The Role of pH, Temperature, Salt Type, and Salt Concentration on the Stability of the Crystalline, Helical, and Randomly Coiled Forms of Collagen*

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SUMMARY

The role of pH, temperature, salt type, and salt concentration on the three possible transformations involving the crystalline, dispersed helical, and randomly coiled forms of collagen has been investigated. The results are given in terms of pseudo phase diagrams where the field of stability of each form is delimited by the curves representing the variation of the three transformation temperatures with salt concentration for a given salt type and pH. The data are adequate to characterize the behavior of the systems corresponding to situations where the solute is an isoelectric protein or a cationic polyelectrolyte.

It is shown that a general mechanism for the interaction between salts and proteins based on the previously defined specific (i.e. binding) and diluent effects, is adequate to describe the transformations crystalline form → randomly coiled form, crystalline form → helical form, helical form → randomly coiled form under isoelectric conditions. The polyelectrolyte behavior included the reversal of the orders of effectiveness of anions with respect to the orders observed under isoelectric conditions. This is explained on the basis of an order for binding of the anions to the solute which remains the same irrespective of whether the protein is isoelectric or cationic.

In dealing with macromolecules such as collagen, deoxyribonucleic acid, polypeptides, etc., in the presence of a solvent, the following situations are often encountered: a solution of randomly coiled molecules; a solution of molecules having a helical conformation; and a crystalline precipitate. At a given polymer concentration, occurrence of one or another of these situations depends upon the pH, the temperature, and the nature of the diluent, which, in the case of a salt solution, implies the specification of both salt type and salt concentration. The object of this paper is that of presenting a unified study of the effect of the four variables, pH, temperature, salt type, and salt concentration, on the physical state of the macromolecules or, generally, the role of these variables on the "equilibria" between the randomly coiled, helical, and crystalline forms. This will be done, in the case of collagen, through the determination of pseudo phase diagrams for the polymer solutions where the field of stability of each form will be defined by curves representing, for instance, the variations of the transformation temperatures with salt concentration at a given pH and salt type. The transformations treated in this paper are schematized in Fig. 1, following the scheme originally given by Flory (1). The range of pH investigated is sufficiently large that our representation offers a description of the role of salts on the stability of the helical, randomly coiled, and the crystalline forms corresponding to situations where the polymer is a polyelectrolyte as well as when it carries a zero net charge.

Separate examples of the effects of charging and of salt on the stability of the three forms have been widely investigated. Von Hippel and Wong (2) and Bianchi, Conio, and Ciferri (3) have extensively studied the effect of neutral salts on the helix-random coil transformation of soluble collagen. These results along with similar studies on DNA (4, 5) polyglutamic acid (6) and polylsine (6) are typical examples of Transformation II (helical to randomly coiled form). In the presence of salts, the above polymers can often precipitate from the helical form directly into a crystalline phase (3, 7) without the intermediate of the randomly coiled form, these being examples of Transformation III (helical to crystalline form). The precipitated phase can often be redissolved, for instance by increasing temperature, into a solution of random coils (3) and this process, along with the shrinking of collagen (8) and fibrous proteins (9, 10) produced by salts or by pH changes, is an example of Transformation I (crystalline to randomly coiled form) for polyelectrolytes or isoelectric proteins. Consequently, not all of our data are original; nevertheless this represents the first attempt to unify the various transformations. The presentation of a unified investi-
nations of such processes arises from the desire (a) to systematize phenomena which have the evident correlations indicated in the pseudo phase diagrams and (b) to establish whether a general mechanism for the interaction between ions and macromolecules can be used for interpreting the role of salts on the three transformations.

Experimental Procedure

Materials—Experiments reported herein have been made starting from a tropocollagen (i.e., the native triple helix unit of collagen) solution obtained by solubilizing carefully washed rat tail tendons in water after prior treatment of the tendons in a 0.5 M aqueous NaH$_2$PO$_4$ according to the procedure described by Dumitru and Garrett (11). The latter salt was eliminated by stirring the tropocollagen solution in the presence of a mixture of anionic and cationic exchange resins. Phosphorus content in the final solution was below 30 p.p.m. Solutions so obtained contained about 0.2% (w/v) of protein determined by dry weight, and had a pH of 4 to 5. The solutions were stored in a cold room before use and storage times longer than 10 days were avoided, no degradation of the protein being evident under these conditions.

Salts of analytical grade used were KSCN, KCl, KF, CaCl$_2$, Ca(SCN)$_2$, and CsCl. For the preparation of protein solutions containing salts at pH = 2.3, 7.5, and 10.7 the pH of the protein water solution and that of the salt solution was adjusted to the required value. When necessary the pH was readjusted after mixing. For the preparation of the protein solution at pH = 6 no adjustment of the pH values of the salt, of the original protein, or of the final solutions was made. Values of the final pH in these cases were 6 ± 0.5. Solutions of salt and protein were generally mixed in the cold room and the proper concentrations were chosen to give final ternary solutions with polymer concentration from 0.01% to 0.1% and the salt concentration specified in each case (in moles per liter).

Determination of Transformation Temperatures—Depending upon alterations of salt type, salt concentration, pH, or temperature the initial tropocollagen solution may undergo a transformation into a solution of random coils, or into a crystalline precipitate. The precipitates encountered in this investigation were crystalline, or at least considerably ordered, because of the fibrous appearance and the high birefringence exhibited when observed in the presence of the supernatant liquid under a polarizing microscope. Results in the literature (12, 13) indicate that the (dried) precipitates obtained under conditions similar to those used here often exhibit the 700 Å spacing of native collagen. However, the results of Schmitt (13) suggest that this is not necessarily true in general, and the possibility of obtaining precipitates differing in detailed structure was not investigated here.

The preferred technique for establishing the transformation temperatures was that of equilibrating the freshly prepared tropocollagen solution of given pH and salt concentration in the cold room for at least 12 hours with moderate stirring; the solution itself being maintained in the bulb of a viscometer. Afterwards the temperature was gradually increased, generally at a rate of 1° per 30 min (particularly near the transformation temperature). At the beginning, two situations were possible; either the solution was still clear or a precipitate had developed. In the latter case, on increasing temperature, the temperature at which the precipitate dissolved was noted by visual observation and by measuring the viscosity of the resulting solution. Invaribly the latter corresponded to that of random coils (parent gelatin) thus indicating that the transformation observed was of type I. When the solution appeared homogeneous in the cold room the high viscosity confirmed the presence of the helical form. On increasing temperature two cases were possible. Either the solution remained homogeneous but a strong decrease of viscosity indicated the occurrence of a helix → coil transformation (Process II), or the direct formation of a crystalline precipitate was visually observed upon heating indicating the occurrence of Transformation III. In this latter case the precipitate could be redissolved on further increase of temperature and the transformation was of type I, similar to that described above.

The above conditions (particularly the storage of the tropocollagen solution in the cold room for 12 hours) were effective in reducing to a minimum the time effects associated with the transformations. In general, time effects were considerably larger for Transformations I and III than for Transformation II. However, with the use of the procedure described, no significant alteration of the temperature of Transformation III was observed, when for instance, for CaCl$_2$ at pH 2.3, the rate of heating was reduced from 1° per 30 min to 1° per 12 hours. Similar results applied to the other transformations. Likewise, the effect of altering the polymer concentration within the range 0.01 to 0.10% on the transformation temperatures was largely inconsequential.

Some arbitrariness was unavoidable in the visual observation of Transformations I and II. However, the possibility of measuring the viscosity just after the melting or just before the formation of the precipitate was extremely useful in narrowing the error associated with the determination of temperature of Transformations I and III which could, in fact, be reproduced within ±3°. It was found that only the final point of Transformation I could be located with adequate reliability, particularly in the case of measurements at pH ≥ 7.4 when an unusual broad melting was noticed (a spread up to 10° was common).

Viscosities were measured with a suspended level viscometer thermostated to ±0.05°. Flow times (±0.1 sec) were always...
FIG. 2. Typical reduced specific viscosity against temperature curves for tropocollagen solutions indicating the helix — coil transformation. \( C_p \), polymer concentration.

greater than 100 sec. Typical reduced specific viscosity curves indicating the helix — coil transformation are reproduced in Fig. 2; Transformation II temperature was taken as the temperature corresponding to the midpoint of the transformation (obtained by extrapolating the linear portion of the curves before and after the drop in viscosity). Under the conditions used they could be reproduced to within \( \pm 1^{\circ} \).

Reversibility—The reversibility of Transformations I and II was not investigated in detail here. It is known that the native crystalline or helical structure is not completely recovered once the randomly coiled form is obtained according to Process I (14) and II (2). Nevertheless the transformations can still be regarded, in principle, as reversible processes since conspicuous evidence of collagen fold re-formation (15) is evident on cooling tendons or dilute solutions of gelatin to just below the temperature of Transformations I and II.

The difficulties of redissolving the precipitate, once the crystalline form has been obtained according to Process III, are also well known (16). Again, these difficulties are not proof of real irreversibility since, for precipitates which have been maintained above the temperature of Transformation III for a considerable time, redissolution can be achieved with a considerable degree of supercooling and prolonged stirring (16). This is analogous to the heat precipitation of poly-L-proline, an unquestionably reversible process (17). In the present investigation redissolution of the precipitate formed by increasing temperature could be easily obtained on cooling just below the temperature of Transformation III provided that the precipitate was not permitted to stand for over 15 min.

RESULTS

Pseudo Phase Diagrams

The effect of pH on the stability of the helical, randomly coiled, and crystalline forms and on the transformation temperatures in absence of salts is represented in Fig. 3. The corresponding effect of salt concentration, for several salt types and pH, is shown in Fig. 4.

We shall refer to the figures as the “pseudo phase diagrams” of the collagen solution where the curves represent the loci of pseudo equilibria crystalline — randomly coiled form, helical — randomly coiled form, and helical — crystalline form and the regions so delimited are the regions of stability of the three forms. The analogy with conventional phase diagrams is evident. The pair salt concentration and temperature (or pH and temperature) can be unequivocally defined either by experiments where salt concentration is constant and temperature varied or vice versa (8) and expressions formally equivalent to the Clausius-Clapeyron equation can be derived (18, 19) to characterize the equilibrium crystalline — randomly coiled form as it is affected by salt concentration and temperature, for a constant value of the other variables influencing the system. Although residual time effects, incomplete reversibility, and limited sharpness were observed in several cases, the occurrence of corresponding equilibrium cooperative processes is not ruled out and we believe that the shape of the equilibrium diagrams would not greatly differ from that reported. It will be noticed that the curves for temperatures of Transformation I, II, and III against salt concentration were not extrapolated to a common triple point. This is, at least in
part, due to the fact that the techniques for determining the transformations were not completely equivalent (cf. "Experimental Procedure")

**Effect of pH (Fig. 3)**

When the pH of the original solution is decreased to about 2 at 25°, the solution of cationic protein remained clear, while on raising the pH, precipitation occurred at pH = 6.9. The isoelectric point was estimated as 7.2 ± 0.2 on the basis of the data of Reference 20 and on the basis of the isoelectric isoelectric pH of the same protein solution at 40°. The latter was determined by passing the protein solution (previously held for about 1 hour at 50°) through a column of Amberlite MB-3, as described elsewhere (21). Under the conditions used the protein was in the randomly coiled form and remained in solution even under isoelectric isoelectric conditions. By contrast the native tropocollagen remained in the crystalline form. A tendency toward resolubilising of the tropocollagen began to decrease continuously on further charging of the protein (cf. also Fig. 2). The same result was obtained by Burge and Hynes (22).

The transformation helical → crystalline form occurred at pH 6.9 practically independently of temperature between 0 and 39°. The transformation temperature of helical → randomly coiled form was independent of pH between pH 6.9 and 4 after which it began to decrease continuously on further charging of the protein (cf. also Fig. 2). The same result was obtained by Burge and Hynes (22).

On the alkaline side, the (final) melting point of the crystalline polymer (crystalline → randomly coiled) was 49°, essentially independent of the pH until pH ~ 11.

**Effect of Salts (Fig. 4)**

At pH = 7.4—Pseudo phase diagrams for KSCN, CaCl₂, and KCl are reported, respectively, in the Columns 1, 2, and 3 of Fig. 4 for different pH values. Column 4 contains typical diagrams for other salts. In all cases the value of the transformation temperature at salt concentration = 0 coincides with the value obtained from the data in Fig. 3. Accordingly, starting with the diagrams at the isoelectric pH, we observe that below 49° the crystalline phase is present. On increasing salt concentration, Transformation I temperature is decreased continuously for the salting in agents (8) KSCN and CaCl₂, while it goes through a minimum for the salting out agents (8) KCl and KF. For the salting in agents the appearance of the helical form is eventually observed on increasing salt concentration. The corresponding helix → coil transformation temperature is continuously depressed on further increasing salt concentration. Thus the field of stability of the randomly coiled form is increased by salting in agents with increasing salt concentration irrespective of whether the ordered forms are crystalline or helical. For the salting out agents no helical form is observed.

While the formation of the dissolved randomly coiled form according to the transformations crystalline → randomly coiled, and helical to randomly coiled forms occurs from left to right on increasing temperature, the formation of the dissolved helical phase according to the process crystalline → helical form occurs with decreasing temperature at a given salt concentration. The temperature of Transformation III is increased on increasing salt concentration, which implies that the field of stability of the helical form with respect to the crystalline form is enlarged with increasing salt concentration.

At pH = 6—We now consider the pseudo phase diagrams at pH = 6 near isoelectric conditions (data for KSCN, CaCl₂, and KCl have been previously published (3)). At salt concentration = 0, the helix is the stable form below 39° for CaCl₂ and KCl. However, in the presence of KSCN the crystalline form is present. At higher salt concentrations no crystalline form appears for CaCl₂ or helical form for KSCN. The temperature for Transformation I for the latter, and Transformation II, for the former, are continuously depressed on increasing salt concentration. In the case of Ca(SCN)₂ no evidence of the crystalline form was found and much larger depressions of the denaturation temperature with salt concentration than those observed for CaCl₂ and KSCN were noticed. On increasing the salt concentration of KCl, the crystalline form is eventually observed. In contrast to the cases observed at pH = 7.4, the helical form is stable at low salt concentrations and the crystalline form at high salt concentrations, and the slope of the helical → crystalline form equilibrium line is negative. Still the transformation helical → crystalline form occurs on increasing temperature. The temperature of Transformation II (for KCl) is depressed until salt concentration ~ 0.1 M and then tends to increase. The temperature of Transformation I is instead raised on increasing salt concentration as for the corresponding diagram at pH = 7.4. Finally, in the case of CaCl₂ a field of stability of the helical form is observed at low temperature with Transformation III temperature going through a maximum at salt concentration ~ 0.6 M. It will be noticed that in this case the helical form can never be directly transformed into the randomly coiled form.

1 E. Bianchi and G. Conio, unpublished experiments.
At pH = 2.3—We finally consider the diagrams at pH = 2.3 where the protein has the maximum net (positive) charge (8). The helix is the stable form at salt concentration near or equal zero and at low temperatures. On increasing salt concentration, the temperature of Transformation II is depressed to a minimum approximately at which point the crystalline form appears and the temperature of Transformation I starts increasing with further increase of salt concentration. It is important to observe that this increase of Transformation I temperature with salt concentration is continuous in the case of the salting out agents KCl and CsCl (even at salt concentrations up to the solubility limit of the salt9), whereas for KSCN and CaCl₂ after salt concentrations of the order of 0.6 to 1 M are reached, the temperature of Transformation I starts decreasing again with salt concentration (at 3 M KSCN, it is ∼ 0°, unreported). In connection with Transformation III we observe that KSCN is more effective than KCl in reducing the stability of the helical form with respect to the crystalline form and that the slope of the salt concentration against Transformation III temperature line is negative in all cases (indicating that the field of stability of the helical with respect to the crystalline form is decreased on increasing salt concentration). Still the transformation helical → crystalline form occurs on increasing temperature. We finally note that a salt, such as KSCN, which is a good solubilizing agent of the helical form at pH = 7.4 turns out to be a good insolubilizing agent at pH = 2.3.

At pH = 10.7—At pH = 10.7 and at room temperature, solutions in KCl were in the gel form while in the case of KSCN a
solution of helices was formed at 0.5 M. The temperature of Transformation II for the latter was ≈ 30° (cf. Fig. 2).

**DISCUSSION**

It is convenient, for purposes of generalization and for clarity, to present a systematic discussion of the factors which we regard as satisfactory interpretations of the effects observed. Some of these interpretations are selected from the literature. They are complemented with our own suggestions as discussed in previous publications. A comparison with data previously obtained for cross-linked collagen tendons (8) is included.

A. **Role of Temperature on Transformations I, II, and III**

Processes I and II occur in the direction from crystalline to randomly coiled form and from helical to randomly coiled form on increasing temperature in analogy to the melting of one-component systems and most cases of crystallization from solutions. The disordered randomly coiled form is stable in the high temperature range because the entropy and enthalpy of the polymer in this form are higher than in the ordered crystalline or helical form. For Process III, we can assume that the way in which increasing temperature brings about precipitation of tropocollagen units is similar to the case of the reversible heat precipitation of poly-L-proline (17). Thus, the fact that the ordered crystalline form is stable in the high temperature range is to be attributed to the fact that both the entropy and enthalpy of the polymer segments in the precipitate phase (S_C and H_C, respectively) exceed the corresponding (partial molar) quantities for the polymer in solution (S_H and H_H, respectively), i.e., both ΔS = S_H - S_C and ΔH = H_H - H_C < 0. A net increase in the entropy per polymer unit upon transfer from solution to the precipitate phase would imply that in the dispersed phase a substantial degree of order prevails probably involving organization of solvent molecules around the solute species (17). This may also occur for the randomly coiled form but the corresponding entropy change may be overshadowed by the large entropy change due to the randomization of the polymer conformation upon melting. The corresponding characteristics of the enthalpic process comply with such interpretations.

B. **Role of pH on Transformations I, II, and III**

On the basis of the titration data of Kenchington and Ward (23) for gelatin, the pH ranges for titration of the significant carboxyl, α-amino + imidazole, and ε-amino groups are, respectively, 1.5 to 6.5, 6.0 to 8.5, and 8.0 to 11.5. Considering our estimated isoelectric pH of 7.2 ± 0.2, pertinent data in Fig. 3 suggest that a certain amount of net cationic charge can exist within the dissolved triple helix without altering the stability of the helical with respect to the randomly coiled form. However, at pH < 4 the number of cationic charges begins to be appreciable and the resulting destabilization of the helix (i.e., reduced temperature of Transformation II) is usually attributed to the electrostatic repulsion of the positive charges.

The same considerations can be extended to the case of a net anionic charge on the alkaline side of the pH scale when only at pH > 10 a depression of Transformation I temperature with pH is observed. Data in Fig. 3 can be compared with those reported in Fig. 5 concerning the variation of the shrinkage temperature with pH for cross-linked collagen tendons (8) when both the presence of cross-links and the large polymer concentration hinder the possibility of observing the helical form (24). A general similarity of pH effects is evident. The difference of about 20° between the absolute values of shrinkage temperature and Transformation I temperature (Figs. 3 and 5) is attributed to the high polymer concentration and to the more perfect crystalline organization in the case of tendons. (The displacement of the Transformation II temperature against pH with respect to the Transformation I temperature against pH curve in Fig. 3 cannot easily be attributed to energetic differences between helical and crystalline forms in view of the experimental difficulty in determining values of the temperature of Transformation I at pH ≥ 7.4.)

Concerning Process III, the fact that a small deviation of about 0.3 pH unit toward the acid side is enough to cause the transformation crystalline to helical form (below 39°) can be interpreted as evidence that even a minute net repulsion between positive charges carried by adjacent tropocollagen units suffices to destroy the intermolecular organization (25). However, it is interesting to note that the apparent building up of a large net negative charge is not effective in stabilizing the helical with respect to the crystalline forms even at high pH values. These observations may be indicative of a different titration behavior for gelatin and native collagen or of a specific, though unspecified, location or function of the functional groups sensitive to the corresponding pH changes.

C. **Role of Salts on Transformations I and II**

Isoelectric Conditions—As pointed out by several investigators (26-28) ions can be ranked according to their power of depressing the denaturation temperature or decreasing the activity coefficient of the solute (generally salting in) according to the following orders

\[ \text{F}^- < \text{SO}_{3}^- < \text{acetate} < \text{Cl}^- < \text{Br}^- < \text{NO}_3^- < \Gamma < \text{SCN}^- \]  
\[ \text{K}^+ < \text{Mg}^{2+} < \text{Na}^+ < \text{Ca}^+ < \text{Li}^+ < \text{Ca}^{2+} \]  

Some results obtained (8) with crosslinked tendons at pH = 7 are included in Fig. 5. The large and continuous depression of the shrinkage temperature (T_s) by KSCN and Ca(SCN)_2 (Fig. 5) is typical of salting in agents while the small depression at low salt concentration (KCl) followed by an increase of T_s at higher salt concentration is typical of salting out agents. An equation of the form (19, 20–31)
\[ \Delta H_f \left( \frac{1}{T_m} - \frac{1}{T_m^*} \right) = A(\xi - \xi_{c*}) + B \ln (1 + KC) \]  

(3)

(where \( T_m^* \) is the melting point of the undiluted protein, \( A \) and \( B \) are positive constants, \( \Delta H_f \) is the heat of fusion, \( \xi \) is the volume fraction of diluent, \( \chi \) is an interaction parameter, \( K \) is an equilibrium constant, and \( C \) is salt concentration) was shown (33, 34) to offer an adequate representation of the experimental behavior of collagen tendons. The second term on the right-hand side represents a contribution from salt binding (specific effect) to the dipoles of peptide (27, 33), bonds in the randomly coiled form, which is predominant for salting in agents. The first term on the right-hand side describes less specific, concentration-dependent types of interactions (diluent effects) (8) which are prevalent with salting out agents (for which \( K = 0 \) (39), and can be described by conventional salting out theories (21, 35).

A comparison of data in Fig. 5 with data in Fig. 4 (pH = 6 and 7.4) shows that the role of salts on the temperatures of Transformations I and II is generally similar and corresponds to that established for fibrous collagen. The similarity of the effects of salts on Transformations I and II can be explained on the basis that both processes are controlled by the interaction of the final randomly coiled form with the salt solution. In fact, it is possible (32) to describe the role of salts on the helix-coil transformation with an equation of the form of Equation 3, minor differences between the dependences of the temperatures of Transformations I and II upon salt concentration being accountable for in terms of numerical differences in the thermodynamic parameters involved.

While binding of salts to the randomly coiled form thermodynamically explains a large melting point depression, the molecular mechanism by which bound salt reduces the conformational stability of the polymer may require elucidation. The Series 1 for anions and 2 for cations for increasing salting in of proteins and related substances coincides with the series for increasing absorption of ions to isoelectric gelatin (37). However, the possibility that the large melting point depression reflects a charging out of the polymer (36) due to unequal anion or cation binding must be rejected on the basis of the results of References 33 and 37. These results indicate that the ionic concentrations are essentially the same inside and outside a swollen collagen gel and therefore Donnan effects must be negligible. Moreover, one finds that the depression caused by a salt where both anions and cations are strongly adsorbed (i.e. Ca(SCN)_2) is larger than for a salt where only one ion is preferentially adsorbed (i.e. KSCN, CaCl_2). Very likely, polyelectrolyte or Donnan effects resulting from unequal ion adsorption play a minor role at relatively high salt concentrations (not necessarily, however, at low pH or low salt concentration) due to the screening action of counterions and co-ions in proximity of the polymer.

These considerations support the view (9) that a local alteration of the electronic conformation of the groups at which binding takes place is a plausible mechanism for the destabilization of the helices.

**Nonisoelectric Conditions**—Under extreme nonisoelectric conditions such as that exhibited at pH = 2.3 (Figs. 4 and 5), the role of salts on Transformations I and II is similar to that observed under isoelectric conditions only for salt concentration \( > 0.8 \) M. For salt concentration \( < 0.8 \) the typical polyelectrolyte effect exhibited can be represented by the competition of two effects (a) a tendency for decreasing the temperature of Transformations I or II prevailing at very low salt concentration and (b) a tendency for increasing the temperature of Transformations I or II prevailing at somewhat higher salt concentration. The latter effect can be associated (38) with a decrease of the electrostatic free energy of the polyelectrolyte due to screening of the fixed charges by the mobile ions. The former effect is less clear although it might be associated (39) with the effect of salts on the apparent pK values of the ionizable groups of the protein. However, one would expect the ionization of \( \varepsilon \)-amino groups to be practically complete at pH \( \sim 2.3 \) while an increase of the apparent dissociation constant of carboxyl groups (40) should decrease (rather than increase) deviations from isoelectric conditions. Even with DNA, when Effect b seems to prevail (38), a small depression of denaturation temperature at salt concentration \( < 0.1 \) M was observed (5). Comparison of the data in Fig. 4 with the data for cross-linked tendons at pH = 2 (Fig. 5) again reveals that occurrence of the helical form does not modify the general form of the dependence of denaturation temperatures upon salt concentration even under nonisoelectric conditions.

We observe from the data in Fig. 4 that KSCN seems more effective than KCl in causing a prevailing Effect b over a. In fact, the denaturation temperature in 0.5 M solutions is about 10° higher for KSCN than for KCl indicating that for cationic polyelectrolytes the order of effectiveness of anions (Series 1) can be reversed at low salt concentration. We interpret this observation as being a further confirmation of the stronger ability of SCN^- over Cl^- to bind to the protein, as discussed in section C "Isoelectric Conditions." In contrast to the case considered in the latter section the effects associated with unequal ion adsorption are considered, in fact, to be very significant for processes which occur at low salt concentration, particularly if the polymer is not isoelectric. Whether binding of SCN^- actually takes place at the peptide bond or represents a more effective screening of the positive charge of the \( \varepsilon \)-amino groups of lysine and hydroxylysine residues is somewhat immaterial from the point of view of causing a decrease in the electrostatic repulsion along the chain. In both cases we can expect that the order of effectiveness for decreasing the electrostatic free energy of the chain is essentially the same as that for ion adsorption. A strong confirmation of this interpretation is offered by the fact that the order for anions for binding to quaternary ammonium anion exchange resins (27, 41) (Duvex 2) is entirely consistent with order in Series 1 which also represents the order for anions binding to isoelectric gelatin (37).

**D. Role of Salts on Transformation III**

**Isoelectric Conditions**—Only in the case of data for CsCl at pH = 6 (Fig. 4) is it possible to characterize the variation of the temperature of Transformation III with salt concentration in a large concentration range. We observe in this case that although the slopes of the curves of Transformation III temperature against salt concentration, and Transformation I temperature against salt concentration are opposite in sign at a given salt concentration, the effect of increasing salt concentration is the same in both cases, i.e. the field of stability of dissolved forms (helical or randomly coiled, respectively) is at first increased and then decreased. This immediately suggests that the role of salts on Process III is similar to that observed for Process I, i.e. an alteration of the activity coefficient of dissolved species, i-
respect to the fact that the dissolved phase is formed on cooling in Process III. This hypothesis is supported by the fact (17) that the order of anions for increasing the stability of dissolved poly-L-proline, a substance which bears close relationship to collagen and also precipitates on heating, was found to be similar to the order of Series 1. From a theoretical standpoint, the fact that the temperature of Transformation III is increased by increasing salt concentration (for a salt which can cause a corresponding decrease of Transformation I temperature) can be simply justified, according to the model embodied in Equation 3 if the over-all heat of transformation $\Delta H$ is a negative quantity in line with the discussion in Section A above. The detailed thermodynamic argument was given by Peller (42). Accordingly, under isoelectric conditions, the same factors considered in Section C, namely, specific and diluent effects, could be advocated for explaining the effect of salts on the temperature of Transformation III. Thus, for instance, considering an isotherm at $10^5$ in Fig. 4, pH = 7.4, the fact that increasing KSCN concentration brings about a stabilization of the helical with respect to the crystalline form, in contrast to the case of KC1 where no helical form is observed, can be attributed to the fact that the former salt is more effective than the latter in decreasing the activity coefficient of the dissolved species in general. (Since Transformation III is generally observed at low salt concentration, a contributing role of unequal ion adsorption will not be excluded.)

It is important to note that a limited salt binding is thus predicted also for the helical form (probably at the less ordered sections). The largest salt binding will occur, however, for the randomly coiled form, as discussed in Section C, “isoelectric conditions,” in line with the greater exposure of active sites by the randomly coiled macromolecules.

Qualitative considerations based on the above discussion and on Equation 3 permit a rationalization of the shape of the pseudo phase diagrams. Thus at the isoelectric pH, when the crystalline precipitate is the form stable at salt concentration = 0 and low temperature, appearance of the helical form depends upon the intersection of the $T_{III}$ against salt concentration and $T_1$ curves (where $T_1$, $T_{II}$, and $T_{III}$ represent, respectively, the temperatures of Transformations I, II, and III). This is clearly possible only for salting in agents (KSCN, CaCl, pH = 7.4) since (cf. Equation 3) $dT_{III}/dC_s > 0$ and $dT_1/dC_s < 0$ when K is large, due to the inversion of the sign of $\Delta H$ for Processes I and III. Even if at high salt concentration, $dT_{III}/dC_s$ becomes negative (cf. dotted curve for KSCN); saturation of the fewer exposed sites will occur at lower salt concentration for the helical than for the randomly coiled form, a stabilization of the crystalline form may be excluded if $dT_{III}/dC_s$ is strongly negative. On reducing the salting in power, $dT_{III}/dC_s$ tends to become generally negative and $dT_1/dC_s$ positive (due to the prevalence of diluent over specific effects) and the two curves will not intersect (CaCl, pH = 6) or, in fact (KCl, KF, pH 7.4) Transformation III may be unobservable unless, perhaps, extremely low temperatures are attained.

**Nonisoelectric Conditions**—Starting from the result at pH = 6 the fact that no helical or crystalline forms are found, respectively, for KSCN and CaCl, is in line with the preferential adsorption of SCN$^-$ and Ca$^{2+}$ over K$^+$ or Cl$^-$. Adsorption of even small amounts of SCN$^-$ by the slightly cationic protein is adequate to compensate for the fixed positive charges and brings about insolubilization, while adsorption of Ca$^{2+}$ will enhance the solubility of the helical form.

As pointed out elsewhere (3), and in agreement with the data by Bensusan and Hoyt (12), a comparison of data for KSCN and KC1 reveals a typical inversion of the order of anions for increasing solubility or depressing the melting described by the usual Hofmeister Series 1. With KSCN, in fact, the crystalline form is invariably more stable than the helical, while with KC1 evidence of the crystalline precipitate is found only when salt concentration > 0.25. This apparent inversion of the order of anions is, again, explained if the order of adsorption of ions is the same in all cases. Since Cl$^-$ is less adsorbed than SCN$^-$, insolubilization of the slightly cationic protein requires higher concentration of KC1 than KSCN. At pH = 7.4, when the protein is isoelectric, KSCN is, however, more effective than KC1 (i.e. no reversal) in stabilizing the helical with respect to the crystalline form. The data at pH = 2.3 are an even stronger proof of the validity of the suggested mechanism since, at variance with the case at pH = 6, no pH drifting was allowed. There again KSCN is more effective than KC1 not only in reducing the polyelectrolyte effect, as discussed in Section C, “Nonisoelectric Conditions” but also in causing stabilization of the crystalline with respect to the helical form. It will be noticed that higher salt concentrations of KSCN and KC1 are required at pH = 2.3 than pH at = 6 for bringing about stabilization of the crystalline with respect to the helical form as would be expected because of the stronger cationic character of the protein at the lower pH. The fact that $dT_{III}/dC_s$ is negative is also in line with the fact that the field of stability of the helical form with respect to the crystalline form is reduced, under nonisoelectric conditions, by increasing the concentration of ions which, through adsorption or screening, reduce the electrostatic repulsion among chains.

We also note that while at pH = 6 in the presence of CaCl$_2$ no crystalline form is observed, this form is however present at pH = 2.3. We attribute this effect to the fact that cation binding (to the native form) is strongly hindered at such low pH where the protein carries a significant positive charge. Accordingly, the insolubilization of the helical form by CaCl$_2$ at pH 2.3 is attributed to the effect of Cl$^-$. In fact, with the use of ionic strength rather than molarity, the concentration of Cl$^-$ required to bring about insolubilization of the helical form is similar for CaCl$_2$ and KCl. This interpretation is supported by measurements which are, in principle, sensitive to selective ion binding. In fact, under near isoelectric conditions, measurements of pH shifts by Bello, Riese, and Vinograd (43) on gelatin indicate that Ca$^{2+}$ is bound more than Cl$^-$, while at pH < 4 we were able to evidence, from similar measurements on tropocollagen, preferential adsorption of Cl$^-$. It would be expected that a reversal of the order of cations analogous to that observed for the anions at low pH should be observed when the protein is anionic. Unfortunately this cannot be tested for tropocollagen which is virtually insoluble at high pH. The fact that at pH 10.7 only KSCN was found effective in stabilizing the helical form, as expected from the more enhanced anionic character of the protein resulting from SCN$^-$ binding, is, however, in line with the above considerations.

**Concluding Remarks**

The variety of effects we have investigated can be most usefully rationalized by the assumption of one primary mechanism of interaction between salts and protein. This is a direct interaction (binding) of the ions with the collagen substrate with the effectiveness orders characterized by Series 1 and 2. The greater
effectiveness of some salt in repressing the polyelectrolyte behavior is also explained along similar lines. Apparent reversal of the order of anions or cations for some processes (44), including the precipitation of tropocollagen considered here, are simply a consequence of the net charge existing at salt concentration – 0, the order of effectiveness for binding in Series 1 and 2 is not reversed.

The description of denaturation processes in terms of pseudo phase diagrams could also be extended to cases where transformations different from the ones considered here occur. A variety of interesting situations may arise in addition to the peculiar case of collagen which precipitates (as tropocollagen) on heating and redissolves (as gelatin) on further heating. Even excluding transformations between different helical forms and different crystalline structures, occurrence of an amorphous precipitate may often occur. For collagen, at high temperatures, the randomly coiled solutions are expected to undergo an additional phase separation yielding an amorphous precipitate, as coherent with the thermodynamics of gelatin solutions (17, 21). Direct precipitation into the amorphous form of some native proteins may often occur. For collagen, at high temperatures, the randomly coiled solutions are expected to undergo an additional phase separation yielding an amorphous precipitate, as coherent with the thermodynamics of gelatin solutions (17, 21). Direct precipitation into the amorphous form of some native proteins (45) and helical DNA seems to occur in some instances with increasing temperature. Proximity of the temperatures for helix → coil transformation and for a liquid-liquid phase separation would readily explain such phenomena.

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The Role of pH, Temperature, Salt Type, and Salt Concentration on the Stability of the Crystalline, Helical, and Randomly Coiled Forms of Collagen
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