A ^{13}C Nuclear Magnetic Resonance and Circular Dichroism Study of the Collagen–Gelatin Transformation in Enzyme Solubilized Collagen†

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ABSTRACT: Natural abundance Fourier transform ^{13}C nuclear magnetic resonance (^{13}C NMR) were obtained for enzyme solubilized collagen at 1° intervals through the transition region. The transition of collagen molecules from the rigid triple helical state to single-stranded, random-coil state is accompanied by a change from broadened carbon resonances unobservable under high-resolution conditions to narrow line spectra. Thus distinction can be made between helical and random-coil states of individual residues. The transition is monophasic, as determined by examination of 14 different carbon resonances, and the entire structure is found to melt cooperatively over a temperature interval of 5 ± 1°. All the residues seem to be involved in the unfolding process concurrently. The transition was also studied by examining the changes in the circular dichroism spectrum brought about by heating. The experiments corroborated the observation that the transition proceeded cooperatively over a temperature interval of 4°. Enzyme soluble collagen is seen to melt less cooperatively than native collagen. The enthalpy change was determined by assuming an equilibrium between three random coil gelatin chains and tropocollogen molecules. From the enthalpy, the average length of the tripeptide sequences (70–85) involved in the transition can be estimated. The shortening of the cooperative unit could arise as a result of some alteration of the native conformation through proctase treatment.

The thermally induced conversion of rigid triple helix collagen molecules (tropocollagen) to random-coil gelatin has been studied by optical rotatory dispersion (Burge and Hynes, 1959), solution viscosity (Boedtker and Doty, 1956), sedimentation velocity (Altgelt et al., 1961; Lewis and Piez, 1964), light scattering (Engel, 1962), kinetics of proteolysis (von Hippel and Harrington, 1959), and small angle laser scattering (Chien and Chang, 1972a). The results have been reviewed by von Hippel (1967) and by Chien (1975). Each technique is responsive to a different aspect of the transition, but none of them are capable of yielding detailed information at the residue level. Therefore, we are still rather ignorant about whether all residues participate simultaneously in the transformation, or whether it is possible for some residues to undergo reactions independently of the remainder of the molecule.

Our earlier ^{13}C nuclear magnetic resonance (^{13}C NMR) study of gelatin (Chien and Wise, 1973) indicated that high resolution spectra are obtainable on the random-coil molecules, and that distinction could be made between carbonyl (C^0), α type (C^α), and side chain carbon resonances. On the other hand, the rigid helical collagen molecules yield no spectra under high resolution conditions because their carbon atom resonances are broadened to the point of unobservability by anisotropic nuclear dipole–dipole interactions. Consequently, discrimination can be made between helical and random-coil residues, or even between mobile and rigid regions of the same molecule. Furthermore, subject to the restriction that the chemically nonequivalent carbons have equal Overhauser enhancements, the integrated intensities of the resonances will in general be directly proportional to the number of carbon atoms contributing to the resonance (Schaefer and Natusch, 1972). Consequently, it seems that ^{13}C NMR can yield detailed information regarding the collagen–gelatin transition at the residue level.

In this study we seek to determine whether all residues melt simultaneously or certain residues, such as those in the helix stabilizing Gly-Pro-X, Gly-Pro-Hyp, and Gly-X-Hyp, melt later than other residues in the Gly-X-Y sequences. We are also interested in finding out whether cis–trans isomerization of the prolyl peptide linkages accompanies the transtion. Finally, the question of the degree of cooperativity of the collagen–gelatin transition will be examined.

Materials and Methods

Materials. The collagen chosen for this investigation has been digested with proteolytic enzyme to remove the telopeptides (Rubin et al. 1968). This material, referred to as ESC, was made by pH 3 proctase treatment of calf-skin collagen. It was purchased from the Japan Leather Company. The amino acid composition has been given earlier (Chien and Chang, 1972b), and the comparison of its properties to native material also described (Rubin et al., 1963, 1965; Chien and Chang, 1973). Subsequent study of the whole protein molecule should reveal the role of telopeptides in the helix–coil transition.

Samples for ^{13}C NMR study at 10.7 weight % were prepared by adding a 0.4% aqueous solution of Carbowax 4000 to the ESC. The Carbowax was added as a chemical shift (123.6 ppm relative to CS_2 = 0) and peak area reference. The sample was not adjusted to any particular pH since the collagen gelatin transition was not strongly dependent on pH except at extreme values (Burge and Hynes, 1959).

^{13}C NMR requires highest possible concentration of collagen for reasons of signal to noise. Therefore, the collagen was merely in the swollen but not dissolved state at low temperatures and the solution was very viscous at elevated temperatures. This contrasts with the usual condition of dilute solution in studies of protein denaturation. However, since collagen exists mostly in the swollen state in nature, our experimental condition is quite relevant. Furthermore,

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the ^{13}C NMR results are consonant with the CD measurement on dilute solutions of ESC.

Nuclear Magnetic Resonance. High-resolution ^{1}H broad-band decoupled, natural abundance Fourier-transform ^{13}C NMR were obtained at 22.63 MHz with Bruker HFX-90 spectrometer. The ^{13}C analytical channel was operated with a 10-mm insert in the single coil configuration where a 17.5-μsec pulse resulted in a $\pi/2$ spin nutation. Hexafluorobenzene or deuterium oxide contained in a 5-mm coaxial tube was used to secure field frequency stabilization. Free induction decays were accumulated in a Nicolet 1080 E time averaging computer using 8192 channels internally swept at a rate of 100 μsec/channel (5 kHz frequency domain). Frequency components greater than 5 kHz were removed by a low-pass filter built into the Nicolet SD 80 signal digitizer. A delay of 100 μsec was introduced between the time of the irradiating pulse and the time the receiver was first sampled by the Nicolet SW 80 sweep generator. Each spectrum represents the accumulation of 16,384 transients using a 16-μsec pulse and a pulse recycle time of 1 sec.

Data manipulation was performed utilizing the Nicolet Fourier transform program (NIC-80/s-7202f). A trapezoidal window correction of 4 addresses was normally applied as well as a time constant of -1.5. Integrated resonance intensities were determined using the nonnormalized integral patch (NUS-80/u-7304b). The samples were equilibrated for 18 hr in an oil bath before being transferred into the spectrometer. The oil bath and insert were maintained at identical constant temperatures regulated to <0.3°. The insert temperature was controlled by a Bruker BST 100/700 variable temperature control unit. The same sample was used in a given series, the experiment began at 38° and progressed at 1° increments to 46°, and then at 10° increments from 50 to 70°.

Circular Dichroism. Circular dichroic (CD) spectra was measured using a Cary recording spectropolarimeter, Model 60.

Optical rotatory dispersion (ORD) measurements were also made with the Cary 60 spectrometer on native acid soluble collagen, ESC, and gelatin. The concentrations were 5×10^{-5} g cm^{-3}. The rms noise level at 600 and 185 nm are 0.4 and 6.5 mdeg, respectively. A cell with a 1-cm path length was used to measure the CD spectrum of a pH 4.5 ESC solution at approximately 4×10^{-5} g cm^{-3}.

Thermal denaturation profiles were observed by monitoring the band at 224 nm. A jacketed cell having a 1-mm path length was used to study pH 3.3 and pH 6.4 solutions at 2×10^{-3} g cm^{-3}. Heating was achieved by circulating water from a Haake water bath (type NBe) through the jacketed cell. The water in the bath was heated at the rate of 18°/hr using a Neslab Instruments Model TP-2 temperature programmer. The relationship between the temperature in the cell and the temperature in the bath was determined by measuring the former with a thermocouple.

Quantitative Analysis of ^{13}C NMR Spectra. The ^{13}C NMR intensity is increased by proton irradiation. The extent of this nuclear Overhauser enhancement (NOE) is given by Natusch et al. (1966), where S_H is the proton saturation parameter ($S_H = 1$ for complete decoupling), the γ's are the gyromagnetic ratios, T_1 is the spin-lattice relaxation time of the ^{13}C nuclei due to all relaxation mechanisms, T_{1CH} is that component of T_1 due to carbon–hydrogen dipole–dipole relaxation, and ρ_{CH} describes the nature and effectiveness of the dynamic coupling between the ^{13}C and ^{1}H spins. When the dipolar relaxation mechanism is dominant, the ratio T_1/T_{1CH} is unity. The situation has been observed for a wide variety of systems by Allerhand et al. (1970, 1971, 1973). Under these conditions, NOE is solely dependent on ρ_{CH}, which is a complicated function of rotational correlation time for the ^{13}C–^{1}H coupling (τ_{CH}), and consequently on the viscosity and temperature of the medium.

$$(\mathrm{NOE}) = 1 + \rho_{CH} S_H (\gamma_H/\gamma_C)(T_1/T_{1CH})$$

Table I: Integrated Intensities of Gly-C$^{\alpha}$ and C$^{\gamma}$ Resonances.

Temp (°C)	A (PEO)	Gly C$^{\alpha}$ Resonances: A (Gly C$^{\alpha}$)	A (Gly C$^{\alpha}$)/A (PEO)	Normalized Ratio	C$^{\gamma}$ Resonances: A (C$^{\gamma}$)	A (C$^{\gamma}$)/A (PEO)	Normalized Ratio
38	7.73				1.84	0.238	0.16
39	2.09	6.56	0.314	0.11	3.18	0.152	0.10
40	16.3	6.18	0.380	0.13	3.04	0.188	0.12
41	17.4	19.0	1.10	0.38	4.75	0.276	0.18
42	21.3	28.5	1.34	0.46	11.8	0.554	0.36
43	9.63	15.8	1.64	0.56	8.33	0.866	0.57
44	8.34	14.8	1.78	0.61	7.37	0.885	0.58
45	9.25	17.3	1.87	0.64	10.0	1.08	0.71
46	8.58	17.0	1.98	0.68	9.85	1.15	0.76
50	8.07	16.8	2.06	0.71	9.31	1.15	0.76
60	6.66	17.0	2.55	0.88	9.05	1.36	0.90
70	6.55	19.1	2.92	1.00	9.97	1.52	1.00

In principle, NOE can be determined by comparing the intensities of decoupled and undecoupled ^{13}C NMR spectra. This determination is, however, not possible for a molecule as complicated as collagen. We chose to overcome this difficulty by the introduction of a water soluble polymeric reference poly(ethylene oxide) (PEO).[1] The basic asumption here is that viscosity and temperature affect similarly the ρ_{CH}'s for poly(ethylene oxide) and collagen. This assumption is valid when either τ_{CH} is equal for the two molecules or $1.0 < \omega_C \tau_{CH} < 0.1$. The validity of the assumption is tested as follows.

There are two resonances, comprised of the largest number of carbon atoms, whose areas can be measured accurately at all temperatures used in this denaturation study. These are the two Gly C$^{\alpha}$ resonances at 150.5 and 151.4 ppm and the C$^{\gamma}$ resonances at 168.7 ppm. The ratio of the areas of these resonances to that of the poly(ethylene oxide) is then proportional to the number of carbon atoms represented by the particular resonance in the random coil state. Effects of resolution, decoupling, and spectrometer tuning are eliminated by this procedure of data reduction. In Table I may be found integrated intensities of poly(ethylene oxide), A (PEO), combined Gly C$^{\alpha}$ resonances, A (Gly C$^{\alpha}$), and C$^{\gamma}$ resonances A (C$^{\gamma}$), along with their ratios to PEO and normalized ratios.

At 70° denaturation can be considered to be complete and the reduced areas should correspond to the residue contents. There are 337/1000 of Gly residues in ESC (Chien and Chang, 1972). The peak at 168.7 ppm arises from the C$^{\gamma}$ resonances of Pro, Arg, and Leu and there are a total of 189/1000 of these three residues. The relative abundances are thus in a ratio of (Pro + Arg + Leu)/Gly = 189/337 = 0.56. The corresponding ^{13}C NMR intensities are in a ratio

[1] Abbreviation used is: PEO, poly(ethylene oxide).

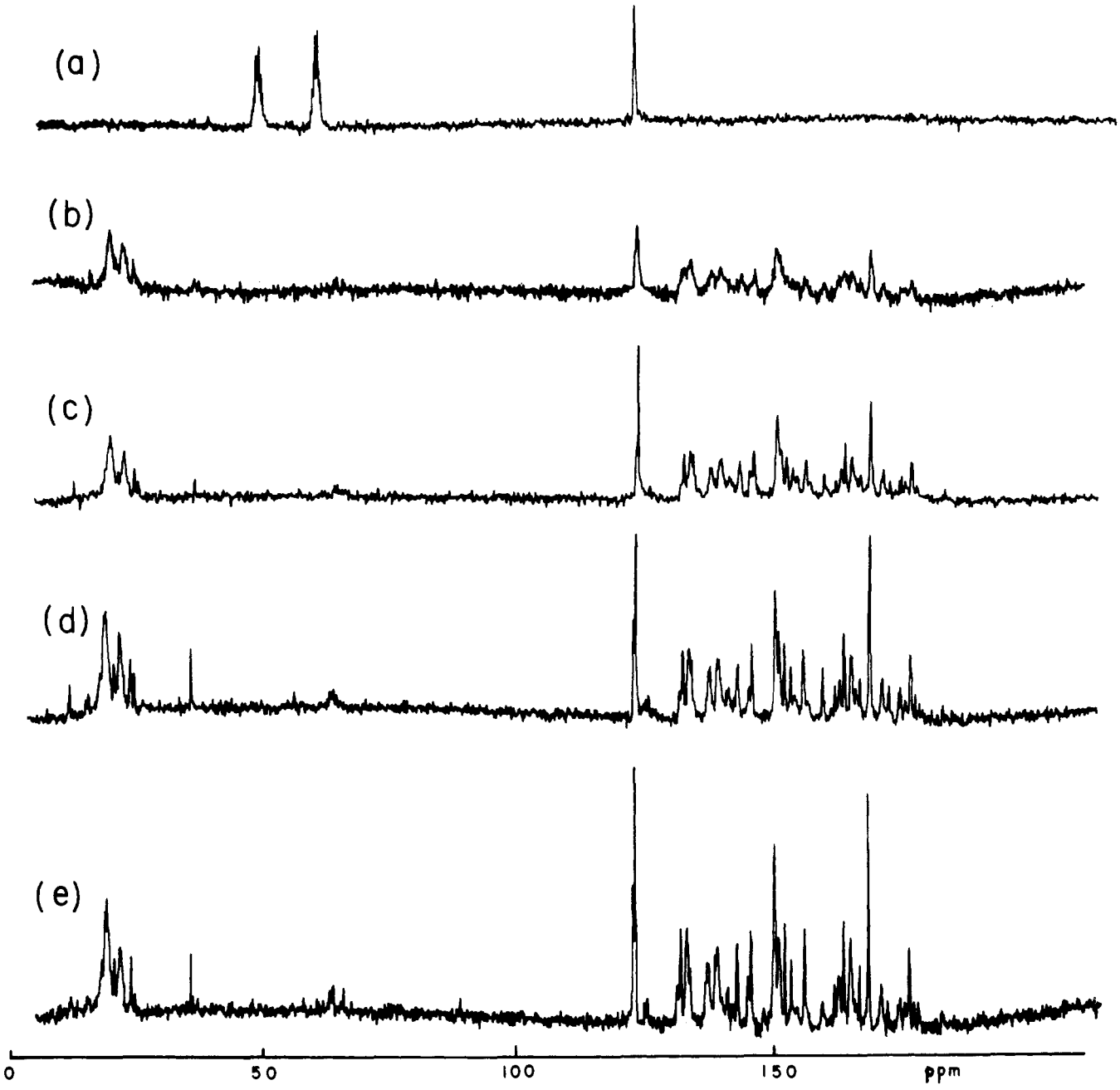

FIGURE 1: ^{13}C NMR spectra of enzyme soluble collagen in water (16 μsec, 1-sec recycle time, 16, 384 transients): (a) 29°; (b) 41°; (c) 43°; (d) 45°; (e) 55°.

of 1.52/2.84 = 0.54. This agreement provides justification for the use of PEO as a reference for quantitative analysis.

Collagen Denaturation Study with ^{13}C NMR. The Fourier transformed spectra (Figure 1) were plotted and used in analysis. Assignments made previously in our study of calf-skin gelatin (Chien and Wise, 1973) were used in interpreting these spectra. By proceeding in the manner described in the preceding section, we obtained and plotted in Figure 2 the fractional increase of the integrated intensities of Gly C^{α} and Pro C^{γ} resonances with increasing temperature. The transition temperature, T_m, is 42.5°; the transition width, ΔT, is 6°.

Information about other residues can be obtained if resonance peak heights (intensities) rather than integrated peak areas are measured. Fourteen lines whose signal to noise ratios were 10:1 or greater in the denatured material were measured from the plotted spectra. The chemical shifts along with their assignments and number residues represented are given in Table II (Chien and Wise, 1973). The spectra were scaled to the same relative peak height for PEO by use of the normalization constant found in the Fourier transformation. The peak heights were expressed as fractions of those expected when all carbons of a given resonance contribute narrow observable resonances. The increase in the weighted least-squares fractional intensity derived from the 14 resonances is plotted vs. temperature. Figure 3 illustrates such a plot for Hyp C^{γ}, Pro C^{δ}, and Arg C^{δ}, and Ala C^{β} resonances.

Figures 2 and 3 exhibit the characteristic sigmoidal shape of a cooperative phenomenon. The transition temperature for the unfolding process is 43° and the width of transition, ΔT, is 5°. Finally no resonance emerged significant-

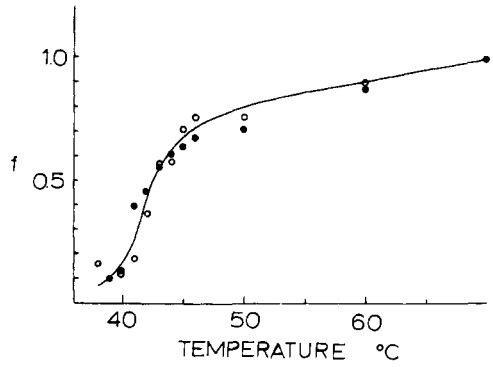

FIGURE 2: Fractional conversion to random coil measured by relative integrated intensities for Gly (●) and C^{γ} resonances (○).

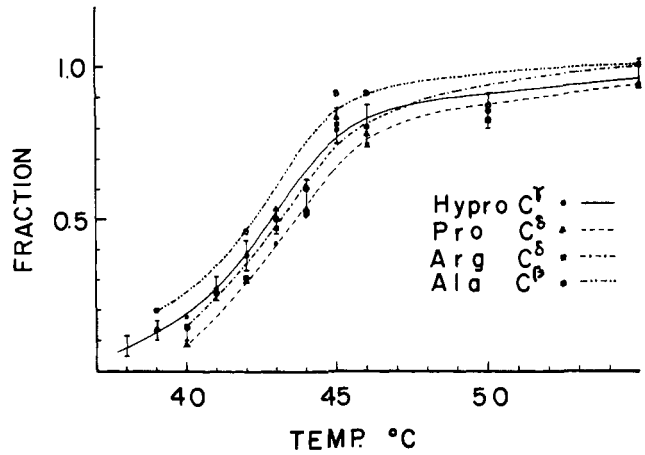

FIGURE 3: Fractional conversion to random coil measured by Hyp C^{γ}, Pro C^{δ}, Arg C^{δ}, and Ala C^{β} resonances.

ly different from the others, indicating uniform melting of the molecule.

Circular Dichroism. The circular dichroism spectra obtained in a dilute solution at pH 4.5 have two bands, a weak positive one at 222 nm and a strong negative one at 198 nm, corresponding to those observed by Timasheff et al. (1967) on native collagen. The weaker positive band was used to monitor the denaturation at pH 3.3 and pH 6.4. The fractional change in band intensity vs. temperature is given in Figure 4. The transition temperature at pH 3.3 is found to be 38° and the width of the transition 3.8°, while the transition at pH 6.4 was 39° and somewhat broader. Included for purposes of comparison on this graph are the smoothed results of the ^{13}C NMR experiment.

The optical rotatory dispersion results are shown in Figure 5. The native acid soluble collagen contains the telopeptides which are believed to be nonhelical. Yet compared with ESC, the native collagen has 10% greater specific rotation. Apparently the structure of ESC has been modified and slightly denatured probably as a consequence of the proctase treatment.

Discussion

In our previous carbon-13 NMR analysis (Chien and Wise, 1973) of random coil gelatin the carbonyl resonance at 24.2 ppm, the shoulder (145.6 ppm) adjacent to the proline C^{δ} resonance, and the resonance at 151.5 ppm were left unassigned. These peaks take on a new significance in the light of the results reported by Torchia et al. (Torchia et al., 1975). Model polypeptide studies by these researchers indicate that the resonance at 24.2 ppm is probably due to the Gly residues which precede Pro residues, the shoulder at 145.6 ppm to C^{α} carbons of Ala residues which precede Hyp residues, and the 151.5 ppm peak to the C^{α} carbons of Gly residues which precede Pro residues. Examination of the $\alpha 1$ chain of calf skin collagen indicates that 34% of the glycl residues are followed by prolyl residues, and that 15% of the alanyl residues are followed by hydroxyprolyl residues.

^{13}C NMR contains information about the percentage of X-Pro and X-Hyp bonds in trans conformations in the random coil state. The small resonance at 125.1 ppm is assigned to the Hyp C^{γ} carbons in cis X-Hyp peptide bonds (Tochia and Lyerla, 1974). The intensity of this resonance shows that ≤13% of the X-Hyp bonds are cis, and the strong resonance at 168.7 ppm shows that at least 95% of the Gly-Pro bonds are trans. The C^{δ}-Pro cis is known to resonate at 170-170.5 ppm (Dorman and Bovey, 1973) and therefore has nearly the same chemical shift as C^{δ}-Leu and C^{γ}-Lys; the doublet at 171.2 ppm is well accounted for by these two types of carbon atoms and thus provides no evidence for a significant number of cis Gly-Pro peptide bonds. If 15% of the Gly-Pro bonds were cis a resonance of nearly the same intensity as the separate members of the doublet should be observed. Thus it is concluded that trans X-Pro and X-Hyp bonds predominate in the coil form. Assuming that all prolines and hydroxyprolines are in the

Table II: ^{13}C Resonance in ESC.

Chemical Shift	Assignment[a]	Residue Abundance[b] per 1000 Residues
123.3	Hyp C^{γ}	95.2
131.8	Pro C^{α}	119
133.7	Ile C^{α}	13.5
	Thr C^{α}	18.2
	Val C^{α}	20.1
134.3	Hyp C^{α}	95.2
	Ser C^{α}	18.2
	Tyr C^{α}	48.2
	Phe C^{α}	12.0
	Hyp C^{δ}	95.2
	Gln C^{α}	
	Met C^{α}	6.3
137.8	Arg C^{α}	44.8
	Glu C^{α}	71.0
	Lys C^{α}	26.1
	His C^{α}	3.7
143.3	Ala C^{α}	112.5
145.6	Ala C^{α}(Ala-Hyp)[d]	
146.1	Pro C^{δ}	119
150.5	Gly C^{α}	337
151.1	Gly C^{α}(Gly-Pro)[d]	
152.4	Arg C^{δ}	44.8
	Phe C^{β}	12.0
	Asp C^{β}	48.1
156.1	Ile C^{β}	13.5
	Tyr C^{β}	3.3
	Hyp C^{β}	95.2
	Asn C^{β}	
163.8	Met C^{γ}	6.3
	Pro C^{β}	119
165.1	Glu C^{β}	71.0
	Arg C^{β}	44.8
	Leu C^{γ}	25.2
168.7	Pro C^{γ}	119
	Arg C^{γ}	44.8
176.5	Ala C^{β}	112.5

[a] Chien and Wise, 1973. [b] Chien and Chang, 1972. [c] This work. [d] Resonance for the carbon in this particular sequence.

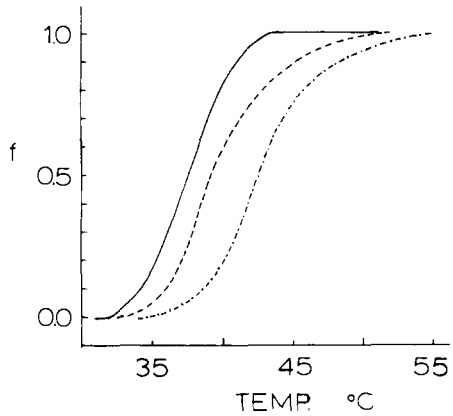

FIGURE 4: Comparison of fractional conversions to random coil measured by CD and ^{13}C NMR; (—) CD at pH 3.3, (- - -) CD at pH 6.4, and (- · - · -) ^{13}C NMR.

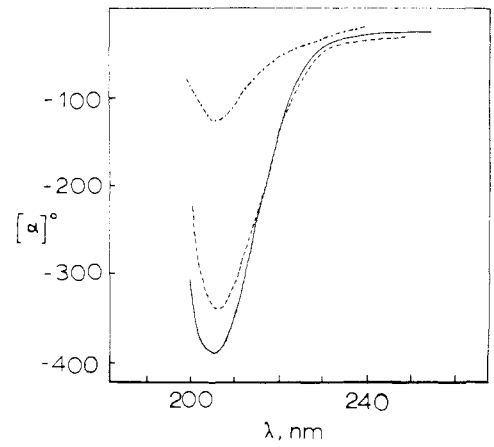

FIGURE 5: Optical rotatory dispersion of (—) native acid soluble collagen; (- - - -) ESC; (- · - ·) denatured ESC.

trans conformation in native collagen then it follows from the discussion above that isomerization of the glycyl–prolyl peptide bonds does not occur in collagen denaturation under our experimental conditions.

We have attempted to quantify the extent of unwinding at each temperature through the transition region by measuring the areas under Gly C^{α} and Pro C^{γ} resonances and comparing them to a resonance containing an invariant number of carbon atoms. For such a procedure to yield valid data it is necessary that the ratio of Overhauser enhancements for the poly(ethylene oxide) and denatured collagen resonances remain the same throughout the transition. The conditions for this to be true are either that τ_{CH} is the same for PEO and collagen or that $0.1 < \omega_C \tau_{CH} < 1.0$ (vide supra). The studies of Allerhand and Oldfield (1973) of the NOE of helical and random coil states of poly(γ-benzyl-L-glutamate) are relevant to this point. In that system an NOE of only 10% for the α carbons of the helical species which increased to 100% intensity enhancement when going to the random coil configuration implied an approach to the extreme narrowing condition. the situation in collagen seems favorable; here the relatively narrower lines (ca. 18 Hz) for both the poly(ethylene oxide) and glycine suggest the correlation times for these residues are already receiving substantial contributions from segmental motion even in the initial stage (38°) of denaturation (no lines are observed for triple helix collagen). Furthermore, as the resonances narrow with increasing temperature at approximately the same rate the NOE's can be expected to maintain comparable ratios through the transition region. Thus the integrated intensity measurements are expected to reflect the increase in random coil content. The melting curve shapes shown in Figure 2 are characteristic of those obtained by other methods, indicating a monophasic and cooperative transition.

The transition width of 5 ± 1° found by ^{13}C NMR for ESC is significantly greater than $\Delta T = 2 \pm 0.5°$ reported by von Hippel and Wang (1963) for native collagen using polarimetric measurements on dilute solutions. To find out whether concentration and viscosity tend to influence the collagen–gelatin transition, CD measurements were made on dilute solutions of ESC. The results in Figure 4 showed two discernible differences between CD and ^{13}C NMR measurements. The T_m observed with CD is 38°. The lower T_m is expected in the light of the experiments of Delisi and Shamos (1972) who showed that T_m of bovine achilles tendon increased as its water content decreased. The lower T_m seen in the CD experiments is thus regarded as the low concentration limit of the denaturation process seen in the ^{13}C NMR experiments. The transformation was also studied at a higher pH (6.4). Basically the same kind of behavior was seen, except T_m is raised and ΔT is increased. The pH 6.4 conditions correspond more closely to the ^{13}C NMR conditions, since at this pH collagen tends to aggregate and precipitate.

The transition width for the homogeneous dilute solution of ESC as measured by CD is 4° which is narrower than the ^{13}C NMR results. It is still greater than the 2° reported for native collagen. Theerefore notwithstanding the effect of concentration in broadening ΔT, denaturation of ESC is definitely less cooperative than the native collagen. This suggests that ESC does not possess the same structural features as native collagen. Either the mild enzyme treatment has affected the perfect interchain alignment necessary to achieve the native structure or the telopeptide is essential in sustaining the highly cooperative native structure. Studies are underway in this laboratory aimed at determining the role of the telopeptide toward preserving the cooperativity of the melting.

If the transformation of collagen is assumed to be reversible (Flory and Weaver, 1960), then we may formulate the conditions for equilibrium assuming the process to be

$$3S_1 \rightleftharpoons S_3 \qquad (1)$$

where S_1 designates a single strand of collagen and S_3 the three-stranded complex. For simplicity the case where no unzipping of the triple helix is allowed (Sćhwarz and Poland, 1974) was chosen as a model for analyzing the data. Under this condition the equilibrium constant K can be written in terms of two parameters: a factor β, which is the equilibrium constant for localizing three independent chains such that the equilibrium can be initiated, and a factor S, which is the equilibrium constant for adding a (Gly-X-Y) tripeptide unit from each of the three chains to the triple helix

$$K = \beta S^n = \theta / 3C^2(1 - \theta)^3 \qquad (2)$$

In eq 2, n is the number of tripeptide segments of a single chain (n = 333 for collagen), θ is the net fraction of helix, and C is the total concentration of single strands in any form. The enthalpy change of the transition may be determined from a plot of ln θ vs. $1/T$ since

$$\left(\frac{\partial \ln \theta}{\partial 1/T}\right)_C = \left(-\frac{\Delta H_0}{R} - \frac{n\Delta H_u}{R}\right)\left(\frac{1 - \theta}{1 + 2\theta}\right) \qquad (3)$$

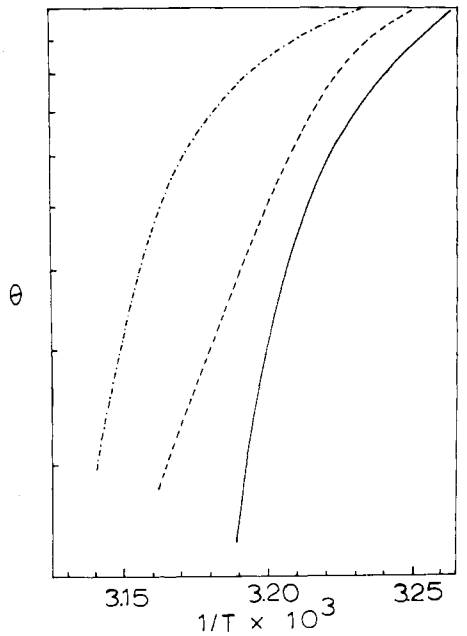

FIGURE 6: Logarithm of θ vs. reciprocal T for CD and ^{13}C NMR; (—) CD at pH 3.3; (- - -) CD at pH 6.4; and (- · - ·) ^{13}C NMR.

where ΔH_0 is the enthalpy required to localize three independent chains such that the triple helix can be initiated, ΔH_u is the enthalpy of unzipping three tripeptide segments of a triple helix, and n is the number of cooperative tripeptide units on each of the three chains. Values of ln θ, obtained from Figure 4, were plotted vs. the reciprocals of the absolute temperatures (Figure 6). The enthalpy changes at $\theta = 0.5$ were computed from the slopes of these curves to give values of $\Delta H_0 + n\Delta H_u$ between -2.0×10^5 and -2.4×10^5 cal/mol. These values agree well with those reported by Burge and Hynes (-2.0 to -2.50×10^5 cal/mol) but differ substantially from the calorimetric value of Privalov (-6.1×10^6 cal/mol) (1969).

The discrepancy between NMR and CD results on the one hand and calorimetric results on the other indicates that the transition is not a two-state process. The number of tripeptide units involved in the transition may be determined if we take the enthalpy of unzipping to be

$$\Delta H_u = 9[(1 - \alpha)\Delta H_a + \Delta H_i] \quad (4)$$

where α is the fraction of imino acids and the subscripts a and i refer respectively to amino and imino acid residues. The values determined for these parameters (Schwarz and Poland, 1974) are

$$\Delta H_a = -80.6 \text{ cal/mol}$$
$$\Delta H_i = -1188 \text{ cal/mol} \quad (5)$$

When these values are substituted into eq 4, along with $\alpha = 0.214$ for ESC, the enthalpy of unzipping is found to be -2.82×10^3 cal/nonet of residues. Thus the enthalpy of melting found in our experiments implies between 70 and 85 tripeptide units are involved in the transition. In this computation ΔH_0 was taken to be zero. This procedure probably establishes an upper limit for the number of tripeptide units involved since ΔH_u appears to be too small to account for either the calorimetric or the polymer melting enthalpy change.

In summary, a more detailed understanding of the thermal denaturation of enzyme soluble collagen has been obtained from ^{13}C NMR and CD studies. Both techniques support the conclusion that ESC does not melt as cooperatively as native collagen. Based on the examination of the ^{13}C resonances of glycine, proline, hydroxyproline, and alanine the melting is found to be monophasic and cooperative. ^{13}C NMR demonstrates that in the random coil state the X-Pro and X-Hyp bonds are predominantly in the trans conformation. All the residues appear to be involved in the unfolding process concurrently. Finally the number of tripeptide units involved the melting of ESC was estimated. The number suggests that the decrease in cooperativeness of ESC results from the alteration of the structure and the removal of telopeptides.

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CORRECTIONS

"Hydrogen Bonding in Derivatives of Adenosine and Uridine", by Josef Pitha, Volume 9, Number 19, 1970, pages 3678–3683.

The weak band at 3245 cm^{-1} observed in the solution of O^2-ethyl-2′,3′-*O*-isopropylideneuridine is due to an impurity or decomposition by solvent. It is not due to intramolecular hydrogen bonding as originally stated. Thus there is no evidence for the syn conformation in this compound. The conclusions drawn on other compounds and the comparison of the above compound and of 2′,3′-*O*-isopropylideneuridine are not affected.

"Comparative Properties of Rat Liver and Sea Urchin Eggs *S*-Adenosyl-L-methionine Decarboxylase", by Carol-Ann Manen and Diane H. Russell,* Volume 13, Number 23, 1974, pages 4729–4735.

Page 4731: the enzyme unit should read 1 pmol of CO_2 liberated per minute, rather than 1 μmol.

"Isolation and Properties of Two Biologically Active Fragments from Limited Tryptic Hydrolysis of Bovine and Ovine Pituitary Growth Hormones", by László Gráf and Choh Hao Li,* Volume 13, Number 26, 1974, pages 5408–5415.

Page 5408: line 6 of the main text should read BGH, not BG′. Page 5413: in Table VIII, the response for RB2b′ at a total dose of 200 μg should read 199, not 119.

"Calorimetric Analysis of Aspartate Transcarbamylase from *Escherichia coli:* Binding of Cytosine 5′-Triphosphate and Adenosine 5′-Triphosphate", by Norma M. Allewell,* Joan Friedland, and Karl Niekamp, Volume 14, Number 2, 1975, pages 224–230.

Page 225: the first sentence in the second paragraph of the Materials and Methods section should read: The concentrations of ATP and CTP.

Page 226, second column, lines 18 and 22: change model 2 to model 1.

Page 227, line 1: change models 2 and 3 to models 1 and 2.

Page 228, second column: the first sentence should read: If ATP .... proposed, our data imply that the overall differences in enthalpy and entropy between the two conformations are small.

"Neutral and Cationic Sulfonamido Derivatives of the Fluorescent Probe 2-*p*-Toluidinylnaphthalene-6-sulfonate. Properties and Mechanistic Implications", by Frank C. Greene, Volume 14, Number 4, 1975, pages 747–753.

Solvent No. 1 in Table I should be methanol–water (3:1, v/v), instead of water–methanol (3:1, v/v).

"Kinetic Studies on Coenzyme Binding and Coenzyme Dissociation in Tryptophanase Immobilized on Sepharose", by Sei-ichiro Ikeda, Yutaka Sumi, and Saburo Fujui,* Volume 14, Number 7, 1975, pages 1464–1470.

The legend for Figure 1B is incorrect. It should read: Apparent K_m of immobilized tryptophanase for Trp . . .