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# The Effect of Neutral Salts on the Melting Temperature and Regeneration Kinetics of the Ordered Collagen Structure*

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The experimental data reported recently by P. H. Von Hippel and K. Y. Wong on the effect of neutral salts on the thermodynamic stability of the ordered collagen structure and on the rate of formation of this structure from the random-coil form has been analyzed. The importance of the structure of water notwithstanding, it is shown, contrary to previous conclusions, that both types of data can be quantitatively explained by the direct binding of ions.

In a recent series of papers Von Hippel and Wong (1962, 1963a,b) have reported the results of studies on the effect of a variety of neutral salts both on the thermodynamic stability of various collagens in dilute aqueous solutions and on the rate at which the ordered structure is regenerated from the disordered one in the same medium. The salts utilized are well known to be capable of transforming all the ordered polypeptides and proteins irrespective of crystallographic structure and amino and imino acid compositions (Mandelkern *et al.*, 1962a,b,c). This ability to disrupt the ordered structure has also been noted in nonaqueous as well as in aqueous media (Mandelkern *et al.*, 1963). The experimental results were explained on the basis that the stability of the ordered protein structure was influenced by the neutral salts through the competitive reorganization of the water that is postulated to be required for the stabilization of the collagen-type helix. The possibility that the results could be explained by the direct binding of ions to the peptide linkages of the protein molecule was considered and dismissed. The refutation of a binding mechanism appears to be based on the fact that the experimental data can be well represented by the empirical relation

$$T_m = T_m^* + K_m \qquad (1)$$

where $T_m^*$ is the melting temperature at a fixed protein concentration in the absence of added salt, $T_m$ is the melting temperature for a salt concentration of molarity M, and $K$ is an experimentally determined constant which is different for each salt. Besides the fact that this relation was obeyed, a binding mechanism was further eliminated because it was observed that for a given salt the constant $K$ was independent of the total protein concentration. These conclusions have been seriously questioned by Bello (1963), who offered qualitative arguments for the serious consideration of a binding mechanism's being involved in the melting process. A major misconception appears to be the supposition that the effect of binding on the melting temperature must be dependent on the total polymer concentration.

The general importance of the structure of water notwithstanding, we wish to point out that most of the experimental data (apart from the two special cases where the melting temperature appears to increase with added salt) can be explained quantitatively by invoking a classical binding mechanism. Equation (1) turns out to be a very good approximation to the more general expression that is applicable. Furthermore, it can be shown that the relative rate at which the ordered collagen structure is regenerated in various salt solutions can be quantitatively explained by utilizing the mechanism proposed by Flory and Weaver (1960) for the similar process in the absence of the added salt.

Following the developments of Schellman (1955) and of Flory (1957), the melting point depression of a pure polymer, when binding occurs, can be expressed as

$$\frac{1}{T_m} = \frac{1}{T^\circ_m} = \frac{R}{\Delta H_u}\frac{V_u}{V_1}(v_1 - \chi_1 v_1^2) + \frac{R}{\Delta H_u} N_A \ln (1 + ka_c) \qquad (2)$$

Here $T_m^\circ$ is the melting temperature of the pure undiluted polymer, $T_m$ is the melting temperature at volume fraction $v_1$ of solvent, and $\Delta H_u$ is the heat of fusion per chain repeating unit, $V_u$ and $V_1$ are the molar volumes of the repeating unit and solvent, respectively, and $\chi_1$ is the thermodynamic interaction parameter between polymer segments and solvent. The mole

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fraction of sites available for binding is given by $N_A$, $a_c$ is the activity of the complexing reagent in the solution, and $k$ is the intrinsic binding constant as defined by Klotz (1953). A basic assumption that is made in deriving equation (2) is that the chemical binding process is restricted to the disordered polymer phase. It is clear from equation (2) that the effect of ion binding on depressing the melting temperature or the transformation temperature will be expected to be independent of total polymer concentration in complete accord with the experimental observations (Von Hippel and Wong, 1962). Since the polymer concentration is held constant in the experiments under present discussion equation (2) can be conveniently rewritten as

$$T_m = T^*_m - \frac{RT_mT^*_m}{\Delta H_u} N_A \ln (1 + ka_c) \qquad (2')$$

where $T_m^*$ is now defined as the melting temperature at the particular fixed protein concentration in the absence of added salt, i.e., in the absence of a binding process. By expanding the logarithm, and approximating the product $T_mT_m^*$ by $T_m^{*2}$ (which is a negligible approximation over the small temperature interval that is involved) one obtains the relation

$$T_m = T^*_m - \frac{RT_m^{*2}}{\Delta H_u} N_A k a_c \qquad (3)$$

which is identical in form to that obtained experimentally (equation 1) by Von Hippel and Wong. The constant $K$ can now be identified with

$$K = \frac{RT_m^{*2}}{\Delta H_u} N_A k \qquad (4)$$

where the activity of the complexing agent is assumed equal to its concentration.

From the above analysis it is therefore now possible to estimate the intrinsic binding constants of the various salts for collagen from the experimentally determined $K$ values. Experimentally $T_m^*$ is found to be 293.9 K. From the work of Flory and Garrett (Flory and Garrett, 1958; Garrett and Flory, 1956) we take $\Delta H_u$ for collagen to be approximately 2000 cal/mole of repeating unit and assume that $N_A$ is unity. Thus we have allowed for binding at each peptide or imide linkage. The results of the calculation are summarized in Table I.

It is apparent from the data in the table that the values of the intrinsic binding constant thus calculated are comparable to those deduced from other independent experiments (Klotz, 1953). We can conclude, therefore, that the neutral salts in question lower the thermodynamic stability of the ordered collagen structure by direct ion binding to the polypeptide chain. Recently (Harrington and Kurtz, 1964) the direct binding of LiBr to poly-L-proline has been demonstrated and further shown to result in a significant configurational change in the polymer.

TABLE I
VALUES OF INTRINSIC BINDING CONSTANT $k$ CALCULATED ACCORDING TO EQUATION (4) FOR VARIOUS SALTS

| Salt | $k$ |
|---|---|
| $(CH_3)_4NBr$ | 0.005 |
| $KCH_3COO$ | 0.009 |
| KCl | 0.016 |
| NaCl | 0.018 |
| CsCl | 0.021 |
| LiCl | 0.048 |
| $CaCl_2$ | 0.10 |
| KSCN | 0.12 |

The analysis and conclusion given here with respect to the melting mechanism also allows for a consistent interpretation of the reversion kinetics. The experimental results for the isothermal rate of coil-to-helix transformation can be expressed in compact form (Von Hippel and Wong, 1962) as

$$\log r = \log r_0 + K_0 m \qquad (5)$$

Here $r$ is the initial rate, $r_0$ is the initial rate in the absence of added salt, and $K_0$ is an experimentally determined constant which has a characteristic value for each different salt. From their studies of the kinetics of the reversion of gelatin (random-coil collagen) to collagen in dilute solutions, Flory and Weaver (1960) found that the rate was apparently first order with respect to concentration and possessed a very marked negative temperature coefficient. In order to reconcile the former of these results with the widely accepted three-chain helical model for the ordered structure of collagen, they postulated that the rate-determining step involved the unimolecular formation of an ordered intermediate. The concentration of this species was postulated always to be very small when compared to that of the random coil. First-order kinetics follows immediately from these assumptions and the rate of reversion is expressed as

$$r = K_1 C \qquad (6)$$

where $C$ is the concentration of random-coil species and $K_1$ is the rate constant. Furthermore, the temperature dependence of the rate constant can be expressed as (Flory and Weaver, 1960)

$$K_1 = \text{Const} \exp\left\{\frac{-A}{kT(T_m^* - T)}\right\} \qquad (7)$$

where $A$ is a constant. Agreement with equation (7) was experimentally demonstrated and the constant $A$ was determined for the particular collagen studied.

If we now assume that the same reversion mechanism is operative in the presence of neutral salts then it immediately follows that

$$\ln r = \ln r_0 + \alpha \left\{\frac{1}{T_m^* - T} - \frac{1}{T_m - T}\right\} \qquad (8)$$

where $\alpha$ equals $A/kT$.

From equations (1), (5), and (8) it is found that

$$K_0 = \frac{\alpha K}{(T_m^* - T)^2\left[1 + \frac{K_m}{(T_m^* - T)}\right]} \qquad (9)$$

or

$$K_0 \cong \frac{\alpha K}{(T_m^* - T)^2} \qquad (10)$$

Hence to a good approximation $K_0$ will be independent of the concentration of added salt when the reversion process is conducted at a fixed temperature.

In addition to explaining the experimentally determined relation embodied in equation (5), the mechanism postulated by Flory and Weaver (1960) also allows for an absolute calculation of $K_0$ when either $K$ or the true intrinsic binding constant is given. The value of the parameter $\alpha$ can be determined from the data of Flory and Weaver (1960) and is found to be 51.4 degrees. The kinetic experiments of Von Hippel and Wong (1962) were conducted at 276.2 K, this temperature being the same irrespective of the nature and concentrations of the added salt. Hence $T_m^* - T$ can be taken to be 17.5°. The results of the calculation are summarized in Table II. The next to the last

TABLE II
CALCULATION OF KINETIC CONSTANT $K_0$ BY MEANS OF EQUATIONS (9) OR (10)

| Salt | $K_0$ (obs) | $K_0$ (calcd) | $K_0$ (calcd) |
|---|---|---|---|
| $(CH_3)_4NBr$ | −0.08 | −0.07 | −0.07 |
| $KCH_3COO$ | −0.08 | −0.13 | −0.13 |
| KCl | −0.42 | −0.24 | −0.25 |
| NaCl | −0.41 | −0.27 | −0.28 |
| CsCl | −0.52 | −0.30 | −0.32 |
| LiCl | −0.66 | −0.69 | −0.78 |
| $CaCl_2$ | −1.82 | −1.48 | −1.98 |
| KSCN | −2.85 | −1.68 | −2.35 |

column is calculated from the approximate relation, equation (10), while the last column is calculated from equation (9) using 0.5 mole/liter for the salt concentration. It is clear from the data in Table II that rather good agreement is obtained between the calculated and observed results. The agreement is perhaps more significant in that the parameter $\alpha$ was determined for another type collagen, and the constant describing the rate was calculated solely from melting point data.

We can conclude, therefore, that a consistent quantitative analysis of the effect of the neutral salts is obtained for both the equilibrium melting temperature and the reversion kinetics by invoking a direct binding process for melting and the Flory-Weaver (1960) mechanism for the reversion kinetics.

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# Studies on the Interaction of Actin with Myosin A*


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The interaction of myosin A and actin was investigated in the presence and absence of various chemical modifiers and with ultraviolet-irradiated myosin A. These studies suggested that the occupancy of an inhibitory group by actin is necessary for the enzymatic interaction between these two proteins. The results obtained with the ultraviolet-irradiated myosin A substantiated the participation of "more than one" group on the active center of the myosin A in the enzymatic interaction of this protein with various modifiers. Physical interaction between myosin A and actin was found to occur also in the absence of any enzymatic interaction between these two proteins.


The association of actin and myosin A at low ionic strength was found to induce a complex change in the enzymatic behavior of the latter (Banga and Szent-Gyorgyi, 1943; Hasselbach, 1952, 1957). Since there was much experimental evidence to show that if ATP and ITP were present together they competed for the same enzyme sites of myosin A (Gergely, 1953; Levy *et al.*, 1962), it was interesting to observe that the association of actin to myosin influenced the splitting of these two substrates rather differently (Kaldor and Gitlin, 1963). A number of compounds were found to accelerate the ATPase activity of myosin A in the presence of $Ca^{2+}$ or $Mg^{2+}$ while ITPase activity was inhibited. Limited similarity in the enzymatic effect of actin and these compounds was thereby suggested. It was shown that DNP competed with actin for the same enzyme sites of myosin A (Chappel and Perry, 1955) and that *p*-mercuribenzoate and DNP did not compete for the same myosin A site, while they stimulated the ATPase in the presence of $Mg^{2+}$ (Levy and Ryan, 1961). We have therefore felt that a systematic study of actin-myosin interaction in the presence and absence of certain chemical modifiers, before and after ultraviolet irradiation–induced molecular changes, may be useful in shedding more light on the nature and function of the enzyme sites involved in the splitting of ATP and ITP.


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## EXPERIMENTAL PROCEDURES

Myosin A and actin were prepared as described by Mommaerts (1958). Myosin A was purified three times by reprecipitation and actin was purified twice by polymerization and centrifugation. Only polymerized actin (F actin) was used in this study.

Myofibrillar suspensions were prepared as previously described (Gergely *et al.*, 1959; Kaldor, 1960).

ATP and ITP were purchased from Sigma Chemical Co., St. Louis, Mo. In this work no attempt was