

The Collagen \rightleftharpoons Gelatin Phase Transition. I. Further Studies of the Effects of Solvent Environment and Polypeptide Chain Composition*

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Received April 29, 1963

In a previous publication (von Hippel and Wong, 1962, *Biochemistry* 1, 664) it was shown that the effects of a wide variety of neutral salts on the rate of formation and stability of the collagen-fold which develops in dilute solutions of cooled gelatin can be expressed by the equations: $T_m = T_m^0 + Km(1)$ and $\log(r/r_0) = k_0m(2)$, where T_m = the mid-point of the phase transition at salt concentration m , r = initial rate of mutarotation (helix formation) following "quenching" of a warm solution of random coil gelatin to a given temperature, and T_m^0 and r_0 are the same parameters for the same conditions at $m = 0$. In this study it is shown that equation (1) also describes the effect of neutral salts on the native collagen \rightarrow gelatin transition for several different collagens, and that in terms of molar effectiveness in reducing T_m the salts tested fall in the same series found with cooled gelatin. However, the values of K obtained are all increased over the cooled gelatin values. It is further shown that the effects of D_2O and urea on the collagen-type helix are very similar to those of neutral salts and can also be described by equations (1) and (2). The initial rate of helix formation (r) is examined as a function of temperature, and it is shown with several types of gelatin samples that $\log r$ is directly proportional $1/T(T_m' - T)$, where T_m' falls several degrees above the mid-point of the transition (T_m), very close to the temperature at which the last vestiges of collagen-type structure melt out. In addition, the relationship between melting temperature and total imino acid content has been re-examined using the values of T_m obtained for calfskin, ichthyocol and earthworm cuticle collagens, and literature values for other collagens corrected to comparable melting conditions. The mechanisms whereby changes in the solvent environment affect the stability of the collagen-type helix are considered in the light of the results obtained.

In a recent publication (von Hippel and Wong, 1962) we described the results of a detailed optical rotatory examination of the effects of a variety of electrolytes on the formation and stability of the collagen-fold in dilute solutions of ichthyocol and calfskin gelatin. Two types of measurement were reported. The effects of ions on the stability of the collagen-fold were examined by measuring the lateral displacement of melting curves obtained with solutions of gelatin cooled for varying lengths of time in the presence of neutral salts. It was found that neutral salts affected the stability of the collagen-type helix in cold gelatin markedly, shifting the transition to higher or lower temperatures to an extent which depends both on the nature and the concentration of the added salt. Quantitatively, it was shown that over a considerable range of salt concentrations the following equation described the situation quite well:

$$T_m = T_m^0 + Km \quad (1)$$

where T_m = the temperature of the mid-point of the phase transition at salt concentration m , $T_m^0 = T_m$ at $m = 0$, and K is a constant characteristic of the particular salt-gelatin system under consideration. The constant K (in deg/mole/liter) was derived from the slope of plots of T_m vs. m , and served as a measure of the molar effectiveness of a given salt in decreasing (or increasing) the stability of the collagen-type helix.

In addition, the initial rate of helix formation (mutarotation) following "quenching" of a warm gelatin solution to temperatures below T_m was studied as a function of the concentration of various salts in order to obtain some insight into the nucleation of the helix.

* Presented in part at the Symposium on the Molecular Biology of Collagen at the 141st National Meeting of the American Chemical Society, March, 1962, Washington, D. C. This research was supported by a research grant (AM-03412) and a career development award (GM-K3-5479) from the U.S. Public Health Service.

It was found that the following relation described the experimental data:

$$\log(r/r_0) = k_0m \quad (2)$$

where $r = (d[\alpha]/dt)_0$ = the initial rate of mutarotation at a given temperature and salt concentration m , $r_0 = r$ at the same temperature and $m = 0$, and k_0 = the slope of the plot of $\log r$ vs. m for the salt-gelatin system under consideration. Thus the constant k_0 (in liters/mole) serves as a useful comparative measure of the effect of various salts on the nucleation step in the formation of the collagen-type helix.

The collagen-fold which develops in cooled gelatin is closely related to the helical conformation of the polypeptide chains of native collagen. (For a summary of the evidence on this point see von Hippel and Harrington, 1960; or Harrington and von Hippel, 1961a.) However, some significant differences do exist (see following paper), and therefore we have carried out a comparable study of the effects of ions on the stability of the helical structure of native collagen.

The results of our previous study on the effects of ions on the collagen-fold were interpreted in terms of a competitive reorganization by the ions of structural water involved in stabilizing the collagen helix. In order to look into this situation further we felt it might also be pertinent to examine the consequences of perturbing the solvent environment with certain nonelectrolytes. Therefore we have considered in detail the effects of urea and D_2O on the stability and rate of formation of the collagen-type helix.

Finally, we also report here similar studies on the effects of temperature and imino acid composition of the polypeptide chains, in order to obtain a unified view of the interaction of all these parameters in their effect on the collagen structure.

MATERIALS AND METHODS

Collagen.—The ichthyocol and calfskin collagens

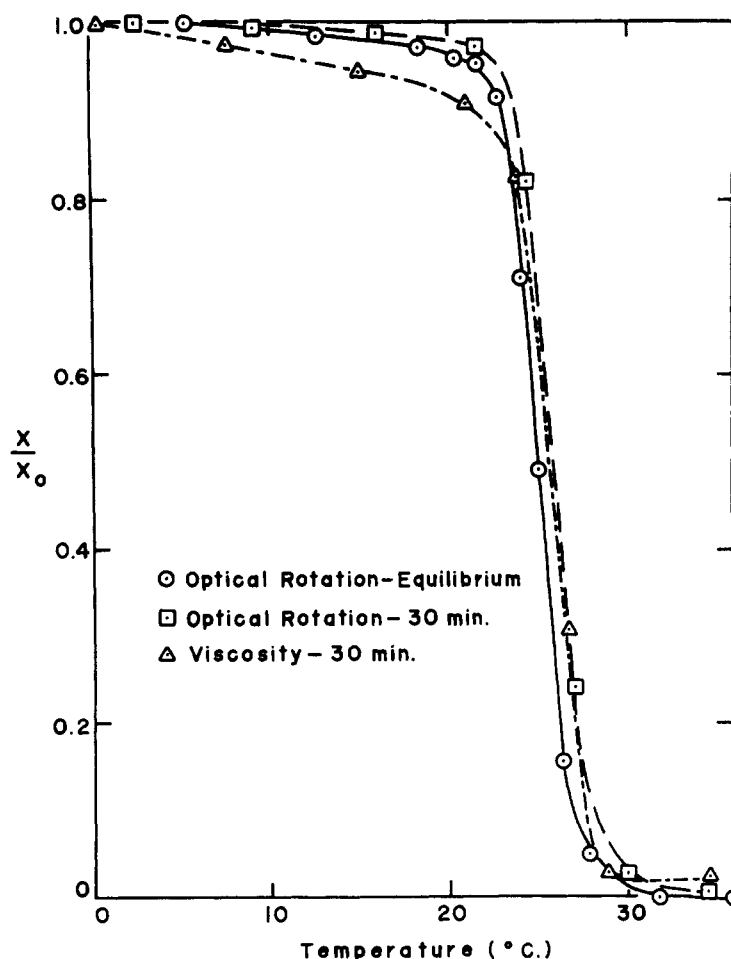


FIG. 1.—Melting curves for ichthyocol gelatin in 0.5 M CaCl_2 , pH 7, protein concentration = 0.7 mg/ml; \circ , optical rotation, “equilibrium”; \square , optical rotation, “30-minute”; \triangle , viscosity, “30-minute.”
 $x/x_0 = ([\alpha]_T - [\alpha]_{40^\circ})/([\alpha]_{25^\circ} - [\alpha]_{40^\circ})$ or $\eta_{sp,T}/\eta_{sp,25^\circ}$

used were prepared from carp swim bladder and calfskin, respectively (Gallop, 1955; Seifter *et al.*, 1959), and kindly provided to us in purified lyophilized form by Dr. Paul M. Gallop of the Albert Einstein College of Medicine. A sample of earthworm cuticle collagen, prepared by the method of Maser and Rice (1962) was generously furnished by Dr. Robert V. Rice of the Mellon Institute. The lyophilized collagens were put into solution, either as native soluble collagen (in neutral 0.5 M CaCl_2) or as random coil gelatin (in neutral 0.025 M CaCl_2), and concentrations were determined as described previously (von Hippel *et al.*, 1960; von Hippel and Wong, 1962). Although CaCl_2 concentrations of approximately 0.5 M were required to dissolve the various collagens in the native form initially, we found that these stocks could be diluted to concentrations of CaCl_2 as low as 0.1 M and carried through melting curves without precipitation. In the presence of some salts a precipitation of the protein analogous to the well-known “heat-gelation” of collagen (e.g., see Gross and Kirk, 1958) was observed as the temperature was raised into the transition region. This occurred particularly with salts characterized by values of K close to zero (see von Hippel and Wong, 1962) making it difficult to obtain T_m and thus K values for native collagen in these salts.

Optical Rotation.—The apparatus used in making the polarimetric measurements, and the techniques used in the measurements of initial rates of mutarotation, have been described elsewhere (von Hippel and Wong,

1962). As before, melting curves were obtained by transferring the cold sample into a precooled polarimeter tube and then raising the temperature in steps of 1–4°, waiting at each temperature until the rotation had essentially stopped changing. As pointed out previously, this often required waiting as long as 18 hours at temperatures in the transition region, but results in the determination of what one might call “equilibrium” melting curves. One potential disadvantage of this procedure is that the sample is exposed to temperatures of approximately 20° for 2–3 days in some cases, and thus there is a finite risk of some breakage of weak covalent bonds. However, the optical rotatory properties of both collagen and gelatin are quite independent of molecular weight over a wide range (see following paper), and we have not found that prolonged exposure to temperatures of this order has any effect on melting curves measured polarimetrically. A gradual decrease in the relative viscosity with time is seen at temperatures below the transition (Boedtker and Doty, 1956; see also Fig. 1).

There are important advantages to measuring melting curves in an equilibrium manner if one is interested in an accurate determination of either T_m or of the actual shape of the transition. In the past most workers have obtained melting curves on dilute solutions of collagen or cooled gelatin (measured either polarimetrically or viscometrically) by a variant of one of the following three nonequilibrium methods: (1) A new solution is used for each point and the

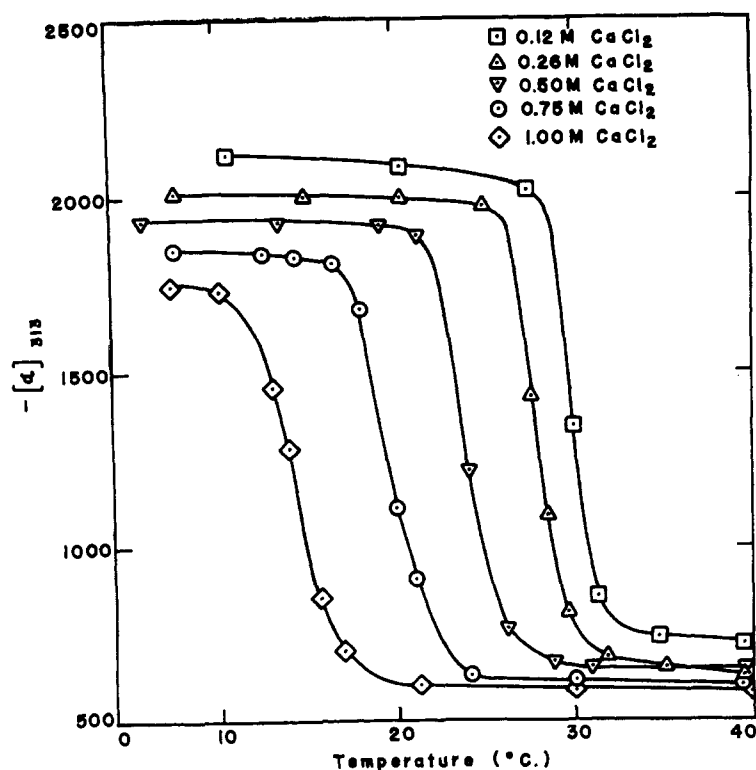


FIG. 2.—Melting curves for ichthyocol collagen in various concentrations of CaCl_2 ; pH 7, ~ 1 mg/ml collagen and: \square , 0.12 M CaCl_2 ; \triangle , 0.26 M CaCl_2 ; ∇ , 0.50 M CaCl_2 ; \circ , 0.75 M CaCl_2 ; \diamond , 1.0 M CaCl_2 .

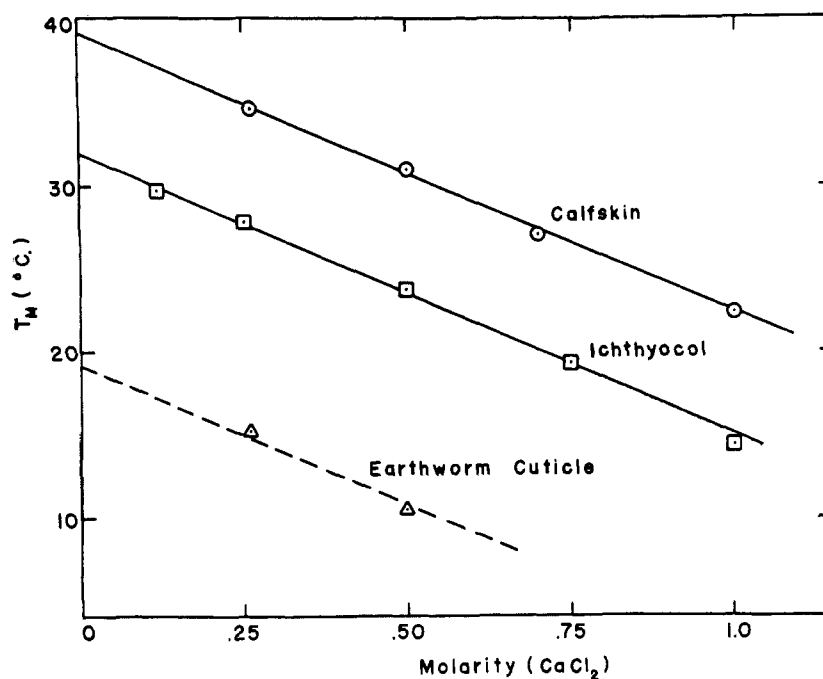


FIG. 3.— T_m for calfskin (\circ), ichthyocol (\square), and earthworm cuticle (\triangle) collagens as a function of CaCl_2 concentration. (Measured from curves such as those shown in Fig. 2).

sample is arbitrarily incubated at each temperature for a fixed time interval (usually 30 minutes) before measurement. (2) The same solution is used for the entire melting curve and the sample is arbitrarily held at each temperature for a fixed interval (again, usually 30 minutes) before measurement. (3) The same solution is used for the entire melting curve and the temperature is raised at a constant rate (usually $2-4^\circ/\text{hour}$). Methods 2 and 3 come closer to being equilibrium methods than method 1, but even with method 2 at

certain temperatures in the transition region values of $[\alpha]_{313}$ may be obtained which are as much as 100° more negative than the corresponding equilibrium values (see Fig. 1). Furthermore, since in these measurements one invariably proceeds from low to higher temperatures, the nonequilibrium melting curves (optical rotation) are generally sharpened at the low temperature end of the transition zone, and may be somewhat rounded at the high temperature end, rather than attaining the more symmetrical shape

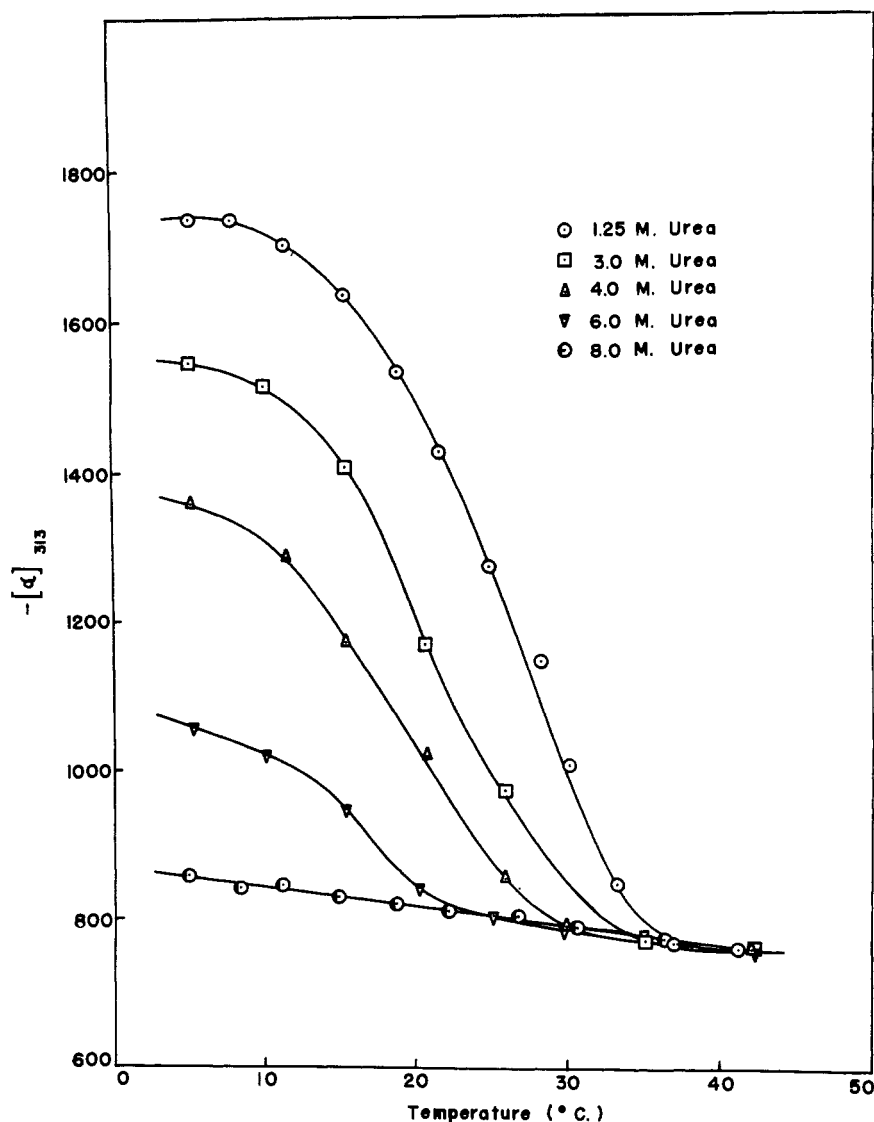


FIG. 4.—Melting curve for calfskin gelatin in various concentrations of urea, cooled at 5° for 7 days in 0.025 M CaCl₂ and: ○, 1.25 M urea; □, 3.0 M urea; △, 4.0 M urea; ▽, 6.0 M urea; ⊙, 8.0 M urea.

characteristic of the equilibrium representation (Fig. 1).

Since in a later portion of this paper (Fig. 10) we utilize a number of values of T_m derived from the literature which were measured by several variants of the "30-minute" procedures described above, we have compared melting curves measured both polarimetrically and viscometrically by method 2 above with an equilibrium (optical rotation) measurement of the same material. The results for a solution of ichthyocol collagen in 0.5 M CaCl₂ are presented in Figure 1, and show that T_m for the "30-minute" procedure is about 1° higher than T_m derived from the equilibrium curve. Similar comparative experiments were carried out with ichthyocol collagen in 0.25 M CaCl₂–1 M LiCl, and with ichthyocol gelatin cooled for 5 days at 5° in 0.025 M CaCl₂. In all cases the equilibrium T_m fell about 1° below the "30-minute" T_m .

Therefore, in compiling Figure 10, "30-minute" T_m values from the literature were corrected to equilibrium conditions by subtracting 1° from the value given. Where necessary, T_m values were also corrected to comparable ionic conditions using K values (equation 1) determined in this and previous studies.

RESULTS AND DISCUSSION

Effect of Ions on the Native Collagen ⇌ Gelatin Transi-

tion.—Figure 2 shows the effect of various concentrations of CaCl₂ on the melting behavior of native ichthyocol collagen. Clearly, as previously shown with dilute cooled gelatin solutions (compare with Fig. 4, von Hippel and Wong, 1962) the transition is shifted toward lower temperatures with increasing concentrations of CaCl₂. Furthermore, as also shown with cooled gelatin, the extent of the displacement of the transition is linearly related to the concentration of added salt (Fig. 3). Thus equation (1) can also be used to describe the relation between T_m and m for the melting of native collagen.

In addition to the ichthyocol data, Figure 3 also presents the results of a comparable series of melting experiments on solutions of native calfskin and earthworm cuticle collagen. Clearly T_m for the melting of calfskin collagen is also linearly related to the CaCl₂ concentration (only two points were obtained with earthworm cuticle collagen) and the slopes (K) for all three collagens in this salt are quite similar (see Table I). On the other hand, the intercepts (T_m^0) obtained for the three collagens are different, and closely related to the total imino acid composition of the collagen (see below).

Aside from examining the effect of varying concentrations of CaCl₂ on the melting of the native collagen

TABLE I

THE EFFECTS OF VARIOUS SALTS ON THE STABILITY OF THE COLLAGEN-FOLD IN NATIVE COLLAGEN AND COOLED GELATIN^a

Material	Salt	K (collagen) (deg/mole/ liter)	K (gelatin) ^c (deg/mole/ liter)
Ichthyocol	(CH ₃) ₄ NCl	~ 0 ^b	+ 2.2
	NaCl	- 3.0 ^b	- 1.6
	LiCl	- 5.7 ^b	- 4.1
	CaCl ₂	-15.8	- 8.8
	KSCN	-21.5 ^b	-10.0
Calfskin	CaCl ₂	-16.7	-12.8
Earthworm cuticle	CaCl ₂	~-17	~-12

^a Collagen concentrations ≈ 1 mg/ml. K defined by equation (1). ^b From measurements made on solutions which also contained 0.25 M CaCl₂. ^c K values for gelatin (except earthworm cuticle) are from von Hippel and Wong (1962).

structure, we have also investigated the effects of several other ions on T_m for native ichthyocol collagen. Values obtained for the molar effectiveness (K) of these salts in altering the stability of the native collagen molecule are listed in Table I and compared with values of K obtained previously for these salts in the melting of cooled gelatin (von Hippel and Wong, 1962). The results show that the order of effectiveness of the salts tested is unchanged, but that the actual values of K obtained with native collagen are substantially larger than those obtained with the same salt and dilute solutions of cooled gelatin.

Effect of Nonelectrolytes on Native Collagen and Gelatin.

—It is well known that concentrated solutions of urea are very effective in bringing about the conversion of native collagen to gelatin (e.g., see Gustavson, 1956, 1960; von Hippel and Harrington, 1960; Steven and Tristram, 1962) and in liquefying gelatin gels (Carpenter and Lovelace, 1938; Bello *et al.*, 1956). The effect of varying concentrations of urea on the stability of the collagen-fold is shown in Figure 4, which presents

TABLE II

EFFECT OF UREA AND D₂O CONCENTRATION ON THE FORMATION AND STABILITY OF THE COLLAGEN-FOLD^a

Material	Conditions	K (deg/ mole/ liter)
Ichthyocol gelatin	Urea (0-2 M) in 0.025 M CaCl ₂ after ~3 days at 5°	-1.7
Calfskin gelatin	Urea (0-4 M) in 0.025 M CaCl ₂ after 7 days at 5°	-2.5
Pigskin gelatin ^b	Urea (1-4 M); 5% gel	-3.1
Ichthyocol gelatin	D ₂ O (0-45 M) in 0.025 M CaCl ₂ after 24 hr at 5°	+0.055
Ichthyocol collagen	D ₂ O (0 and 44 M) in 0.5 M CaCl ₂	+0.04

Material	Conditions	k_0 (liters/ mole)
Ichthyocol gelatin	Urea (0-2 M) in 0.025 M CaCl ₂ , 3°	-0.73
Ichthyocol gelatin	D ₂ O (0-45 M) in 0.025 M CaCl ₂ , 4°	+0.005

^a K defined by equation (1), k_0 by equation (2); protein concentration in all experiments ≈ 1 mg/ml. ^b Estimated from Bello *et al.* (1956).

equilibrium melting curves obtained with calfskin gelatin after cooling for 7 days at 5° in 1.25-8.0 M urea. As shown previously (Harrington and von Hippel, 1961b), 8 M urea completely suppresses helix formation. However, Figure 4 shows that in intermediate concentrations of urea some regeneration of the poly-L-proline II-type helix does take place, though as the molarity of urea increases both the amount of helix formed in 7 days and the melting temperature shift toward lower values.

In Figure 5 we plot T_m obtained from these curves vs. m_{urea} . Clearly in solutions in which the urea concentration is ≤ 4 M, T_m and m_{urea} are linearly related and yield a value of K (equation 1) of $-2.5^\circ/\text{mole/liter}$. This value is close to the K estimated from data

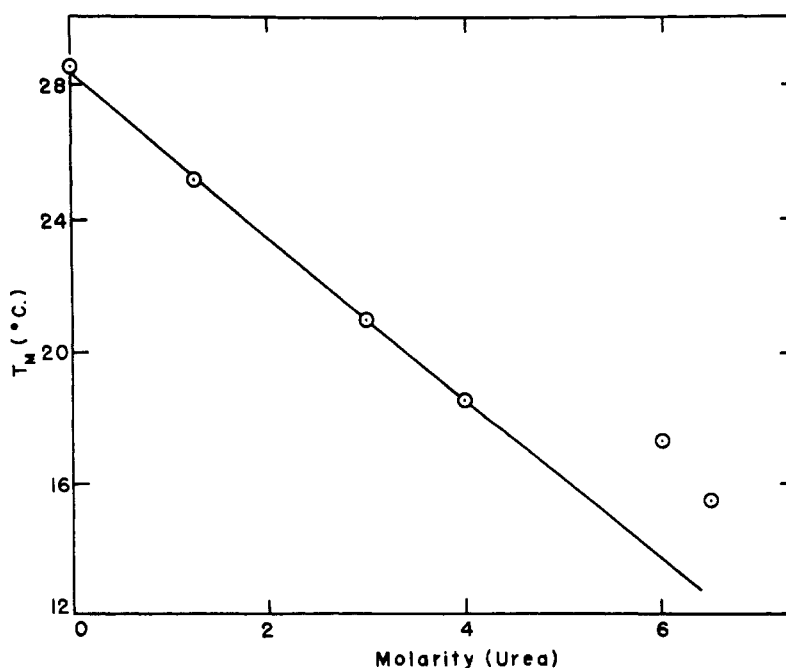


FIG. 5.— T_m for calfskin gelatin as a function of urea concentration (data from Fig. 4).

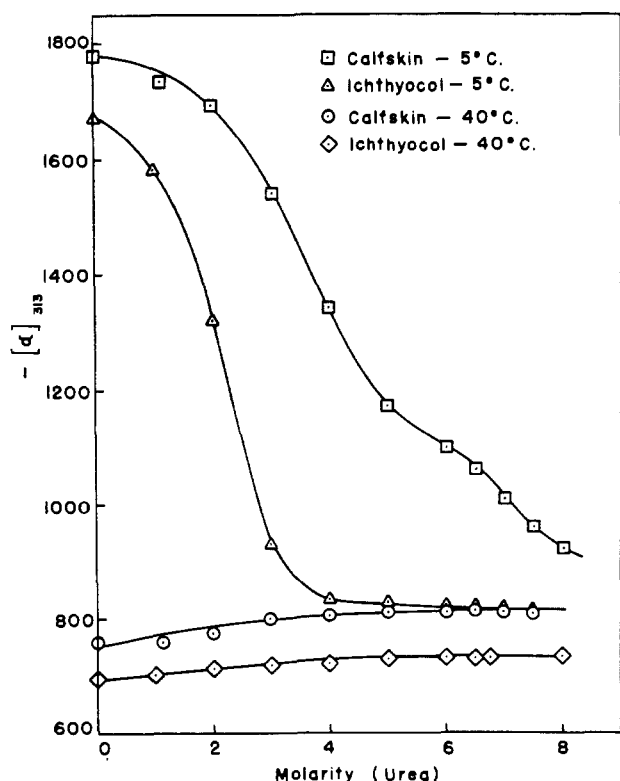


FIG. 6.—Specific rotation of calfskin (\square) and ichthyocol (Δ) gelatin at 5° after cooling 7 and 5 days respectively at 5°; and at 40° (\circ calfskin; \diamond , ichthyocol) as a function of urea concentration. All solutions also 0.025 M in CaCl_2 , pH 7, gelatin concn. \approx 1 mg/ml.

obtained by Bello *et al.* (1956) on the effect of urea on the melting of 5% pigskin gelatin gels, and to K obtained with cooled solutions of ichthyocol gelatin (Table II).

However, Figure 5 also clearly shows that the points obtained in 6.0 and 6.5 M urea do not fall on the line which applies to the data for the lower urea concentrations. In this connection it is interesting to examine the "isothermal melting curve" in which the abscissa is urea concentration rather than temperature (Fig. 6). If measured at an equivalent time after cooling, such a plot corresponds to passing a vertical line at 5° through a series of melting curves such as those shown in Figure 4. However, in general such isothermal melting curves are not equilibrium representations, since at higher concentrations of urea (or electrolyte, see Fig. 2, von Hippel and Wong, 1962) equilibrium is attained extremely slowly.¹

In Figure 6 we plot (upper curves) the specific rotation for calfskin and ichthyocol gelatins after cooling at 5° for 7 and 5 days, respectively, vs. urea concentration. The lower curves represent the specific rotation of the same samples after heating to 40°. Clearly, in ichthyocol gelatin at urea concentrations greater

¹ It is important to distinguish the various uses of "equilibrium" made here. As used in this and previous publications, an "equilibrium" melting curve is one obtained by waiting at each temperature until the parameter being measured (generally optical rotation) has stopped changing with time (see Materials and Methods). However, this does not imply that the melting curve represents the melting of the total or "equilibrium" amount of helix which might have been regenerated after infinite cooling. Rather, it means only that the amount of helix present when melting was begun was carried through the phase transition in an "equilibrium" fashion.

than 4 M, no poly-L-proline II-type helix is regenerated (the residual difference in rotation between the 5° and 40° curves above 4 M urea is simply due to the effect of temperature on the random coil; see von Hippel and Wong, 1963). For calfskin gelatin the situation appears to be somewhat different. At urea concentrations of 4 M or less, the curve is very similar to that obtained with ichthyocol. However, instead of becoming parallel to the 40° curve at higher concentrations of urea, a definite "shoulder" is seen between 4 and 8 M urea, giving the entire low-temperature calfskin curve the appearance of a biphasic melting curve. Furthermore, it is in this range of urea concentration that the anomalous values of T_m are observed (Fig. 4). One might speculate that this structure in cooled calfskin gelatin, which is especially resistant to destruction by urea, might correspond to particularly stable elements of the poly-L-proline II-type helix in the immediate vicinity of nuclei. Alternatively, the stability of these regions in calfskin gelatin might be related to the fact that this gelatin contains many more covalent interchain cross links than are present in ichthyocol gelatin.

The effect of various concentrations of urea on the initial rate of mutarotation following "quenching" to 3° of urea-containing ichthyocol gelatin solutions has also been examined. The data obtained are plotted as $\log (d[\alpha]/dt)_0$ vs. molarity of urea in Figure 7. Clearly these data (like those for electrolytes; von Hippel and Wong, 1962) fit very well on a straight line in this representation, and a value of $k_0 = -0.73$ liter/mole may be obtained from the slope (equation 2).

An analogous examination of the effects of varying concentrations of D_2O on T_m and on the initial rate of mutarotation has also been carried out. Previously it had been shown (von Hippel and Harrington, 1960; Harrington and von Hippel, 1961b) that T_m obtained for the collagen-fold \rightleftharpoons gelatin transition in cooled ichthyocol gelatin in 0.5 M CaCl_2 was approximately 4° higher with D_2O as solvent than with H_2O . Similarly, mutarotation experiments in these solvents had shown that the initial rate of mutarotation in D_2O was approximately 3-fold greater than the rate in H_2O under the same conditions. We have investigated this situation in more detail, carrying out equilibrium melting experiments on cooled ichthyocol gelatin in 0.025 M CaCl_2 and 0, 20, 40, 60, and 80% D_2O . The results showed, as expected, that T_m in this system is a linear function of D_2O concentration, and the value of K obtained from these experiments is recorded in Table II. The initial rate of mutarotation (r) for these samples after quenching to 4° was also measured, and as expected (equation 2) $\log (d[\alpha]/dt)_0$ was found to be directly proportional to $m_{\text{D}_2\text{O}}$. The value of $k_0(\text{ichthyocol}-\text{D}_2\text{O})$ obtained is also recorded in Table II. T_m for native ichthyocol collagen dissolved in 0.5 M CaCl_2 was measured with pure H_2O and 80% D_2O as solvent (Fig. 6, following paper). From these experiments a value of $K(\text{ichthyocol}-\text{D}_2\text{O})$ was estimated for the melting of the native collagen structure also (Table II).

Effect of Temperature on the Initial Rate of Helix Formation.—The rate at which the collagen-type helix is regenerated following quenching of a warm gelatin solution to a temperature (T) below T_m depends markedly on the value of T chosen (von Hippel and Harrington, 1960; Flory and Weaver, 1960; Harrington and von Hippel, 1961b). Over a relatively narrow temperature interval a plot of $\log (d[\alpha]/dt)_0$ (initial rate of mutarotation) or $\log t_{1/2}$ (half-time for the (mutarotation process) vs. $1/T$ may appear linear,

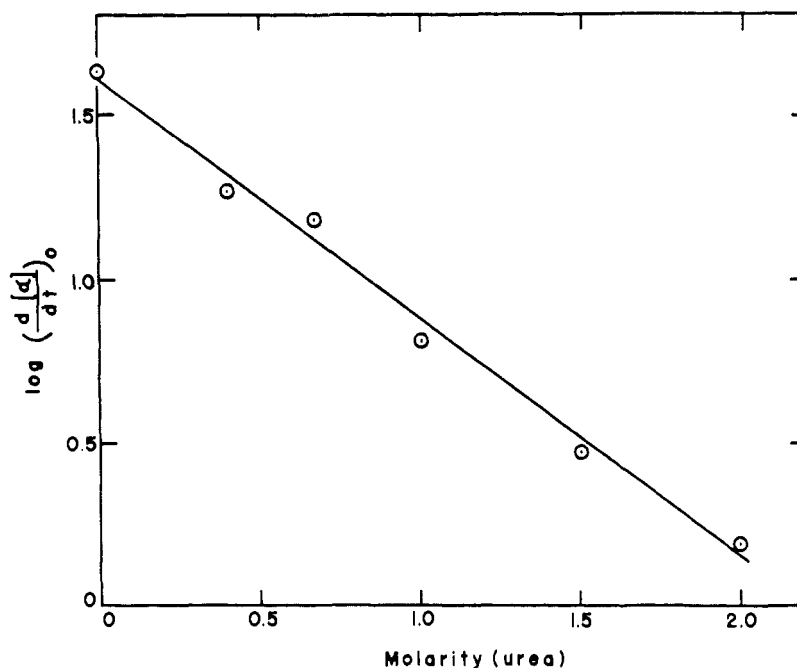


FIG. 7.—Logarithm of initial rate of mutarotation of ichthyocol gelatin at 3° (and 313 $m\mu$) as a function of urea concentration. All solutions also 0.025 M in CaCl_2 , pH 7, gelatin concn. \approx 1 mg/ml.

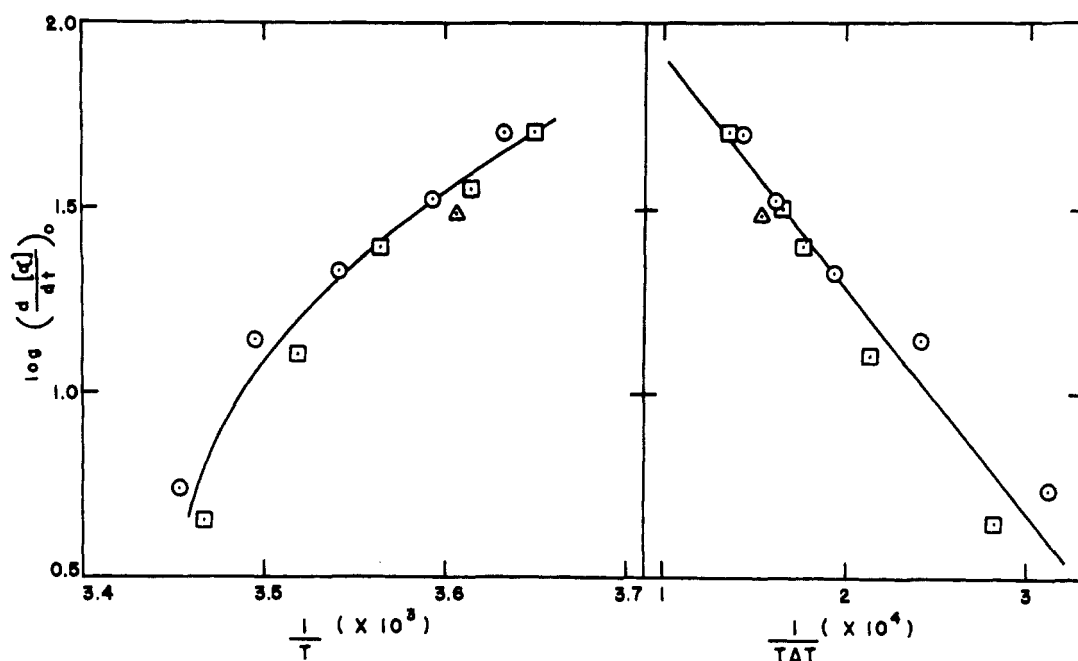


FIG. 8.—Initial rate of mutarotation at 3° (313 $m\mu$) of three different preparations of ichthyocol gelatin as a function of (a) $1/T$ and (b) $1/T\Delta T$ (see text). 0.025 M CaCl_2 , pH 7, gelatin concn. \approx 1 mg/ml.

but with more data over a broader range of temperatures such plots deviate markedly from linearity. Figure 8a shows a typical example of such a plot, in this case for the initial rate of mutarotation of three different preparations of dilute ichthyocol gelatin in 0.025 M CaCl_2 , following quenching to temperatures between 0 and 17°. Clearly this plot is concave downward, the slope and thus the apparent energy of activation increasing progressively as T_m is approached.

Flory and Weaver (1960) and Flory (1961) have pointed out on theoretical grounds that at temperatures below T_m the rate constant for a coil \rightarrow helix transformation such as the gelatin \rightarrow collagen-fold

transition should depend on absolute temperature as follows:

$$k' = Ce^{-(A/kT\Delta T)} \quad (3)$$

where C and A are constants, k = Boltzmann's constant, T (in $^\circ\text{K}$) = the temperature of the experiment and $\Delta T = T_m' - T$, where T_m' = the melting temperature of the crystalline phase (see below).²

Experimentally, Flory and Weaver (1960) showed

² For other discussions of equation (3) as applied to the kinetics of formation of the crystalline phase in linear polymers and synthetic polynucleotides, see Mandelkern (1956), Saunders and Ross (1960), Ross and Sturtevant (1962).

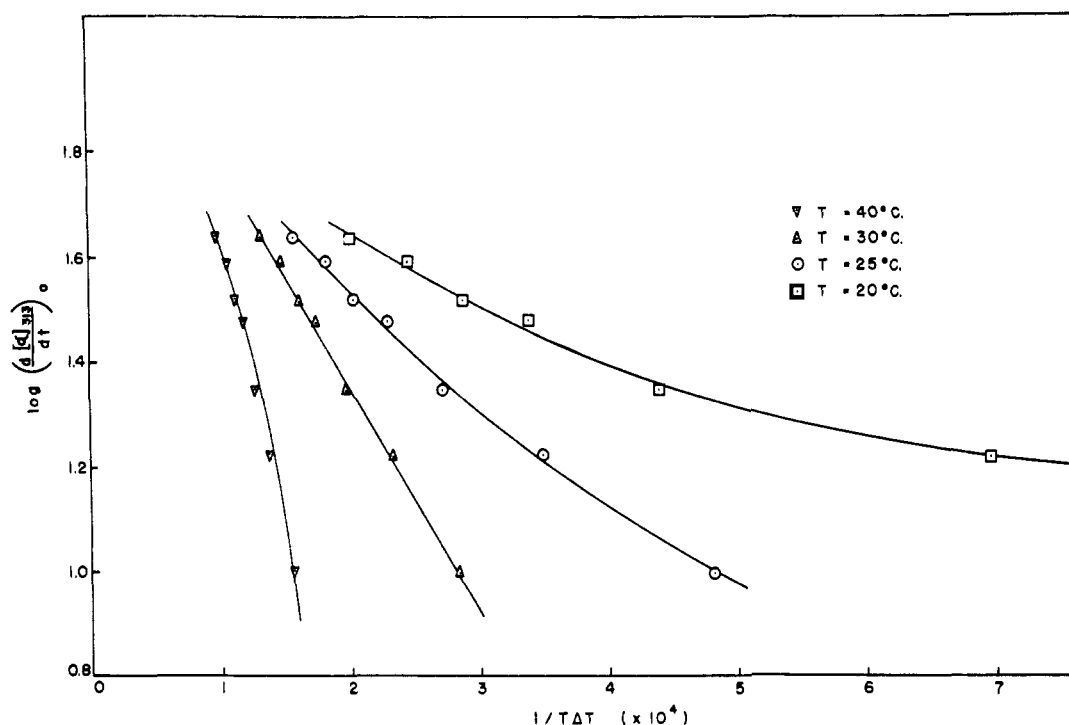


FIG. 9.—Initial rate of mutarotation at 3° (313 μM) of "de-esterified" calfskin gelatin ($\bar{M}_w \approx 94,000$) as a function of $1/T\Delta T \cdot T_m' = 40^\circ$ (∇); 30° (Δ); 25° (\circ); 20° (\square).

that their data for the half-time of the mutarotation process which follows the quenching of solutions of rat tail tendon gelatin to various temperatures fitted reasonably well on a straight line in a plot of $\log t_{1/2}$ vs. $1/T\Delta T$ (using $T_m' = 35^\circ$).

In Figure 8b the ichthyocol gelatin data shown in Figure 8a are replotted as $\log (d[\alpha]/dt)_0$ vs. $1/T\Delta T$, using the value of T_m' which by trial and error yields the best straight line. For Figure 8b this value is 27.5° , which is markedly higher than T_m ($\sim 20.5^\circ$; defined as the mid-point of the melting curve under these conditions). Examination of the 0.025 M CaCl_2 melting curve for cooled ichthyocol gelatin (Fig. 4, von Hippel and Wong, 1962) shows that $T_m' \approx 27.5^\circ$ corresponds closely to the temperature at which the most perfectly ordered portion of the crystalline phase melts.

Such trial and error attempts to fit initial rate of mutarotation data to the best straight line in plots of $\log (d[\alpha]/dt)_0$ vs. $1/T\Delta T$ may be used to arrive at a crude measure of T_m' . Curves obtained with several trial values of T_m' using mutarotation data obtained on a partially "de-esterified" calfskin gelatin (Gallop *et al.*, 1959) in 0.025 M CaCl_2 are shown in Figure 9. Clearly the best value of T_m' for these data is 30° ($\pm 3^\circ$). An equilibrium melting curve for this material shows that T_m is $\approx 23^\circ$; thus again T_m' falls approximately 7° above the midpoint of the transition and lies very close to the temperature at which the last vestiges of collagen-type structure melt out. Therefore, as pointed out previously (von Hippel and Harrington, 1960), in some respects this melting point (T_m') provides a more relevant measure of the stability of the crystalline phase than T_m .

Effect of Imino Acid Content of Polypeptide Chains.—Some years ago Gustavson (1953, 1956) noted the existence of a close correlation between the shrinkage temperature (T_s) of a collagen fiber and its hydroxyproline content. This observation led Gustavson to propose that interchain hydrogen bonds involving the hydroxyl groups of hydroxyproline might be primarily

responsible for stabilizing the collagen structure. More recently, as more total amino acid analyses of various collagens have become available, Piez (1960) and Piez and Gross (1960) showed that the correlation of T_s with total imino acid content (hydroxyproline plus proline) was, in fact, statistically somewhat better than the correlation with either imino acid alone. A similar relation between T_m (the melting temperature of collagen in solution) and either proline or hydroxyproline content alone, or total imino acid content, was demonstrated by Burge and Hynes (1959) for five different collagens.

Several different lines of evidence suggested that the total number of pyrrolidine rings in the chain should be the key element in controlling the stability of the collagen structure (for a discussion of this point see von Hippel and Harrington, 1960). However, since, the ratio of proline to hydroxyproline for most collagens is very similar (1.5 ± 0.3), it was relatively difficult to show directly that the correlation between T_s (or T_m) and total imino acid content was more than marginally better than that with either proline or hydroxyproline alone (especially since there was no *a priori* justification for the usual assumption that these relationships should be linear). However, the recent isolation and purification of earthworm cuticle collagen by Maser and Rice (1962) has conclusively settled this question. This collagen differs markedly from all others previously examined in that it contains very little proline (11 residues/1000) and a great deal of hydroxyproline (153 residues/1000). Maser (1962) and Maser and Rice (1963) have carried this material through viscometric and polarimetric melting curves and obtained values of T_m which, even qualitatively, are compatible only with total pyrrolidine content as a determinant of collagen stability.³

³ A similar conclusion has also been reached by Josse and Harrington (1963) on the basis of a comparison of the melting behavior of ascaris cuticle (high proline, low hydroxyproline) and earthworm cuticle (low proline, high hydroxyproline) collagens.

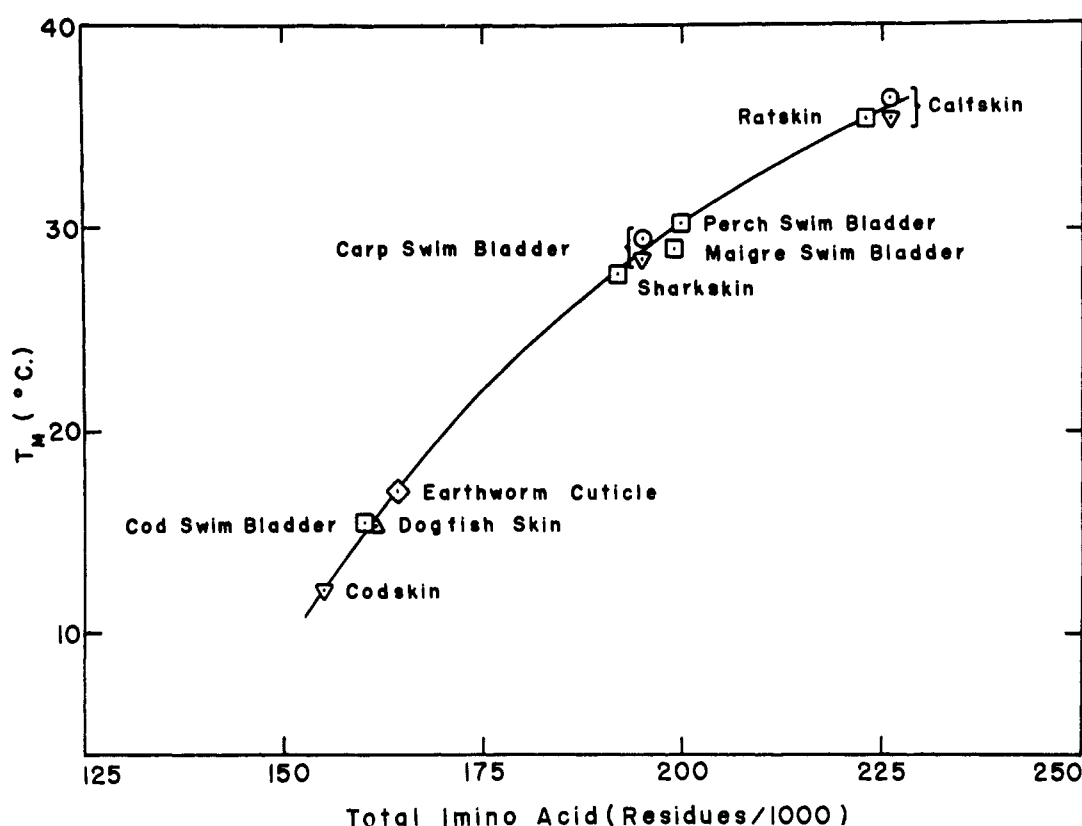


FIG. 10.—Relation between total imino acid content and T_m for a number of collagens. All T_m values adjusted to "equilibrium" conditions (see text). Data derived from following sources:

Symbol	Imino Acid Content	T_m
○	Piez and Gross (1960); Piez <i>et al.</i> (1960); and Piez <i>et al.</i> (1963); average	This study
□	Burge and Hynes (1959)	Burge and Hynes (1959)
▽	Piez and Gross (1960) and Piez <i>et al.</i> (1960); average	Doty and Nishihara (1958)
△	Lewis and Piez (1961) and Piez <i>et al.</i> (1963); average	Lewis and Piez (1961)
◇	Maser and Rice (1962)	This study

With this question settled it seemed worthwhile to attempt to establish the exact form of the relationship between T_m and total imino acid content. In the course of the present studies, T_m has been obtained from equilibrium melting curves for several collagens (including earthworm cuticle collagen). In addition, a number of measurements of T_m for a variety of collagens have been culled from the literature and corrected to "equilibrium" and equivalent solvent conditions as described under Materials and Methods (and Fig. 1). These data are plotted against total imino acid content in Figure 10. This figure shows that the relationship between these parameters is not quite linear, but that all the points fall very close to a curve that is somewhat concave to the imino acid content axis.

Extrapolating this curve to lower values of T_m suggests that a total pyrrolidine content of 130–140 residues is required to attain a value of T_m of approximately 0°, and that therefore stable poly-L-proline II-type helices should not be expected in proteins containing less than this number of imino acid residues. This conclusion is in good agreement with the suggestion of Szent-Gyorgyi and Cohen (1957) that proteins such as the caseins (with 90–130 proline residues per 1000; Tristram, 1953) occur under all conditions in the random coil form (as judged by rotatory dispersion, e.g.,

Jirgensons, 1958) because they contain too much proline to form stable α -helices, and too little to form stable elements of the poly-L-proline II helix.

It should be pointed out that the close fit of all the points in Figure 10 to a single curve, taken in conjunction with the fact that only monophasic melting curves of essentially identical "sharpness" are seen for all collagens investigated (von Hippel and Wong, 1963), indicates that the imino acids are distributed in a relatively uniform manner along the polypeptide chains. This point is considered further in the following paper.

GENERAL DISCUSSION

The effects of ions on the melting behavior of soluble native collagen (Figs. 2 and 3 and Table I) are very similar to their effects on the collagen-fold \rightleftharpoons gelatin transition in cooled gelatin (von Hippel and Wong, 1962). In both systems T_m appears to be a linear function of ion concentration (equation 1) and, though a smaller number of electrolytes were tested on native collagen, the rank order of effectiveness of these ions in reducing T_m seems to be the same for both. This finding reinforces the concept that the poly-L-proline II-type helices in native collagen and in cooled gelatin are stabilized by basically similar mechanisms, and

that ions perturb these mechanisms by similar means.⁴

One striking quantitative difference has been observed between the values of K obtained with native collagen and those obtained with cooled gelatin (Table I). The K values for native collagen are all 50–100% larger than the equivalent ones obtained with cooled gelatin. However, from certain points of view this discrepancy seems not unreasonable. In many ways increasing the concentration of a neutral salt (with a negative K) is quite analogous to increasing the temperature, and thus just as native collagen undergoes thermal melting in a more “highly cooperative” fashion than cooled gelatin, it is not unreasonable that destabilization of the helical structure by a given salt should also be more “cooperative” for native collagen, as evidenced by a larger value of K . Such differences between the melting of native collagen and cooled gelatin are considered in some detail in the following paper.

It is clear that no single, specific and unequivocal interpretation of the effects of neutral salts on the collagen-type helix can be made at this time. However, possible mechanisms can be subdivided into two broad categories and considered critically in the light of recent experimental developments. These categories might be labeled *direct mechanisms*, in which the stability of the collagen helix is altered as a consequence of direct binding of ions to specific sites on the polypeptide chain; and *indirect mechanisms*, in which the ions perturb the helix by a competitive reorganization of the structure of water involved in some way in stabilizing it. The following remarks are intended to supplement and bring up to date the discussion in our previous paper (von Hippel and Wong, 1962).

Proposed mechanisms of the direct type generally invoke specific binding of ions to the peptide grouping itself. Mandelkern *et al.* (1962) have suggested that the effect of concentrated solutions of lithium bromide on the shrinkage of α -keratin fibers might be due to such binding, resulting in an electronic reorganization of the peptide bond which decreases its double-bond character and thus facilitates rotation of the chain about it. Direct binding of perchloric acid (Steinberg *et al.*, 1960) and lithium bromide (Harrington and Kurtz, personal communication) to polyproline in nonaqueous solution has been demonstrated, and the associated conformational changes have been interpreted in terms of a direct effect on the partial double-bond character of the peptide bond. It has been rather convincingly shown by Bello *et al.* (1956), in their study of the effect of ions on the melting of gelatin gels, that ion-binding to the various polar residues of gelatin is probably *not* involved in the neutral salt effect. These workers used specific chemical blocking techniques to prevent ion-binding to various types of residues, and found no change in the effects of the salts on the melting temperature of the gel. In this connection we may also point out that Cu^{++} and Ni^{++} , which clearly *do* alter the stability of the collagen-type helix by direct binding to peptide groups, behave quite differently from the neutral salts considered in this and

our previous paper. Unlike these salts, Cu^{++} in particular is characterized by a *curved* dependence of T_m on ion concentration, and a marked dependence of the effect on pH (see Discussion in von Hippel and Wong, 1962).

Any general mechanism which requires that direct binding of ions to the peptide grouping be responsible for the effects of neutral salts on the stability of the collagen-type helix must be compatible with the following experimental facts (as, of course, must any other proposed mechanism): (1) The binding constant for the proposed interaction must be very small, since the neutral salt effect seems to be independent of gelatin (and peptide bond) concentration over at least two orders of magnitude (see von Hippel and Wong, 1962; Bello, 1963). (2) The extent of *effective* binding must be very temperature dependent, and equilibrium must be reached very rapidly, since helix formation in a solution of a given salt begins with maximal velocity (no lag period) immediately after “quenching” a sample of random coil gelatin from a temperature above to a temperature below the T_m which applies to that particular gelatin-salt system (von Hippel and Wong, 1962). (3) The proposed ion-binding must modify the stability of the helical form without introducing a unique destabilizing mechanism, since there exists a definite transition temperature for the collagen-type helix in the absence of salt which is related linearly and without discontinuity to the values of T_m obtained at finite salt concentrations (von Hippel and Wong, 1962). (4) Both anions and cations are effective in altering T_m , the net change in mixed salt solutions approximating roughly the algebraic sum of the effects expected from the various ions present (Bello *et al.*, 1956; von Hippel and Wong, 1962). (5) Some ions raise T_m , and others lower it. These facts, considered together, seem rather incompatible with a *general* mechanism which seeks to attribute the effects of neutral salts on the collagen-type helix solely to direct binding of the ions to the polypeptide chain. In our opinion, these facts are more readily interpreted in terms of an “indirect” mechanism of the type described above. (However, see Bello, 1963.)

In this connection, it seemed pertinent to investigate the generality of these neutral salt effects. Therefore we studied the effect of various concentrations of neutral salts on the temperature of the mid-point of the well-known thermal transition which occurs in ribonuclease in water at about 60° (e.g., see Harrington and Schellman, 1956). We have found that T_m for this transition is altered by neutral salts in a fashion very similar to the effect these salts have on the collagen-type helix (von Hippel and Wong, in preparation). The salts we have studied to date are listed in Table III in order of increasing molar effectiveness in depressing the temperature of the ribonuclease transition, and compared with the anion and cation series obtained for collagen and gelatin. Clearly the order of effectiveness is closely similar for the destabilization of the left-handed poly-L-proline II-type helix in collagen and cold gelatin, and the destabilization of the presumably right-handed structure which is melted out in the ribonuclease transition (this transition is accompanied by an *increase* in specific levorotation).

It is also interesting to consider the effects of ions on the thermally-induced helix \rightleftharpoons coil transition in a macromolecule which does not contain the peptide linkage. Hamaguchi and Geiduschek (1962) have recently published a study of the effects of high concentrations of neutral salts on the transition temperature of various species of DNA. Their studies are also summarized in Table III, in terms of the order

⁴ It has recently come to our attention that Weir and Carter (1950), in the course of a study of the shrinkage behavior of kangaroo tail tendon, also observed a roughly linear relation for some salts between the concentration of neutral salt added and the shrinkage (or melting) temperature (T_m) of the tendon. In particular, they showed that LiCl , NaSCN , BaCl_2 , CaCl_2 , and MgCl_2 markedly lowered T_m , while Na_2SO_4 substantially elevated the shrinkage temperature. “ K ” values estimated from their data appear to be somewhat larger than those obtained with native collagen in solution in this study.

TABLE III
ORDER OF EFFECTIVENESS OF VARIOUS IONS IN REDUCING T_m FOR VARIOUS MACROMOLECULES

←—Helix (Salting-out)	Coil—→ (Salting-in)
<i>Collagen-gelatin^a</i>	
$\text{SO}_4^{--} < \text{CH}_3\text{COO}^- < \text{Cl}^- < \text{Br}^- < \text{NO}_3^- < \text{ClO}_4^- < \text{I}^- < \text{CNS}^-$ $(\text{CH}_3)_4\text{N}^+ < \text{NH}_4^+ < \text{Rb}^+, \text{K}^+, \text{Na}^+, \text{Cs}^+ < \text{Li}^+ < \text{Mg}^{++} < \text{Ca}^{++} < \text{Ba}^{++}$ $(\text{CH}_3)_4\text{N}^+ < (\text{C}_2\text{H}_5)_4\text{N}^+ \ll (\text{C}_3\text{H}_7)_4\text{N}^+, (\text{C}_4\text{H}_9)_4\text{N}^+$	
<i>Ribonuclease^b</i>	
$\text{SO}_4^{--} \ll \text{Cl}^- < \text{Br}^- < \text{ClO}_4^- < \text{CNS}^-$ $(\text{CH}_3)_4\text{N}^+, \text{Na}^+ < \text{Li}^+ < \text{Ca}^{++}$ $(\text{CH}_3)_4\text{N}^+ < (\text{C}_2\text{H}_5)_4\text{N}^+ < (\text{C}_3\text{H}_7)_4\text{N}^+ < (\text{C}_4\text{H}_9)_4\text{N}^+$	
<i>DNA^c</i>	
$\text{Cl}^-, \text{Br}^- < \text{CH}_3\text{COO}^- < \text{I}^- < \text{ClO}_4^- < \text{CNS}^-$ $(\text{CH}_3)_4\text{N}^+ < \text{K}^+ < \text{Na}^+ < \text{Li}^+$	
<i>Benzoic acid^d</i>	
$\text{Cl}^- < \text{Br}^- < \text{I}^- < \text{NO}_3^- < \text{ClO}_4^- < \text{CNS}^-$ $\text{Li}^+ < \text{Na}^+ < \text{K}^+ < \text{NH}_4^+ \ll (\text{CH}_3)_4\text{N}^+ < (\text{C}_2\text{H}_5)_4\text{N}^+$	

^a Data from von Hippel and Wong (1962), Bello *et al.* (1956), and Carpenter (1938). ^b Data from von Hippel and Wong (in preparation). ^c Data from Hamaguchi and Geiduschek (1962); differences between cations are small. ^d Data from compilation of Long and McDevit (1952); in increasing order of effectiveness in salting-in.

of effectiveness of the various ions investigated; note again the marked similarity to the series obtained with collagen and gelatin and with ribonuclease. Hamaguchi and Geiduschek have also interpreted their results in terms of a competitive reorganization of the structure of water by the added salts, resulting in a weakening of the "hydrophobic bonds" between "stacked" nucleotide bases.

The findings summarized in Table III, which suggest that these salt effects are due to something beyond the chemical or conformational details of the specific macromolecules involved, make all general mechanisms which depend on such details seem less likely. Therefore these findings seem to mitigate against both mechanisms which postulate direct binding of the ions to specific chemical groups, and mechanisms which propose that water molecules stabilize the helix by fitting directly into the macromolecular conformation in some specific way (e.g., the model proposed previously by von Hippel and Harrington, 1960, and Harrington and von Hippel, 1961b).

It would seem that the ions must act primarily on some general property of the aqueous solution, perhaps perturbing macromolecular conformations indirectly by weakening or strengthening "hydrophobic bonds" (e.g., see Némethy and Scheraga, 1962) by competitively disorganizing or further organizing the water clusters or "cages" upon which all current theories of "hydrophobic bonding" are based (Frank and Evans, 1945).

The ions listed in Table III are, of course, arranged basically in the classical Hofmeister series which has been found to apply to so many properties of ionic solutions. We may note that the same salts which are particularly effective in destabilizing the collagen-type helix are also most effective in solubilizing collagen fibers (see Gallop *et al.*, 1957). Also it is shown in Table III that the same anion series applies to a (comparatively) simple process such as the salting-out of benzoic acid from water (Long and McDevit, 1952) though it should be noted that the order of effectiveness of the cations is reversed.

A more satisfying general interpretation of these salt effects must probably await further developments in our knowledge of the structure of water and the effect of ions upon it. However, the general qualitative similarities evidenced in Table III should not blind

us to the marked quantitative differences which do exist between these systems, and which suggest that perhaps specific ion effects are involved as well.

If we assume for the moment that neutral salts do operate on the collagen-type helix indirectly through competitive interactions with structurally involved water, then nonelectrolytes which are known to influence the structure of water should also be effective in altering the stability of collagen. The experiments with D₂O and urea which are described in this paper amply bear out this expectation. Not only are the forms of the equations relating the melting temperature and the initial rate of mutarotation to the molarity of added nonelectrolyte the same as those derived from electrolyte data (equations 1 and 2), but the data for the ichthyocol-urea and the ichthyocol-D₂O systems both fit quite well on the empirical curve relating the kinetic parameter (k_0) and the equilibrium parameter (K) for the various salts tested (Fig. 8, von Hippel and Wong, 1962). In terms of its molar effect on T_m and $(d[\alpha]/dt)_0$, urea falls between LiCl and CsCl, and D₂O between (CH₃)₄Br and (CH₃)₄NCl on this curve. We may also note that the effects of D₂O and urea on the thermal transition in ribonuclease are qualitatively and semiquantitatively the same as the effects on the collagen-gelatin transition (Hermans and Scheraga, 1959; Foss and Schellman, 1959; von Hippel and Wong, in preparation).

The total imino acid content of the polypeptide chain has an appreciable effect on both the stability of the collagen structure with respect to thermal denaturation and on the rate of formation of the collagen-type helix. As has been shown elsewhere (von Hippel and Harrington, 1960; Harrington and von Hippel, 1961b), the initial rate of mutarotation of various gelatins following quenching to a given temperature increases markedly with increasing total pyrrolidine-ring content. The data available suggest that the rate for a given gelatin depends primarily on ΔT (the difference between the temperature of the experiment and the transition temperature for that gelatin). This suggestion, of course, is in good accord with the results of Flory and Weaver (1960) and those described here, which show that the initial rate of mutarotation for a particular gelatin at a particular temperature also depends primarily on ΔT .

The effects of ions and of total imino acid content on the thermal stability of collagen appear to be roughly additive, at least in terms of the effect of CaCl_2 on T_m for calfskin, ichthyocol, and earthworm cuticle collagen. Figure 1 shows that the slopes of the T_m vs. m_{CaCl_2} plots (and thus the values of K) are roughly similar for these collagens, while the intercept with the T_m axis varies markedly with imino acid content as required by Figure 10.

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