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D. Puett[ab]; L. V. Rajagh[a]
[a] Chemstrand Research Center, Inc. Durham, North Carolina [b] C.R.C.: Department of Biochemistry, University of North Carolina Medical School, Chapel Hill, North Carolina

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The Effect of Aqueous Salt Solutions on the Melting of Collagen and the Viscosity of Gelatin

D. PUETT* and L. V. RAJAGH†

Chemstrand Research Center, Inc.
Durham, North Carolina

Summary

Measurements of shrinkage temperatures (T_s) for fibrous collagen, of equilibrium degree of swelling (v^{-1}) for the same in the amorphous state, and of intrinsic viscosity ($[\eta]$) for randomly coiled gelatin are reported. The data were obtained for a large variety of salts which are typical representatives of lyotropic series. The order of anions and cations for decreasing T_s and increasing both v^{-1} and $[\eta]$ (generally, for increasing salting in) is $F^- < SO_4^{2-} <$ acetate $< Cl^- < Br^- < NO_3^- < I^- < SCN^-$ and $K^+ < Mg^{2+} < Na^+ < Cs^+ < Li^+ < Ca^{2+}$. For salts of ions at the extreme left of the series (salting-out agents) the shrinkage temperature in solutions of different salt concentration (C_s) is controlled by the amount of diluent in equilibrium swelling with the molten network, and the shape of T_s vs. C_s curves can be represented by conventional theories valid for binary polymer diluent systems based on a lattice model with a single interaction parameter. In such cases the salt–water solution behaves as a single component diluent and its effect on the measured properties is described as a diluent effect.

For salting-in agents, however, the shrinkage temperature is not controlled by the amount of diluent and the said theories lose their validity. The salt is said to have a specific effect over and above the simple diluent effect. Critical analysis of the role of ions on (1) binding to the peptide groups, (2) Donnan effects, (3) activity of water, (4) water structure, (5) internal pressure of solutions, and (6) classical electrostatic interaction with the protein clearly indicate that binding (which can be measured independently and is effectively negligible for salting-out agents) can best justify the above series and the overall behavior.

*On leave of absence from C.R.C. Present address: Department of Biochemistry, University of North Carolina Medical School, Chapel Hill, North Carolina.

†Present address: 2412 Remington Road, Raleigh, North Carolina.

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It has been shown [1–3] that at least four different factors should be considered in assessing the role of salt and salt concentration on the shrinkage temperature of fibrous collagen under isoelectric conditions. These were the (1) specific effect, (2) diluent effect, (3) cross-linking effect, and (4) nonequilibrium effect. In addition, a polyelectrolyte effect had to be considered when the protein was not under isoelectric conditions. The object of this paper is to present results concerning the diluent and specific effects which will allow a more detailed description of them.

In reviewing the essential facts which allowed us to define [1, 3] these two effects, it is convenient to consider the melting behaviour of binary polymer–diluent systems. For these we use Flory's equation [3, 4]

$$\frac{1}{T_m} - \frac{1}{T_m^0} = \frac{R}{\Delta H_u} V_u/V_1 (\xi_1 - \chi_1 \xi_1^2) \qquad (1)$$

where T_m^0 and T_m are the melting points of the undiluted and diluted polymer, ΔH_u the melting enthalpy, V_u the molar volume of the repeating unit, V_1 and ξ_1 the molar volume and the volume fraction of diluent, and χ_1 a polymer–diluent interaction parameter. In these cases as well as in classical melting-point-depression theory, the *diluent effect*, in depressing T_m^0, depends upon both the quantity of diluent, expressed by ξ_1, and its quality (diluent power), a sum of interactions which can be represented by χ_1.

In formulating a theory for the effect of salt solutions on the melting of polymers, the question arises as to the possibility of describing the interaction between the polymer and a given salt solution, tentatively regarded as a single component diluent, by Eq. (1) with a χ_1 parameter determined by swelling or viscosity measurements. We found [3] that for solutions with KCl it was possible to measure ξ_1 and χ_1 and predict the dependence of the shrinkage temperature on salt concentration for cross-linked tendons, whereas, for solutions with KSCN, the approach was inconsistent. Thus for salts such as KCl the polymer–diluent interaction is sufficiently weak and can be satisfactorily approximated by conventional polymer–diluent theories. In contrast, for salts such as KSCN the interaction is considerably stronger, so another effect, the *specific effect*, must be introduced.

The present work describes additional attempts to correlate the thermodynamic interaction of salt solutions with the randomly coiled polymer to the shrinkage behavior of collagen. The results tend to support the view that the two effects can indeed be operationally defined.

EXPERIMENTAL

Material

Unfractionated purified pigskin gelatin, similar to that used be-

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fore [1], was used for all viscosity measurements. Quinone cross-linked rattail tendons (all with the same degree of cross-linking), obtained as described elsewhere [2], were used for the determination of shrinkage temperatures and equilibrium swelling in the amorphous state. Salt concentrations C_s, expressed in molarity M, were determined by standard titration techniques. Gelatin concentrations C_g are given in units of grams dry protein per deciliter, no correction being made for bound water or salt. The pH of the solutions was always maintained between 7 and 9.3, within the isoelectric plateau of the protein [3].

Viscosity

The technique and treatment of the data are the same as those described elsewhere [1]. The reduced viscosity was measured at no less than four different polymer concentrations and could easily be extrapolated to $C_g = 0$ to obtain the intrinsic viscosity $[\eta]$; see the typical data in Fig. 1. [An apparent exception was noted on unreported measurements in pure water or in 0.2 M Ca $(SCN)_2$ when the reduced viscosity exhibited an upturn at about $C_g = 0.3\%$.] To

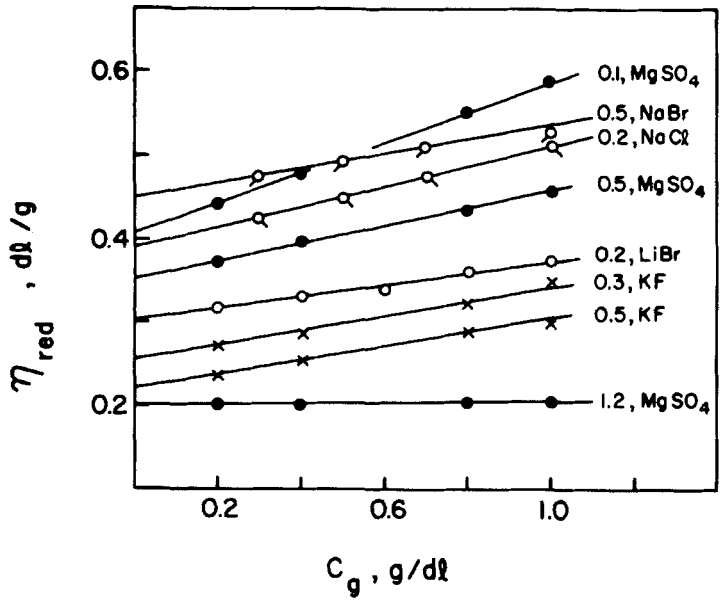

FIG. 1. Typical reduced viscosity vs. gelatin concentration curves for salt solutions of the indicated molarity.

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prevent bacterial degradation, a drop of toluene in about 500 ml of solution was found to be effective. For the systems KSCN, KBr, KNO_3, KCl, NaBr, NaCl, NaF, LiCl, and CsF, data were obtained only at 50°C, while for the systems KF, $MgSO_4$, and LiBr, data were obtained at four different temperatures (40, 50, 60, and 70 ± 0.01°C). For the latter three salts a thermodynamic analysis of the viscosity data, similar to that described earlier [1] for KCl and KSCN, was made. Pertinent data are assembled in Table 1. The thermodynamic quality of the solvent controls the intrinsic viscosity through the relation [4] (see the expanded form in [1]):

$$[\eta] = f[\psi_1(1 - \frac{\theta}{T})] \tag{2}$$

TABLE 1. Viscosity Results for Gelatin

C_S, M	$\lvert\eta\rvert_{50°}$, dl/g	$-\frac{d\lvert\eta\rvert}{dT} \times 10^3$	θ, °K	$-\psi_1$	$-k_1$(55°C)	$-(k_1 - \psi_1)$
			$MgSO_4$			
0.1	0.405	1.72	408	0.020	0.025	0.005
0.3	0.386	1.20	420	0.015	0.019	0.004
0.5	0.363	1.18	439	0.009	0.012	0.003
0.8	0.300	0.98	355	0.006	0.007	0.001
1.0	0.249	0.96	276	0.006	0.005	−0.001
1.2	0.203	0.68	222	0.006	0.004	−0.002
			KF			
0.1	0.339	0.50	405	0.009	0.011	0.002
0.3	0.258	0.68	295	0.008	0.007	−0.001
0.5	0.220	0.66	214	0.005	0.003	−0.002
1.0	0.160	0.38	167	0.005	0.002	−0.003
			LiBr			
0.2	0.304	1.56	334	0.013	0.014	0.001
0.4	0.295	1.60	333	0.017	0.017	0
0.6	0.280	1.04	323	0.008	0.008	0
0.8	0.278	0.78	319	0.008	0.008	0
1.0	0.280	0.60	320	0.007	0.007	0

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where

$$\theta = k_1 T/\psi_1 \tag{3}$$

$$\psi_1 = \Delta\bar{S}_1/R\xi^2 \tag{4}$$

$$k_1 = \Delta\bar{h}_1/RT\xi^2 \tag{5}$$

$$-(k_1 - \psi_1) = \psi_1\left(1 - \frac{\theta}{T}\right) = \frac{\Delta\bar{F}_1}{RT\xi^2} = \frac{1}{2} - \chi_1 \tag{6}$$

ξ being the volume fraction of polymer; θ the theta temperature, at which the excess chemical potential of the diluent, $\Delta\bar{F}_1$, is zero; and ψ_1 and k_1 the entropy and enthalpy components which add up to the term $\frac{1}{2} - \chi_1$ according to Eq. (6). From the viscosity temperature data, the quantities θ, χ_1, and k_1 can be evaluated [1] with the assumption that the salt solution can be regarded as a single-component diluent.

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Swelling

Equilibrium degree of swelling, v^{-1} (volume swollen polymer/volume dry polymer), determinations for amorphous cross-linked tendons immersed in an excess of salt solution were carried out as described elsewhere [2] through microscopic observation of the sample dimensions. Values of v and of dv/dT, corresponding to situations where the tendon is completely amorphous, are reported in Table 2 for the systems KF, NaBr, $MgSO_4$, and LiCl. The value of χ_1 is calculated from the equation [1, 4]

$$(2C_1/RT)V_1[v^{1/3} - v(\langle r_0^2\rangle/\langle r_i^2\rangle) = -[\ln(1 - v) + v + \chi_1 v^2] \tag{7}$$

where $2C_1$ is the rubber elasticity modulus of the network obtained from independent stress-strain measurements [2] ($2C_1 = 2.5$ kg/cm^2 at 333°K) and V_1 is the molar volume of diluent obtained as described earlier [1]. The parameter $\langle r_0^2\rangle/\langle r_i^2\rangle$ (ratio of the unperturbed mean-square end-to-end distance for a chain unbound and bound by the cross-linkages) is taken to be unity and its temperature coefficient assessed as has been discussed earlier [1] where it was also shown that k_1[(Eq. (5)] is equal to $-T(d\chi_1/dT)$. Values of χ_1 and k_1 thus obtained are included in Table 2.

TABLE 2. Swelling Data for Isotropic Collagen

C_s, M	v	$\frac{dv}{dT} \times 10^3$	χ_1	$-k_1$
		H_2O (80°C)		
0	0.303	1.02	0.624	0.220
		NaBr (80°C)		
1.0	0.281	1.55	0.612	0.322
2.0	0.315	0.98	0.632	0.209
3.0	0.330	0.73	0.642	0.156
		LiCl (80°C)		
1.0	0.300	1.31	0.623	0.275
2.0	0.300	0.91	0.623	0.189
3.0	0.309	0.82	0.628	0.172
4.0	0.328	0.82	0.640	0.176
		KF (86°C)		
1.0	0.334	0.17	0.644	0.031
2.0	0.408	—	0.695	—
		$MgSO_4$ (86°C)		
0.2	0.220	—	0.576	—
0.4	0.225	0.11	0.579	0.016
1.0	0.333	—	0.634	—

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Shrinkage

The shrinkage temperatures (T_S) of cross-linked tendons in the presence of an excess of salt solution were determined as described earlier [2] following the variation of length with temperature using rates of heating of the order of 5°C/hr. The shrinkage temperature can be measured within at least ±0.5°C as a result of the considerable sharpness of the transition.

RESULTS

The variation of $[\eta]$ with C_S in Fig. 2 for several salt systems exhibits the general feature of an increase of $[\eta]$ at relatively low C_S

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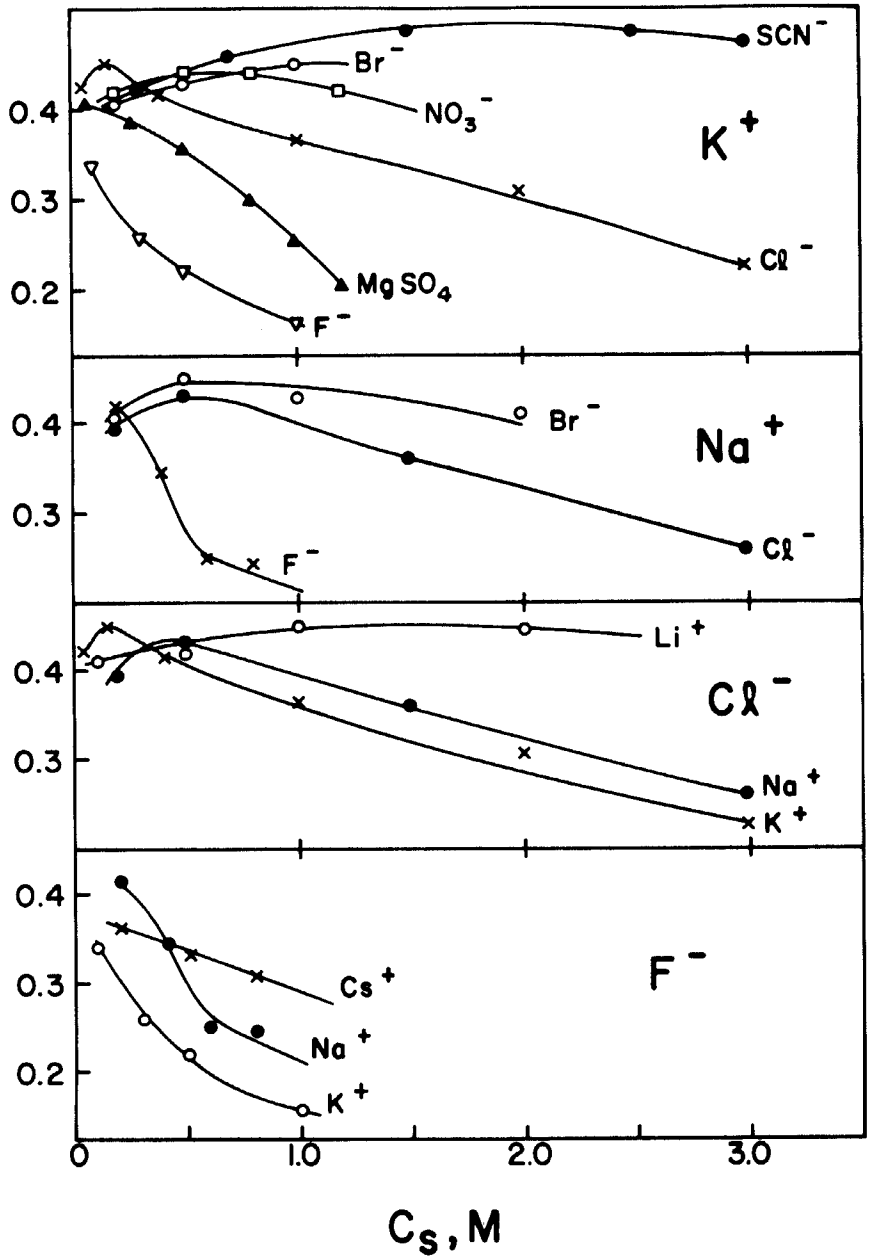

FIG. 2. Variation of intrinsic viscosity of gelatin with salt concentration for several salt systems at 50°C.

followed by a decrease at still higher C_S. This is similar to the behavior previously observed [1], and we can refer to this as a salting in followed by a salting out of the solute. Although there is an overlapping of the various curves, especially at low C_S, it is still possible to establish a well-defined order for both types of ions for favouring salting in or salting out. Considering viscosities at $C_S = 1$ M, all data admit the following order (salting in increasing from left to right):

$$F^-, Cl^-, NO_3^-, Br^-, SCN^- \quad (A)$$

$$K^+, Na^+, Cs^+, Li^+ \quad (B)$$

$MgSO_4$ exhibiting a considerable salting-out power. [An anomaly was observed with LiBr, which should be ranked as a strong salting in agent according to the above series (see also Fig. 3). In fact, the intrinsic viscosities in LiBr (cf. Table 1) are usually small, even though they do not decrease appreciably with C_S, as in the case of salting-out agents. An additional anomaly for this system was observed in the variation of the length of amorphous tendons with temperature, when C_S was greater than 1 M. For all other systems investigated, the length–temperature coefficient was negative, yet for this system it was strongly positive.]

The thermodynamic analysis of viscosity for the systems KF, LiBr, and $MgSO_4$ (Table 1) reveals the general features found [1] for both KCl and KSCN. The enthalpy of dilution parameter, k_1, is negative at low C_S and continuously decreases on increasing salt concentration. The entropy parameter is also negative at low C_S and its absolute value also continuously decreases on increasing C_S. This indicates that, with increasing salt concentration, the thermodynamic quality of the solvent is improved from the entropy of dilution standpoint. The balance of heat and entropy is embodied in the quantity $-(k_1 - \psi_1)$ which, according to Eq. (6), represents the negative excess chemical potential of the solvent and controls directly the intrinsic viscosity [cf. Eq. (2) and (6)]. As pointed out earlier [1], limitations implicit in the overall approach suggest that the trend of ψ_1 and k_1 with C_S, rather than their absolute values, should be considered. Consequently, comparison of the values of ψ_1 and k_1 for the various salts at a given C_S cannot be made with the required confidence. Swelling data (Table 2) are in general agreement with the viscosity results, as was found [1] for KCl and KSCN (decreasing viscosity corresponds to decreasing v^{-1}).

Shrinkage curves are collected in Fig. 3. Above $C_S = 1$ M all data admit the following order of ions for increasing salting in (i.e., reduced T_S) from left to right (note that the order can be altered at lower C_S):

$$F^-, SO_4^{2-}, \text{acetate}, Cl^-, NO_3^-, Br^-, SCN^- \quad (C)$$

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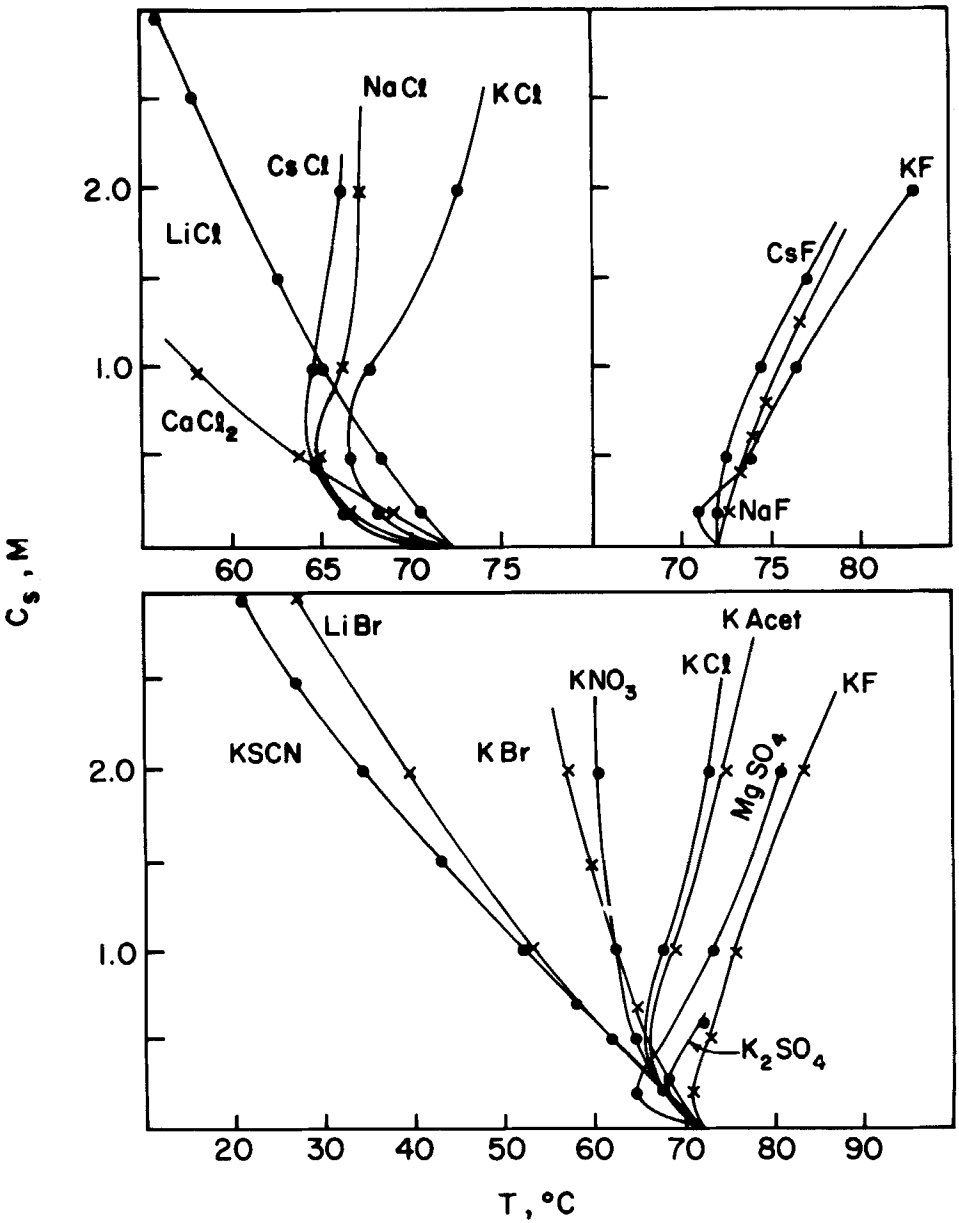

FIG. 3. Shrinkage temperature vs. salt concentration for cross-linked rattail tendons in equilibrium with salt solutions.

$$K^+, Mg^{2+}, Na^+, Cs^+, Li^+, Ca^{2+} \qquad (D)$$

the effects of various ions for a given salt being largely additive. These data are in general agreement with those reported by Weir [5].

Comparison of (A) and (B) with (C) and (D) immediately reveals full agreement between the order for salting in deduced from viscosity and shrinkage data. However, there is a difference between the two sets of results in the dependence of the effect considered upon C_S. For salts such as KCl, KF, and $MgSO_4$ there is an apparent

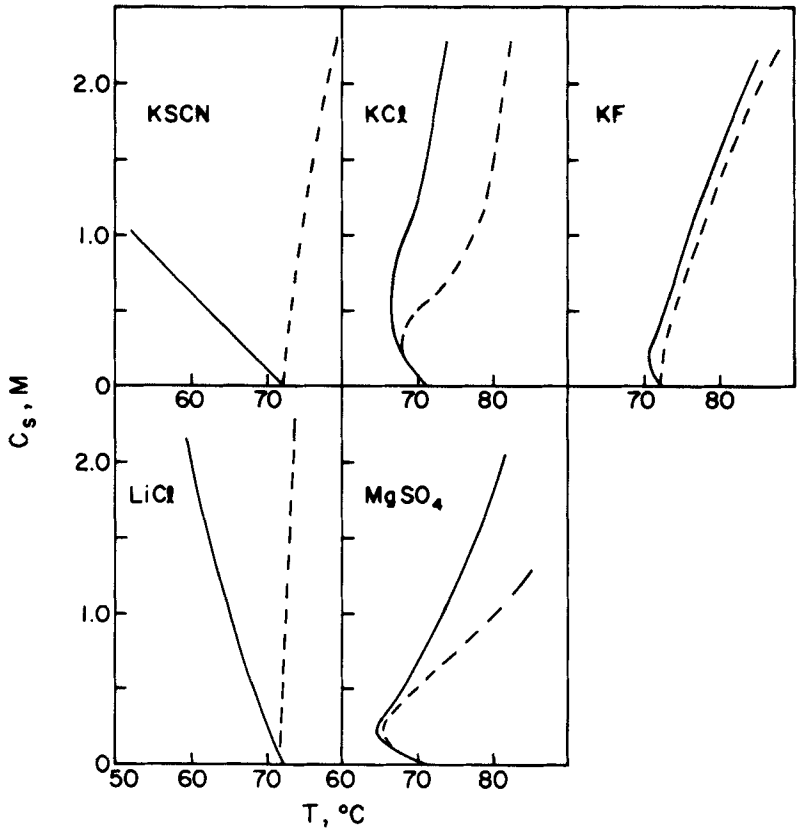

FIG. 4. Experimental shrinkage curves (cf. Fig. 3) for cross-linked tendons in several salt solutions compared with theoretical curves (dashed lines) obtained, as explained in the text, from Eq. (1) and the equilibrium swelling data, extrapolated at the shrinkage temperature, collection in Table 2. Curves for KSCN and KCl recalculated from data [1].

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correlation between the shape of the T_s vs. C_s curves and the shape of the isothermal viscosity (or swelling) vs. C_s curves in the sense that in the range of the salt concentration where the viscosity increases T_s is depressed, and when $[\eta]$, or v^{-1}, decreases T_s is raised. However, with increasing salting in power, this correlation becomes less evident. The lack of correlation between the shrinkage temperature and the isothermal degree of swelling for strong salting in agents was also observed by Gustavson [6]. A more quantitative correlation between the degree of swelling and shrinkage temperature is reported in Fig. 4 for KSCN, KCl, KF, LiCl, and $MgSO_4$. The theoretical melting curves (dashed lines) were calculated from Eq. (1) using values of χ_1 and ξ_1 corresponding to the equilibrium degree of swelling extrapolated to the shrinkage temperature using the data collected in Table 2. Values of ΔH_u and T_m^0 were chosen to fit the experimental shrinkage curves at $C_s = 0$ coherent with the necessity of attaching more significance to the trend of the thermodynamic parameters with C_s than to their absolute values. They were, respectively, 40 cal/g and 200°C, and are not unduly high in view of the inaccuracy in the absolute values of χ_1, the difference [7] between T_s and T_m and the fact that Eq. (1) cannot be expected to reproduce with great precision the absolute values of T_m^0 and ΔH_u in the case of swollen cross-linked networks [8]. [In fact, in the latter case an extra term should be added [8] to the rightside of Eq. (1); however, in the absence of an external tensile force, this term never exceeds 10% of the term $(R/\Delta H_u)(V_u/V_1)(\xi_1 - \chi_1\xi_1^2)$ for collagen.] It is clear from Fig. 4 that there is a satisfactory correlation between the shapes of the experimental and theoretical curves for salting-out agents. However, as salting in becomes more pronounced, a large and continuous depression is experimentally observed, although the theoretical curve, which reflects the variation of v^{-1} and χ_1, would predict a small increase of T_s with C_s.

DISCUSSION

A coherent complex of theories, which successfully represents the melting, viscosity, and swelling behavior of binary polymer–diluent systems, describes the behavior of ternary systems involving strong salting-out agents, at least within the same limits valid for conventional systems. In the case of salting-in agents, however, the complex of theories loses its coherence and the melting-point depression is no longer determined by the amount of diluent available to the molten network. This immediately suggests that in the latter case a selective adsorption of salt may occur, while in the former case this unusually strong interaction can be neglected. This is indeed a satisfactory interpretation and we shall show its validity by critically analyzing the various effects which have been suggested for describing the interaction of solutes in water–salt solutions.

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Binding

If the assumption is made that salt binding occurs only in the amorphous state [9], a depression of the melting temperature will generally result. This can be properly regarded as a specific effect [10]. In more quantitative terms, we may assume that the Schellmann–Flory–Mandelkern [9] equation applies:

$$\frac{1}{T_m} - \frac{1}{T_m^0} = \frac{R}{\Delta H_u}\,\frac{V_u}{V_1}\,(\xi_1 - \chi_1 \xi_1^2) + \frac{Rp}{\Delta H_u} \ln(1 + Ka_S) \qquad (8)$$

where K is the equilibrium constant for the reaction, amorphous protein + salt $\rightleftharpoons$ complex (salt–protein), a_S the activity of the salt, and p the mole fraction of available sites. This equation exemplifies a separation of specific and diluent effects—respectively, the second and the first term on the right side.

An alternateive method of expressing the reduction of the melting temperature, as influenced by the selective adsorption of salt, was recently given by Katchalsky and Oplatka [11]. In this less restrictive theory no distinction is made between salt which is bound to the protein and salt which is part of the diluent, the specific effect being attributed simply to an enrichment of salt in the swollen tendon.

The earlier, excellent data of Docking and Heymann [12] are pertinent in assessing the validity of the binding hypothesis. These authors determined the apparent adsorption of salt by dry, insoluble gelatin by equilibrating the gel in a salt solution of known initial concentration C_S^i. If the final concentration of the excess solution in equilibrium with the gel is indicated as C_S^f, the difference $C_S^i - C_S^f$ represents the variation due to selective binding of salt and water when it is assumed that the ratios of free water to salt molecules inside and outside the gel are equal. Selected data from Docking and Heymann are reproduced in Fig. 5. High, positive values of $C_S^i - C_S^f$ mean, of course, that a large adsorption of salt overshadows the normal adsorption of water by the initially dry gelatin, while high negative values indicate that the salt adsorption is small or negligible. The deduced order for increasing salt adsorption is

$$SO_4^{2-},\ \text{acetate},\ Cl^-,\ NO_3^-,\ Br^-,\ SCN^- \qquad (E)$$

$$K^+,\ Mg^{2+},\ Na^+,\ Cs^+,\ Li^+,\ Ca^{2+} \qquad (F)$$

Thus a more satisfactory agreement between the orders of both anions and cations for increasing adsorption and for increasing salting in, (C) and (D), could not have been expected. Furthermore, it is clear that salting-out agents tend to have a small or negligible salt adsorption.

Since Eq. (8) predicts a continuous depression of the melting tem-

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perature with C_S due to binding, the reversal of the T_S vs. C_S curves for salting-out agents can be ascribed to a diluent effect. In fact, a reversal can generally be expected when the diluent effect prevails over a limited or negligible binding, or when a binding saturation occurs. Thus the data reported in Fig. 5 offer strong evi-

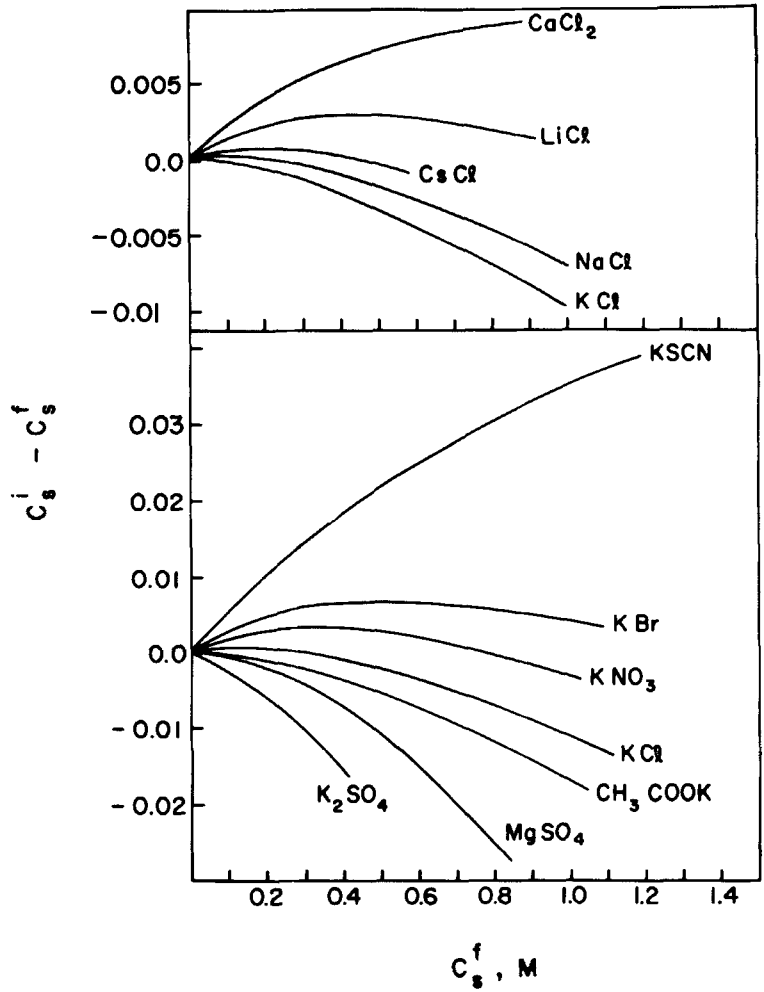

FIG. 5. Apparent adsorption of salts on gelatin plotted as a function of the concentration of the (outside) solution in equilibrium with the gel. Data obtained from the work of Docking and Heymann [12].

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dence for the hypothesis that binding is the effect to be associated with the large salting in at high concentrations of most salts.

A comparison of equilibrium constants obtained from the data in Fig. 3 using Eq. (8) and from the data in Fig. 5 has been presented elsewhere [13a]. Also, using a radioisotope comparison technique [13a, b] we have found that denatured collagen binds about 60 and 7 molecules (per 1000 residues) of KSCN and KCl, respectively, at $C_S = 1M$. These data are in agreement with the curves shown for KSCN and KCl in Fig. 5. In addition, using a new technique of ultracentrifugation, we have found [13c] that KSCN is bound much more than KCl by soluble gelatin; the extent of binding was found to be in essential agreement with our results [13a] using the radioisotope comparison technique and with those of Docking and Heymann [12]. The location of salt binding along the polymer chain is believed to be the peptide bonds themselves [14].

It should be noted that insofar as a specific interaction is generally invoked when the components involved have an abnormally great attraction for each other [10], our operational definition of binding (from the data in Fig. 5) will not necessarily imply that the association of ions to the protein originates, *in all instances*, in what could be strictly defined as a typical chemical compound. In the most restrictive sense, the addition of a term involving an equilibrium constant to Eq. (8) merely illustrates the necessity of adding a term characterizing the strong polymer–salt interaction. The interpretation advanced here differs mainly from that advanced below in that the specific effect is associated with a disproportion of the water–salt mixture and not with a physical property of the whole salt–water solution.

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Donnan Effect

The series for unequal adsorption of anions and cations to gelatin obtained by Bello et al. [15] from pH shifts coincides with series (E) and (F). Thus an electrostatic repulsion of anions or cations unequally adsorbed with respect to their counterions could be considered responsible for the depression of the denaturation temperature (in analogy to the effect observed when the protein is no longer isoelectric as a result of pH changes in absence of salts [3]).

However, Ciferri et al. [13a], Northrop and Kunitz [16], and Docking and Heymann [12] have attempted to analyze the problem quantitatively and concluded that an appreciable Donnan effect is improbable even with strongly adsorbed thiocyanates, although it might be appreciable for salts such as $AlCl_3$ and $CaCl_2$.

Hydration

The classical Hofmeister series [17], applicable to the precipitation of (negative) egg albumin, predicts for the salts considered

here an order of increasing coagulation power (salting out) from right to left as follows:

$$SO_4^{2-}, \text{acetate}, Cl^-, NO_3^-, I^-, SCN^- \quad \text{(G)}$$

$$Li^+, Na^+, K^+, Mg^{2+} \approx Ca^{2+} \quad \text{(H)}$$

Each ion in this series was characterized by a lyotropic number, and McBain [17] ascribed the series to the respective size of the anhydrous ions. By stipulating that the water of hydration is not available to the protein, the lyotropic effect was ascribed to a removal of solvent (dehydration). Although the order for the anions which we have experimentally determined can qualitatively be brought into correspondence with this theory, its failure is obvious when attention is focussed on the cations [cf. series (B) and (D). Lack of a general correlation between the thermodynamic activity of water and the denaturation temperature of DNA was noted by Hamaguchi and Geiduschek [18], and the same seems to be true for the shrinkage of collagen. In fact, the activities of water (calculated as indicated by Hamaguchi and Geiduschek) in 4 M solutions of LiCl, NaCl, and KCl, are, respectively, 0.794_3, 0.834_0, and 0.847_5. Yet the shrinkage temperature in pure water is 70°C and in the corresponding salt solutions is 50.0, 67.4, and 74.5°C, respectively.

Water Structure

The effect of ions on the denaturation of DNA [18], ribonuclease [19], and collagen [14, 19] has been associated with their general ability to act as "breakers" or "formers" of the water structure, which is described in terms of ice-like "flickering clusters" [20, 21]. The prevailing view, dating from the studies of Frank and Evans [20b] is that the population of water molecules having a more ice-like structure is increased in the neighborhood of nonpolar molecules or groups such as those belonging to exposed side chains or proteins (whether polar or not). Formation of icebergs bordering these solutes is considered to be responsible for their anomalous high negative entropy of solution. When ions are added to pure water the entropy of dilution is increased or decreased by some ions in the order (cf. Frank and Robinson [20a], $\bar{S}_1 - \bar{S}_0$ for 1 molal chlorides and potassium salts)

increased	decreased	
Cs^+, K^+, Li^+, Ca^{2+}, Mg^{2+}		(I)
NO_3^-, I^-, Br^-, Cl^-, F^-		(J)

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Similarly, the viscosity B-coefficients are decreased or increased by some ions in the order (cf. Kaminsky [22])

decreased				increased		(K)
		Cs^+,	K^+,	Na^+,	Li^+, Ca^{2+}, Mg^{2+}	

$$I^-,\ NO_3^-,\ Br^-,\ Cl^-,\ SO_4^{2-} \qquad \text{(L)}$$

The shift of the maximum of infrared reflection towards the position corresponding to the vapor phase increases in the order (Boswell et al. [23])

$$SO_4^{2-},\ Cl^-,\ Br^-,\ SCN^-,\ I^- \qquad \text{(M)}$$

Because of this, ions such as Li^+, Na^+, Ca^{2+}, Mg^{2+}, F^-, SO_4^{2-} are said to be *net* structure formers and ions such as K^+ Cs^+, Cl^-, Br^-, I^-, NO_3^-, and SCN^- are said to be *net* structure breakers. If one attempts a qualitative correlation between the order of ions which characterize their net structure breaking or forming power with the order of ions for producing salting in or salting out, it is clear that there is a satisfactory correlation between the order of anions (A) and (C) for increasing salting in and for increasing their structure-breaking power (J), (L), and (M). This could be qualitatively justified, since the structure-breaking anions are expected to increase the liquid character of the diluent (or its entropy of dilution) and to destabilize the cage of icebergs (and water–carbonyl bridges [14]), which in turn may increase the polymer conformational entropy. The salting-out effect of anions which are net structure formers should then be ascribed to the fact that in the more ordered solution (even though the structure may not be ice-like), mixing with the polymer is hindered. If, however, a similar picture is applied to the case of cations, one finds that no correlation exists. Thus, although the liquid character of the diluent is expected to influence the solubility and melting behavior of the polymer through the diluent effect, there is no general justification for attributing the specific effect to it.

Internal Pressure

The internal-pressure theory of Long and McDevitt [24] correlates the extent to which water is compressed or loosened when ions are present, to the salting-out behavior of nonpolar substances. According to this theory, salting out increases on increasing the difference $V_S - \overline{V}_S^0$, where V_S is the molar volume of pure "liquid" salt and $\overline{V}_S^0$ its partial molar volume at infinite dilution. This quantity is positive for most salts (salting out) and decreases approxi-

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mately with ionic size until it becomes negative for very large ions (salting in). The salting-out constant is defined as

$$K_S = \bar{V}_1^0(V_S - \bar{V}_S^0)/2.3\beta_0 RT$$

where β_0 is the compressibility of water and $\bar{V}_1^0$ the partial molar volume of the solute. The theory would predict positive K_S (i.e., salting out) for all the salts we have used, with the possible exception of the acetate ion. This in contrast with our experimental findings; however, Long and McDevitt [24] pointed out that if a more proper account of the size of large polar molecules was made, the K_S should be considerably reduced. Still, the theory would rank the effect of chlorides in the order (cf. Table 2 of [24])

$$Cs^+, Li^+, K^+, Na^+ \qquad (O)$$

salting out being greater at the extreme right. This order is verified in the case of salting out of benzene, but again, it is in contrast to the order found for collagen, (D).

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Electrostatic Theories of Salting In and Salting Out

Kirkwood's ion-dipole interaction theory [24, 25] for salting in of dipolar substances (zwitterions) cannot justify the specific effect responsible for the large melting-point depression, since the same depression is exhibited by acetylated, nitrated, or esterified gelatins [14, 15]. Furthermore, both the electrostatic theory of salting in and the Debye–McAulay [24, 25] theory for salting out of nonpolar substances do not explain the wide variety of effects caused by the different salts, although they do offer a clear description of the solute polarity on the salting in–salting out behavior. Since these theories cannot be associated with the specific effect, it is plausible to ask what is the role of the electrostatic contribution described by these theories neglecting the binding of ions to the peptide bond. If the polymer can be regarded as a mixture of zwitterions and hydrophobic groups (a simplification which neglects both the polymeric nature of the protein and the need of more complicated models than the simple dipole enclosed within a sphere used for glycine [25]), it can be assumed that the former will be responsible for a salting-in tendency (described in terms of the Kirkwood theory) and the latter for a salting-out tendency (described in terms of the Debye–McAulay theory). Although, according to the original theories, both salting in and salting out should increase with increasing C_S, it is known [25] that even for zwitterions such as cystine the solubility is increased at low C_S and decreased at higher C_S. This decrease

of the solubility could be theoretically justified, as discussed elsewhere [1]. If we assume that for salts such as $MgSO_4$ and K_2SO_4 binding at the peptide bond is indeed negligible, the above considerations suggest that the salting-in tendency observed for these systems at $C_S < 0.2$ M (Fig. 3) can be attributed to ion–dipole interactions involving the charged group of the protein.

From the above discussion it is apparent that binding can best be correlated with the behavior of anions and, particularly, of cations. Moreover, binding can be used for a satisfactory theoretical description of the specific effect. We conclude this section with a consideration of the diluent effect.

When salt binding is negligible, as for salting-out agents such as KCl, CH_3COOK, $MgSO_4$, K_2SO_4, and KF, the results collected in Tables 1 and 2 (cf. also Part I) should give a satisfactory description of the diluent effect for the water-bound polymer (the decrease of $C_S^i - C_S^f$ with C_S in such instances is, in fact, coherent with the adsorption of about two water molecules for each peptide residue [13]). In attempting a molecular description of the thermodynamic parameters, one can consider for the diluent effect all interactions considered above aside from binding at the peptide bond. The "liquid" character of the diluent in the case of the strongest salting-out agents such as K_2SO_4 and KF, which are structure formers and exhibit high internal pressure, is not as favorable to dilution, from an entropy standpoint (i.e., ψ_1), as with weaker salting-out salts, such as CH_3COOK and KCl. Also, for the strongly hydrated salting-out agents we expect diluent-diluent contacts to be energetically more favourable than for weaker salting-out agents, and this would justify the greater reduction of the exothermicity of dilution (i.e., k_1). In fact, the role of increased cohesive energy with increasing salting out, in the absence of binding, is well illustrated by Eq. (9), and it is known [10] that as the cohesive energy of a diluent is progressively increased over that of the solute, the solution becomes more endothermal. It should be pointed out that those theories, which suggest that on increasing C_S solubilization of hydrophobic groups is favored, owing to the disordering effect on the water structure, in effect fail to consider that the concommitant reduction of exothermicity may more than offset the increase in the entropy of dilution.

Electrostatic contributions would also affect the salting-out behavior, as discussed above. The fact that the ion–dipole interaction at the charged groups (at least in the limited concentration range in which it causes salting in) is lumped into the diluent effect can be questioned. Alternatively, the effect could be regarded as a binding at the charged groups, distinct, however, from binding at the peptide bond, which probably involves an interaction of the mobile ions with

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the dipoles. This aspect of the problem is simplified in the case of substances which do not carry charged substituents. For polyproline [26] and for uncharged model peptides [27], no salting in at very low salt concentrations is observed for KCl and KF.

CONCLUDING REMARKS

As early as 1933, Katz and Weidinger [28] suggested that in lyotropic action we have to distinguish between an adsorption effect and a salting-out effect, the former being prevalent with weakly hydrated anions at one end of the series, the latter with strongly hydrated anions at the other end. In more recent times the problem has become highly controversial [14]. Yet Robinson and Jencks [27], as a result of an excellent analysis of the effects discussed above, have recently concluded that the effect of salts on the denaturation and solubility of proteins and model peptides can be summarized in terms of a salting out and of a direct interaction of salts with amide groups. Our conclusions that the specific effect can be described as a binding, whereas the diluent effect reflects a sum of relatively weak interactions of the polymer with the environmental solution, is in essential agreement with that of these investigors.

As far as a detailed molecular description of the binding process is concerned, only speculations can be made. On proceding from left to right along the anion series (A), several factors favorable to salting in may begin to play a role: binding, Donnan effects, reduced hydration, increasing disruption of the water structure, or reduced cohesive energy for the water–salt solution. While this suggests that binding is to be superimposed upon a diluent effect which has, in itself, a salting in tendency, still the above coincidence is singular (and it is probably a reason for much controversy!). Likewise, the generality of the salt effects on the denaturation of such diverse macromoles as DNA [18], collagen, and ribonuclease [19] would seem to indicate that an indirect property of the salt solution is responsible for the salting in. We believe that these facts indicate that anion binding *is primarily controlled by factors which pertain to the hydration of ions and to the structure of the solutions,* at least for the relatively loose ionic complexes which are generally involved. Hence, it is likely that an ion such as SCN^-, which is a strong structure breaker and is poorly hydrated, may have more tendency than a Cl^- ion to reach the surface of the polar solute and to establish an electrostatic interaction which results in a destabilization of the helix. This is because the water "cage" bordering the macromolecule [21] is intrinsically more disordered in the case of SCN^- and and because, in agreement with an interpretation advanced by Sinano-

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glu and Adbulnur [29] for DNA, the reduced requirement for hydration favors the concentration of the ion at the polymer–water interface. As found by Robinson and Jencks [27] for an uncharged peptide, the order for decreasing exclusion of anions from a water–air interface is in close agreement with the order for increasing salting in of collagen. The same authors, in a similar vein, state that binding reflects more the poor interaction of the ions with water than a specific preference of the ions for the solute.

The above interpretation could account for the behavior of the anions but fails to explain the large salting in by strongly hydrated, net-structure-former cations such as Li^+ and Ca^{2+}. For these, the hydration layer may have the required orientation and structure, so that formation of more definite classes of compounds [14, 27], involving a more specific role of the polymer structure, could be advocated [the anomalous behavior of LiBr and $Ca(SCN)_2$ indicated above could be a reflection of a more specific action of these cations].

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Zusammenfassung

Es wird über Messungen der Schrumpfungstemperaturen (T_S) von faserförmigem Kollagen, des Gleichgewichts-Quellungsgrades (ν^{-1}) für dasselbe Material im amorphen Zustand und der grundmolaren Viskosität ($[\eta]$) von statistisch gefaltetem Gelatin berichtet. Daten wurden für eine grosse Anzahl verschiedener Salze erhalten, die typische Vertreter lyotroper Serien sind. Die folgende Reihe von Anionen und Kationen ergibt sich für abnemendes T_S und zunehmendes ν^{-1} als auch $[\eta]$ (im allgemeinen auch Reihe zunehmenden Einsalzens): $F^- < SO_4^{2-} <$ Azetat $< Cl^- < Br^- < NO_3^- < I^- < SCN^-$ $K^+ < Mg^{2+} < Na^+ < Cs^+ < Li^+ < Ca^{2+}$ Für die Salze aus Ionen in

der extrem linken Seite der Serie (Aussalzmittel) wird die Schrumpfungstemperature in Lösungen verschiedener Salzkonzentration (C_S) durch die Menge an Lösungsmittel in dem Quellungsgleich-gewicht mit dem geschmolzenen Netzwerk bestimmt und die Form der T_S vs C_S Kurven kann nach konventionellen Theorien für binäre Polymer-Verdünnungsmittel-Systeme dargestellt werden, basierend auf einem Gittermodel mit einem einzigen Wechselwirkungsparameter. In solchen Fällen verhält sich die Salz-Wasser Lösung wie ein einkomponentiges Verdünnungsmittel, dessen Effekt auf die gemessenen Eigenschaften als Verdünnungseffekt beschrieben werden kann.

Für Einsalzmittel ist dagegen die Schrumpfungstemperatur nicht durch die Menge an Lösungsmittel bestimmt und die angegebenen Theorien verlieren ihre Gültigkeit. Das Salz hat dann einen spezifischen Effekt der über die einfachen Verdünnung hinausgeht. Eine kritische Analyse der Rolle der Ionen in Bezug auf (1) die Bindung zu Peptidgruppen, (2) Donnan-Effekte, (3) die Aktivität des Wassers, (4) die Struktur des Wassers, (4) die Struktur des Wassers, (5) den internen Druck von Lösungen, und (6) klassiche elektrostatische Wechselwirkung zwischen dem Protein, weisen klar dar auf hin, dass eine Bindung (welche unabhängig gemessen werden kann und im Falle von Aussalzmitteln effektiv vernachlässigbar ist) am besten die obige Reihe als auch das allgemeine Verhalten erklären kann.

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Résumé

On fait un rapport sue des mésures de températures de la contraction (T_S) du collagène fibreux, du degré du gonflement à l'équilibre (V^{-1}) dans l'état amorphe et de la viscosité intrinsèque ($[\eta]$) pour une gelatine pelotonée au hasard. On a obtenu des donneés pour une grande varieté de sels, représentants typiques de séries lyotropes. L'ordre des anions et des cations avec des Ts decroissants et V^- et $[\eta]$ augmentant (Géneralement pour des salinités augmentées) est $F^- < SO_4^{2-} <$ acetate $< Cl^- < Br^- < NO_3^- < I^- < SCN^-$ $K^+ < Mg^{2+} < Na^+ < Cs^+ < Li^+ < Ca^{2+}$. Pour des sels des ions à l'extrème gauche des séries (agents desalants) la température de la contraction dans des solutions de sels avec des concentrations différentes (C_S) est controlée per le montant du diluant en gonflement equilibré avec le réseau fusé, et la forme des courbes de T_S vs. C_S peut être representée par des théories conventionelles, valables pour un système binaire polymère-diluant, basé sur un modèle de réseau avec un seul paramètre d'interaction. Dans ces cas la solution sel-eau se comporte comme un diluant à seul composant et son effet sur les proprietés mesurées est décrit comme l'effet diluant.

Pour des agents salants, cependant, la température de la contraction n'est pas controlée par le taux du dilant et les théories mentionnées perdent leur validité. On dit que le sel possède un effet

specifique superieur au simple effet diluant. L'Analyse critique du role des ions sur (1) la liaison des groupes peptidiques, (2) effets Donnan, (3) activité de l'eau, (4) structure de l'eau, (5) pression interne de solutions, et (6) l'interaction électrostatique classique avec les protéines, indique clairement que la liaison (qu'on peut mesurer independament et qui est negligible pour des agents de salants) peut au mieux justifier ces séries et leur comportement global.

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