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Thermodynamic Study of Shrinkage in Fibers Made from Insulin[1,2]

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Studies have been carried out on the thermodynamic properties of insulin fibers prepared by the following method: the spinning solution was made by dissolving a Zn-insulin–sodium lauryl sulfate mixture in a mixture of N,N-dimethylformamide, formic acid and water at an insulin concentration of about 50 g./100 ml. This solution was extruded through the needle of a hypodermic syringe into a coagulation bath which contained sodium sulfate, magnesium sulfate and formaldehyde. The fiber, thus pre-hardened with formaldehyde, was permanently crosslinked in *p*-benzoquinone solution and then elongated over 200% at elevated temperature in water or in other suitable media to orient the chains. With these fibers, studies were made of the dependence of the phase transition temperature on *p*H and medium composition by means of measurements of length and force. The degree of shrinkage at the transition temperature was about 40%. This shrinkage may be considered to be due to a phase transition between crystalline and amorphous forms. The *p*H-dependence of the transition temperature was given by a curve which has a maximum at a *p*H of about 5.5. These experimental results were satisfactorily accounted for by a molecular model for insulin and theoretical equations for the effect of side-chain hydrogen bonding on the phase transitions. In this calculation, the numerical values for the heat and entropy changes per peptide residue for the dissociation of one backbone hydrogen bond were obtained from experiments in urea solution.

Introduction

In an accompanying paper[4] a thermodynamic study of shrinkage in ribonuclease films was presented. In this paper we report on a similar study of shrinkage in insulin fibers, the primary aim being to provide experimental evidence for previous theoretical considerations[5] of the effect of side-chain hydrogen bonds on the elastic properties of protein fibers.[5] Insulin was chosen for this study because its covalent structure is completely known.[6] Fibers were prepared from insulin and cross-linked with *p*-benzoquinone. The chain configuration in such fibers need not be the same as in native insulin. As in the accompanying paper,[4] the point of view of Flory[7] was adopted, *viz.* that the shrinkage of fibrous proteins involves a crystalline-to-amorphous phase transition.

Experimental

Preparation of Fibers.—Crystalline beef Zn-insulin, kindly donated by Dr. O. K. Behrens of the Eli Lilly Company (Lot 693,502) was used for preparing fibers. In order to obtain a very concentrated solution for spinning, the following mixture was prepared: 75.2 mg. of Zn-insulin, 5.6 mg. of sodium lauryl sulfate and 0.17 ml. of a solvent consisting of water, N,N-dimethyl formamide and formic acid in the ratio 10:4:3; the *p*H of the solution was 5.5–6.0. The choice of this solvent was made after many trials based on earlier work of various investigators.[8-17] The spinning solution was allowed to stand for 20 hr. and then extruded through the needle (No. 23 gauge) of a hypodermic syringe into a coagulation bath, containing formaldehyde to provide a small degree of cross-linking during coagulation. The bath contained 150 g. of Na_2SO_4, 150 g. of $MgSO_4 \cdot 7H_2O$ and 15 g. of HCHO in 1000 ml. of aqueous solution (the *p*H being adjusted to 6 by addition of NaOH).

The brittle fibers thus formed were kept in the coagulation bath for about 2 days, then immersed in hot water for 20 minutes and washed with cold water for 3 days to remove detergent.

The fibers were subsequently cross-linked in 0.1–0.5% *p*-benzoquinone solutions (containing phosphate buffer at *p*H 8.86) for a period of 0.5–1.5 hr., then washed with water and elongated in water or in 1 *M* urea solution at temperatures up to 75° by hanging a weight of 2.25 g. on one end of the fiber. With this weight the fiber was extended about 200%. The extended fibers were cooled to room temperature and then kept in water at room temperature under tension for 1 day. After removal of the weight, the fibers maintained their extended length and were stored at 4°.

From the effect on the transition temperature (to be defined later), shown in Fig. 1, it appears that the reaction with formaldehyde reaches equilibrium within 2 or 3 days and that with *p*-benzoquinone in 2 to 3 hours.

Force and Length Measurements.—The measurements of force were carried out with the apparatus previously described.[4] The length measurements were made with a Leitz petrographic microscope equipped with an electrically-heated hot stage and a micrometer eyepiece (Bausch and Lomb Optical Company). A piece of fiber about 2 mm. long was equilibrated with solvent in the hot stage at the starting temperature. The hot stage was then heated, the temperature being indicated to ± 0.1° by a thermometer in a hole in the metal block and the length being measured to better than ± 0.5% of the initial length.

X-Ray Measurements.—X-Ray diffraction measurements were made on the fiber at various temperatures in the presence and absence of solvent. Diffraction patterns of Zn-insulin powder were also obtained. A Norelco X-ray unit was used (35 kv., 18 ma., Ni-filtered Cu radiation) with a Type 52055 micro camera manufactured by Philips Electronic, Inc. The film-to-specimen distance was 1.5 cm. and the fiber was mounted in a thin, flat Pyrex glass tube with its axis vertical. When dry fibers were used, the glass tube was omitted. The sample was heated with a nichrome wire wound around the thin glass tube. The temperature was determined with a thermocouple and recorder. Exposure times ranged from 4 to 5 hr.

The spacings were computed from the Bragg law ($\lambda = 2d \sin\theta$), where λ is the wave length of the X-rays ($CuK\alpha$ = 1.54 Å.), d is the spacing and θ is the angle of incidence given by $\tan 2\theta = r/a$ where r is the radius of the ring on the diffraction pattern and a is the film-to-specimen distance.

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(2) Presented before the Division of Biological Chemistry at the 136th Meeting of the American Chemical Society, Atlantic City, N. J., September, 1959.

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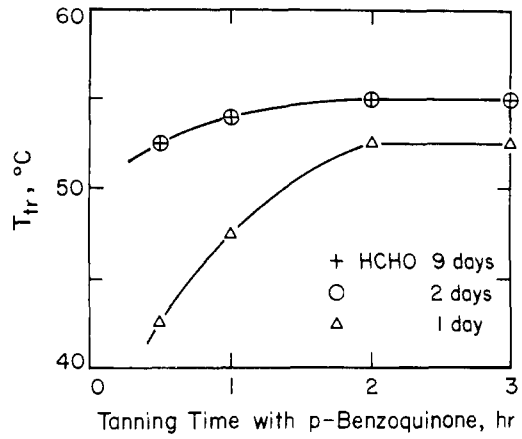

Fig. 1.—Effect of tanning with formaldehyde and *p*-benzoquinone on the transition temperature of insulin fibers in a buffer solution of *p*H 6.92. The fibers, after treatment with formaldehyde for the period shown, were tanned in a 0.5% quinone solution of *p*H 8.86 for various times and then stretched 200%. The transition temperature was determined from length measurements on the extended fibers.

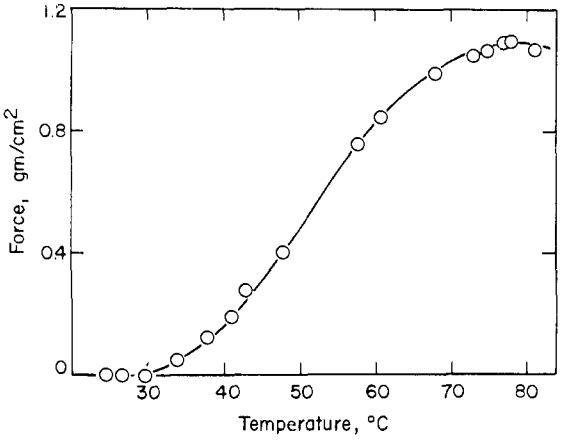

Fig. 2.—Force-temperature curve at constant length for an insulin fiber in a buffer solution of *p*H 3.04.

Results

Thermally Induced Change in Length and Stress.—When a partially oriented fiber is heated, it shrinks over the temperature range at which melting occurs.[7] If the length is fixed so that the fiber cannot shrink, then a contractile force will be generated over the melting range.[7] Figure 2 shows the change in the contractile force with temperature as an insulin fiber is heated at constant length in a buffer solution of *p*H 3.04. Figure 3 shows the change in length of the fiber at zero force in the same buffer solution. Here, the ratio of the length at a given temperature to that at the starting temperature was plotted against temperature. Figures 2 and 3 indicate the corresponding changes in force and length with increasing temperature. The inflection points of these curves are taken as the transition temperature, T_{tr}, for the melting process. The values of T_{tr} determined from both the force-temperature and length-temperature measurements agree fairly well. Most of the values of T_{tr} reported in this paper were obtained from length measurements.

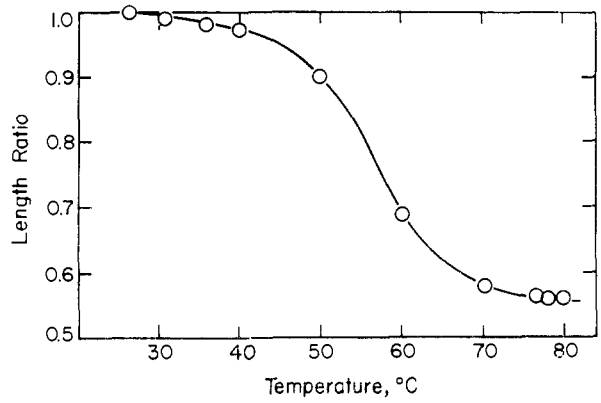

Fig. 3.—Length-temperature curve at zero force for an insulin fiber in a buffer solution of *p*H 3.04.

X-Ray Diffraction.—Since the change in length during the heat-induced transition is thought to accompany a change in molecular configuration, it should be possible to detect this configurational change by X-ray diffraction. Figure 4 shows the diffraction patterns obtained, together with idealized diagrams to point out the important features of the patterns. Figure 4a was obtained with Zn-insulin powder in a thin, flat Pyrex glass tube. The inner intense sharp ring (d_1 in Table I) is considered to arise from the inter-chain spacing[18] and the outer intense ring (d_2 in Table I) from an overlap (leading to broadening) of diffractions (due to random orientation of crystallites) from a repeat distance[19] of 5.1 Å. along the α-keratin structure and a backbone spacing[20] of 4.7 Å. in the β-configuration, because the sample is not completely composed of α-keratin type structures. The pattern of an insulin fiber stretched 200% and air dried (mounted in the camera without a glass tube) is shown in Fig. 4b. Here, too, the sharp inner ring and diffuse outer ring appear and probably arise from the same spacings as in Fig. 4a. The pattern of Fig. 4d was obtained from a fiber prepared under the same conditions as that of Fig. 4b, except that the fiber was stored in a *p*H 3.04 buffer solution and then sealed in a thin, flat Pyrex glass tube containing the same buffer (keeping the lower end of the fiber in the buffer). The X-ray photograph was taken at 25° on the upper end of the fiber. Here, too, the same two rings appear as in Fig. 4b. Figure 4e is a pattern of the same fiber as in Fig. 4d, except that the fiber was fully immersed in the buffer in the glass tube. Although there is considerable X-ray scattering due to the water, the outer ring of about 4.6 Å. spacing still remains. The weakening of the inner 10 Å. ring is probably due to swelling by the diluent. Figure 4f shows a pattern of sample 4e at 80°. Here the rings corresponding to the 10 Å. and 4.8 Å. spacings were weakened and the intensity of the ring corresponding to a 3.4 Å. spacing was strengthened. After experiment 4f, the fiber was

(18) B. W. Low, "The Proteins," edited by H. Neurath and K. Bailey, IA, Academic Press, Inc., New York, N. Y., 1953, p. 290.
(19) Ref. 18, p. 384.
(20) Ref. 18, p. 257.

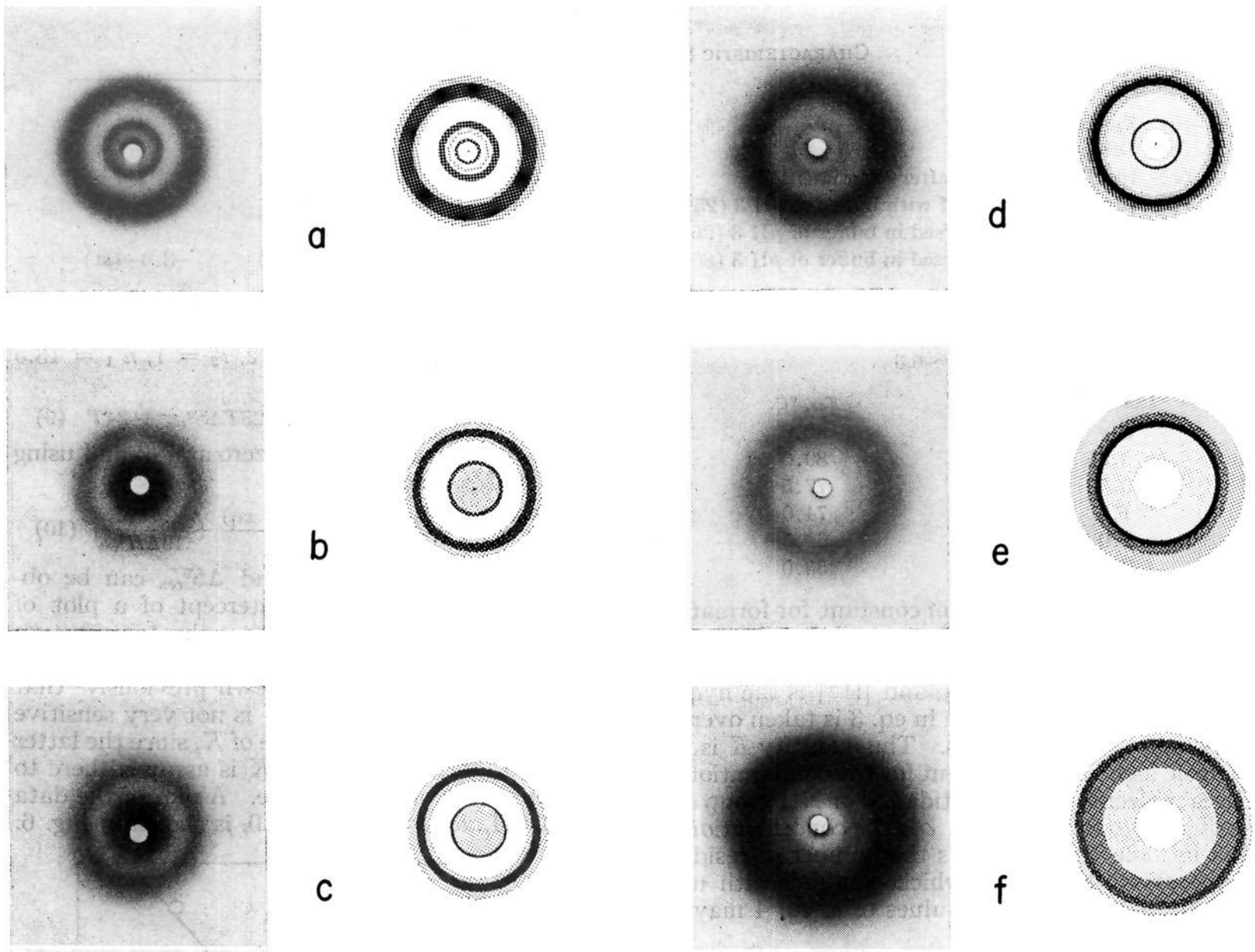

Fig. 4.—Wide angle X-ray diffraction patterns of insulin: (a) Zn-insulin powder; (b) insulin fiber stretched 200% and air dried; (c) insulin fiber dried after experiment f; (d) insulin fiber wetted with a buffer solution of *p*H 3.04, at 25°; (e) insulin fiber immersed in a buffer solution of *p*H 3.04, at 25°; (f) insulin fiber immersed in a buffer solution of *p*H 3.04, at 80°.

washed with water and dried. Figure 4c shows the resulting pattern, obtained without a glass tube. This pattern was the same as that of Fig. 4b. In all these experiments the fiber was mounted in the camera without any applied stress.

While the chains were not sufficiently oriented to give a diffraction pattern characteristic of oriented crystalline material, nevertheless, the appearance of two fairly sharp rings below the transition temperature[21] indicates the presence of crystalline material of a very low degree of orientation.

Above the transition temperature, *i.e.*, at 80°, we may assume that the randomly coiled form predominates, since the 3.4 Å. spacing corresponds to the diffraction from single peptide units.[22]

Effect of Urea on the Transition.—Length-temperature measurements were made in urea solutions, the data obtained being shown in Table II. In contrast to corresponding experiments of the previous paper,[4] no salt was added to the urea.

As discussed previously,[4,23] the observed standard free energy of unfolding the helix, ΔF^0_{obsd} in the presence of urea is given by

$$\Delta F^0_{obsd} = \Delta F^0_B + \Delta F^0_H + \Delta F^0_{comb} - T\Delta S^0_x \quad (1)$$

(neglecting electrostatic effects), where ΔF^0_B, ΔF^0_H and ΔF^0_{comb} denote the standard free energy changes from the unfolding of the backbone chain, the rupture of the side-chain hydrogen bonds and the combination of the extended polypeptide chain with urea, respectively. ΔS^0_x is the molar entropy decrease of the random coil resulting from the presence of cross-links.[7] These may be written[5,7,23] as

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

$$\Delta F^0_H = -RT\Sigma \ln (1 - x_{ij}) \quad (3)$$

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

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

where n is the number of residues in the helical region, ΔH^0_{res} and ΔS^0_{res} are the heat and entropy change, respectively, per residue for the unfolding of the infinitely long helix, x_{ij} is the fraction of the molecules having a side-chain hydrogen bond between an i^{th} donor and j^{th} acceptor and is given by eq. 6 for heterologous single bonds.[24]

$$x_{ij} = \frac{K_{ij}}{1 + K_{ij} + K_1/[H^+] + [H^+]/K_2} \quad (6)$$

(21) The transition temperature at *p*H 3.04 is about 55°.

(22) Ref. 18, p. 273.

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

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

TABLE I

CHARACTERISTIC SPACINGS IN INSULIN FIBERS

	Spacing (Å.)		
	d_1	d_2	d_3
(a) Zn-insulin powder	7.8–10.0 (st)	~4.8– (st)	
(b) Dried insulin fiber	9.8 (st)	~4.8– (st)	
(c) Insulin fiber dried after experiment (f)	9.8 (st)	~4.8– (st)	
(d) Insulin fiber wetted with buffer of *p*H 3 (25°)	10.7 (st)	~4.6– (st)	
(e) Insulin fiber immersed in buffer of *p*H 3 (25°)	10.7 (wk)	~4.6– (st)	
(f) Insulin fiber immersed in buffer of *p*H 3 (80°)	10.7 (wk)	~4.8– (wk)	~3.4– (st)

TABLE II

TRANSITION TEMPERATURES IN AQUEOUS UREA SOLUTIONS AT *p*H ~6.3

Urea, concn., (mole/l.)	T_{tr}, °C.
0	82.0
0.25	80.0
0.50	77.5
1.0	74.0
2.0	62.5
3.0	53.0

where K_{ij} is the equilibrium constant for formation of the ij^{th} bond, K_1 and K_2 are the ionization constants of the non-hydrogen bonded donor and acceptor groups, respectively, and $[H^+]$ is the hydrogen ion activity. The sum in eq. 3 is taken over all side-chain hydrogen bonds. The quantity K is the intrinsic association constant for the combination of a urea molecule with a peptide CO or NH group and is equal[4,23] to 0.025 at 25°; c is the concentration of urea in mole/liter, and p is the number of residues in the crystalline chain which combine with urea after unfolding. At low values of c, eq. 4 may be approximated by

$$\Delta F^0_{comb} = -2pRTKc \qquad (7)$$

The quantity ν is the number of cross-linked helices, and n' is the number of statistical elements (assumed here to be equal to the number of peptide residues) between cross-links.

In order to evaluate these contributions to ΔF^0_{obsd} it is necessary to postulate a model. For this purpose we have used the model shown in Fig. 5 for

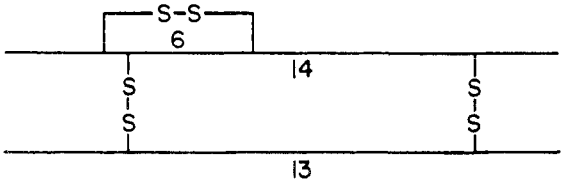

Fig. 5.—A model for the helical portion of the insulin fiber in the system.

the helical portion of the insulin fiber. This model is based on the Sanger structure.[6] The 14 residues in the A-chain and 13 residues in the B-chain are assumed to be in the α-helical configuration. Three S–S bridges are also present[6] as shown. The remaining parts of the A- and B-chains are assumed to be in the amorphous region. According to this model, eq. 2 and 5 become

$$\Delta F^0_B - T\Delta S^0_x = [(n_1 - 4) + (n_2 - 4)]\Delta H^0_{res} - [(n_1 - 1) + (n_2 - 1)]\, T\Delta S^0_{res} - T(\Delta S^0_{x1} + \Delta S^0_{x2}) \qquad (8)$$

Here, n_1 and n_2 denote the number of residues in the helical parts of the A- and B-chains, respectively, and ΔS^0_{x1} and ΔS^0_{x2} are the contributions from the two loops shown in Fig. 5. For the assumed model $n_1 = 14$, $n_2 = 13$, $\nu_1 = 2$, $\nu_2 = 1$, $n'_1 = 13.5$ and $n'_2 = 6$. Thus

$$\Delta F^0_B - T\Delta S^0_x = 19\Delta H^0_{res} - 25T\Delta S^0_{res} + 24T \qquad (9)$$

Equating ΔF^0_{obsd} of eq. 1 to zero at T_{tr}, and using eq. 3, 7 and 9, we obtain

$$\frac{1}{T_{tr}} = \frac{25\Delta S^0_{res} - 24 + R\Sigma \ln(1 - x_{ij})}{19\Delta H^0_{res}} + \frac{2pRKc}{19\Delta H^0_{res}} \qquad (10)$$

Thus, the values of ΔH^0_{res} and ΔS^0_{res} can be obtained from the slope and intercept of a plot of $1/T_{tr}$ against c. While data on the temperature dependence of K are required for this interpretation of eq. 10, it has been shown previously[4] that the computed value of ΔH^0_{res} is not very sensitive to the temperature dependence of K, since the latter is not too large. Therefore, K is assumed here to be independent of temperature. A plot of the data of Table II, according to eq. 10, is shown in Fig. 6.

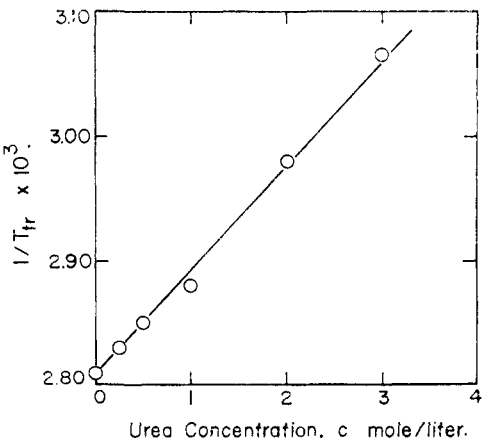

Fig. 6.—Data for the dependence of the transition temperature on urea concentration, plotted according to eq. 10.

A value of $\Delta H^0_{res} = 1720$ cal./mole is obtained from the slope. This compares very well with the value of 1700 cal./mole, determined from similar experiments with ribonuclease films.[4] In the case of ribonuclease, the experiments were carried out at low *p*H, where the term $R\Sigma \ln(1 - x_{ij})$ was zero, permitting a determination of $\Delta S^0_{res} = 4.83$ e.u. from the intercept of a curve similar to that of Fig. 6. In order to avoid additional assumptions about the *p*H-dependence of $R\Sigma \ln(1 - x_{ij})$, which is not zero at the *p*H (6.3) of the experiments of Table II, we shall take $\Delta S^0_{res} = 4.83$ e.u. for insulin, the same as found for ribonuclease. With this assumption, we shall be able to consider (in the next section) the *p*H dependence of the transition temperature as arising from the *p*H dependence of ΔF^0_H.

Effect of *p*H on the Transition.—Having determined ΔH^0_{res} and ΔS^0_{res} from the experiments on ribonuclease films and insulin fibers in urea, we can now consider the *p*H dependence of the transition temperature *in the absence of urea.* For this purpose we consider eq. 1 without the term ΔF^0_{comb} and assume[5] the *p*H-dependence of ΔF^0_{obsd} to arise from the *p*H-dependence of ΔF^0_H. Thus, setting ΔF^0_{obsd} equal to zero at T_{tr}, we obtain

$$19\Delta H^0_{res} - 25T_{tr}\Delta S^0_{res} + 24T_{tr} - RT_{tr}\Sigma \ln (1 - x_{ij}) = 0 \quad (11)$$

which can be solved[5] for T_{tr} as a function of *p*H. To complete the calculation, we need only to introduce the side-chain hydrogen bonds explicitly into the model of Fig. 5. Thus, we assume the helical unit of Fig. 5 to contain one histidyl-carboxylate ion side-chain hydrogen bond with parameters shown in Table III. Such a bond might be one between residues 10 (histidyl) and 13 (glutamic acid) of the same or adjacent B-chains.[25]

TABLE III

ASSUMED VALUES AT 25° FOR MODEL CALCULATION INVOLVING HISTIDYL-CARBOXYLATE ION HYDROGEN BOND

K_1 (histidyl)	$10^{-6.5}$
K_2 (carboxyl)	$10^{-4.5}$
ΔH_1^0, kcal./mole	7
ΔH_2^0, kcal./mole	0
K_{ij}	70[a]
ΔH^0_{ij}, kcal./mole	−6
ΔH^0_{res}, kcal./mole	1.7
ΔS^0_{res}, e.u.	4.83

[a] The value of K_{ij} was taken as a rather high value here since, in contrast to a tyrosyl-carboxylate ion bond,[26] a histidyl-carboxylate ion bond has an electrostatic contribution to the free energy of formation. The value of $\Delta F^0 = -RT \ln 70$ is −2.55 kcal./mole. If ΔF^0 is regarded as $\Delta F^0_{ij} + \Delta F^0_{elec}$, where $\Delta F^0_{ij} \sim 0$[26] and $\Delta F^0_{elec} = -N\epsilon^2/Dr$ (N being Avogadro's number, ϵ the electronic charge, D the dielectric constant and r the distance between charges), then ΔF^0_{elec} will be −2.55 kcal./mole for $D = 50$ and $r = 2.5$ Å. or for $D = 40$ and $r = 3.2$ Å. Since these are reasonable values of D and r, the magnitude of ΔF^0_{elec} (and therefore, K_{ij}) is reasonable.

The *p*H-dependence of T_{tr}, calculated according to eq. 11, is shown as a dashed curve in Fig. 7. The experimental data, obtained from curves such as that of Fig. 3 at various *p*H values, are also shown. The agreement seems reasonable. However, this investigation of insulin is not as complete as our previous one on ribonuclease.[4] In particular, we have not added excess salt to suppress electrostatic effects, nor have we investigated the *p*H-dependence of swelling. Both of these effects were measured for ribonuclease.[4] We may add that the experimental curve for the *p*H-dependence of T_{tr} for ribonuclease was independent of the salt concentration. It thus seems reasonable to conclude that the *p*H-dependence of T_{tr} for insulin arises from

(25) H. Lindley and J. S. Rollett, *Biochim. Biophys. Acta*, **18**, 183 (1955).

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

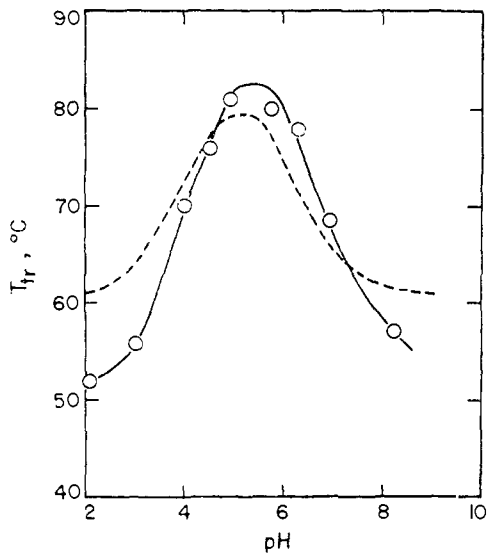

Fig. 7.—Relation between the transition temperature and *p*H of the diluent; the dashed curve was obtained from the model calculation, using eq. 11.

side-chain hydrogen bonding, as it appears for ribonuclease. In the case of insulin the maximum side-chain stabilization occurs at around *p*H 5.5. The agreement between the two curves of Fig. 7 provides additional confirmation of the theory previously presented.[5] Presumably, the agreement could be improved if electrostatic effects were included.

Discussion

According to our X-ray measurements, our insulin fibers contained crystallites but, even at 200% extension, were not very oriented. The insulin molecule is very small with very few sites for reaction with cross-linking reagents such as *p*-benzoquinone. Therefore, it is not surprising that it was not possible to obtain a network structure with a high degree of orientation. Further, unlike our previous studies of ribonuclease,[4] the shrinkage measurements were not reversible. Despite this fact the data still have some validity since they were obtained on the heating part of the cycle. In the case of ribonuclease, under conditions where hysteresis could be observed, the data obtained from the heating part of the cycle appeared to approach the equilibrium values. Taken alone, the insulin data are not very convincing; however, in conjunction with the more extensive measurements on ribonuclease, these results illustrate the role which side-chain hydrogen bonds can play in stabilizing the crystalline form of a protein, either in molecularly dispersed species in solution, or in a network, as shown here for insulin and previously for ribonuclease.

Acknowledgment.—We are very grateful to Dr. G. I. Loeb for many helpful discussions and also for aid in the experimental work.