 40–50% of the film. The remaining parts (60–50%) are in the amorphous regions. The assumed presence of one tyrosyl-carboxylate and one histidyl-carboxylate side-chain hydrogen bond per helical unit gives fairly good agreement with the experimental results.

TABLE V
ESTIMATIONS OF ENTHALPIES IN CAL./PEPTIDE UNIT
$W_0 = 0.0024$ g.; $M_{unit} = 100$

ΔH (M_{unit}/W_0)	0.25
ΔH_{sol}	−765 to −910 (from Table III)
ΔH^0_{res} (calcd.)	+765 to +910
Eq. 24	− 16 to −18

Acknowledgments.—The authors wish to thank Mrs. Miriam Taylor for able assistance in the experimental work and Dr. George Loeb for many helpful discussions.