

## **EFFECT OF NEUTRAL SALTS ON THE FORMATION AND DISSOCIATION OF PROTEIN AGGREGATES**

**WAYNE MELANDER and CSABA HORVATH**

*Chemical Engineering Group  
Department of Engineering and Applied Science  
Yale University  
New Haven, Connecticut 06520*

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A theoretical framework is presented for the treatment of solvent-mediated behavior observed with polymerizing systems. It has been found that the surface tension and the bulk dielectric constant of the medium play a major role in determining the energetics of the polymerization or depolymerization process. The effect of salt is accounted for by specific binding, by electrostatic effects, which are treated on the basis of the Debye-Hückel theory, and by salt-induced changes in the surface tension. Literature data on the effect of various salts on the depolymerization of apo-D(-)- $\beta$ -hydroxybutyrate dehydrogenase observed chromatographically, on the actin G-F equilibrium, and in flagellin polymerization show the theoretically predicted behavior. The influence of organic solvents in the medium can be treated in a similar fashion as demonstrated by observed dependence of the activation energy of actin-F polymerization on the concentration of ethanol.

### **INTRODUCTION**

The aggregation of macromolecules to form polymeric supramolecular structures is a pervasive and important phenomenon in biological systems. The polymerization of actin to form filaments and that of tubulin to form microtubules, for example, are found throughout both living kingdoms. However, to date, the thermodynamic basis of polymerization has not been treated in general terms.

We have found that several related phenomena can be adequately described using a simple treatment of hydrophobic interactions as a solvent effect (1-3). The theoretical approach pioneered by Sinanoglu and his co-workers (4-11) imagines the process of bringing a species into solution to proceed in two steps. First, a cavity suitable for the solute is prepared. Second, the solute is placed into the cavity where it interacts with the surrounding solvent molecules. The energy required for the first process is given by the product of the cavity surface area and the surface tension of the solvent converted from macroscopic to molecular dimensions. The correction is frequently small and vanishes when the size of the solvent

molecules becomes much greater than that of the solvent molecules. The energy of the solute-solvent interactions is imagined mainly to consist of two terms representing the corresponding van der Waals and electrostatic interactions. Sinanoglu et al. (4,6,8,9) have calculated the van der Waals term and the interaction energy of a simple dipole with its environment. For solutes which have a net charge or which cannot be considered as simple dipoles, *i.e.*, if the solute is a zwitterion or the dipole moment is large, there have been developed suitable expressions for the electrostatic interaction (2,3).

This approach can be easily extended to develop a theory for the polymerization of proteins. The corresponding terms for the energetics of cavity formation and interaction can be calculated for the polymer in the same way as one would for the monomeric species, and thus the equilibrium constant for the process can be determined. In this communication, we shall use this method in order to treat the effect of salt, solvent, and temperature variations on the polymerization process. The results explain many experimentally observed phenomena and shed light on salt properties which are qualitatively expressed by the Hofmeister series.

## THEORY

### *Fundamental Aspects*

The unitary free energy of solution from a hypothetical gas phase at atmospheric pressure  $P$  is given by the equation (4)

$$\Delta G^0 = \Delta G_c + \Delta G_{\text{int}} + RT \ln(RT/PV) \quad (1)$$

where  $\Delta G_c$  and  $\Delta G_{\text{int}}$  are the terms for the energy of cavity formation and interaction, respectively, in a solvent of molar volume  $V$ . The cavity term is expressed as

$$\Delta G_c = NA\gamma\kappa^e(r)(1 - W) \quad (2)$$

where  $N$  is Avogadro's number,  $A$  is the area of the cavity,  $\gamma$  is the bulk surface tension,  $\kappa^e(r)$  is a factor which depends on the solute dimensions, and  $W$  is a correction factor for the relationship between the entropy and enthalpy of cavity formation (10). Usually  $W$  can be safely neglected for large solutes; this is especially true in aqueous solutions. Therefore, assuming that the molecules may be treated as spheres as given elsewhere (7), we can write for the free energy for cavity formation that

$$\Delta G_c = NA\gamma + (\kappa^e - 1)NA_1\gamma \quad (3)$$

where  $\kappa^e$  is the value of  $\kappa^e(r)$  for a neat solvent and  $A_1$  is the corresponding area of a solvent molecule.

The interaction energy is given by

$$\Delta G_{\text{int}} = \Delta G_{\text{vdw}} + \Delta G_{\text{es}} \quad (4)$$

where  $\Delta G_{\text{vdw}}$  and  $\Delta G_{\text{es}}$  are van der Waals and electrostatic contributions, respectively.

The magnitude of the electrostatic contribution can be estimated from the Debye-Hückel theory for ions, or from the expressions given by Kirkwood (12,13) and Linderstrøm-Lang (14) for large dipoles in solution. The electrostatic interaction energy for ions is given by

$$\Delta G_{\text{es}} = (Z_i^2 e^2 / 2 \epsilon k T b_i) - [Z_i^2 \kappa e^2 / 2 \epsilon (1 + \kappa a)] \quad (5)$$

where  $Z_i$  is the valence,  $e$  is the electronic charge,  $\epsilon$  is the bulk dielectric constant,  $b_i$  is the radius of the particle,  $I$  is the ionic strength,  $k$  is Boltzmann's constant, and  $a$  is the distance of closest approach, which is usually taken as 3 Å. The value of  $\kappa$  is expressed by

$$\kappa = e(8\pi NI / 1000 \epsilon k T)^{1/2} \quad (6)$$

Depending on the geometry of and the charge distribution within the solute molecule several expressions have been derived to describe the energy of dipoles in electrolyte solutions. For ellipsoids of revolution this energy is generally given by (13)

$$\Delta G_{\text{es}} = -D\mu^j m \quad (7)$$

where  $m$  is the salt molality of the medium,  $j = 1$  or 2, depending on the charge distribution,  $\mu$  is the dipole moment of the solute, and  $D$  is a constant accounting for the particle shape. Upon examination of the salting-out of proteins, we found that  $j = 1$  adequately describes the data (3). In the case of a long rod, the ionic strength dependence of the energy is given by (14)

$$\Delta G_{\text{es}} = (e^2 / \epsilon R)(\kappa R - 1 + e^{-\kappa R}) \quad (8)$$

where  $\kappa$  is the Debye-Hückel screening parameter and  $R$  is of the distance between the two charge centers in the dipole. Equation (8) becomes for moderately concentrated electrolytic solutions of fibrous particles

$$\Delta G_{\text{es}} = (2e^3 / \epsilon)[(2\pi N / 1000 \epsilon k T)]^{1/2} I^{1/2} - e^3 / \epsilon \mu \quad (9)$$

where  $\mu$  is the dipole moment.

The van der Waals contributions can be evaluated, in principle, from the theory of Sinanoglu (4,6,8,9). However, it requires a knowledge of parameters, such as the acentric factor, which are not generally available for macromolecules. An alternative formulation due to Hamaker is available for large spherical molecules (15,16). In practice, we shall use neither

formulation for this term because the magnitude of the change of this term with changing environmental conditions is expected to be small.

### *Effect of Salt Binding on Polymerization*

Salts can bind to macromolecules via specific electrostatic interactions. Schellman (17) *inter alios* has considered the chemical potential of a macromolecule which binds  $l$  ligands of the same kind. He finds that the energy change due to binding,  $\Delta G_b$ , can be expressed by

$$\Delta G_b = RT \ln \left( 1 + \sum_{i=1}^{\epsilon} K_i m^i \right) \quad (10)$$

where  $K_i$  is the binding constant for binding of  $i$  ligands of molality  $m$ . If the binding sites are independent and equivalent, the expression becomes

$$\Delta G_b = lRT \ln(1 + Km) \quad (11)$$

where  $K$  is the intrinsic binding constant for each of the  $l$  ligands.

Several kinds of polymerizing systems have been considered by Oosawa and Asakura (18). For simplicity we shall consider only two schemes. In many cases the condensation of protein to form polymers resembles a crystallization. That is, condensation does not occur until a critical concentration is reached. All the monomer in excess of that critical concentration condenses to form polymers. In ideal solutions the thermodynamic requirement for the critical concentration,  $X_c$ , is given by

$$\Delta G = \Delta G^0 + RT \ln X_c = 0 \quad (12)$$

$$\ln X_c = -\Delta G^0 / RT \quad (13)$$

The combination of Eqs. (1), (3), (11), and (13) yields

$$\begin{aligned} \ln X_c = & -[NA\gamma + NA_1\gamma(\kappa^e - 1) + \Delta G_{es} + \Delta G_{vdw}](RT)^{-1} \\ & + l \ln(1 + Km) - \ln(RT/PV) \end{aligned} \quad (14)$$

where all the terms have their earlier meaning.

An alternative system is that of finite polymerization which can be represented by the equilibrium



where  $n$  monomer units  $M$  combine to form a polymer  $P$ . The corresponding free energy change for the process is

$$\begin{aligned} \Delta G^0 = & N\gamma(A_p - nA_M) + (\kappa^e - 1)A_M\gamma(1 - n) + \Delta\Delta G_{vdw} + \Delta\Delta G_{es} \\ & - RT(l_p - nl_M) \ln(1 + Km) + (1 - n)RT \ln(RT/PV) \end{aligned} \quad (16)$$

where the subscripts P and M refer to the properties of the polymer and monomer, respectively. The meaning of the terms  $\Delta\Delta G_{\text{vdw}}$  and  $\Delta\Delta G_{\text{es}}$  is given by

$$\Delta\Delta G_i = \Delta G_{i,P} - n\Delta G_{i,M} \quad (17)$$

If the solution is ideal, the equilibrium ratio of monomer to polymer can be expressed by

$$\ln X_M^n/X_P = \Delta G^0/RT \quad (18)$$

### *Kinetics of Polymerization*

In the transition state theory of chemical kinetics, the rate constant  $k$  is given by

$$k = (kT/h)K^\ddagger \quad (19)$$

where  $K^\ddagger$  is the "equilibrium constant" between the transition state complex and the reactants. The expression for the rate constant can be rewritten as

$$\begin{aligned} k &= (kT/h) \exp(-\Delta G^\ddagger/RT) \\ &= (kT/h) \exp(-\Delta H^\ddagger/RT) \exp(\Delta S^\ddagger/R) \end{aligned} \quad (20)$$

where  $\Delta G^\ddagger$ ,  $\Delta H^\ddagger$ , and  $\Delta S^\ddagger$  are the respective standard free energy, enthalpy, and entropy for the formation of the complex from the reactant (19). The application of this expression in the calculation of rate constants *a priori* or rationalization of them *ex posteriori* is not straightforward because a knowledge of the properties of the transition state is necessary. This condition can be relaxed if we assume that the transition state resembles the final state. In this event the energies will be approximated by that of the final state for the reverse reaction and by the difference between the two states for the forward process. This very crude approximation allows one to estimate in a qualitative fashion the effect of solvent variation on the rate of polymerization and depolymerization of aggregating systems.

### *Salt Effects*

The presence of added salt may be expected to moderate each of the terms in Eq. (14). However, in dilute and moderately concentrated salt solutions the change in the van der Waals term can be expected to be small. Similarly, the change in apparent molar volume of the solvent can be taken to be small. As a consequence, for ease of analysis, we shall neglect the effect of added electrolyte on the penultimate and ultimate terms of that expression. The effect of added electrolyte on the energy of dipole

interaction with the circumambient solvent is explicitly given. Electrolytes are known usually to increase the surface tension. The increase is approximately linear over a wide range of concentration. We have called the coefficient of proportionality the molal surface tension increment,  $\sigma$ , and have tabulated the values for some common salts (3). Since the surface tension is given approximately by

$$\gamma = \gamma^0 + \sigma m \quad (21)$$

where  $\gamma^0$  is the surface tension of pure water, Eq. (14) can be rewritten as

$$\begin{aligned} \ln X_c = & -\Delta G^0/(RT)^{-1} - N[(A + A_1(\kappa^e - 1)]\sigma m/(RT)^{-1} \\ & - \Delta G_{es}/(RT)^{-1} + l \ln(1 + Km) \end{aligned} \quad (22)$$

where  $\Delta G^0$  is the corresponding value of  $\Delta G$  in pure water. If  $X_c^0$  indicates the hypothetical value of the critical concentration in pure water, we obtain that

$$\ln (X_c/X_c^0) = -N[A + A_1(\kappa^e - 1)]\sigma m(RT)^{-1} - \Delta G_{es}(RT)^{-1} + l \ln(1 + Km) \quad (23)$$

Before examining experimental data in light of this hermeneutics, it is of some value to examine the qualitative effects predicted by Eq. (23). As written, the solubility of a monomer has two different power dependencies; the specific salt binding term leads to a linear dependence on salt concentration whereas the other terms predict an exponential dependence. The ultimate term is always positive; therefore, a linear increase in the solubility with salt would be expected. The rate of increase would be salt specific. The remaining terms generate an exponential salt dependence leading to increased or decreased solubility depending on the magnitudes of the area changes and dipole moment changes upon condensation. In general the third term of Eq. (23) will be positive; thus it implies an increasing solubility with increasing salt concentration. The terms involving the area will be shown to generate a salting-out effect.

A graph illustrating the expected solubility behavior over a broad range of salt concentration is given in Fig. 1. The parameters used were 3 for the coefficient of  $\sigma$  in the first term, 1.54 for  $\sigma$ , 1 for  $l$ , and 25 for  $K$ . The curve represents the fraction in soluble protein as a function of the salt concentration. The value of  $\sigma$  corresponds to that of aqueous KCl solutions. No attempt was made to calculate the electrostatic term. The curve is very similar to data obtained by Kasai (20) for the dependence of the birefringence of actin solutions on KCl concentration at three temperatures. The slope is similar to that of a plot obtained for the kinetics of polymerization as a function of salt concentration.

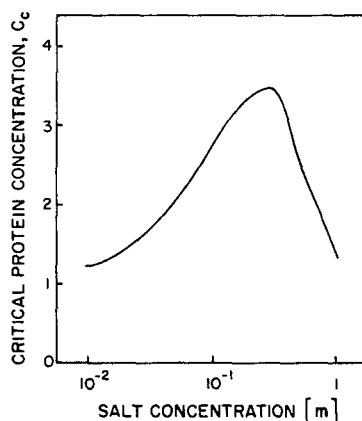


FIG. 1. The general dependence of the critical concentration in a polymerizing system on salt concentration according to Eq. (23). The curve was drawn with  $K = 25 \text{ m}^{-1}$  and the other salt-dependent terms corresponding to an area change of  $800 \text{ \AA}^2$  with no electrostatic contribution and assuming  $\sigma = 1.54 \text{ dynes/cm}$ .

The addition of agents other than salt can also modify the critical concentration. The result can be formally written as

$$\begin{aligned} X_M/X_M^0 = e^l(1 + Km) \exp\{(-NA\delta\gamma - N\delta[(\kappa^e - 1)A_1\gamma] - (\mu_M - \mu_P)m\delta\phi \\ - \delta\Delta G_{\text{vdw}})(RT)^{-1}\} \end{aligned} \quad (24)$$

where  $\delta$  expresses the change in the corresponding solvent property and  $\delta\sigma$  represents the solvent-dependent change in the electrostatic contribution. The dipole moments of the monomer and polymer molecules are given by  $\mu_M$  and  $\mu_P$ . For simplicity the salt concentration and the intrinsic binding constant were assumed to be unchanged with the change of solvent.

## RESULTS

### *Salt Effects: Exclusion Chromatography*

The effect of salts on the concentration of monomeric and polymeric species in a finitely polymerizing system can be expressed by using Eqs. (16) and (17). Subtracting the corresponding equations for the two species we obtain that

$$\begin{aligned} \ln(X_M/X_P) = \ln(X_M^0/X_P^0) + (N\sigma M/RT)(A_P - nA_M) \\ - (A_1 N\sigma m/RT)[(n-1)(\kappa^e - 1)] \\ - n\Delta G_{\text{es},M} + \Delta G_{\text{es},P} + (l_P - nl_M) \ln(1 + Km) \end{aligned} \quad (25)$$

If the polymerization proceeds without a net change in electronic charge, the concentration ratio of the monomer and polymer can be written as

$$\begin{aligned} X_M/X_P &= \tau \exp(\Delta A \sigma m / RT) \\ \Delta A &= N(A_P - nA_M) \end{aligned} \quad (26)$$

The mole fraction of monomer,  $\zeta$ , is given by

$$\zeta = X_M / (X_P + X_M) \quad (27)$$

$\zeta$  can be expressed, by using Eq. (26), as

$$\zeta = \tau \exp(\Delta A \sigma m / RT) / [1 + \tau \exp(\Delta A \sigma m / RT)] \quad (28)$$

In the chromatographic system to be considered, the column is packed with controlled pore glass and only the monomer can enter the intraparticulate space of the porous glass. Consequently, the capacity factor for the protein,  $k_R$ , provided it does not adsorb on the glass surface, is given by

$$k_R = \theta X_M / (X_P + X_M) = \theta \zeta \quad (29)$$

where  $\theta$  is the volume ratio of the intraparticulate and interstitial void spaces in the column. If adsorption also occurs,  $\theta$  represents the volume ratio times the pertinent adsorption coefficient. Substituting  $\zeta$  from Eqs. (27) and (28) into Eq. (29) we obtain that

$$k_R = \theta \tau \exp\{[N(A_P - nA_M)\sigma m / RT]\} / \{1 + \tau \exp[N(A_P - nA_M)\sigma m / RT]\} \quad (30)$$

When the term  $\tau \exp N(A_P - nA_M)\sigma m / RT$  is small the denominator of Eq. (30) approaches unity and we can express the logarithm of the capacity factor simply by

$$\ln k_R = \ln \theta \tau + N(A_P - nA_M)\sigma m / RT \quad (31)$$

Equation (31) predicts that if  $\theta$  does not change appreciably with the salt concentration, or  $\ln \theta$  is proportional to the salt concentration, the logarithm of the capacity factor increases linearly with the concentration of the salt. When different salt solutions having the same concentration are used as eluents,  $\ln k_R$  is expected to be linearly dependent on the molal surface tension increment,  $\sigma$ .

Recently Bock et al. (21) found that the chromatographic retention of apo-D(-)- $\beta$ -hydroxybutyrate dehydrogenase (EC 1.1.1.30) on controlled pore glass depends on the nature of different salts present in the eluent at a fixed ionic strength. The results, which were ascribed to salt effects on the deaggregation of the protein, can serve as a test of Eqs. (20)–(31), since the



electrostatic effect was invariant under the conditions employed in the experiments.

The elution volume,  $V_e$ , in each run was calculated from the experimental data by using the relationship

$$V_e = \sum v_i f_i / \sum f_i \quad (32)$$

where  $f_i$  is the protein fraction in tube  $i$  and  $v_i$  is the sum of the eluent volume, which passed through the column before tube  $i$  was collected, and the half-volume of the eluent in tube  $i$ . In this case the capacity factor  $k_R$  can be considered proportional to the elution volume  $V_e$  because  $V_e$  is much greater than the void volume of the column. According to Eq. (3) plots of  $\log V_e$  versus either the salt concentration  $m$  or the surface tension increment  $\sigma$  should yield a straight line. Since the data of Bock et al. (21) were obtained at fixed salt concentrations, the values of  $\log V_e$  have been evaluated for the eluents containing 1 M NaSCN, NaI, NaNO<sub>3</sub>, KBr, LiBr, and plotted against the corresponding  $\sigma$  values given by Melander and Horvath (3). The results are shown in Fig. 2, and it is seen that the linear relationship predicted by Eq. (31) indeed holds.

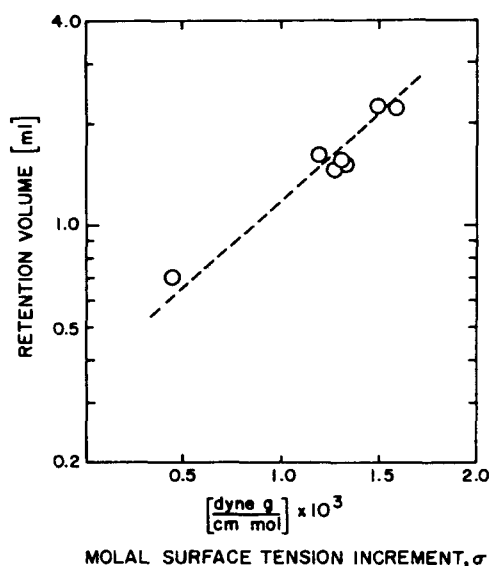


FIG. 2. Plot of the retention volume of apo-D(-)- $\beta$ -hydroxybutyrate dehydrogenase versus the molal surface tension increment of the salts in the eluent. Column: controlled pore glass 10-350. Eluents: 0.1 M Tris-acetate, pH 8.15, containing various salts at 1.0 M. From the data of Bock et al. (21).

### *Salt Effects: Infinite Polymerization*

In many studies to be considered subsequently, a protein is either polymerized or depolymerized to a defined extent, usually 50%, and the salt concentration required to achieve this degree of change is recorded. In these experiments the final concentration of monomer is identical in each case. As a consequence, Eq. (23), with the salt binding constant  $K$  set equal to zero, may take the approximate form

$$\ln(X_c/X_c^0) = a\sigma m + bm + c \quad (33)$$

where  $a$ ,  $b$ , and  $c$  are salt-independent constants. Equation (33) can be rearranged to obtain the expression

$$\sigma m = bm + c' \quad (34)$$

where  $m$  is the salt molality at the final state and  $c'$  is a constant.

If salt binds to the monomer, Eq. (23) can be rewritten as

$$\sigma m = Bm + C - \lambda' \ln(1 + Km) \quad (35)$$

where  $\lambda' = \lambda kT/[A + A_1(\kappa^e - 1)]$ . In this expression  $\lambda$  is the number of ions bound per mole of monomer and the area change upon condensation is given by  $A + A_1(\kappa^e - 1)$ . The value of  $\lambda'$  is nearly unity if  $A + A_1(\kappa^e - 1)$  is approximately  $400 \text{ \AA}^2$ .

We have analyzed, using Eq. (33), the data of Nagy and Jencks (22) obtained with the depolymerization of F-actin. The data fit this expression fairly well with a correlation coefficient of 0.903. The value of the parameter  $b$  together with the sum of the terms  $\sigma m + bm$  are presented in Table 1. According to Eq. (33) these should be constant. As seen from Table 1 this expectation is fairly well satisfied.

The effect of halide salts on F-actin depolymerization was also examined. The salt concentrations required to reduce the viscosity by 50% were measured by Nagy and Jencks (22) and are reproduced, in part, in Table 2. The data were found to conform to Eq. (34) with the parameters  $b = 1.7$  and  $c' = -0.2$  and the correlation coefficient was 0.974. In the last column of Table 2 are shown those values of  $c'$  which were calculated from Eq. (33) with the assumption that  $b = 1.7$ .

The reversal of the depolymerizing effect by  $\text{Ki}$ ,  $\text{LiI}$ , and urea can also be examined in the light of Eq. (33) if the assumption is made that the equilibrium concentration of G-actin,  $C_c$ , is related to the specific viscosity of F-actin,  $\eta_{\text{SP}}$ , by

$$C_c = (100 - \eta_{\text{SP}})/\eta_{\text{SP}} \quad (36)$$

The corresponding plot of  $\ln C_c$  vs.  $\sigma m$  has a slope of  $-0.34$  with a correlation coefficient of 0.868.

TABLE 1. Data from the Analysis of the Depolymerization of Actin According to Eq. (33) Which Relates the Surface Tension Increment  $\sigma$  to the Salt Molality,  $m$  at a Certain Degree of Polymerization<sup>a</sup>

Salt	$\sigma$ 10 <sup>3</sup> (erg-cm/mol)	$m$ (mol/kg)	$\sigma m - bm$ (erg/cm <sup>2</sup> )
KSCN	0.45	0.26	-0.11
NaClO <sub>4</sub>	0.55	0.35	-0.11
NH <sub>4</sub> NO <sub>3</sub>	0.85	0.40	-0.064
LiNO <sub>3</sub>	1.16	0.40	0.12
NaNO <sub>3</sub>	1.06	1.80	0.34
(CH <sub>3</sub> ) <sub>4</sub> NCl	0.8	2.30	-0.512
(CH <sub>3</sub> ) <sub>4</sub> NBr	0.76	0.75	-0.079
(C <sub>2</sub> H <sub>5</sub> ) <sub>4</sub> NCl	-1.6	0.32	0.23

<sup>a</sup>The parameters obtained by least squares analysis are  $b = 0.866 \pm 0.27$  cm/dyne · mol and  $a = 0.29 \pm 0.12$  cm/dyne with a correlation coefficient  $r = 0.9316$ . The last column should be nearly constant, as discussed in the text. The experimental data are from Nagy and Jencks (22) and the values of  $\sigma$  are from Melander and Horvath (3).

TABLE 2. Concentration of Halide Salts Required to Reduce the Viscosity of F-actin Solution by 50%<sup>a,b</sup>

Salt	$\sigma$ 10 <sup>3</sup> (erg-cm/mol)	$m_{\text{exp}}$ (mol/kg)	$m_{\text{exp}}$ (mol/kg)	$c'$ (erg/cm <sup>2</sup> )
LiCl	1.63	1.00	2.0	-0.078
NH <sub>4</sub> Cl	1.39	1.10	0.61	-0.35
LiBr	1.26	0.25	0.43	-0.112
NaBr	1.32	0.75	0.49	-0.29
KBr	1.31	1.10	0.49	-0.44
CsBr	1.83	1.25	—	0.153
NH <sub>4</sub> Br	1.44	0.45	0.72	-0.121
LiI	0.79	0.25	0.21	-0.23
NaI	1.02	0.25	0.28	-0.172
KI	0.84	0.44	0.23	-0.382
CsI	1.39	0.30	0.61	-0.095
NH <sub>4</sub> I	0.74	0.12	0.20	-0.12

<sup>a</sup> Taken from Nagy and Jencks (22).

<sup>b</sup> The data, when tested against Eq. (34), were found to have a correlation coefficient  $r = 0.9732$  with  $b = 1.708 \pm 0.232$  and  $c' = -0.1994 \pm 0.0889$  where  $c' = \sigma m - bm$ . The third column tests the fit by calculations of the salt molality predicted by this regression. The  $\sigma$  values are from Melander and Horvath (3). The large deviation from the observed value is in part due to the uncertainty in  $c'$ , shown in the last column.

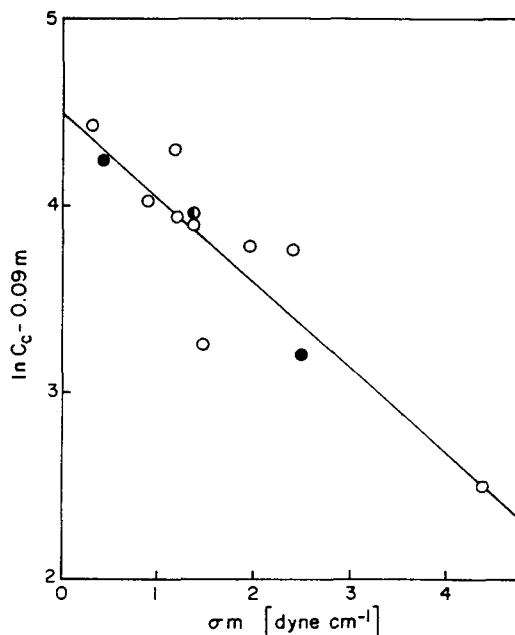


FIG. 3. The dependence of the critical concentration of actin on the surface tension of the solutions containing urea, LiI, or KI. The G-actin concentration in partially depolymerized solutions was estimated by Eq. (36). The data were found to conform to Eq. (33) with the parameters  $a = -0.453$ ,  $b = 0.09$ ,  $c = 4.44$ , and a correlation coefficient  $r = 0.913$ . Data obtained with 4 M urea are shown by solid circles and that with LiI by the half-solid circle.

When a more detailed analysis is made according to Eq. (34), a better correlation ( $r = 0.913$ ) is obtained. The data have been replotted as  $\ln C_c - b_m$  vs.  $\sigma m$  as shown in Fig. 3. The good linearity demonstrates that the effect of several different agents on the energetics of the depolymerization can be described by the treatment given earlier. This, along with the reasonably good accord of the results shown in Tables 1 and 2, is in agreement with the qualitative predictions of the theory and indicates that it can satisfactorily account for salt and solvent effects observed in the actin F-G equilibrium.

#### *Actomyosin Formation*

G-actin and myosin aggregate to form actomyosin. Holtzer et al. (23) examined the effect of salts on this process at constant ionic strength maintained with NaCl or KCl. The concentration of the different Na or K salts required for the dissociation is given in Table 3. In the light of our

TABLE 3. Concentration of Na or K Salts Required for the Dissociation of Actomyosin<sup>a</sup>

Salt	$\sigma$ 10 <sup>3</sup> (erg-cm/mol)	$m_{\text{obs}}$ (mol/kg)	$\sigma m$ (erg/cm <sup>2</sup> )	$m_{\text{calc}}$ (mol/kg)
SCN <sup>-</sup>	0.45	0.2	0.73	0.16
I <sup>-</sup>	1.02	0.3	0.78	0.3
ClO <sub>4</sub> <sup>-</sup>	1.4	0.3	0.90	0.9
Br <sup>-</sup>	1.3	0.6	0.78	0.6
NO <sub>3</sub> <sup>-</sup>	1.06	0.65	0.69	0.33
Cl <sup>-</sup>	1.6	>2		(0.49)

<sup>a</sup> According to the data of Holtzer et al. (23). The ionic strength was maintained constant with KCl or NaCl. For all salts the surface tension increase,  $\sigma m$ , should be constant. The calculated value for this increase, in the fourth column, includes the effect of the chloride salt present. The fifth column shows the expected concentration of the salt if the surface tension increase is 0.78 dyne/cm.

interpretative framework we would expect that the main effect of the added salt is to increase the surface tension and thus modify the cavity term. Under conditions of the experiments the electrostatic terms are not expected to change with the nature of the salt because the ionic strength was held constant. If this is so, the effect of the different salts on the equilibrium should be due to their property of raising the surface tension linearly with concentration. This in fact occurs as can be seen in the fourth column of Table 3. The increase in the surface tension of the solutions compared to neat water is very nearly 0.75 erg/cm<sup>2</sup>. The only significant exception occurs with ClO<sub>4</sub><sup>-</sup>; we suspect on other grounds that the literature value of  $\sigma$  for this species is too large and that a more realistic value would cause this point to conform. It is interesting that the chlorides do not dissociate actomyosin at any salt concentration between 0.6 and 2.0 M. This is expected since they always will have a surface tension increment greater than 0.75 if the ionic strength is held at a value of 0.6 or greater. In fact, the smallest value of  $\sigma m$  they can have is about unity under conditions of the experiment.

In view of Eq. (23), the critical concentration for condensation of a polymeric protein which has the features of a crystallization can be expressed in general terms as

$$\ln X_c = -NA\gamma + f(\mu, Z, I)/\epsilon RT - \ln(RT/PV) + \text{const} \quad (37)$$

where  $f(\mu, Z, I)$  is a function of the dipole moment  $\mu$  and the net charge  $Z$  appropriate to the macromolecule and the ionic strength  $I$  of the solution. Equation (37) is expected to hold over a relatively small range of temperature.

The data obtained by Taniguchi (24) on the formation of tobacco mosaic virus (TMV) coat protein trimers can be used to examine the validity of Eq. (37). Similarly to actin, it behaves like a crystallizing system, and the formation of TMV protein trimer begins when a critical concentration is obtained. The critical concentration is very temperature dependent. In the study of this effect by Taniguchi (24) the logarithm of the critical concentrations was plotted versus the reciprocal temperature to obtain the enthalpy from a van't Hoff plot. Since the product of the temperature and the dielectric constant of water is very nearly temperature invariant, the dielectric constant can be assumed constant. If  $f(\mu, Z, I)$  does not change appreciably in the experimental temperature interval, we may expect the critical concentration to vary with the temperature due to the effect of temperature on the surface tension. The data of Taniguchi have been replotted in Fig. 4 as  $RT \ln C_c$  vs.  $\gamma - 73$  where  $C_c$  is the critical

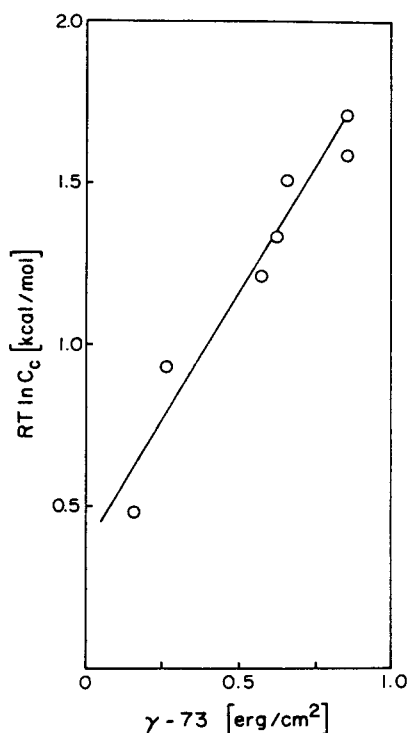


FIG. 4. Dependence of tobacco mosaic virus protein trimer formation on the surface tension of the medium. The logarithm of the critical concentration  $C_c$  when multiplied by  $RT$  is expected to be linearly dependent on the surface tension according to Eq. (23). This provides a phenomenological explanation for the observed van't Hoff behavior in this system. The data are from Taniguchi (24) and the values of surface tension  $\gamma$  were taken from the International Critical Tables (25).

concentration and  $\gamma$  the surface tension. The resultant plot is nearly linear and in fact, the correlation coefficient  $r = 0.963$  is identical with that found for the van't Hoff plot. An estimate of the area change per particle upon condensation can be made on the basis of the plot and is found to be approximately  $1200 \text{ \AA}^2/\text{molecule}$ .

#### *Kinetics of the Condensation of 1,2-Flagellin*

In a study of the condensation of 1,2-flagellin as measured by the specific viscosity, Wakabayashi et al. (26) found that the rate of polymer formation was markedly modified by the presence of salts. To examine these salt effects in light of our framework we define an apparent rate constant by  $10^3/t_{0.4}$  where  $t_{0.4}$  is the time required for the specific viscosity to increase to 0.4 of the final value in the experiments. In light of transition state treatment the logarithm of the rate constant plotted versus the molarity of the salt should give a straight line. In Figs. 5 and 6 the rate data are plotted versus the concentration of  $\text{Na}_2\text{SO}_4$  and phosphate, both at pH 6.5, and indeed an approximately linear relationship between the logarithm of the rate constant and the salt molarity is observed. The slopes in the two cases, however, are markedly different, the difference being caused by electrostatic effects. One would anticipate that the slope obtained with  $\text{Na}_2\text{SO}_4$  would be greater by about 50% than that obtained with phosphate. The observed difference, however, is much greater than that and strongly suggests that the effect of salts on the surface tension plays the major role in determining the rate constant.

#### *Effect of Salt and Solvent Variation on Transition State Parameters with Actin*

Monomeric G-actin aggregates to form F-actin. This process has been the object of much research and the polymerization has been described as a phase change. In this case, we would expect Eq. (23) to properly describe the energetics of the system. The addition of salts and organic solutes is known to modify the energetics of the system. The most extensive data on these phenomena have been obtained by Nagy and Jencks (22) and Kasai (20) and the results have been summarized by Oosawa and Asakura (18).

The addition of ethanol causes the activation enthalpy for polymerization to be reduced, ultimately changing sign at 15% v/v ethanol. If we examine this in the framework of transition state theory with the additional assumption that we can use an expression for the energy of the form shown in Eq. (35), we find that the activation enthalpy is given approximately by

$$\Delta H^\ddagger = A^\ddagger \gamma [1 - (d \ln \gamma)/(d \ln T)] + (U^\ddagger/\epsilon) [1 + (d \ln \epsilon)/(d \ln T)] + V^\ddagger \quad (38)$$

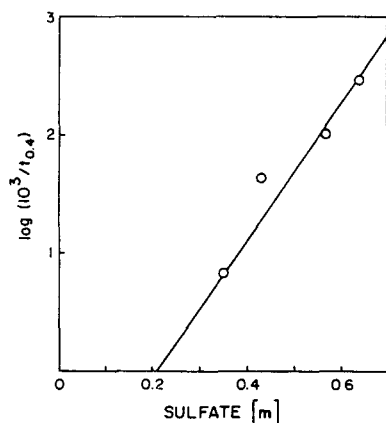


FIG. 5. Dependence of the apparent first-order rate constant for flagellin polymerization on the concentration of  $\text{Na}_2\text{SO}_4$ . In the light of Eq. (20) and (23), the logarithm of the rate constant should be a linear function of the salt concentration in the absence of specific salt effects. The rate constant is defined in the text as  $1000/t_{0.4}$  where  $t_{0.4}$  is the time required for the reaction to proceed to a fixed extent and is proportional to the first-order constant. The data are taken from Wakabayashi et al. (26).

where  $A^\ddagger$ ,  $U^\ddagger$ , and  $V^\ddagger$  are constants corresponding to the area change, the change in dipole moment upon activation, and  $V^\ddagger$  includes the van der Waals term and other terms largely independent of solvent properties. Since the thermal variations of the surface tension and the dielectric constant are nearly independent of temperature, we can regard them as

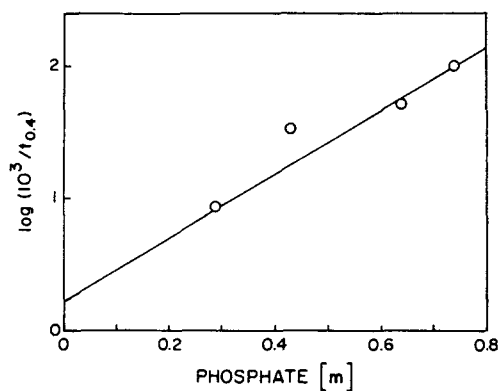


FIG. 6. Dependence of apparent first-order rate constant for flagellin formation on the concentration of phosphate buffer. The data are taken from Wakabayashi et al (26).



constant and rewrite the expression as

$$\Delta H^\ddagger = A^{\ddagger'}\gamma + (U^{\ddagger'}/\epsilon) + V^{\ddagger'} \quad (39)$$

The results were analyzed by linear regression. The coefficients and the backcalculated values of the enthalpy are presented in Table 4 where the activation energy is given in kilocalories per mole and the surface tension in dynes per centimeter. The values observed are in accordance with the model. The area change is about  $67 \text{ \AA}^2$ , which is constant, with only a small fraction of the solvent shell being decomposed in the transition state. Actin-F and actin-G are not yet fully joined in the transition state, and consequently the circumambient solvent is only partially expelled. The corresponding change in the dipole moment depends on the distance between actin-F and actin-G. If  $40 \text{ \AA}$  is used as the estimate, the change in the moment is about 2000 D. This large change is consistent with the observation that the dipole moment of actin-G is large whereas the moment per monomer in actin-F is relatively small (29,30). The final term corresponds to a large attractive energy ( $\sim 100 \text{ kcal}$ ) between the two particles, as expected from the size and the identity of the particles under investigation.

The calculated and observed values for the heat of activation are not wholly in agreement. The disparity is reduced, however, when one considers the crudity of this approach and assigns an uncertainty of  $\pm 2 \text{ kcal/mol}$  to the calculated value based on error analysis. Furthermore the terms included in the constant  $V^{\ddagger'}$  can vary somewhat with solvent composition. However, this variation is less easily described by bulk properties of the solvent and a more precise calculation would require a more accurate picture of the geometry of particles in the states of interest.

TABLE 4. Comparison of Observed Enthalpy of Actin Polymerization in Hydro-organic Solvents to That Obtained by Use of Eq. (38) with  $A^{\ddagger'} = -0.222$ ,  $U^{\ddagger'} = -1.22 \times 10^4$ , and  $V^{\ddagger'} = 160.7^a$

% ethanol	$\gamma^b$ (erg/cm <sup>2</sup> )	$\epsilon^c$	$\Delta H_{\text{obs}}$ (kcal/mol)	$\Delta H_{\text{calc}}$ (kcal/mol)
0	72.8	80.4	25	25.2
5.3	58.5	77.4	17	15.9
11	47.7	73.6	4	4.9
16.6	41.4	70.6	-4	-3.8
20.7	38.2	68.7	-9	-9.4

<sup>a</sup> The experimental data are from Kasai (20). The values tabulated are appropriate to 20°C.

<sup>b</sup> From Timmermans (27).

<sup>c</sup> Calculated according to Åkerlöf (28).

Nevertheless, the assumption of a constant  $V^{*}$  is probably justified in this case. In a detailed investigation of the individual terms of a very similar expression derived for the binding of solutes in reversed phase liquid chromatography, these terms were found to be independent of solvent composition in hydroorganic media up to 0.8 mol fraction of methanol or acetonitrile (1).

### DISCUSSION

In the preceding sections we have presented a simple conceptual framework for the analysis of some important phenomena regarding the polymerization of proteins. The effect of the solvent is described largely in terms of macroscopic solvent properties.

According to the theory three solvent properties are important in determining the energetics of the process: the surface tension for calculating the cavity energy, the dielectric constant for calculating the mean electrostatic energy, and the solvent molecular size, which is used for the van der Waals energy calculation (4,6,8,9).

Polymerization of proteins is known to follow one of two general schemes. It may proceed via the reversible formation of a complex such that an equilibrium constant with the concentrations of the monomeric and polymeric forms can be derived. Alternatively, the process can be likened to a crystallization insofar as the process occurs only when the concentration of the monomers meets or exceeds a critical concentration and is independent of the amount of protein in the polymers; i.e., the polymer behaves like another phase. The first form of polymerizing system can be described by an appropriate combination of equations in which the terms are modified to account for the area, dipole moment, or charge and of the monomer and polymer molecules as well as for size-specific ion binding or solvent effects. This has been done for the general case and is presented in Eq. (14). In the second case, the standard free energy of the crystallizing system can be calculated on the basis of Eq. (16), which is a modification of the general expression given in Eq. (14). In either case, the equilibrium composition of a polymerizing mixture is determined by the surface tension and dielectric constant among other solvent parameters.

This proposition has been examined in several cases. In the first case, the polymerization of apo-D(-)- $\beta$ -hydroxybutyrate dehydrogenase occurred at fixed ionic strength. Thus the term in the dielectric constant was constant. However, the term in the surface tension at a given molar concentration was different for each of the salts. As a consequence, the equilibrium was shifted and the shift could be determined by the molal surface tension increment. In terms of the actual chromatographic

experiment one expects the retention values to increase exponentially as the surface tension increases, and this effect is clearly shown in Fig. 1. The role of the surface tension has been shown elsewhere to be of importance in accounting for the interactions of proteins. The salting out of proteins, for example, depends on the nature of salts and has been used as a basis of the Hofmeister series. By using a similar treatment as presented here, we have shown that this series can be quantitatively described in terms of the molal surface tension increment of the individual salts (3).

The actin G-F transformation offers another partial test of the theory. In many cases, data for the solvent conditions have been obtained under conditions when the G-F equilibrium was fixed; e.g., the specific viscosity, a measure of the F-actin content at a given protein concentration, was constant. According to the present theory, at fixed ionic strength the equilibrium condition should be a simple function of the surface tension in the absence of specific salt effects. However, if the data are not obtained at fixed salt concentration and if the electrostatic interaction is due to dipole interactions with the bathing medium, the free energy change should be a simple function of both the surface tension increment and the salt molality. Equation (34) approximately describes such equilibria and predicts that the sum  $\sigma m - bm$  is constant and has a negative sign. These predictions are satisfactorily met as shown in Tables 1-3.

The most dramatic example of solvent effects on the actin system is found with added ethanol which decreases and eventually changes the sign of the enthalpy of activation. This phenomenon can be interpreted by the corresponding changes in the surface tension and dielectric constant of the medium, and Eq. (38) reproduces the original data within the accuracy of the linear regression. The sign of the term in the surface tension is negative which is surprising at first glance because it corresponds to an increase in the area of the transition state compared to the area of the incoming G-actin molecule and the F-actin with which it is combining. In retrospect it is perhaps less surprising because one would expect the transition state for the combination of two rigid rods to have the geometry of a dumbbell which has a significantly greater area-to-volume ratio than the rods. The dipole moment of the complex can only be estimated because its calculation requires knowledge of the geometry of the transition state. However, with this caveat we estimate that it is in excess of 2000 D. This number is in agreement with the dipole moments measured for fragments of G-actin and F-actin (29,30).

A decrease in the activation energy is observed with ethanol but no change in the enthalpy of the G-F transition or in the activation energy is observed with salts and urea. At first this may appear startling. However, a partial explanation is found in the expressions presented within this paper.

The addition of urea or salts, at the levels normally used, increases the surface tension by 1–2 ergs/cm<sup>2</sup> whereas the addition of ethanol will decrease it very rapidly by about 50 ergs/cm<sup>2</sup>. That is, the two different treatments modify the term for the energy of cavity formation by less than 3% and more than 60%, respectively. Similarly, the addition of ethanol also greatly affects the dielectric constant of the medium. The result is that the pertinent solvent properties change drastically and the effect is a decrease in the activation energy with increasing organic composition. According to the corresponding theoretical expression the addition of salts and urea causes only negligible changes in the enthalpy. In contradistinction, these changes, small as they are, are sufficient to cause significant changes in the G–F equilibrium. This is not unexpected when we recall that an energy shift of 1.5 kcal/mol will change the equilibrium ratio by a factor of 10. According to the theory, this would correspond to an increase in the concentration of KCl from nearly zero to 4 M if the molecular surface area of the solute is 1000 Å<sup>2</sup>, without considering other effects. Since the surface area is in fact much larger, a small increase in salt concentration can be responsible for a large shift in the equilibrium toward polymer formation.

It should be noted that changes in the surface tension can affect not only the extent of polymer formation but also the chemical forms of the polymer. Oosawa (31) showed that different forms can be expected with changing solvent conditions, especially surface tension, because the requirement of energy minimization implies that forms with different surface area-to-volume ratios will be found as the surface tension and other parameters of the solvent change.

In conclusion, we have presented a theory for the energy of macromolecular polymerization, which is based on macroscopic properties of the solvent. This theory appears to describe adequately several polymerizing systems which either show finite or infinite degrees of polymerization. Both the modification of the polymer–monomer equilibrium by organic solvent and the effect of various salts in modifying the polymerization can readily be explained on the basis of the theory.

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