The Collagen ⇄ Gelatin Phase Transition. II. Shape of the Melting Curves and Effect of Chain Length*

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The shapes of optical rotatory equilibrium melting curves obtained for the collagen-fold ⇄ gelatin transition under a variety of conditions are examined in terms of three parameters: $\Delta[\alpha]$, T_m, and ΔT. It is shown that $\Delta[\alpha]$, the total change in rotation (corrected for solvent effects) across the transition, can be used as a measure of the helix content of the ordered structure. T_m, the temperature at the mid-point of the transition, measures the over-all stability of the ordered phase, and ΔT, the difference in temperature between the points at which the transition is one-fourth and three-fourths complete, defines the sharpness (or "degree of cooperativeness") of the transition. It is shown that the collagen-fold which develops on cooling primarily single-chain gelatin, either in the presence of a wide variety of added electrolytes and nonelectrolytes or after partial degradation with proteolytic enzymes, is invariably characterized by a value of $\Delta T = 7 \pm 1°$, and a maximum $\Delta[\alpha]_{313} \simeq 1000°$ (equivalent to a collagen-type helix content of ~70%). The melting behavior of native collagen (and cross-linked γ-type gelatin) is quite different; under all conditions $\Delta T = 2 \pm 0.5°$ and $\Delta[\alpha]_{313} \simeq 1400°$ (100% helix by definition). Helix formation in gelatin is also studied as a function of molecular weight, using ichthyocol gelatin chains that have been partially degraded with collagenase, trypsin, and hydroxylamine, and it is shown that a minimum chain length of 40–80 residues is required to generate stable elements of the collagen-type helix. These results are considered in terms of current views of the structure of the collagen molecule, and it is concluded that in primarily single-chain gelatin the transition is dominated by factors involved in *intra*chain stabilization, and that the "cooperative units" in this material are probably "interband" segments of the gelatin chains.

In preceding papers (von Hippel and Wong, 1962, 1963) we reported the results of a series of experiments on the effects of electrolytes, nonelectrolytes, temperature, and imino-acid content on the stability and rate of formation of the collagen-type helix. In this paper we examine the actual shapes of the collagen ⇄ gelatin melting curves and the effects of reducing the length of the gelatin chains, in an effort to obtain further insight into the nature and interactions of the forces involved in forming and stabilizing the collagen structure.

MATERIALS AND METHODS

A number of the methods employed in this investigation, including the preparation and purification of collagen and gelatin, measurement of protein concentration, polarimetric measurement of equilibrium melting curves and initial rates of helix formation, etc., have been described in detail in previous papers (von Hippel and Wong, 1962, 1963). In addition, the following materials and techniques were used:

Enzymes and Inhibitors.—The collagenase used in this study, isolated from cultures of *Cl. histolyticum* and purified as described by Gallop *et al.* (1957), was kindly provided by Dr. Paul M. Gallop of the Albert Einstein College of Medicine. Trypsin (2 × crystallized, salt-free) and soybean trypsin inhibitor (crystalline) were purchased from Worthington Biochemical Corp. (Freehold, N. J.) and used without further purification.

pH-Stat and "De-esterification" Measurements.—The hydrolysis of peptide bonds in gelatin chains by trypsin and collagenase was followed by *p*H-stat methods, using a Radiometer automatic titrator (TTTlb) and titrigraph (SBR2C). Reactions were run in a thermostated cell (± 0.05°) and temperatures were measured directly in the cell by means of a thermistor arrangement. A constant stream of prepurified nitrogen (bubbled through NaOH and H_2SO_4 and then saturated with water at the temperature of the experiment) was passed over the solution to exclude CO_2. Before adding enzyme, the system was always incubated for a short time to test the efficacy of this procedure and to establish a base line of NaOH uptake in the absence of enzyme.

After the reaction had proceeded to the desired extent the solution was quickly withdrawn from the cell with a cold syringe, and the enzyme was inactivated by expelling the solution into a tube heated to 80° (collagenase) or into a tube containing a 3-fold excess of soybean trypsin inhibitor (trypsin). Model experiments were run in the *p*H-stat to show that these methods actually inactivated the enzymes instantly. The heating step precipitated the collagenase as insoluble aggregates, which were then removed by centrifugation.

The "ester-bonds" in gelatin were broken by treatment with 1 M hydroxylamine-HCl, *p*H 8, at 40° for various lengths of time. The excess hydroxylamine was removed by dialysis in the cold, and the number of bonds broken was determined by measuring protein-bound hydroxamate, using a modification of the procedure of Bergmann and Segal (1956) described by Seifter *et al.* (1960).

Sedimentation Equilibrium.—Molecular weights of the degraded gelatin fractions were determined by sedimentation equilibrium techniques, using a Spinco Model E analytical ultracentrifuge. For these measurements the schlieren optical system of the ultracentrifuge was modified by substituting an 0.005-inch diameter platinum wire for the phase plate supplied

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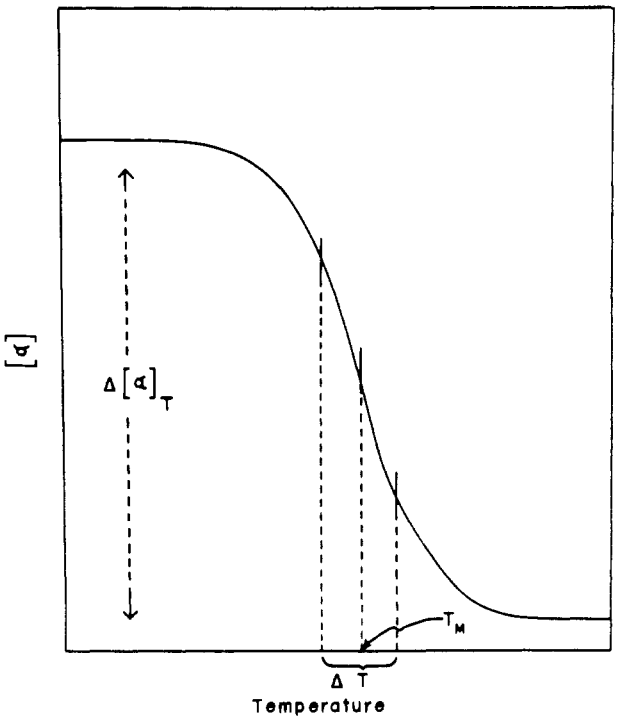

FIG. 1.—Schematic representation of a phase transition showing the parameters $\Delta[\alpha]_T$, T_m, and ΔT (see text).

with the instrument. We found after trying a series of different phase plates that asymmetries in the distribution of fringes on either side of the central fringe, resulting from imperfections in the phase plate, made it very difficult to locate the center of the fringe system in an unambiguous fashion. This difficulty was circumvented by using a wire as the schlieren diaphragm. Experiments with wires of varying thickness showed that a fringe system of minimum width is obtained with an 0.005-inch wire.

Experiments were run in standard Spinco double-sector cells or in the multichannel cells designed by Yphantis (1960), using columns ranging in height from 0.6–2.0 mm. The photographic plates were measured using a Bausch and Lomb two-dimensional comparator. Weight-average molecular weights were determined by the method of Yphantis (1960) and *Z*-average molecular weights by method II of van Holde and Baldwin (1958).

RESULTS AND DISCUSSION

There are a number of parameters, useful in characterizing a phase transition, which may be derived from a plot of specific rotation versus temperature. Three such parameters are indicated on the phase transition diagramed in Figure 1, and may be defined as follows: T_m = the temperature of the mid-point of the transition. This temperature serves as a measure of the stability of the helical structure in a given solvent environment. $\Delta[\alpha]_T$ = the difference between the specific rotation at the high and the low temperature ends of the transition, corrected for solvent and temperature effects. This quantity is proportional to the number of residues which pass from a helical to a random coil conformation over the temperature interval under consideration. ΔT = the difference in temperature between the points at which the helix-coil transition is one-fourth and three-fourths complete. This temperature difference, which is directly related to the "sharpness" of the phase transition, serves as a convenient measure of the "degree of cooperativeness" of the helical structure. Before proceeding to a consideration of the effects on the phase transition of various modifications of the polypeptide chains and the solvent environment, we summarize the available evidence bearing on the interpretation of these parameters as applied to the collagen-gelatin system.

T_m.—In previous publications (von Hippel and Wong, 1962, 1963) we have shown that the value of T_m, which characterizes the melting of either native collagen or the collagen-fold re-formed from random-coil gelatin by cooling, is linearly dependent on the concentration of various electrolytes and nonelectrolytes added to the solution. In other words, in a three-component system consisting of solvent, a solute capable of undergoing a helix-coil transition, and a third component, the entire transition is shifted horizontally along the temperature axis to an extent that is directly proportional to the amount of the third component added. The proportionality constant which applies to any particular third component serves as a measure of the extent to which that component interacts with the solute (or solvent) to alter the stability of the helical phase. Such a linear relationship between T_m and the concentration of a third component has been predicted on theoretical grounds by Peller (1959) and also has been shown for related parameters such as the temperature of melting of gelatin gels (Bello *et al.*, 1956) and the shrinkage temperature of collagen fibers (Flory and Garrett, 1958).

$\Delta[\alpha]_T$.—A number of authors (e.g., Schellman and Schellman, 1961; Urnes and Doty, 1961) have pointed out the danger inherent in using the change in specific rotation at a single wavelength as a measure of the helix content of a particular protein without further information on the rotatory dispersion of that system in both the helical and the random coil form.

The situation with collagen and gelatin fortunately appears to be quite straightforward, and the use of the change in specific rotation across the transition, appropriately corrected for solvent and temperature effects (see below), seems to be a valid measure of helix content. The argument may be summarized as follows: The individual chains of the native collagen structure occur as helices characterized by a left-handed, 3-fold screw axis with three residues per turn and a pitch of approximately 9 A. In collagen these helices are

TABLE I

OPTICAL ROTATORY PROPERTIES OF ICHTHYOCOL COLLAGEN AND GELATIN

Conditions	$[\alpha]_{589}$	λ_c	a_c	a_0	b_0
Gelatin, 40°, 0.5 M $CaCl_2$	−116°	214 mμ	−770°	−550°	−20°
Gelatin, 40°, 0.025 M $CaCl_2$	−122	213	−810	−590	−20
Gelatin, 0 min at 8°, 0.025 M $CaCl_2$[a]	−128	212	−860	−620	0
Gelatin, 10 min at 8°, 0.025 M $CaCl_2$[a]	−177	205	−1290	−860	+50
Gelatin, 24 hr at 8°, 0.025 M $CaCl_2$[a]	−320	203	−2370	−1490	+100
Collagen, 8°, 0.5 M $CaCl_2$	−360	202	−2710	−1780	+160
Poly-L-proline II, 25°, 0.1 M KCl[b]	−540	202	−4060	−2840	+250

[a] Time after quenching a random coil solution of gelatin from 40° to 8°. [b] Calculated from Harrington and Sela (1958).

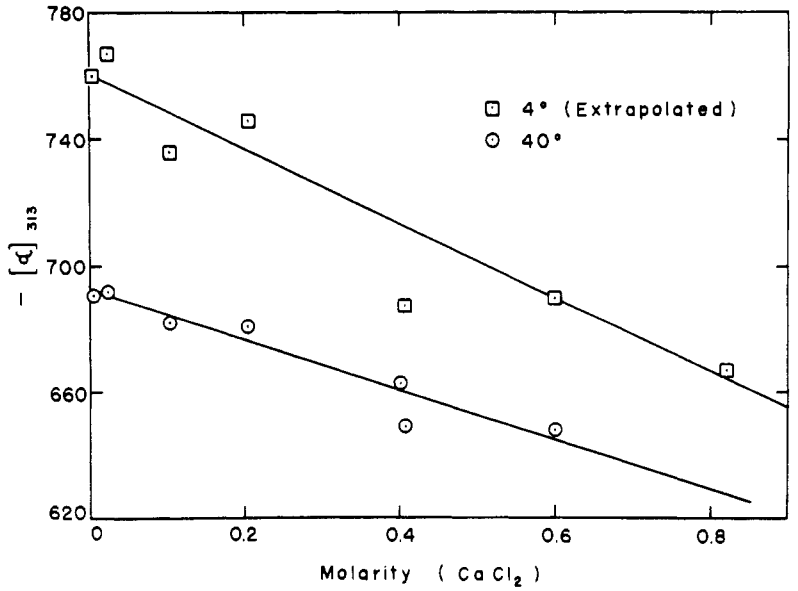

FIG. 2.—Specific rotation of random coil ichthyocol gelatin as a function of $CaCl_2$ concentration. O, 40°; □, 4°. Gelatin conl.c. = 6 mg/ml; *p*H 7.

further deformed into a very gradually coiling right-handed superhelix involving groups of three chains, but the effects of this deformation on the optical rotatory properties of the structure are relatively marginal. Cowan *et al.* (1955) showed that the individual chains of collagen have the same backbone conformation as that exhibited by poly-L-proline in the so-called poly-L-proline II form (Cowan and McGavin, 1955). Harrington and Sela (1958) then showed that poly-L-proline exists in aqueous solution as poly-L-proline II, and that this conformation is characterized by a linear Drude plot with λ_c and $[\alpha]_{589}$ values as shown in Table I. Subsequently Harrington (1958) pointed out that the rotatory dispersion properties of collagen are very similar to those of poly-L-proline II, and that the difference in specific rotation between either of these materials in the helical form and the mean residue rotation of its constituent amino acids is approximately the same. For both, $\Delta[\alpha]_{589} \simeq -250°$ (and $\Delta[\alpha]_{366} \simeq -760°$). Furthermore, when transferred from conditions favoring the random coil to those favoring the formation of the helix, both gelatin and poly-L-proline mutarotate toward rotatory parameters characteristic of collagen and poly-L-proline II, respectively. The various lines of evidence which indicate that random coil gelatin mutarotates to a conformation having many of the characteristics of the collagen-fold (or poly-L-proline II helix) have been summarized elsewhere (von Hippel and Harrington, 1960; Harrington and von Hippel, 1961b).

In Figure 2 we present data illustrating the nature and magnitude of the solvent and temperature effects which must be considered in evaluating $\Delta[\alpha]_T$; the specific rotation (at 313 mμ), measured at 40° and at 4° for random coil ichthyocol gelatin, is plotted as a function of $CaCl_2$ concentration. Since 4° is well below the helix-coil transition for ichthyocol gelatin at these salt concentrations, these rotations were obtained by plotting $[\alpha]_{313}$ vs. time after "quenching" a gelatin solution from 40° to 4° and extrapolating back to zero time (see von Hippel and Wong, 1962). Figure 2 shows that $[\alpha]_{313}$ for random coil gelatin is approximately 60° more negative at a temperature of 4° than at 40° at the same salt concentration, and therefore measured values of $\Delta[\alpha]_T$ (Fig. 1) must be corrected for this factor. It is interesting to note that this change in rotation with temperature, corresponding to about +0.25°/° at 589 mμ, is closely similar to the values of +0.25 to +0.30°/° which have been obtained with oxidized ribonuclease (Harrington and Schellman, 1956), clupein (Schellman, 1958), and ribonuclease, chymotrypsinogen, and α-chymotrypsin in 8 M urea (Foss and Schellman, 1959; Schellman, 1958). Thus in terms of flexibility as reflected in the temperature

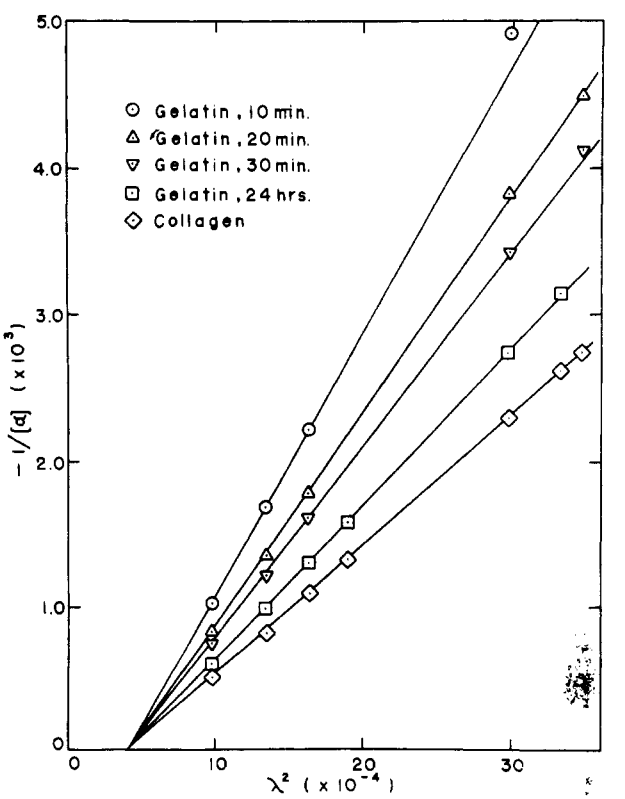

FIG. 3.—Rotary dispersion of ichthyocol gelatin and collagen at various stages of formation of the poly-L-proline II-type helix. Times after quenching a 1.2 mg/ml solution of ichthyocol gelatin (0.025M $CaCl_2$, *p*H 7) to 8°: O, 10 min; Δ, 20 min; ▽, 30 min; □, 24 hr; ◇, collagen, 1.4 mg/ml in 0.5 M $CaCl_2$.

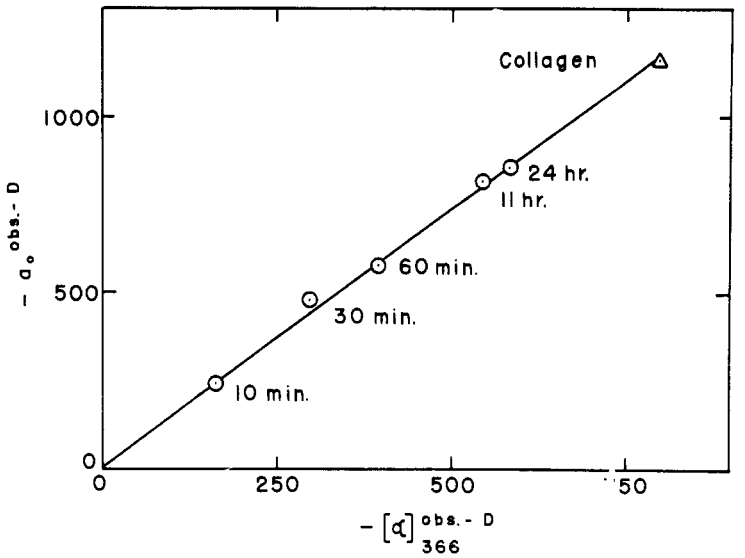

FIG. 4.—Plot of $-a_0^{obs-D}$ versus $-[\alpha]_{366}^{obs-D}$ (see text.) Times represent mutarotation time at 8° (see Fig. 3).

dependence of rotation, random coil gelatin resembles closely other proteins in the random coil state.

The rotatory dispersion of collagen and gelatin is of a particularly simple type (e.g., see Cohen, 1955; Harrington, 1958). The data obtained between 300 and 600 mμ can be represented by a single-term Drude equation such as the following:

$$[\alpha]_\lambda = \frac{a_c\lambda_c^2}{\lambda^2 - \lambda_c^2} \qquad (1)$$

where λ is the wavelength at which the measurement is made, $[\alpha]_\lambda$ is the specific rotation at that wavelength and, a_c and λ_c are the constants obtained by fitting the data to this equation. Furthermore, λ_c for both the helical and the random coil form of this protein are essentially the same. Values of λ_c and a_c obtained both by plotting λ^2 against $1/[\alpha]_\lambda$ (Lowry, 1935) and $\lambda^2[\alpha]_\lambda$ against $[\alpha]_\lambda$ (Yang and Doty, 1957) are compiled in Table I for collagen, random coil gelatin, and gelatin containing varying amounts of helix. This table shows that λ_c, at a *constant temperature*, is essentially the same for collagen and for gelatin in various stages of mutarotation (~203 mμ at 4° and ~213 mμ at 40°). λ_c does not appear to vary with salt concentration; $[\alpha]_\lambda$, on the other hand, does vary with both temperature and salt concentration as shown in Figure 2.

Figure 3 illustrates the rotatory dispersion behavior of gelatin in various stages of mutarotation, clearly demonstrating that the points pivot on a constant value of λ_c and that the slopes of the $-1/[\alpha]_\lambda$ vs. λ^2 lines decrease monotonically (and therefore $-a_c$ *increases* monotonically) with increasing helix content.

Both Schellman and Schellman (1961) and Urnes and Doty (1961) have pointed out that even for systems characterized by simple dispersion it is sometimes convenient to represent the data by the two-term equation proposed by Moffitt and Yang (1956) to deal with polypeptides exhibiting anomalous dispersion:

$$[m']_\lambda = \frac{a_0\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \qquad (2)$$

where a_0, b_0, and λ_0 are constants derived by fitting the data to equation (2) and $[m']_\lambda$ is the reduced mean residue rotation defined as follows:

$$[m']_\lambda = \left(\frac{3}{n^2 + 2}\right)\frac{MRW}{100}[\alpha]_\lambda \qquad (3)$$

(n = the refractive index of the solvent at wavelength λ, and MRW = the mean residue weight.)

Linear Moffitt-Yang plots for both collagen and gelatin in various stages of mutarotation were obtained by setting $\lambda_0 = 212$ mμ. Since this value of λ_0 differs somewhat from λ_c (especially at 4°), nonzero values of b_0 result even though the system shows simple dispersion. Values of a_0 and b_0 obtained with the various forms of collagen and gelatin are collected in Table I. Clearly increasing poly-L-proline II helix content is associated with a large change toward more *negative* values of a_0, and a qualitative trend toward more *positive* b_0 values.

It is well known that the formation of a right-handed α-helix from a random coil is accompanied by a progressive decrease in b_0 from approximately 0 to about −630° (see Yang, 1961; Schellman and Schellman, 1961; Urnes and Doty, 1961). Furthermore, b_0 provides a useful direct measure of α-helical content since this quantity is relatively unaffected by changes in the solvent environment. However, for the collagen helix b_0 turns out to be relatively small (Table I) and the errors involved in determining it from the slope of a plot of

$$[m']_\lambda \frac{\lambda^2 - \lambda_0^2}{\lambda_0^2} \text{ vs. } \frac{\lambda_0^2}{\lambda^2 - \lambda_0^2}$$

are rather large. Therefore we have chosen to use either a_0 or $[\alpha]_\lambda$ as a measure of helix content. Since both of these parameters are sensitive to changes in temperature, solvent, etc., one must use $a_0^{obs} - a_0^D$ (or $[\alpha]_\lambda^{obs} - [\alpha]_\lambda^D$) where a_0^{obs} and $[\alpha]_\lambda^{obs}$ are the observed values and a_0^D and $[\alpha]_\lambda^D$ are the same parameters for the completely denatured (or random) form of the chain *under identical environmental conditions* (see Urnes and Doty, 1961). In Figure 4, $a_0^{obs} - a_0^D$ (written a_0^{obs-D}) is plotted against $[\alpha]_\lambda^{obs} - [\alpha]_\lambda^D$ (written $[\alpha]_\lambda^{obs-D}$). Clearly these quantities are directly related and the fraction of the residues which exist in the poly-L-proline II-type helix at any given time may be calculated from either a_0^{obs-D}/a_0^{H-D} or $[\alpha]_\lambda^{obs-D}/[\alpha]_\lambda^{H-D}$, where a_0^H (and $[\alpha]_\lambda^H$) apply to the 100% helical structure, which for present purposes we equate with native collagen. In the remainder of this paper we will use $\Delta[\alpha]_T$ (Fig. 1), after correction for solvent and temperature effects (Fig. 2), as a

TABLE II

OPTICAL ROTATORY PARAMETERS FOR THE COLLAGEN-FOLD → GELATIN TRANSITION IN ICHTHYOCOL GELATIN IN THE PRESENCE OF VARIOUS ELECTROLYTES[a]

Electrolyte		T_m (°C)	$(\Delta[\alpha]_{313})_T$	ΔT (°C)
$CaCl_2$	0.025 M	20.2	−910°	7.6
	0.15	18.9	−880	8.0
	0.30	17.2	−810	6.9
	0.50	16.4	−650	6.5
	0.70	14.1	−270	5.9
KSCN	0.08	18.4	−900	5.2
	0.50	14.2	−680	7.3
LiCl	0.48	18.4	−960	8.1
	2.4	11.2	−460	5.2
KCl	0.48	19.6	−940	6.7
	2.4	17.7	−790	7.4
NaCl	0.4	18.3	−840	6.6
	1.6	16.4	−760	6.3
CsCl	2.4	16.6	−840	6.5
KCH_3COO	0.20	21.3	−950	8.6
	1.4	20.4	−900	8.3
$(NH_4)_2SO_4$	0.40	21.8	−910	8.5
	0.64	22.8	−890	8.7
$(CH_3)_4NCl$	0.32	20.8	−900	7.7
	3.2	27.1	−810	11.2
$(CH_3)_4NBr$	2.0	18.8	−800	7.3
	1.0	18.4	−910	7.2
$(C_2H_5)_4NBr$	2.0	15.4	−740	6.9

[a] Melting began in all cases after 24 hr at 5°. All solutions contained 0.025 M $CaCl_2$ in addition to the salts listed. Protein concn., ~1 mg/ml, pH = 7.

measure of poly-L-proline II helix content. As defined, $\Delta[\alpha]_T$ is equivalent to $[\alpha]_\lambda^{obs-D}$.

ΔT.—In theory a phase transition is infinitely sharp; that is, it goes to completion over an infinitesimal temperature interval. However, it can be shown on theoretical grounds that a formally one-dimensional phase transition, such as the helix-coil transitions which occur in polypeptides and polynucleotides, must have a finite breadth (see Flory, 1961). Furthermore, a number of alterations in the system can broaden the transition still further. These include a decrease in ΔH_u, the enthalpy change accompanying the transfer of one chain unit from the helical to the random coil state, or a decrease in l, the length of the chain element which undergoes the helix-coil transition as a "cooperative unit." In fairly short chains this length generally corresponds to the entire molecule, though in very long chains both helical and random sections can coexist. Several excellent theoretical discussions of these matters are available (e.g., see Flory, 1961).

Zimm *et al.* (1959) have shown experimentally, using samples of poly-γ-benzyl-L-glutamate with $\bar{M}_w$ = 3350, 6000, and 194,000, that the breadth of the α-helix-coil transition depends markedly on the molecular weight (or, in the above terms, on the length of the "cooperative unit"). Furthermore, it is well known from studies of helix-coil transitions in DNA that the breadth of the transition in that system also depends on the length of the "cooperative unit"; native calf thymus DNA melts very sharply while heat-denatured and recooled calf thymus DNA, in which hydrogen bonds are reformed essentially at random and which seems to consist of very short helical segments separated by amorphous regions, melts over a very broad temperature interval (e.g., see Felsenfeld and Sandeen, 1962).

In what follows, ΔT (Fig. 1) will be used as a convenient measure of the "degree of cooperativeness" of the ordered regions which undergo melting in any particular phase transition.

Effects of Changes in Solvent Environment.—Table II summarizes values of T_m, $\Delta[\alpha]_T$, and ΔT obtained from equilibrium melting curves measured on dilute solutions of ichthyocol gelatin cooled for 24 hours at 5° in the presence of various electrolytes. The amount of helix (measured by $\Delta[\alpha]_T$) formed in these various salt solutions in this time varied markedly, since the rate of mutarotation is also a sensitive function of salt concentration (von Hippel and Wong, 1962). At equilibrium, the amount of helix regenerated seems to be independent of the molarity of the salt, but at high concentrations of salts, which are very effective in reducing the rate of helix formation, many days are required to attain equilibrium. Thus it should be clearly understood that the data compiled in Table II apply to the "equilibrium" melting of the amount of

TABLE III

OPTICAL ROTATORY PARAMETERS FOR THE COLLAGEN-FOLD → GELATIN TRANSITION IN ICHTHYOCOL, CALFSKIN, AND EARTHWORM CUTICLE GELATIN IN VARIOUS SOLVENT ENVIRONMENTS[a]

Gelatin	Solvent	T_m (°C)	$(\Delta[\alpha]_{313})_T$	ΔT (°C)
Ichthyocol	D_2O (in 0.025 M $CaCl_2$)			
	20%	20.4	−870°	6.7
	40%	21.2	−900	7.3
	60%	21.1	−900	7.9
	80%	22.8	−910	8.1
Ichthyocol	D_2O (in 0.5 M $CaCl_2$)			
	100%	20.0	−890	7.0
Calfskin	Urea (in 0.025 M $CaCl_2$)			
	1.25 M	25.2	−920	10.0
	3.0	21.0	−730	8.5
	4.0	18.6	−540	9.1
	6.0	17.3	−260	7.4
	6.5	15.5	−200	7.3
Calfskin	$CaCl_2$ 0.025 M	28.5	−950	9.9
	0.5	23.4	−880	9.9
Earthworm cuticle	$CaCl_2$ 0.005 M	9.2	−760	7.0
	0.025	8.7	−720	7.9

[a] Melting began after 24 hr at 5°, except urea data after 8–12 days at 5°.

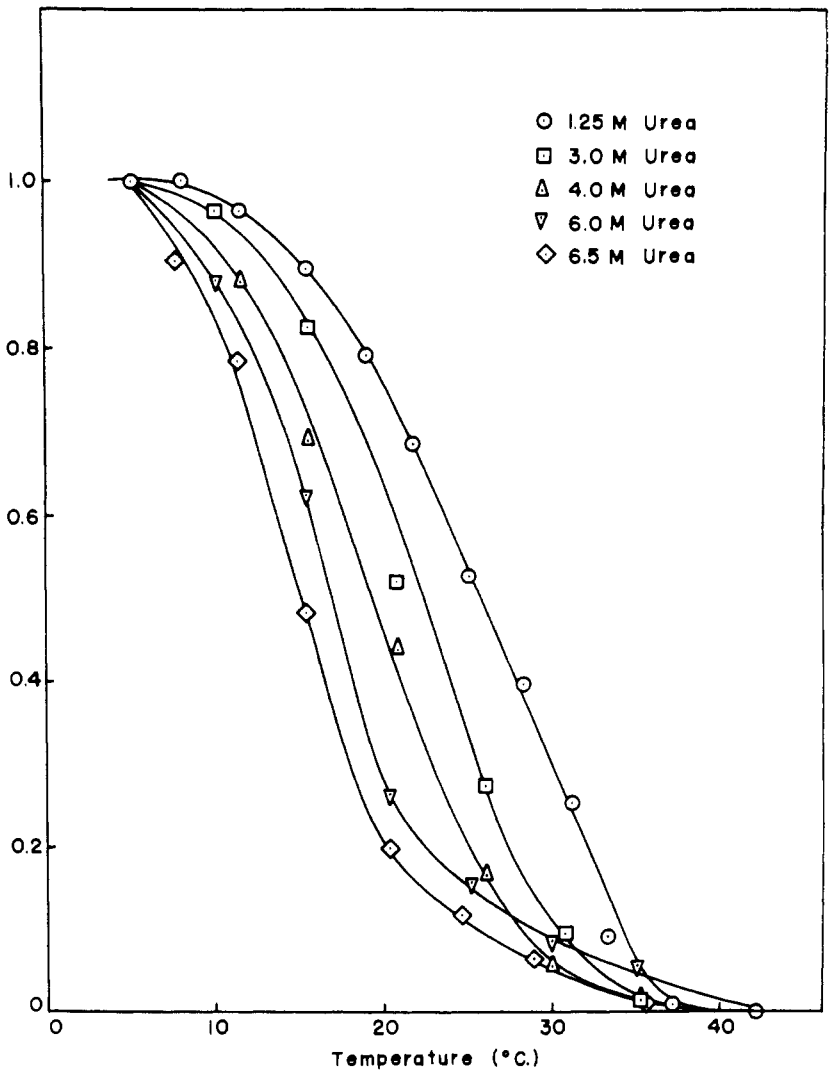

FIG. 5.—Melting curves for calfskin gelatin in various concentrations of urea, cooled at 5° for 7 days, $\Delta[\alpha]_T$ normalized to unity; 0.025 M $CaCl_2$, pH 7, 1.0 mg/ml gelatin and: O, 1.25 M urea; □, 3.0 M urea; △, 4.0 M urea; ▽, 6.0 M urea; ◇, 6.5 M urea.

helix which forms in 24 hours at 5°. Comparison of the largest values of $\Delta[\alpha]_T$ in Table II with $\Delta[\alpha]_T$ for the melting of native ichthyocol collagen (Table IV) shows that a maximum of 65–70% of the helical content of native collagen is regenerated in cooled gelatin under optimal conditions (in terms of $[\alpha]_\lambda^{obs-D}/[\alpha]_\lambda^{H-D}$, see above). The last column of Table II, in which we tabulate ΔT, the parameter which serves as a measure of the "sharpness" of the phase transition, shows that approximately the same value of ΔT (7.3 ± 0.9°) is obtained for all the transitions examined.

Table III presents similar data on collagen-fold → gelatin transitions with ichthyocol, calfskin, and earthworm cuticle gelatins dissolved in aqueous solutions containing various charged and uncharged additives (D_2O, urea, $CaCl_2$). These substances affect T_m in much the same way as the neutral salts (see von Hippel and Wong, 1962, 1963) and some, particularly elevated concentrations of urea, also depress markedly the amount of helix regenerated in a finite time. But again it should be noted (compare with Table IV) that the *maximum* helical content observed in cooled gelatin samples corresponds to about 70% of the values obtained with the corresponding collagens, and that again ΔT is approximately invariant under all these conditions, at 8.1 ± 0.9° (average of all the ΔT data in Table III). If one averages only the ichthyocol data (in various concentrations of D_2O) a value of 7.4 ± 0.5° is obtained, which is remarkably close to the average ΔT of 7.3 ± 0.9° found for ichthyocol in the presence of various salts.

Approximately invariant ΔT values suggest that the shape of at least the mid-portion of the phase transition is the same for all the melting curves examined. That this is approximately true for the entire transition, even when markedly different amounts of helical structure are being transformed, is shown in Figure 5 for the melting of cooled calfskin gelatin in the presence of various amounts of urea. In order to facilitate comparison all the curves have been normalized to a total $\Delta[\alpha]_T$ of unity, though the actual $(\Delta[\alpha]_{313})_T$ values varied between −920° and −200° (see Table III).

Table IV presents a similar analysis of transitions involving the melting of native soluble collagen (ichthyocol, calfskin, and earthworm cuticle). T_m values for these transitions are somewhat higher than those obtained in the same media with cooled gelatin samples, but vary in roughly the same manner with salt concentration (see von Hippel and Wong, 1963). As pointed out in connection with Tables II and III, the $\Delta[\alpha]_T$ values obtained with these collagen → gelatin transitions are about 35% larger than the maximum values measured on cooled gelatins.

However, the most striking difference between phase

TABLE IV

OPTICAL ROTATORY PARAMETERS FOR THE COLLAGEN → GELATIN TRANSITION IN ICHTHYOCOL, CALFSKIN, AND EARTHWORM CUTICLE COLLAGEN IN VARIOUS SOLVENT ENVIRONMENTS

Collagen	Solvent	T_m (°C)	$(\Delta[\alpha]_{313})_T$	ΔT (°C)
Ichthyocol	$CaCl_2$ 0.12 M	29.8	−1420°	1.3
	0.26	27.9	−1310	1.7
	0.50	23.8	−1230	2.2
	0.75	19.4	−1180	2.5
	1.00	14.4	−1100	2.5
Ichthyocol	0.4 M KSCN + 0.25 M $CaCl_2$	18.9	−1170	2.4
	1.0 M LiCl + 0.25 M $CaCl_2$	23.8	−1230	2.2
Ichthyocol	0.5 M $CaCl_2$ (79% D_2O)	26.5	−1330	2.2
Calfskin	$CaCl_2$ 0.26 M	34.7	−1360	1.9
	0.50	31.1	−1340	2.0
	0.70	27.2	−1300	1.7
	1.00	22.4	−1300	2.0
Calfskin	Citrate 0.15 M (*p*H 3.7)	33.8	−1320	2.8
Earthworm cuticle	$CaCl_2$ 0.26 M	15.5	−1180	3.7
	0.50	10.4	− 830	4.0

transitions which involve the melting of native collagens (Table IV) and those involving the melting of cooled gelatins (Tables II and III) lies in ΔT. Inspection of Table IV reveals that the values of ΔT obtained with native collagens are markedly smaller than those for cooled gelatins. In fact, an average ΔT of 2.1 ± 0.3° is obtained for the data on Table IV, which is approximately one-fourth the average ΔT measured with the cooled gelatins (see Tables II and III.)[1]

This dramatic difference in ΔT (and $\Delta[\alpha]_T$) between collagen and cooled gelatin is shown graphically (for two samples of ichthyocol collagen and gelatin in D_2O and H_2O) in Figure 6. It is interesting to note in this experiment (carried out with identical samples of protein diluted in the one case with D_2O and in the other with H_2O) that even though T_m for the D_2O samples is higher than T_m for the H_2O samples and thus presumably the over-all helical structure is more stable in D_2O, $\Delta[\alpha]_T$ for the D_2O-collagen sample is somewhat decreased. It would appear that during the exchange of deuterium for hydrogen which follows the change of solvent, a certain fraction of the helical content of the macromolecule was lost in this case.

Effect of Changes in Chain Length.—In addition to altering the size and position of the phase transition by manipulating the solvent environment, it also seemed that it might be revealing to examine the transition in lower molecular weight fractions of gelatin. Several ways of reducing the molecular weight were employed, including: (1) Tryptic digestion at 40°. This enzyme, which catalyzes the cleavage of peptide bonds on the carboxyl side of lysine and arginine, would be expected to attack the "band" regions of the gelatin chain which contain primarily the polar amino acids. (2) Collagenase digestion at 40°. This enzyme, with its unusual specificity requirements (e.g., see Gallop and Seifter, 1962; Grassmann *et al.*, 1962) attacks the gelatin chain only in the pyrrolidine-rich "interband" regions containing primarily the nonpolar amino (and imino) acid residues. (3) Collagenase digestion at 15°. The kinetics of collagenase digestion of gelatin and collagen are very different at temperatures above and below T_m (von Hippel and Harrington, 1959; von Hippel *et al.*, 1960) presumably in part because the preference of the enzyme for particular residue sequences within the limits of the over-all specificity depends on chain conformation. Thus one might expect that different bonds would be broken in a brief exposure to collagenase at 15° and 40°. (4) "De-esterification" with 1 M hydroxylamine at 40° and *p*H 8.0 (Gallop *et al.*, 1959; Blumenfeld and Gallop, 1962).

The enzymatic degradations were followed by use of a *p*H-stat method, and the de-esterification process by measuring protein-bound hydroxamate formation as described under Materials and Methods. The number of breaks per chain (and number-average molecular weights) were calculated from such data. The weight- and *Z*-average molecular weights of the reaction products were measured using short-column sedimentation equilibrium methods as described under Materials and Methods.

Data obtained from representative melting curves on gelatins degraded in various ways and then cooled at 5° for varying periods of time are assembled in Table V. Note that there is a slight decrease in T_m which accompanies the decrease in weight average molecular weight ($\bar{M}_w$) and that the amount of helix regenerated decreases markedly as the molecular weight is lowered. However, as before ΔT remains remarkably constant for all values of $\bar{M}_w$ and methods of poly-

TABLE V

MOLECULAR WEIGHT AND OPTICAL ROTATORY PARAMETERS FOR THE COLLAGEN-FOLD → GELATIN TRANSITION IN ICHTHYOCOL GELATIN AFTER CHAIN DEGRADATION[a]

Treatment	$\bar{M}_w$ (× 10^3)	$\bar{M}_z$ (× 10^3)	T_m (°C)	$(\Delta[\alpha]_{313})_T$	ΔT (°C)
Control	85.9	146	20.0	−915°	6.6
Trypsin (40°)	36.2	43	18.3	−830	6.7
	28.6	34			
	20.4	32	17.2	−570	6.5
	9.1		17.0	−320	5.9
	6.3	8.6	16.5	−180	5.9
Collagenase (40°)	42.3	53	18.1	−650	6.3
	25.2	45			
	13.2	25	16.9	−360	7.4
	6.9		16.2	−130	6.5
Collagenase (15°)	26.3		17.5	−560	7.9
	19.6	39	17.7	−440	8.0
	4.1	7.1			
De-esterified (hydroxylamine, 40°)	35		17.2	−750	7.4

[a] Melting experiments begun after cooling samples at 5° in 0.025 M $CaCl_2$ for varying periods ranging from 3 days to 4 weeks. Estimated uncertainty in molecular weight measurements: $\bar{M}_w$, ~ ± 5%; $\bar{M}_z$, ~ ± 10%.

[1] The average ΔT calculated from the data in Table IV does not include the values for earthworm cuticle collagen, which seem anomalously large. This material is particularly difficult to handle because of its extremely low melting temperature, and it seems not unlikely that it may have become partially denatured in the course of putting it into solution (see von Hippel and Wong, 1963).

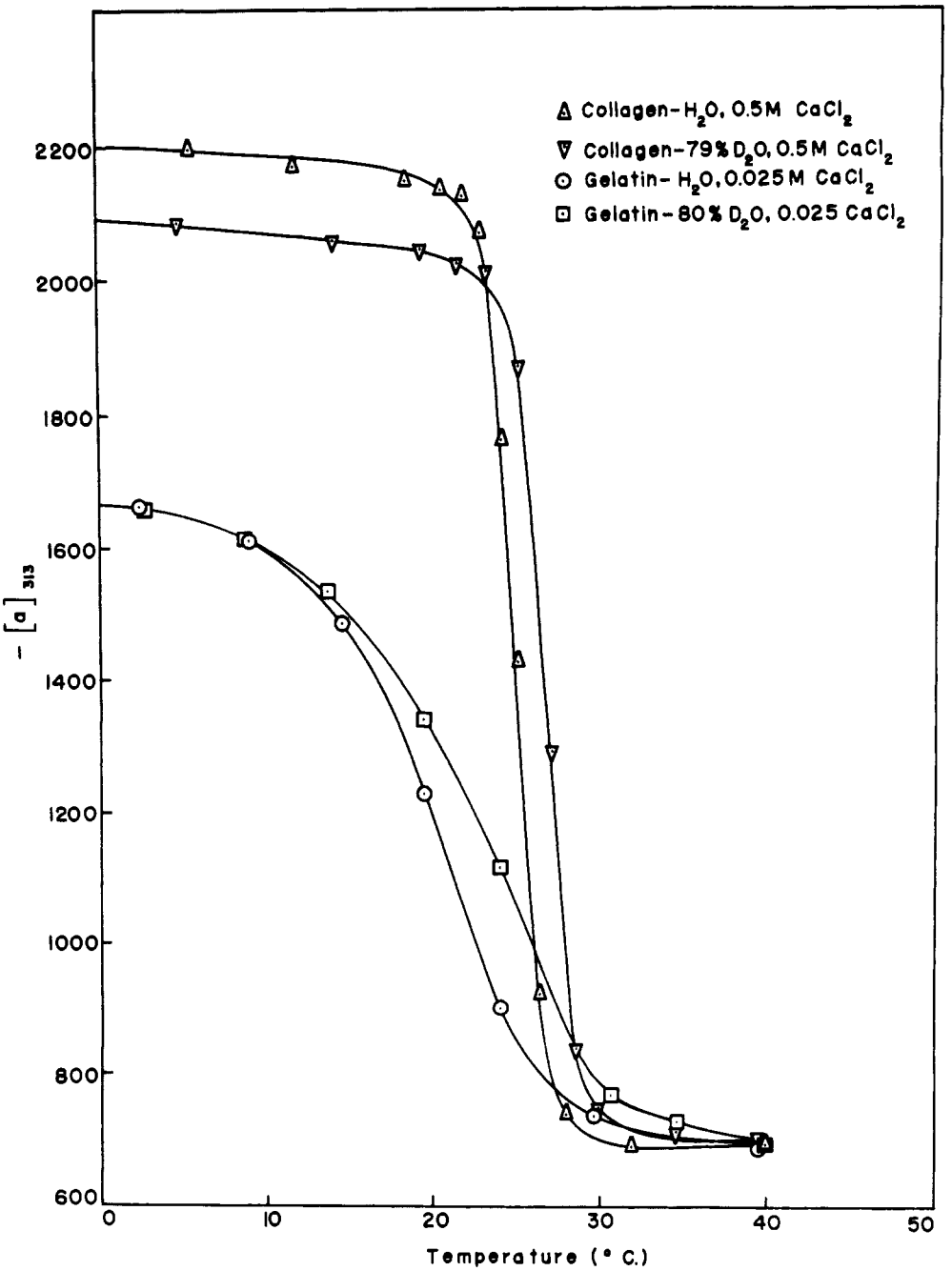

FIG. 6.—Melting curves for ichthyocol collagen and gelatin. Protein conc. ≃ 1.0 mg/ml, *p*H 7: Δ, collagen, 0.5 M $CaCl_2$ in H_2O; ▽, collagen, 0.5 M $CaCl_2$ in 79% D_2O; ○, gelatin, 0.025 M $CaCl_2$ in H_2O, cooled at 5° for 24 hr; □, gelatin, 0.025 M $CaCl_2$ in 80% D_2O, cooled at 5° for 24 hr.

peptide chain breakage (average ΔT for all data in Table V is 6.8 ± 0.6°), and very close to the values of ΔT obtained for cooled gelatin in various solvent environments (Table II and III). Figure 7a shows the actual transitions observed with the fractions degraded with trypsin, and in Figure 7b these data are normalized to a helix content ($\Delta[\alpha]_T$) of unity to facilitate direct comparison of the shapes of the melting curves. Clearly, as suggested by the ΔT data, the curves are very similar, even though they correspond to values of $(\Delta[\alpha]_{313})_T$ ranging between −915° and −180° (Fig. 7a).

Considerable additional information bearing on the mechanism of formation of the collagen-fold can be derived from a further examination of the mutarotation and melting properties of gelatin preparations of varying molecular weight. In Figure 8, values of T_m obtained from melting curves of these gelatin fractions are plotted as a function of the weight average molecular weight of the sample ($\bar{M}_w$). Clearly T_m shows a slight but definite linear dependence on $\bar{M}_w$, though a decrease from ~42,000 to ~7000 only reduces the melting temperature of *that fraction of the material which mutarotates to the helical form* by about 2°. It is interesting to note that all the data fall very close to the same straight line in Figure 8, suggesting that T_m depends only on $\bar{M}_w$ and is independent of the method used to cleave the polypeptide chain. However, in considering these data, it should be borne in mind that the values of T_m plotted in Figure 8 apply only to the

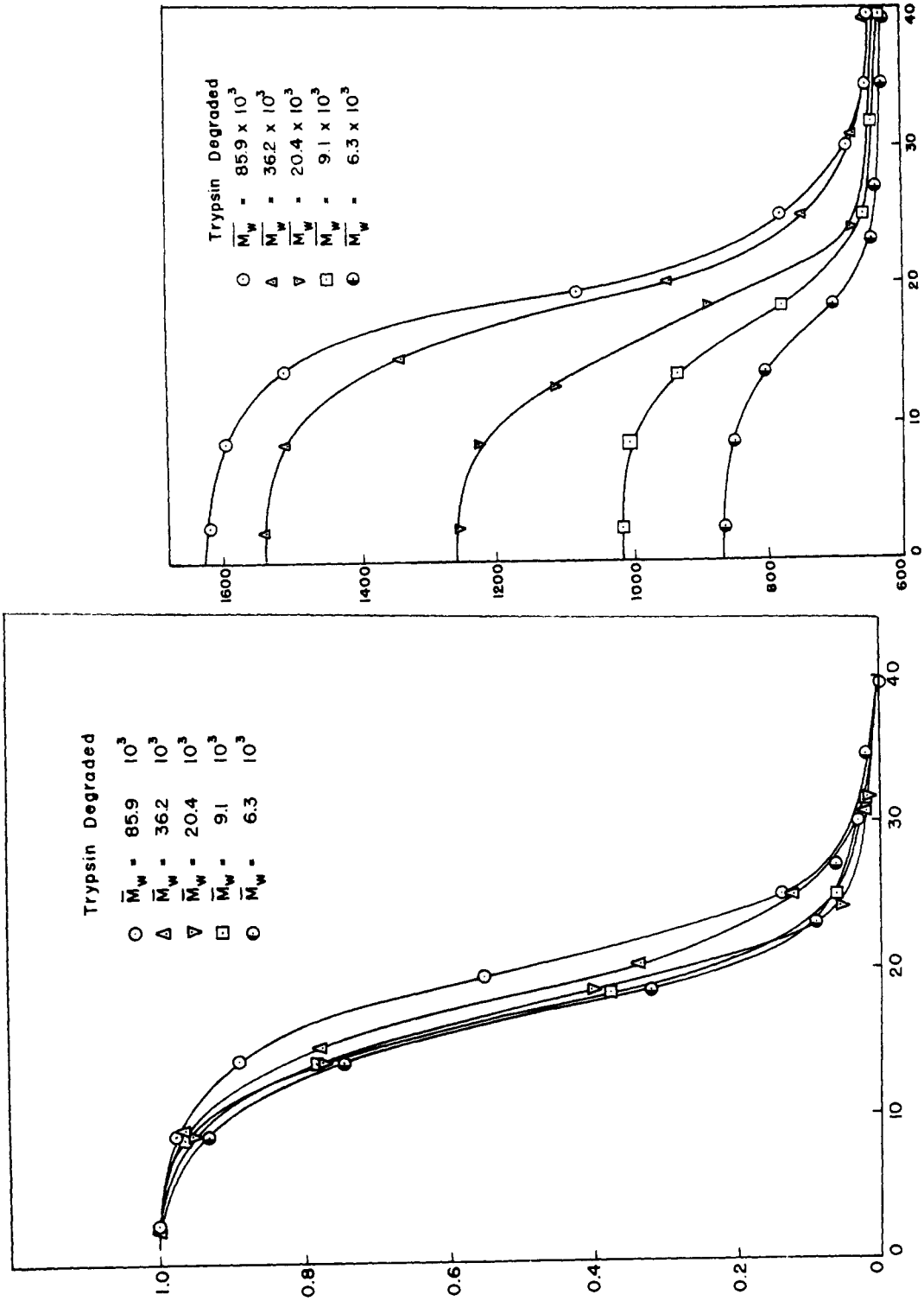

FIG. 7.—(a, upper) Melting curves for trypsin-degraded ichthyocol gelatins in 0.025 M $CaCl_2$, *p*H 7, gelatin conc ≃ 1.0 mg/ml, cooled for 11–16 days at 5° prior to melting. ○, $\bar{M}_w = 85.9 \times 10^3$; △, $\bar{M}_w = 36.2 \times 10^3$; ▽, $\bar{M}_w = 20.4 \times 10^3$; □, $\bar{M}_w = 9.1 \times 10^3$; ◐, $\bar{M}_w = 6.3 \times 10^3$. (b, lower) Melting curves for trypsin-degraded ichthyocol gelatins, $\Delta[\alpha]_T$ normalized to unity. Same data as in (a).

helical portions of each sample, while $\bar{M}_w$ applies to all the material present. As may be seen from Table V and Figure 9a, in the lower molecular weight fractions large portions of the samples were *not* initially helical.

It was also of interest to examine the final amount of poly-L-proline II-type helix generated in the samples of various molecular weight. The results of such studies are presented in Figure 9a,b, where we plot $\Delta[\alpha]_{313}$ (equal to $[\alpha]_{313}^{obs\,-D}$ or $\Delta[\alpha]_T$ as defined above) against $\bar{M}_w$ and n (moles of breaks per 10^5 g of gelatin), respectively. In the experiments $\Delta[\alpha]_{313}$ was measured for each sample after 4–7 days of incubation at 5°. Further standing at this temperature resulted in very little further increase in specific levorotation. The results reveal that, like T_m, $\Delta[\alpha]_{313}$ seems to be independent of the method of chain breakage employed; breaks produced by trypsin, collagenase at 40°, or collagenase at 15° all apparently reduce the final amount of helix formed to the same extent. Figure 9b shows that $\Delta[\alpha]_{313}$ is a linear function of n, extrapolating to complete suppression of helix formation ($\Delta[\alpha]_{313} = 0$) at about 42 breaks per chain of molecular weight 100,000. Figure 9a shows that

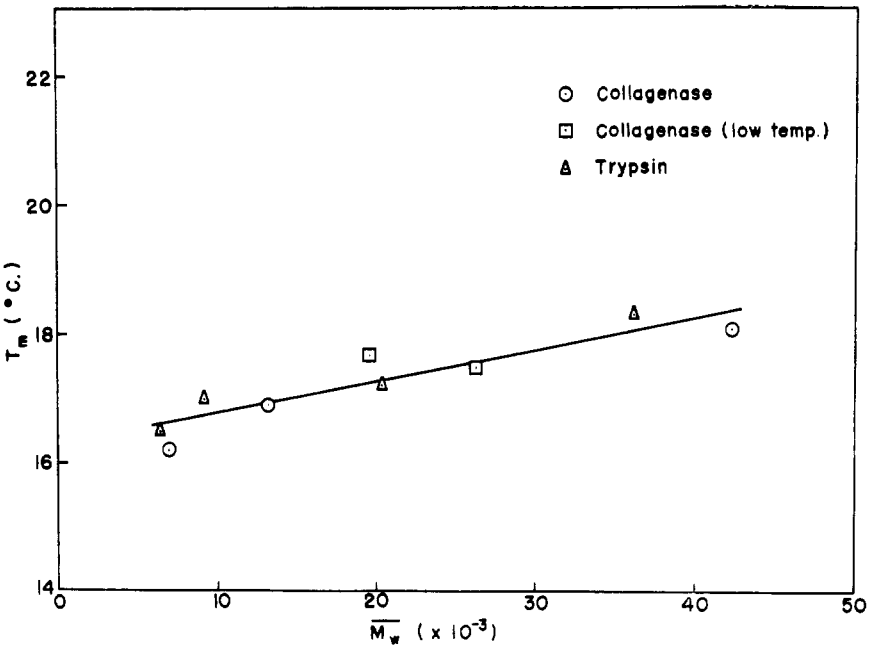

FIG. 8.—Melting temperature as function of $\bar{M}_w$ for various degraded ichthyocol gelatin samples: O, collagenase at 40°; □, collagenase at 15°; Δ, trypsin at 40°. Conditions as in Table V.

$\Delta[\alpha]_{313}$ extrapolates to zero at $\bar{M}_w \simeq 4{,}000$. The rate of increase of $\Delta[\alpha]_{313}$ with increasing molecular weight becomes progressively smaller as $\bar{M}_w$ gets larger, becoming essentially independent of this parameter at $\bar{M}_w > 50{,}000$.

We have also examined the initial rate of helix formation as a function of chain length, in order to obtain further information on the nature of the nucleation process which initiates the formation of the collagen-fold. Figure 10a,b presents the data obtained, in the form of $(d[\alpha]_{313}/dt)_0$, as a function of $\bar{M}_w$ and n, respectively. Again the same line may be passed through all the points in each case, suggesting that the initial rate of helix formation also depends only on the number of breaks and not on where in the chain the breaks occur.[2]

Figure 10a shows clearly that the initial rate of helix formation for all samples of $\bar{M}_w < 50{,}000$ can be represented as a linear function of $\bar{M}_w$. These data extrapolate to $(d[\alpha]_{313}/dt)_0 = 0$ at $\bar{M}_w \simeq 7{,}000$.[3] As might be predicted from Figure 10a, $(d[\alpha]_{313}/dt)_0$ is *not* proportional to n; the first breaks in the chain are clearly much more effective than later ones in reducing the initial rate of mutarotation. Figure 10b shows that no detectable helix formation is observed in 30 minutes at $n > 35$ moles of breaks per 10^5 g of gelatin.

In order to make valid interpretations of these results it is important to know something about the *distribution* of molecular weights in the degraded gelatin samples. To this end we have determined the number average ($\bar{M}_n$) and Z-average ($\bar{M}_z$) molecular weights for most of the degraded gelatin fractions studied. Number average molecular weights were calculated from n (the number of breaks measured with the pH-stat) using 100,000 as the molecular weight of the uncleaved chain. Z-Average molecular weights were measured by sedimentation equilibrium (see Materials and Methods).

Previous information on the mode of action of trypsin and particularly collagenase (von Hippel *et al.*, 1960) suggested that an approximately random attack of these enzymes on the substrate might be expected (i.e., a ratio of $\bar{M}_n:\bar{M}_w:\bar{M}_z$ of approximately 1:2:3 should be obtained). The experimental results are in reasonable accord with these expectations (see Table V); an average of all the data gives $\bar{M}_n:\bar{M}_w:\bar{M}_z$ = 1:2.2(±0.3):3.7(±1.0), where $\bar{M}_n$ in each case is taken as unity. This average includes all the data on the fractions degraded with trypsin (at 40°) and with collagenase (at 15° and 40°). The results show rather large standard deviations since the determinations of $\bar{M}_n$ and $\bar{M}_z$ both involve errors of approximately ±10%. However, they do suggest that peptide bonds were broken approximately at random by these enzymes (within the limitations imposed by the somewhat nonrandom distribution of potentially susceptible bonds along the gelatin chains; see General Discussion). Furthermore, no noticeable trend in the $\bar{M}_n:\bar{M}_w:\bar{M}_z$ ratio was observed in any of the digestions as the molecular weight decreased, indicating that an approximately constant relative distribution of molecular sizes was maintained throughout most of the degradation process in each case.

In the course of these studies the weight average molecular weights of samples of ichthyocol gelatin after *complete* digestion with either collagenase or trypsin were also determined. For complete collagenolytic digests, $\bar{M}_w$ = 810 (±40); for complete digestion with trypsin, $\bar{M}_w$ = 2,400 (±100). These values are in reasonably good accord with present knowledge of the distribution of potentially susceptible bonds in the gelatin chains (see General Discussion).

GENERAL DISCUSSION

On examining the data summarized in Tables II, III, and V, one is immediately struck by two facts. First, no matter what values of T_m and $\Delta[\alpha]_T$ apply to a

[2] The points corresponding to de-esterified gelatin fall marginally lower than the other data in Figure 10a, suggesting that decreasing $\bar{M}_w$ by breaking "ester-type" bonds might be slightly more effective in reducing the initial rate than an equivalent amount of peptide bond breakage.

[3] This means no detectable helix formation in 30 minutes at 2.4°. Of course, we have shown that some helix does form in longer times at weight average molecular weights down to 4000 (see Fig. 9a).

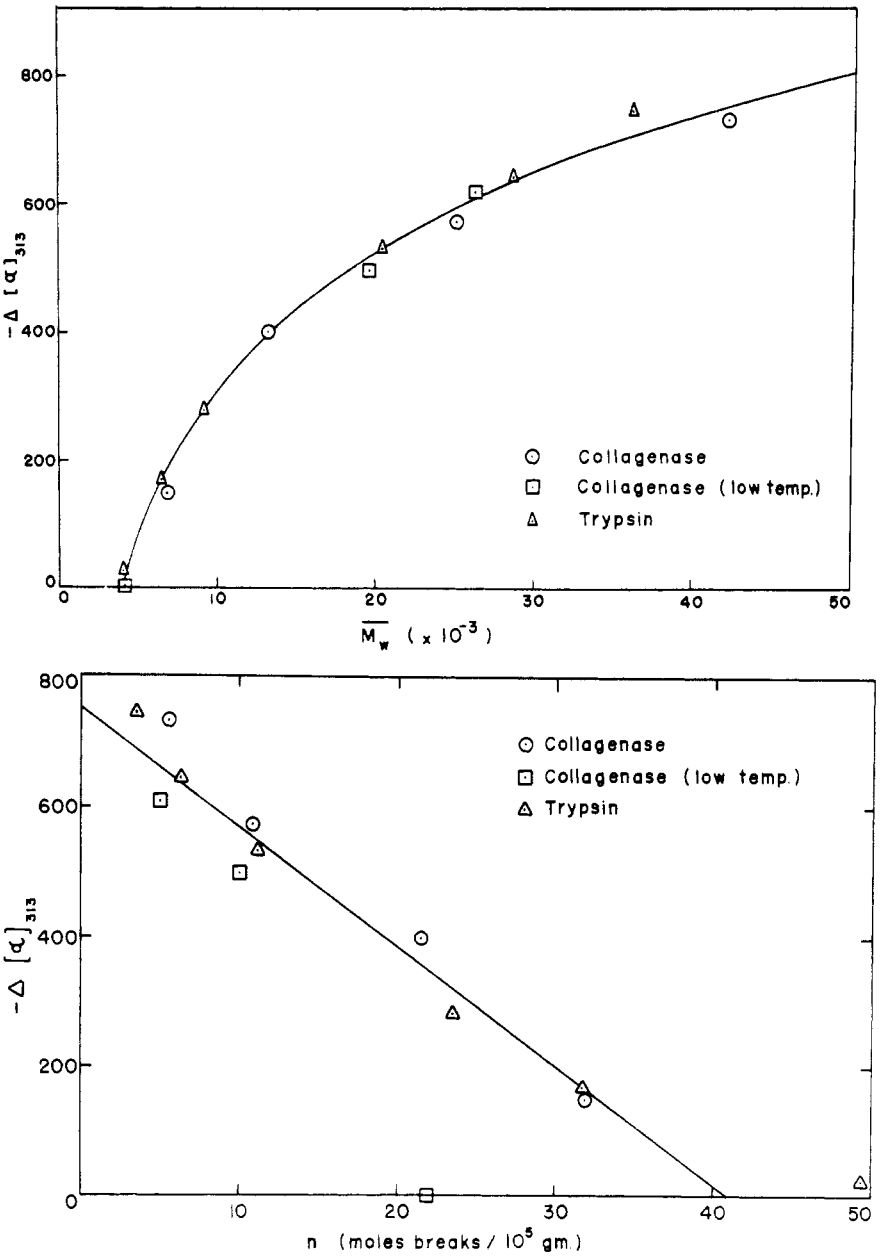

FIG. 9—(a, upper) $-\Delta[\alpha]_{313}$ as a function of $\bar{M}_w$ for various degraded ichthyocol gelatin samples in 0.025 M $CaCl_2$, *p*H 7, gelatin conc. $\simeq$ 1.0 mg/ml. Cooled for 4–7 days at 5°, degraded with: O, collagenase (40°); □, collagenase (15°); △, trypsin (40°). (b, lower) $-\Delta[\alpha]_{313}$ as a function of n (moles of breaks per 10^5 g of gelatin). Same conditions and symbols as in (*a*).

particular transition, ΔT (for the cooled gelatin samples) remains constant at approximately 7°. Also, the maximum value of $\Delta[\alpha]_T$ attained never exceeds $\sim$ 70% of the value characteristic of native collagen. The latter is not a kinetic effect, since it appears to be true not only for the samples cooled for 24 hours which have been examined in this study, but also applies to samples cooled for as long as 44 days (e.g., see Fig. 2, von Hippel and Wong, 1962).

Previously, the general decrease in height and sharpness which characterizes the melting of cooled gelatin when compared with the transition seen in native collagen itself has generally been attributed to rather nonspecific "imperfections" in the crystalline state introduced when the collagen-fold is regenerated from random coil gelatin by cooling. This concept is doubtless correct in the broadest sense; clearly the melting of a somewhat "less cooperative" and thus less "perfect" structure is observed when one carries cooled gelatin through a thermally induced helix → coil transition. However, the constancy of ΔT and the discrete maximum in $\Delta[\alpha]_T$ obtained for this transition under all circumstances investigated suggests that the melting of some very specific, though less highly organized, structure is being observed. The recent isolation of a fraction called γ-gelatin from calfskin gelatin by Veis *et al.* (1961, 1962) and Altgelt *et al.* (1961) has important implications in this connection.

These workers showed that the molecular weight of γ-gelatin is very close to that of the native collagen molecule. They therefore suggested (see also Grassmann *et al.*, 1961) that this material is a denatured version of the original three-stranded molecule in which the individual chains are held together by covalent interchain linkages. When cooled, the specific levorotation of this fraction increased much more

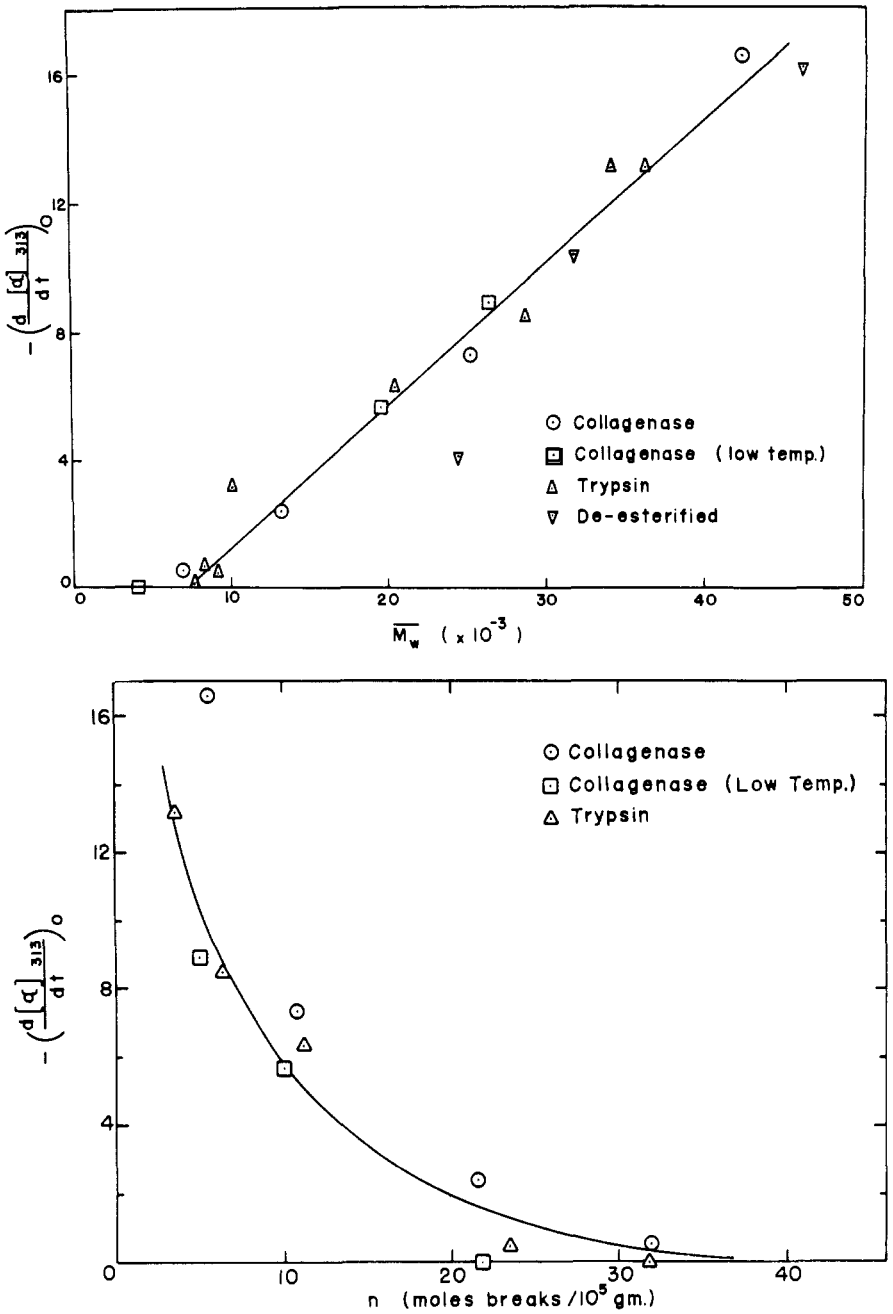

FIG. 10.—(a, upper) Initial rate of mutarotation as a function of $\bar{M}_w$ for various degraded ichthyocol gelatin samples after quenching to 2.4°. 0.025 M $CaCl_2$, *p*H 7, gelatin conc. $\simeq$ 1.0 mg/ml. Degraded with: O, collagenase (40°); □, collagenase (15°); Δ trypsin (40°); ▽, hydroxylamine (1 M, *p*H 8, 40°). (b, lower) Initial rate of mutarotation as a function of *n* (moles of breaks per 10^5 g of gelatin). Same conditions and symbols as in (a).

rapidly than that of single-chain gelatin, attaining a final specific rotation very close to that characteristic of native collagen (Altgelt *et al.*, 1961). Furthermore, this material showed many of the specific higher-order properties of native collagen, including a rodlike appearance in the electron microscope and the ability to aggregate into the specific segment-long-spacing (SLS) pattern in the presence of ATP (Rice, 1960; Veis and Cohen, 1960; Veis *et al.*, 1961). These findings suggested that the interchain cross links in γ-gelatin hold the three chains together in a way which insures appropriate interaction between them and the total regeneration of the original, crystalline, three-chain structure on cooling.

This interpretation of the structure of γ-gelatin has recently been reinforced by the elegant demonstration by Veis and Drake (1963) that the three chains of native ichthyocol collagen can be covalently linked via interchain methylene bridges by treating the native molecule with formaldehyde in the cold, resulting in the production of a synthetic γ-gelatin. (Ichthyocol, in its natural form, is only cross-linked to a small degree and yields no γ-gelatin at all on heating.) Veis and Drake (1963) showed that this synthetic γ-gelatin, on cooling, forms molecules which are indistinguishable from the native material in terms of their ability to reconstitute the highly specific native and SLS interaction patterns observed with the electron microscope. We have made some synthetic γ-gelatin in this way, and find that the melting behavior of this specifically

cross-linked gelatin is essentially the same as that of native collagen in terms of ΔT and $\Delta[\alpha]_T$ (von Hippel and Wong, in preparation). Thus the melting curves we have examined in this study, though clearly arising from the melting of basically similar structures, may be divided into two distinct groups: (a) *the "collagen-type" transition*, obtained with collagen and cooled γ-gelatin and characterized by $\Delta T \simeq 2°$ and 100% helix content (by definition);[4] and (b) *the "gelatin-type" transition*, obtained with cooled, primarily single-chain gelatin and characterized by $\Delta T \simeq 7°$ and a maximum helix content of ~70%.

Previous considerations have suggested that the reformation of the collagen structure from random coil gelatin might be viewed *formally* as a three-step process: (1) nucleation of the poly-L-proline II helix in imino acid–rich regions of the gelatin chain; (2) growth of the poly-L-proline II-type helix along individual chains; and (3) stabilization via specific interchain hydrogen-bonding (see von Hippel and Harrington, 1959; von Hippel and Harrington, 1960; Flory and Weaver, 1960; Harrington and von Hippel, 1961a).[5]

In these terms the present findings might be interpreted by assuming that the "gelatin-type" transition represents the melting of *primarily* intrachain-stabilized elements of the poly-L-proline II helix generated in step (2) above. The "collagen-type" transition would then apply only to the melting of structures in which step (3) had gone to completion in a perfectly ordered fashion. The results suggest that this occurs with sufficient precision to provide the additional element of "cooperativeness" required to reduce ΔT from 7° to 2° only in native collagen or in γ-gelatin (where the three chains are maintained in the proper juxtaposition in the denatured form by covalent linkages). The less regular formation of interchain hydrogen bonds which generally accompanies (and accelerates) step (2)[5] apparently does not introduce sufficient additional "cooperativeness" into the structure to appear as an increase in the sharpness of the transition.

It therefore appears that the "gelatin-type" transition is dominated by factors involved in *intrachain* stabilization of the helix. The following observations reinforce and refine this view, and also suggest that the "cooperative unit" is a relatively short segment of the gelatin chain.

1. Elevated concentrations of salts such as KSCN, $CaCl_2$, LiCl, etc., slow the rate of formation of the poly-L-prolind II helix so greatly that in some cases less than 20% of the amount of helix expected at equilibrium has formed in 24 hours at 5°. This fraction of the total potential helix melts with the same T_m and ΔT as the equilibrium structure (see Table II; also von Hippel and Wong, 1962).

2. Unlike the renaturation of DNA (e.g., see Mamur and Doty, 1961) the transition parameters for the "gelatin-type" helix are not affected by the rate of cooling of the solution. Samples "quenched" instantly and samples cooled slowly to 5° yield virtually identical melting curves.

3. The helix which forms on cooling partially digested samples of gelatin also melts with the same ΔT and almost the same T_m as the undegraded chain in the same solvent environment (Table V).

The banded appearance of oriented aggregates of collagen molecules in the electron microscope have generally been attributed to aligned sequences of polar residues (responsible for the darkly staining, electron dense "band" regions) alternating with sequences of nonpolar residues (producing the relatively nonstaining "interband" regions). Amino acid sequence studies, particularly by Grassmann and co-workers (e.g., see Grassmann *et al.*, 1960, 1962), collagenase digestion experiments (Nishigai *et al.*, 1960; Franzblau, 1962), and other lines of evidence have all led to the view that the "interband" regions are 20–30 residues long and consist largely of the repeating sequence gly.pro.X (where X is often hypro) while the interdigitated "band" regions are approximately 15 residues long and contain primarily glycyl, aspartyl, glutamyl, seryl, alanyl, and lysyl residues (see Franzblau, 1962). In terms of this analysis the "interband" regions represent 60–70% of the collagen molecule.

If we assume that the "gelatin-type" transition arises from the essentially independent melting of individual "interband" segments of the gelatin chain, the above model leads to a predicted minimum length for the "cooperative unit" of approximately 25 residues ($M \simeq 2500$) and a maximum helix content of 60–70% of that of native collagen. These values are, of course, close to the limits we observe (Tables II, III, and V and Figs. 9 and 10). This in turn suggests that perfect interchain alignment (achieved only in native collagen and γ-gelatin) is required to "drive" the helix through the nonimino acid–containing "band" regions of the chain to achieve 100% helix and simultaneously couple the melting of the "interband" segments to make possible the more highly cooperative "collagen-type" transition.

As pointed out under Results, in order to interpret the data obtained with the enzymatically degraded gelatin samples it is necessary to consider the degree of polydispersity of the sample. All the available data indicate that both enzymes attack the potentially susceptible peptide bonds of the substrate essentially at random. The degree of polydispersity (as measured by the ratio $\bar{M}_n:\bar{M}_w:\bar{M}_z$) remains relatively constant throughout the degradation in each case, showing that each enzymatic attack occurs as an independent event and that all the chains are degraded essentially simultaneously (rather than sequentially). Furthermore, as will be shown, all but the smallest chains contribute to the optical rotatory effects observed. An extreme example makes this clear. If one assumes that only intact chains contribute to the initial rate of mutarotation, it can easily be shown that when the initial rate is reduced by 50% (because one-half the chains have been cleaved at least once), the *maximum* possible decrease in $\bar{M}_w$ is only 16%, a result clearly incompatible with the data presented in Figure 10a.

Further insight into the mechanisms of nucleation

[4] Melting curves for a variety of native collagens (rat skin, perch swim-bladder, shark skin and cod swim-bladder) have been examined at *p*H 3.7 and 1.2 by Burge and Hynes (1959). Though their methods are not strictly comparable to ours (they used viscometry and incubated only 30 minutes at each temperature rather than waiting for equilibrium), examination of their data shows that all the curves are very similar in shape with a constant value of $\Delta T \simeq 1.5°$.

[5] Recently we have shown by simultaneous optical rotatory and sedimentation equilibrium measurements that under certain conditions a substantial amount of helix can be generated in solutions of single-chain gelatin without any increase in molecular weight (von Hippel and Wong, unpublished data). Thus clearly stable intrachain elements of poly-L-proline II helix can develop without stabilization by interchain associations. (Of course, experiments such as this cannot be used to rule out mechanisms involving the doubling or "tripling" back on itself of a single chain.) On the other hand, it is also clear that interchain associations *are* very effective in increasing both the rate and the extent of helix formation (e.g., γ-gelatin; see also Veis and Legowik, 1963) and thus steps (2) and (3) should clearly not be considered to be entirely independent of each other in any case.

and growth of the poly-L-proline II helix in gelatin chains can be derived from a detailed consideration of the data obtained with the partially digested gelatin samples.

It is clear that the stability of elements of the poly-L-proline II helix in relatively short chains is not greatly decreased relative to helical regions in long chains. Specifically, Figure 8 shows that T_m decreases approximately linearly with decreasing molecular weight for $\bar{M}_w < 50{,}000$, but only about 1° per 20,000 decrease in $\bar{M}_w$. (However, see comments under Results on this point.)

Figure 9b shows that the final amount of helix attained in partially digested samples of gelatin appears to be a linear function of the number of breaks produced in the polypeptide chain by the enzyme. This may be written:

$$-\Delta[\alpha] = k_i(n - n_0) \qquad (4)$$

for $n < n_0$, where n = moles of breaks per 10^5 g of gelatin, $n_0 = n$ at $\Delta[\alpha] = 0$ and k_i = the slope of the plot of $\Delta[\alpha]$ vs. n. From Figure 9b, $n_0 \simeq 40$ moles of breaks per 10^5 g, which corresponds to $(\bar{M}_n)_0 \simeq 2500$ or $(\bar{M}_w)_0 \simeq 5000$ (see Fig. 9a). As pointed out previously, these molecular weights correspond roughly to the average length of an "interband" segment of the gelatin chain.

Taken together, these observations may be interpreted by assuming that all residues in each gelatin chain which fall within N residues (~20–30) of either end of the chain are inactivated as potential sites of helix formation. Each additional break creates a new pair of chain ends, and thus inactivates another 2 N residues. One might expect that breaks toward the end of the digestion would be less effective than earlier ones in inactivating portions of the chain, since the probability of cleaving through a previously inactivated segment becomes appreciable as digestion proceeds. Some indication of this may be seen in Figure 9b.

The fact that ΔT remains essentially constant for melting curves obtained with gelatin samples of progressively lower molecular weight (Table V and Fig. 7a,b) can be explained by assuming that helix formation in an individual "cooperative unit" is an "all-or-none" proposition. Thus one break through the chain would inactivate the entire unit as a site of potential helix formation. Furthermore, since breaks produced by either collagenase or trypsin seem to have equivalent effects on helix formation (Fig. 9a,b), one must hypothesize that a break through either an "interband" segment (collagenase) or an adjacent "band" segment (trypsin) serves to suppress helix formation in that unit of the chain. Thus the previous suggestion that the "cooperative unit" is an "interband" segment and that the "band" segments serve primarily to isolate these units from one another must be modified by adding that a necessary requirement for helix formation in an "interband" segment is that it be attached to a "band" segment at either end. This interpretation, of course, is consistent with our finding that a gelatin sample digested to completion with trypsin (and thus presumably composed exclusively of isolated "interband" segments) shows no mutarotation whatsoever. It is also pertinent to note that the short segments of poly-L-proline which Yaron and Berger (1961) examined in attempting to find the shortest sequence of this homopolymer capable of forming helix, were all coupled by one end to an inactive (D,L) polypeptide backbone (see below).

The dependence of the initial rate of mutarotation on n and $\bar{M}_w$ differs significantly from the dependence of $\Delta[\alpha]$ on these parameters. Figure 10a shows that $(d[\alpha]/dt)_0$ is a linear function of $\bar{M}_w$, suggesting that the *rate* of mutarotation depends directly on the length of the entire chain.[6] Thus the initial rate of growth of the poly-L-proline II helix, even in segments located at some distance from the ends of the chain, is slower than that in very long chains. The relationship between $\bar{M}_w$ and $(d[\alpha]/dt)_0$ may be written as follows:

$$-(d[\alpha]/dt)_0 = k_i\,[\bar{M}_w - (\bar{M}_w)_0] \qquad (5)$$

where $(\bar{M}_w)_0 = \bar{M}_w$ extrapolated to $(d[\alpha]/dt)_0 = 0$ and k_i is the slope of the plot of $(d[\alpha]/dt)_0$ vs. $\bar{M}_w$. Figure 10a shows that $(\bar{M}_w)_0 \simeq 7000$ (corresponding to ~ 35 moles of breaks per 10^5 g of gelatin; Fig. 10b). This relationship may be interpreted either by assuming that the initial rate of mutarotation of all segments of a gelatin chain of given length is directly proportional to the length, or by assuming that the rate of mutarotation of a given segment of chain is directly proportional to its distance from the nearest end of the chain (for all segments located within approximately 250 residues of an end). In either case this would make $(d[\alpha]/dt)_0$ much more sensitive than $\Delta[\alpha]_T$ to chain length and lead to a departure from linearity in a plot of $(d[\alpha]/dt)_0$ vs. n at a much lower value of n than is seen in a plot of $\Delta[\alpha]$ vs. n (compare Figs. 9b and 10b). It should be noted that collagenase- and trypsin-induced breaks are equivalent in their effect on the initial rate of mutarotation as well as on $\Delta[\alpha]$.

In summary, these results suggest that fairly short segments of polypeptide chain, rich in proline and hydroxyproline, are capable of generating a poly-L-proline II-type helix which is very similar in stability and "degree of cooperativeness" to the helix as it occurs in much longer chains. In this connection, it is pertinent to recall that Yaron and Berger (1961) showed that a sequence of six proline residues, attached to an optically inactive back-bond chain, was sufficient to form a completely stable segment of poly-L-proline II helix.

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[6] Data not shown in Figures 9a and 10a suggest that both $\Delta[\alpha]$ and $(d[\alpha]/dt)_0$ become virtually independent of $\bar{M}_w$ for values of $\bar{M}_w$ greater than approximately 50,000.

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