Elson, E. L., and Edsall, J. T. (1962), *Biochemistry 1*, 1.
Garfinkel, D., and Edsall, J. T. (1958), *J. Am. Chem. Soc. 80*, 3823.
Grafius, M. A., and Neilands, J. B. (1955), *J. Am. Chem. Soc. 77*, 3389.
Josien, M. L., Dizabo, P., and Saumagne, P. (1957), *Bull. Soc. Chim. France*, 423.
Kohlrausch, K. W. F. (1943), Ramanspektren, Leipzig, Akademische Verlagsgesellschaft p. 211.
Martin, R. B., and Edsall, J. T. (1958), *Bull. Soc. Chim. Biol. 40*, 1763.
Pauling, L. (1960), The Nature of the Chemical Bond, 3d ed., Ithaca, N. Y., Cornell Univ. Press, p. 90.
Wright, W. B. (1958), *Acta Cryst. 11*, 632.

Polymerization-Depolymerization of Tobacco Mosaic Virus Protein. IV. The Role of Water*

Charles L. Stevens and Max A. Lauffer

ABSTRACT: Previous investigations have shown that the polymerization of tobacco mosaic virus protein is a spontaneous endothermic process, presumably because of an interaction between the protein subunits and solvent. Specifically, it is an increase of entropy associated with the loss of a bound solvent component which appears to be decisive. Utilizing a new quartz spring-balance, we have measured directly the amount of water lost by the protein upon polymerization and have found it to be about 150 moles of water from each 1×10^5 g of polymerizing material. This amount may be sufficient to explain the entire observed entropy of polymerization. In addition, we find an increase of about 7 moles of salt bound to each 1×10^5 g of protein and an increase of 0.0060 ml/g in the partial specific volume. The data seem to be consistent with electrostrictive binding of water at zwitterionic groups, but the loss of icelike domains of water about hydrophobic groups on the protein is an alternative hypothesis.

Protein from tobacco mosaic virus (TMV),[1] even when freed from nucleic acid, has the remarkable property of polymerizing spontaneously into a rod similar to the intact virus. The apparent physical differences between the polymerized protein and the intact virus are minor (Klug and Caspar, 1960); polymerization of protein in the absence of nucleic acid, however, does not produce infective particles.

Polymerization is thermodynamically reversible (Lauffer *et al.*, 1958; C. E. Smith and M. A. Lauffer, data to be published). Positive increments of temperature, ionic strength, and protein concentration and a decrease of *p*H favor polymerization. Protein in 0.1% solution buffered at *p*H 6.5 by 0.1 ionic strength sodium phosphate exists predominantly in the polymerized state at room temperature and is depolymerized at refrigerator temperature. Although the basic subunit has a molecular weight of about 17,500, the polymerizing unit under the conditions mentioned seems to have a molecular weight of about 50,000–100,000 (Schramm and Zillig, 1955). Complete dissociation is attained probably only under conditions of extreme dilution (Ansevin and Lauffer, 1963), extremes of *p*H, or by chemical modification or denaturation of the subunit.

Lauffer *et al.* (1958) found that the polymerization reaction is endothermic and has a positive entropy change. The last result might be regarded as unexpected because polymerization involves the ordering of protein units into a highly specific structure. Accordingly, the authors state that the polymerization of protein from TMV can be understood only in terms of interaction between the protein and solvent; they propose that the protein monomer loses water of hydration upon polymerization. Thus, the over-all reaction is: hydrated monomer yields polymer plus water with a net increase of enthalpy and entropy.

The purpose of this investigation is to provide a direct test of the hypothesis that water is released from

* From the Department of Biophysics, University of Pittsburgh, Pittsburgh, Pa. *Received September 25, 1964.* Publications I, II, and III of this series are Ansevin and Lauffer (1963), Lauffer (1964), and Ansevin *et al.* (1964), respectively. This is publication No. 111 of the Department of Biophysics, University of Pittsburgh. Work was supported by a U.S. Public Health Service grant (GM 10403-02). The data presented in this paper are taken in part from the dissertation presented by C.L.S. to the University of Pittsburgh in partial fulfillment of the requirements for the degree of Doctor of Philosophy, April 18, 1962.

[1] Abbreviation used in this work: TMV, tobacco mosaic virus; RNA, ribonucleic acid.

the protein concomitantly with polymerization. The change of hydration of protein associated with the reaction monomer → polymer has been measured with a quartz spring-balance. The measurement depends upon the fact that the apparent weight of a solute in aqueous solution is a function of the water of hydration associated with it. To measure the change of hydration, intact virus has been used as a control substance. Thus, the difference of hydration changes of protein and virus is obtained and should reflect the change associated with the polymerization reaction.

Materials and Methods

Virus and Protein. The method of purifying the common strain of TMV and the preparation of protein by alkaline degradation of virus are similar to those used by Ansevin and Lauffer (1963) and have been described (Stevens, 1962). In order to remove any RNA which might remain in the protein preparations, the *p*H of the solution was adjusted to 6.0 with monobasic sodium phosphate. The slightly opalescent precipitate was removed by centrifugation at 40,000 rpm in a Spinco No. 40 rotor. Absorption spectra of the supernatant fluids showed that the amount of RNA was less than 0.1% (with respect to protein) (Ansevin, 1958). Protein was concentrated by slowly adding 0.1 M hydrochloric acid to the protein solution while in an ice bath. When the *p*H had reached 4.7 or 4.8, the precipitated protein was removed by centrifugation. The pellets were dissolved in an appropriate volume of 0.1 M solution of sodium bicarbonate (*p*H 8.23), centrifuged at 40,000 rpm for 2 hours, and dialyzed against a 5×10^{-5} M solution of sodium hydroxide. The final *p*H of the protein was 8.0–8.5.

The concentrations of virus and protein were determined both by refractometry and by absorption spectroscopy. The specific refractive increments of virus and protein were measured on a Bryce-Phoenix differential refractometer. The equilibrium dialyzing fluids were used for the reference fluids in the instrument. Concentration was determined by drying a known weight of solution for 26 hours in an oven at 50–60°, then drying over phosphorus pentoxide to constant weight in an Abderhalden dryer (110°). The specific refractive increment of virus could not be distinguished from that of protein, so the values were averaged. The average of five observations at 546 mμ was 0.1856 (concentration units: g/g) with a standard deviation of 0.00095. The concentration range was 9–19 mg/g.

Absorption spectra were obtained from a Beckman Model DU instrument. For virus solutions, the absorption was corrected for scattering by the method of Englander and Epstein (1957). Concentrations were determined refractometrically. The solvent for virus was water; that for protein was 0.033 M sodium phosphate solution with a *p*H of 7.1. Solutions containing 0.1 mg of virus or protein per g had optical densities of 0.26 and 0.13 at the respective absorption maxima, 263 mμ for virus and 281 mμ for protein. These values compare well with those obtained by Fraenkel-Conrat and Williams (1955), 0.27 and 0.13.

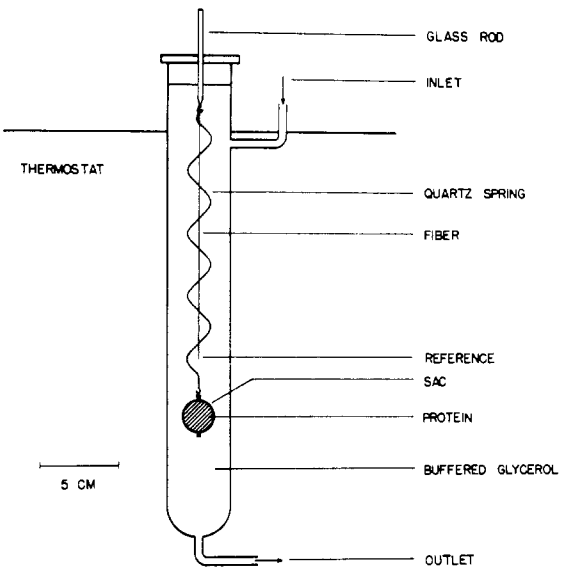

FIGURE 1: A schematic diagram of the assembled spring, dialysis sack, and chamber.

It was necessary to prepare four buffer solutions for each set of experiments. Two had a density of 1.0044 g/ml at 4°, one with a *p*H of 7.59 and the other with a *p*H of 5.50. The other two had a density of 1.0665 and *p*H values of 7.50 and 5.43. Both pairs of solutions contained sodium phosphate at an ionic strength of 0.025. The solutions with the higher *p*H contained 0.075 mole of sodium chloride per liter; the others contained 0.0533 mole of the salt. Although it was desirable to have 0.1 ionic strength salt in all solutions, the proper match of densities could not be obtained under this condition. The high-density solutions were made by placing the dry salts in a volumetric flask and dissolving them in an appropriate volume of 25% (w/v) glycerol solution from a common supply.

The concentrations of virus and protein solutions were determined refractometrically, and carefully measured aliquots containing 100 mg of material were introduced into dialysis sacks. Care was taken that no air bubbles were introduced. Boiled distilled water was used for the solutions so that if a small bubble was introduced it dissolved away during subsequent dialysis. The materials were dialyzed against large volumes of buffer solution with a *p*H of 7.5. Under these conditions, the protein was not polymerized.

The Quartz Spring-Balance. A schematic diagram of the assembled spring, dialysis sack, and chamber appears in Figure 1. The spring was formed from a fine fiber of quartz. Protein or virus was in a cellophane dialysis sack tied with nylon thread. The sack was attached to the lower hook of the spring with a loop of fine platinum wire. The weight of cellophane in the sacks and length of wire were kept uniform throughout.

The assembly consisting of spring, reference fiber, sack, and supporting glass rod was immersed in an appropriate buffer solution in the cylindrical glass chamber. The spring supported the sack at a position about 1 cm below the tip of the reference fiber, a fine glass rod coaxial with the spring. The extension of the spring was always determined with respect to the tip of the reference fiber.

The glass chamber was fitted with an inlet and an outlet so that solutions could be drained and replaced with a minimum disturbance of the system. The chamber was supported in a thermostated water bath controlled at 4.0 ± 0.05°. The water bath was fitted with a port to permit viewing of the position of the sack with a cathetometer.

Initially, the sack contained virus or protein essentially pre-equilibrated to buffer or buffered glycerol solutions with a *p*H of 7.5. There was always a small redistribution of secondary solutes; after a day or two (without mechanical stirring) the system equilibrated. After the position of the sack was determined, the solution was drained from the chamber. The chamber was refilled with solution of *p*H 5.5 which caused the protein to polymerize. A dropwise flow of fresh solution was maintained for several days in order to wash away materials diffusing from the sack. The position of the sack was measured periodically until the system equilibrated again. The change of apparent weight of the sack and its contents was calculated from the change of position of the sack and the spring constant. In order to determine whether any irreversible changes had taken place, buffer solution with a *p*H of 7.5 was returned to the chamber and the equilibrium position of the sack was compared with the initial position.

The quartz spring was calibrated by hanging platinum weights on its lower hook and immersing the assembly in water at 4.0°, the temperature of the experiments. (The weights had been calibrated on an analytical balance and corrected for buoyancy.) Deflections were measured with a cathetometer and could be read to within 0.002 mm; the measurements were reproducible to within 0.01 mm. Two springs were used in the experiments. One had first- and second-order coefficients of 0.423 mg/mm and 2.0 × 10^{-4} mg/mm², respectively; the other had coefficients of 0.430 mg/mm and 2.7 × 10^{-4} mg/mm². The second-order coefficient was not needed for deflections encountered within an experiment, but the position of the sack varied from experiment to experiment.

The Dilatometer. The change of partial specific volume of virus and protein was measured by dilatometry. The experimental conditions paralleled those pertaining to the spring-balance experiments except that buffered solutions were not used. The dilatometer was similar to those described by Linderström-Lang (1950) and by Kauzmann (1958). Two tubes were joined to form a chamber with the shape of an inverted V. The legs were sealed, and a 1.0-mm capillary was sealed to the chamber at the apex of the V. The chamber contained a glass ball so that the contents could be mixed easily. The dilatometer had a volume of 32 ml.

Seven ml of a virus or protein solution was introduced into one leg of the dilatometer. The solution was 0.1 M with respect to sodium chloride, and the *p*H had been adjusted to 7.6 with dilute sodium hydroxide. The other leg contained a solution 0.1 M with respect to sodium chloride and just enough hydrochloric acid to bring the *p*H of the protein or virus solution to 5.5 when mixed. The liquid in the legs of the dilatometer was chilled, then a 0.1 M solution of sodium chloride was layered over the others. The last solution filled the dilatometer up to a point in the capillary. The position of the meniscus with respect to a reference line on the capillary was determined with a cathetometer after thermal equilibration in a 4.0° bath. The liquids were then mixed, and the position of the meniscus was remeasured after thermal equilibration. The change of volume was calculated from the change of position of the meniscus and the volume per unit height of the capillary. A control experiment consisted of diluting protein in the dilatometer without acid in order to determine whether a significant proportion of the observed volume change could be attributed to dilution alone.

Experimental Results

A representative plot of the time course of equilibration of protein in the spring-balance appears in Figure 2. The equilibrium weight changes between *p*H 7.5

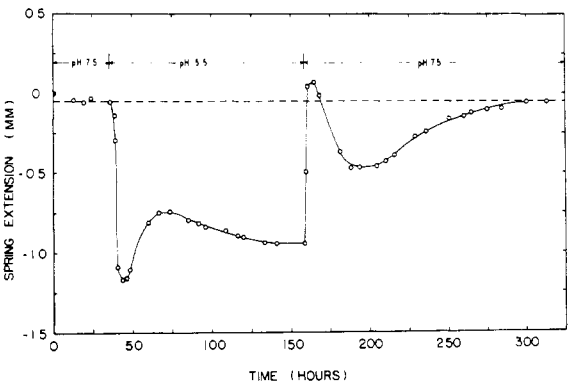

FIGURE 2: The time course of equilibration of TMV protein in the spring-balance. The protein was equilibrated successively to buffered glycerol solutions with a *p*H of 7.5, 5.5, and 7.5 again.

and 5.5 appear in Table I. Entries are included for experiments with virus and with protein each equilibrated to buffer and to buffered glycerol solutions. Also, the densities of the equilibrating solutions are given. Upon reequilibration to the original solution with a *p*H of 7.5, the sacks always returned to within 0.1 mm or less of the starting position.

The results of the experiments with the dilatometer appear in Table II. The change of volume observed upon merely diluting the protein is included in the

TABLE I: Tabulation of the Change of Apparent Weight of the Virus and Protein Systems in the Quartz Spring-Balance.[a]

	Virus (mg)	Protein (mg)
Buffer solution (density 1.0044)	−0.338	−0.638
Buffered glycerol solution (density 1.0665)	−0.204	−0.368

[a] Each entry gives the change of weight of the sack between *p*H 7.5 and 5.5. Each sample contained 0.1 g of virus or protein.

table but not in the calculation of ΔV. The experiment shows that the volume change is small; to include the correction in the calculation, one should include data for the dilution of virus and data for the subsequent concentration of the materials.

The weight, W, of material in the sack is given by equations (1) and (2) (Lauffer, 1964)

$$W = \gamma_2 G[1 - (V_1)\rho] + \gamma_2 G'[1 - (V_4)\rho] + \gamma_2 [1 - (V_2)\rho] + \frac{M_3\gamma_1}{1000} m_3'' (H_3 - 1)[1 - (V_3)\rho] + \frac{M_4\gamma_1}{1000} m_4'' \left[\sqrt{H_4 + \left(\frac{q}{2m_4''}\right)^2} - \frac{q}{2m_4''} - 1\right] \times [1 - (V_4)\rho] \quad (1)$$

$$W = \varphi(G) + \varphi_1(G') + \varphi_2(V_2) + \varphi_3(H_3) + \varphi_4(H_4,q) \quad (2)$$

where the appropriate functions are given by equation (1). In equation (1), subscripts 1, 2, 3, and 4 refer to water, protein salt, glycerol, and NaCl, respectively; γ means mass inside sack; ρ, density of external solution; (V_i), average partial specific volume; G, "bound" water and G', "bound" salt in grams per gram of 2; M, molecular weight; m'', molality in external solution, and q, molality of positive ion (Na^+) inside sack from ionization of protein salt. H_3 and H_4 are defined by the relationships:

$$H_3 = \frac{f_1' f_3''}{f_3' f_1''} \exp(h_1 - h_3) \quad \text{and}$$

$$H_4 = \frac{(f_1')^2 f_{4+}'' f_{4-}''}{f_{4+}' f_{4-}' (f_1'')^2} \exp(2h_1 - h_4)$$

where f means activity coefficient. Single prime refers to "free" components (Lauffer, 1964) inside the sack, and double prime to components in the external solution.

TABLE II: Change of Apparent Partial Specific Volume of Virus and Protein.[a]

	Virus	Protein	Protein (dilution)[b]
Δh (mm)	+0.27	+1.21	−0.061
	+0.32	+1.23	
w (g)	0.169	0.128	0.105
	0.169	0.131	
ΔV (cc/g)	+0.00127	+0.00744	−0.00046
	+0.00149	+0.00738	
Final *p*H	5.3	5.5	
	5.3	5.5	

$\Delta V_a - \Delta V_b = +0.0060$ (cc/g)

[a] A double entry represents an experiment in duplicate. The diameter of the capillary bore of the dilatometer is 1.0 mm; Δh is the change of height of the meniscus (+ represents an expansion), w is the weight of material (virus or protein), and ΔV is the change of apparent partial specific volume. [b] Explained in text.

$$h_i = (P'\bar{V}_i' - P''\bar{V}_i'')/RT$$

where P is pressure and $\bar{V}_i$ is partial molar volume; h_i is approximately equal to $m_2'\bar{V}_i$.

The change of weight of protein between the two *p*H values, ΔW_a, is given by equation (3):

$$\Delta W_a = \varphi(\Delta G_a) + \varphi_1(\Delta G_a') + \varphi_2[\Delta(V_{2a})] + \Delta\varphi_3(H_{3a}) + \Delta\varphi_4(H_{4a},q_a) \quad (3)$$

A similar equation with subscript b can be written for the control virus. The difference between the two yields equation (4) when γ_2 and γ_1 are the same in all experiments.

$$\Delta W_a - \Delta W_b = \varphi(\Delta G_a - \Delta G_b) + \varphi_1(\Delta G_a' - \Delta G_b') + \varphi_2[(\Delta V)_{2a} - (\Delta V)_{2b}] + \Delta\varphi_3(H_{3a}) - \Delta\varphi_3(H_{3b}) + \Delta\varphi_4(H_{4a},q_a) - \Delta\varphi_4(H_{4b},q_b) \quad (4)$$

Since $\Delta q_a = \Delta q_b$, the final term reduces to

$$\varphi_4\left(\Delta\sqrt{H_{4a} + \left(\frac{q_a}{2m_4''}\right)^2} - \Delta\sqrt{H_{4b} + \left(\frac{q_b}{2m_4''}\right)^2}\right)$$

which can be expanded into a power series and reduced to approximately

$$\varphi_4(1/2\,\Delta H_{4a} - 1/2\,\Delta H_{4b})$$

Since there is no change in osmotic pressure when control virus changes charge, $\Delta H_{3a} = \Delta H_{4b} = 0$. If W' is now defined as W/γ_2, weight per gram of 2, equation (4) can be transformed into equation (5):

$$\Delta W_a' - \Delta W_b' = (\Delta G_a - \Delta G_b)(1 - V_1\rho) + (\Delta G_a' - \Delta G_b')(1 - V_4\rho) - \rho[\Delta(V)_{2a} - \Delta(V)_{2b}] + \frac{C_3}{C_2}(1 - V_3\rho)(\Delta H_{3a}) + 1/2\,\frac{C_4}{C_2}(1 - V_4\rho)\,\Delta H_{4a} \quad (5)$$

where $\Delta W'$ is the change of apparent weight (in grams per gram of virus or protein) registered by the spring-balance between the two *p*H values utilized; C_3 and C_4 are, respectively, the concentrations of glycerol and salt in the external solution, and C_2 is the concentration of virus or protein, all in grams per gram of water. The next to last term of equation (5) is included in the expression in order to correct for the diminished concentration of glycerol as a result of osmotic redistribution. The value of the last term, the corresponding term for electrolyte, is negligible under the conditions of the experiments.

Experiments have been conducted in media of different densities so that two independent equations are generated from equation (5). The equations are solved simultaneously for $\Delta G_a - \Delta G_b$, the change of hydration associated with the polymerization, and for $\Delta G_a' - \Delta G_b'$, the corresponding change of amount of ions bound. Here V_1 is approximately equal to 1. For simplicity, it will be assumed that the ions which may be bound or released during polymerization are sodium and chloride; V_4 and V_3 have been calculated for this salt and glycerol from tables of density and concentration, and respective values of 0.29 and 0.79 ml/g have been used. Also C_3 was 0.31 g/g, and C_2 was approximately 0.022 g/g; m_2 was approximately 3.4×10^{-4} M. The next to last term of equation (5) is much smaller than the preceding terms, so only an approximate value of the term is required. Using these values, one calculates that for each gram of virus and protein there are 0.0043 g of salt bound and 0.027 g of water released.

Discussion

The theoretical basis of the present study originated in earlier treatments of hydration by Schachman and Lauffer (1950) and Lauffer and Bendet (1954). A theory of the hydration of macromolecules has been developed from thermodynamic principles by Lauffer (1964). Accordingly, the theory is not based on any model and therefore does not imply a mechanism by which the macromolecule becomes hydrated. Such an interpretation of hydration or changes of hydration must be made on the basis of further considerations. The theory treats the general case of interactions between a macromolecular solute and any other component of the solution and is directly applicable to the present experiment. Hydration is defined operationally according to the following scheme: If one adds an anhydrous macromolecular (primary) solute to an aqueous solution containing one diffusible solute, the ratio of activity of water to activity of diffusible solute will, in general, be changed. When this ratio is restored to its original value by adding water, by definition the macromolecules have become hydrated, and the amount of water added is defined as the water of hydration. When the aqueous solution contains more than one diffusible solute, it may be necessary to add solutes and water to restore activity ratios to their original value. In this case, the binding of secondary solutes is defined in a manner analogous to the definition of hydration (Lauffer, 1964). If, now, the solution containing macromolecules is enclosed within a semipermeable membrane and maintained at equilibrium with solvent and diffusible solutes, the apparent weight of the system can be expressed in terms of the hydration of macromolecules, among other parameters.

The polymerization of TMV protein was carried out within a cellophane dialysis membrane and was brought about by lowering the *p*H of the environment. From titration data (Ansevin *et al.*, 1964), it was found that protein and virus change net charge to the same extent if the *p*H range is 7.5–5.5, the range over which the transition of protein from the depolymerized to the polymerized state occurs. By performing a second set of measurements with intact virus over this range, it is possible to cancel the effect of the Donnan equilibrium on the change of apparent weight. (It is estimated that the magnitude of this effect is of the same order as the change of hydration). This is in addition to the two measurements with protein, each performed in a medium of different density. Furthermore, it was necessary to measure independently the change of partial specific volume of protein and virus.

It was found that, as a result of polymerization, an equivalent of 0.0043 g of sodium chloride is bound and 0.027 g of water is released per gram of TMV protein. One would like to express these numbers on a mole-per-mole basis; however, a satisfactory value for the molecular weight of the polymerizing unit of protein does not exist. The measurement of the molecular weight of the protein is very difficult, because it depends upon the concentration. Indeed, if one plots the sedimentation coefficient of protein as a function of concentration, the curve has a maximum value at about 1% protein (Stevens, 1962). Although the result is likely to have little meaning, one can extrapolate to zero concentration the points representing concentrations greater than 1%. (The concentration of protein in the spring-balance experiments was a little less than 1.7%.) A value for $s_{20,w}$ of about 4.9 S was found. This is slightly larger than 4.6, which was obtained by Schramm and Zillig (1955) for their "A protein." They obtained a molecular weight by sedimentation and diffusion of 87,000 for the material. A calculation of molecular weight using Schramm's diffusion coefficient and the extrapolated $s_{20,w}$ obtained for this study gives a value close to 100,000. On the other hand, if the latter figure is near the actual weight-average molecular weight, and if the reaction is a condensation polymerization (Flory, 1936), then the number-average molecular weight of the polymerizing unit is about 50,000. Although a value of 100,000 will be assumed here, it will be remembered that the value may be half that. With these restrictions, then, we are led to the following values: For every mole of protein polymerizing, 7

moles of sodium chloride are bound and 150 moles of water are released.

In considering the origin of the water released during polymerization, one should perhaps begin with a discussion of the dilatometer results. It was found that 0.14 meq of acid per gram of material was required to lower the *p*H of solutions of virus or protein (Ansevin *et al.*, 1964) from *p*H 7.5 to 5.5. Over the same range, the change of partial specific volume was 0.00138 and 0.00741 ml/g, respectively, for virus and protein. One calculates that the respective volume changes represent 10 and 53 ml for each equivalent of acid neutralized. These numbers may be compared to one obtained by Kauzmann (1958). He found that the expansion of ovalbumin is 11 ml for each equivalent of acid neutralized. It is likely that the value corresponds to the volume change of 10.3 ml per equivalent resulting from the neutralization of a carboxylate ion by a proton (Weber, 1930). Apparently the entire volume change of TMV can be explained on the basis of the neutralization of charged groups and probably is a result of the release of electrostricted water at the ionic groups. On the other hand, the expansion of TMV protein is almost six times greater and must arise from an additional mechanism. The observation is similar to that observed for the enzymatic digestion of proteins for which the change of volume cannot be completely explained by the electrostriction of water resulting from the splitting of peptide bonds (Linderström-Lang, 1950). This line of thought, however, suggests that perhaps water can be released by masking zwitterionic groups. Such groups when transferred from an aqueous environment to a "protein" environment as a result of polymerization would have a tendency to lose electrostricted water and bind salt by ionic linkage. The lower dielectric constant of the "protein" environment might allow the structure to be stable. The values for the electrostriction associated with dipolar α-amino acids range between 11.5 and 14.7 ml/mole of dipoles. For peptides, the value increases as the separation of the charges increases and appears to approach a limiting value of about 20 ml/mole for compounds containing a single charged amino group and single charged carboxyl group (Cohn and Edsall, 1943). The change of volume of the protein minus the virus (Table II) is 300 ml/5 $\times$ 10^4 g of protein. If each mole of zwitterion makes up 20 ml of the total, the observed volume change could result from the abolishment of 15 zwitterions/molecule. This is not an unreasonable number. Although the results of the experiment are consistent with the binding of 7 molecules of salt, a one-to-one correspondence between zwitterions abolished and salt bound does not seem to be required. This interpretation is not meant to suggest that a stabilizing effect arises from salt linkages formed between subunits. The response of the system to a change of ionic strength indicates that, if the effect exists, it is not important.

An alternative hypothesis for the increase of volume and release of water with polymerization might be based upon the existence of icelike domains of water associated with protein in the dissociated state. The possibility that nonpolar solutes stabilize such structures was pointed out by Frank and Evans (1945). Klotz (1958) has considered the possibility that some of the properties of proteins can be explained on the basis of the existence of such icelike domains about hydrophobic groups on the protein. Kauzmann states that the transfer of such groups from a nonaqueous ("proteinlike") environment to an aqueous one is accompanied by a decrease of volume (Kauzmann, 1959). If this means that the additional structure in water induced by the nonpolar group is associated with a decrease of volume, then the structured-water hypothesis is consistent with our dilatometric experiments. The validity of this volume relationship, however, does not seem to be firmly established.

A portion of the water released during polymerization might be attributable to an excluded volume effect. Glycerol and water have different molecular radii, therefore the distance of closest approach of the molecules to protein is different. This results in an apparent shell of water surrounding the protein subunit, the region from which glycerol is excluded but not water. When the effective surface area of the protein decreases upon polymerization, the volume of this region decreases, giving rise to a loss of water of hydration registered by the spring-balance. Schachman and Lauffer (1949) calculated the effect of excluded volume on the sedimentation of TMV in solutions of sucrose and bovine serum albumin. For TMV protein, the difference of excluded volume for glycerol and for water is equivalent to about 148 molecules of water per subunit (molecular weight, 17,500).[2] The maximum possible contribution to the measured change of hydration must be considerably less than this because the area change per subunit is much less than the total area. The protein is partially polymerized at the beginning of an experiment (between 3 and 7 subunits), and the polymerized protein has a larger surface area than the equivalent cylinder.

The results of the experiments clearly substantiate the hypothesis concerning the source of positive entropy of polymerization. Moreover, in light of a subsequent report of this series, the loss of water of hydration may be sufficient to explain the entire observed entropy of polymerization (C. E. Smith and M. A. Lauffer, data to be published).

References

Ansevin, A. T. (1958), Master of Science thesis, University of Pittsburgh.

Ansevin, A. T., and Lauffer, M. A. (1963), *Biophysic. J.* *3*, 3.

[2] This calculation is based on the following values: The molecular radius of water is 1.95 A. The ratio of molecular radii of glycerol to water is 1.57. The subunit of TMV protein is approximately a prolate ellipsoid of revolution with major axis of 70 A and minor of 23 A and area of 4140 A^2. The excluded volume is the product of surface area of the subunit and molecular radius of glycerol or water.

Ansevin, A. T., Stevens, C. L., and Lauffer, M. A. (1964), *Biochemistry 3*, 1512.
Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, Reinhold, p. 157.
Englander, S. W., and Epstein, H. T. (1957), *Arch. Biochem. Biophys. 68*, 144.
Flory, P. J. (1936), *J. Am. Chem. Soc. 58*, 1877.
Fraenkel-Conrat, H., and Williams, R. C. (1955), *Proc. Natl. Acad. Sci. U.S. 41*, 690.
Frank, H. F., and Evans, M. W. (1945), *J. Chem. Phys. 13*, 507.
Kauzmann, W. (1958), *Biochim. Biophys. Acta 28*, 87.
Kauzmann, W. (1959), *Advan. Protein Chem. 14*, 1.
Klotz, I. M. (1958), *Science 128*, 815.
Klug, A., and Caspar, D. L. D. (1960), *Advan. Virus Res. 7*, 225.
Lauffer, M. A. (1964), *Biochemistry 3*, 731.
Lauffer, M. A., Ansevin, A. T., Cartwright, T. E., and Brinton, C. E. (1958), *Nature 181*, 1338.
Lauffer, M. A., and Bendet, I. J. (1954), *Advan. Virus Res. 2*, 241.
Linderström-Lang, K. (1950), *Cold Spring Harbor Symp. Quant. Biol. 14*, 117.
Schachman, H. K., and Lauffer, M. A. (1949), *J. Am. Chem. Soc. 71*, 536.
Schachman, H. K., and Lauffer, M. A. (1950), *J. Am. Chem. Soc. 72*, 4266.
Schramm, G., and Zillig, W. (1955), *Z. Naturforsch. 10b*, 493.
Stevens, C. L. (1962), Doctor of Philosophy dissertation, University of Pittsburgh.
Weber, H. H. (1930), *Biochem. Z. 218*, 1.

Protonation of Polypeptides in "Helix-Breaking" Solvents: Spectral and Optical-Rotatory Properties in Solutions Containing Strong Organic Acids*

Sue Hanlon† and Irving M. Klotz

ABSTRACT: Poly-γ-benzyl-L-glutamate, poly-L-alanine, and poly-L-leucine have been examined spectroscopically in the near infrared in mixed solvents containing strong organic acids. The optical-rotatory-dispersion properties of the alanine and leucine polymers have also been determined in the same solvents. The results indicate that a sizable fraction of the peptide groups of these polyamino acids are protonated by these solvents.

The transition in b_0, commonly interpreted as a helix-coil transition, cannot in these particular cases involve the breakage of intramolecular peptide hydrogen bonds. Rather, the parallel changes observed in the spectral properties support the conclusion that the optical-rotatory transitions reflect the transformation of the protonated polypeptide chain to a form which is partially hydrogen bonded to the organic acid of the solvent.

A knowledge of the conformation of polyamino acids in solution under a variety of conditions is important to the understanding of the forces responsible for the maintenance of protein structure in solution. Yet this knowledge is not easily obtained. Many techniques that might be applied to the problem are limited either by their lack of applicability in a multicomponent system or by the absence of a sound theoretical basis. Thus the interpretation of data from these techniques is a rather hazardous venture, particularly in those cases where a given transformation is effected by the addition of an appreciable quantity of a third strongly interacting component.

Since a direct determination of conformational change in such a system is so difficult, a more fruitful approach to the problem would seem to be a direct assessment of the chemical state of the peptide groups under conditions where other data have been interpreted in terms of a helix-coil transition. Presumably this transition should be reflected in a transformation of the hydrogen-bonded peptide groups to solvent-interacting peptide groups.

On the basis of results for *N*-methylacetamide in a variety of solvents (Klotz and Franzen, 1962; Hanlon *et al.*, 1963; Klotz *et al.*, 1964), it was felt that an identification of the various interactions of the peptide

* From the Department of Chemistry, Northwestern University, Evanston, Ill. *Received August 28, 1962.* This investigation was supported in part by a grant (GM 09280) from the National Institute of General Medical Sciences, U.S. Public Health Service. It was also assisted by support made available by a U.S. Public Health Service training grant (5TI-GM-626) from the National Institute of General Medical Sciences.

† Postdoctoral Fellow of the U.S. Public Health Service, 1962–1963. Present address: Department of Biochemistry, Chicago Medical School, Chicago, Ill.