Polymerization–Depolymerization of Tobacco Mosaic Virus Protein. VIII. Light-Scattering Studies*

Carl E. Smith† and Max A. Lauffer

ABSTRACT: In the temperature range of about 15–20°, tobacco mosaic virus (TMV) protein polymerizes endothermically and reversibly when the temperature is changed very slowly. The reaction can be followed by means of turbidity measurements. Data obtained over about a 5° temperature range can be analyzed in terms of the equations developed by Flory for linear condensation polymerization. Data obtained at higher temperatures, corresponding to greater degrees of polymerization, fit this theory only when it is modified to take into account chain terminators. Mean values of $\Delta H°$ and $\Delta S°$ computed for polymerization of TMV protein in pH 6.5 phosphate buffer at 0.10 ionic strength, respectively, were found to be 206,000 cal/mole and 739 eu. Decreasing ionic strength favored depolymerization.

Urea at a concentration of 1.8 M causes depolymerization and denaturation. Urea at a concentration of 0.40 M in pH 6.5 phosphate buffer at 0.10 ionic strength favors depolymerization. Dioxane at a concentration of $^1/_{15}$ ml/ml in 0.10 ionic strength phosphate buffer at pH 6.5 favors depolymerization; at higher concentrations it causes both denaturation and depolymerization. Tetra-*n*-butylammonium bromide at pH 6.5 and total ionic strength of 0.10 at concentrations up to 0.03 M shifts the equilibrium toward depolymerization and at 0.04 and 0.05 M causes both depolymerization and denaturation. At the same concentrations, tetramethylammonium bromide shows little, if any, effect.

In a suitable solvent, the protein of tobacco mosaic virus (TMV) undergoes polymerization, the extent of which depends upon temperature (Lauffer *et al.*, 1958). Polymerization into high molecular weight rodlike (and virus like) particles is favored by an increase in temperature. In the direction of polymerization, the reaction is thus endothermic with both enthalpy and entropy increasing. Lauffer *et al.* (1958) postulated that the entropy increase arose from the release of water molecules from the protein during polymerization, and water release has, in fact, been measured as a concomitant of polymerization (Stevens and Lauffer, 1965).

The present work continues the investigation of the polymerization reaction. It has been shown that the reaction can be carried out in a thermodynamically reversible manner. The effects of temperature, ionic strength, urea, dioxane, and quaternary ammonium compounds on the reaction have been studied. Preliminary references to some of the data of the present publication were made in two review articles (Lauffer, 1962, 1964b).

Materials and Methods

Virus. The common strain of tobacco mosaic virus was purified by differential centrifugation. In most cases it was depigmented (Ginoza *et al.*, 1954) at the first high-speed pellet stage.

Protein. Nucleic acid free, low molecular weight protein (A protein) was prepared from virus both by a method similar to the alkaline degradation method of Schramm and Zillig (1955) and by the acetic acid method of Fraenkel-Conrat (1957). No obvious difference was noted between the proteins prepared by the two methods.

Concentration Determination. Concentrations of TMV and tobacco mosaic virus protein (TMV-P) solutions were determined with a Beckman Model DU spectrophotometer. In each case the ultraviolet absorption curve was measured from 400 to about 230 mμ. The solvent was distilled water for TMV and 0.033 M (pH 7) potassium phosphate buffer for TMV-P (Ansevin, 1958). In this buffer the protein does not polymerize at room temperature.

Measured optical densities were corrected for scattering according to Englander and Epstein (1957). Virus solutions had broad maxima at 260 mμ and minima at 248 mμ. Protein solutions had maxima at 281 mμ and minima at 251 mμ. The concentrations

* From the Department of Biophysics and Microbiology, University of Pittsburgh, Pittsburgh, Pennsylvania. *Received February 17, 1967.* Publications I–VII of this series are Ansevin and Lauffer (1963), Lauffer, (1964a), Ansevin *et al.* (1964), Stevens and Lauffer (1965), Lauffer (1966a), Banerjee and Lauffer (1966), and Lauffer (1966b), respectively. This is publication No. 129 of the Department of Biophysics and Microbiology, University of Pittsburgh. Work was supported in part by a U. S. Public Health Service Grant (GM 10403).

† Present address: Department of Health, Education, and Welfare, U. S. Public Health Service, National Institutes of Health, Bethesda, Md. 20014. The data presented in this paper are taken from the dissertation of C. E. S. submitted to the University of Pittsburgh in partial fulfillment of the requirements of the degree of Doctor of Philosophy, 1961.

of TMV were calculated from the optical density at the maximum using an extinction coefficient of 27 (g/100 ml)$^{-1}$ (Fraenkel-Conrat and Williams, 1955). Protein solution concentrations were similarly obtained by use of an extinction coefficient of 13 (g/100 ml)$^{-1}$ (Fraenkel-Conrat and Williams, 1955).

Extent of Polymerization. The extent of polymerization was estimated from turbidity measurements made with a Beckman Model DU spectrophotometer.[1] Standard cuvets of 1-cm path length were used. The optical density attributable to scattering was read at 320 mμ, the wavelength of choice. At shorter wavelengths there is true absorption; at longer wavelengths there is a rapidly decreasing sensitivity. Occasionally, optical densities were read also at 400 mμ. Electron microscopy demonstrated, as shown previously (Lauffer *et al.*, 1958), that in extreme cases such observed turbidities were caused by high molecular weight, rodlike particles similar to the virus itself.

Temperature Control and Measurement. Turbidity *vs.* temperature measurements were made by equipping the cell compartment of the spectrophotometer with thermospacers through which water circulated from a bath of adjustable temperature. Temperatures were controlled by a Brownwill mercury thermoregulator. The protein solution temperatures were determined with a bead-type, calibrated thermistor immersed in a cuvet containing the same volume of distilled water as the protein solution cuvets. Temperatures are accurate to $\pm 0.1°$.

Experimental Procedure

Solutions were usually made up in an ice bath and poured into cold 1-cm cuvets. The filled cuvets were put into their holder, also cold. The thermistor was then installed in the distilled water cuvet and the entire assembly was placed in the spectrophotometer cell compartment maintained at the desired initial temperature. This latter was usually in the range of 10–13°, where experience showed the optical density to have a low-temperature plateau. The protein solutions were allowed to come to thermal equilibrium as determined with the calibrated thermistor. Optical density readings were then made as a function of time until equilibrium was reached. Operationally, this was taken to mean an unchanging optical density. The temperature was then raised 0.5–2.5° and the process was repeated. This was continued until the final high temperature (*ca.* 25°) was reached. The process was then reversed, the protein solutions being slowly cooled to the initial low-temperature region, thus completing a cycle of heating and cooling. Such a cycle has taken 12–16 hr for completion. Part of this time was taken up in reaching temperature equilibrium, and part in assuring that reaction equilibrium was attained. For all temperature changes, for both polymerization and depolymerization, the reaction is close to, but not at, the new equilibrium in a fairly short time. But attempts to reduce the over-all time any considerable amount resulted in hysteresis loops of optical density *vs.* temperature, the warming half of the cycle falling below the cooling portion. The key, then, to obtaining thermodynamically reversible results is very gradual change in temperature. Coincidence or near coincidence of the heating and cooling curves was regarded as evidence for a reversible reaction.

Results

I. Polymerization as a Function of Ionic Strength. The polymerization reaction was studied at three ionic strengths (0.05, 0.08, and 0.10 μ), all of the ionic strength coming from potassium phosphate buffer (pH 6.5). As seen in Figure 1, the temperature corresponding to a given optical density, *i.e.*, extent of polymerization, decreases with increasing ionic strength.

With the 0.08 μ solution, the turbidity did not return quite to its initial value in the low-temperature region during the cooling portion of the cycle. In the computations to follow, only those points coincident to the heating portion of the cycle have been used.

II. Effect of Urea upon the Polymerization Reaction. Urea, added to a final concentration of 1.8 M to TMV-P in polymer form at pH 6.5, 0.1 μ, and 25°, causes an immediate, complete loss of turbidity which is followed in a few minutes by a heavy, temperature-irreversible turbidity. If, instead, the solution is chilled to about 4° immediately after the initial disappearance of turbidity and dialyzed at that temperature against phosphate buffer (pH 6.5, 0.1 μ), the protein still exhibits temperature-reversible polymerization. Examination with the electron microscope after such dialysis shows high molecular weight, rodlike particles present at 25° and absent at 4°.

At pH 5.3 and 0.1 μ, TMV-P is in the polymerized form at all temperatures from 0 to 30° (Lauffer *et al.*, 1958). Under these conditions at ice-bath temperature, 0.7 M urea depolymerized the protein almost completely, but turbidity returned slowly over a period of 45 min. This is probably the result of denaturation because the turbid material could be removed by low-speed centrifugation.

The equilibrium shift caused by urea was studied quantitatively. Optical density was measured as a function of temperature as in the ionic strength experiments. As can be seen in Figure 2, 0.4 M urea in pH 6.5 buffer at 0.1 μ favors depolymerization of TMV-P.

III. The Effect of Quaternary Ammonium Salts on Polymerization. The effects of two quaternary ammonium salts on the polymerization reaction have been studied qualitatively by visual observation of turbidity and by electron microscopy. The salts were tetra-*n*-

[1] Data obtained by Dr. Charles L. Stevens and Dr. Severo Paglini of our laboratory indicate that reasonably reliable turbidity measurements can be made with this spectrophotometer. The ratio of turbidity to concentration at infinite dilution of a TMV solution was determined by integrating over all directions the intensity of scattered light as measured in a Brice-Phoenix light-scattering apparatus. The same ratio was then determined for the same TMV solution with the spectrophotometer. Agreement within 3% was obtained.

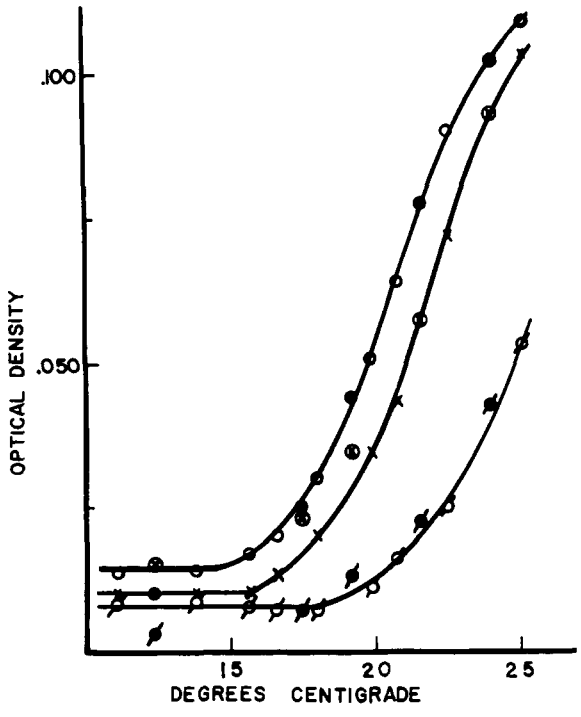

FIGURE 1: Optical density (320 mμ) *vs.* temperature. Ionic strength dependence of 0.05 μ: (⌀) increasing and (●) decreasing temperature; of 0.08 μ: (x) increasing and (⊗) decreasing; and at 0.1 μ: (O) increasing and (●) decreasing.

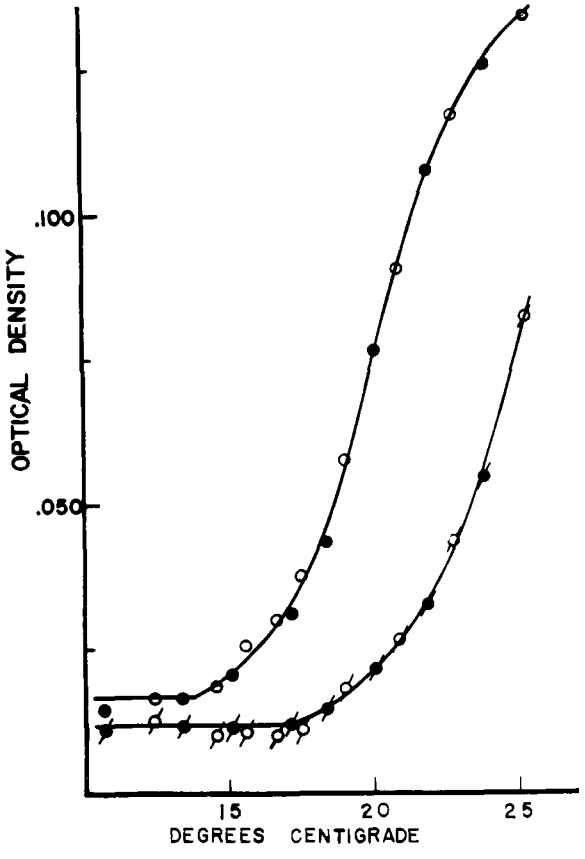

FIGURE 2: Optical density (320 mμ) *vs.* temperature. Effect of urea on polymerization at 0.1 μ. Control: (O) increasing and (●) decreasing; plus 0.4 M urea: (⌀) increasing and (●) decreasing.

butylammonium bromide (TBABr)[2] and tetramethylammonium bromide (TMABr). These salts are regarded as "structure makers" in the sense emphasized by Frank and Evans (1945).

A. THE EFFECT OF TBABR. Experiments were done at pH 6.5, 0.1 μ. It will be recalled that under these conditions the polymerization reaction (in phosphate buffer) is reversible with temperature.

The effect of the tetra-*n*-butylammonium (TBA^+) ion was studied at five concentrations, beginning at 0.01 M and increasing in steps of 0.01–0.05 M. The bromide ion concentration was kept constant at 0.05 M by adding appropriate amounts of potassium bromide. The balance of the ionic strength was supplied by potassium phosphate buffer which maintained the pH at 6.4–6.5. The protein concentration in all cases was 0.81 mg/ml, and all solutions were made up at ice-bath temperature.

The solutions were then brought to about 25°. It was found that at 0.04 and 0.05 M TBABr, the protein develops temperature-irreversible turbidity, while at 0.01, 0.02, and 0.03 M TBABr, the protein develops turbidity which is completely, or almost completely, reversible in the cold. In all five cases the turbidity develops more slowly than a phosphate buffer control (at 0.09 μ) or a potassium bromide control (0.05 M KBr and 0.05 μ phosphate buffer), the latter two solutions developing turbidity at about the same rate. Observation in the electron microscope at the three lower concentrations of TBABr showed that the temperature-reversible turbidity consists of high molecular weight rodlike particles.

The effect of TBABr on TMV-P already in the polymer form was also studied. The conditions of the experiment were exactly like those described above except that the TBABr was added to polymer–protein at 25°. The result of such addition was immediate disappearance of turbidity at 0.04 and 0.05 M TBABr, followed by a fairly rapid increase of turbidity which became far greater than that of either the KBr control or phosphate buffer control. This turbidity was not temperature reversible, indicating that at these concentrations extensive denaturation has occurred. At 0.02 and 0.03 M TBABr turbidity decreased immediately and then increased to levels equal, or nearly equal, to the control turbidities. The turbidities were temperature reversible. At 0.01 M TBABr slight, if any, initial decrease of turbidity was noted and the turbidity after TBABr addition again was readily temperature reversible.

[2] Abbreviations used: TBABr, tetra-*n*-butylammonium bromide; TMABr, tetramethylammonium bromide.

The immediate loss of turbidity followed by temperature-irreversible turbidity with 0.05 M TBABr was investigated further in a dialysis experiment identical with that previously employed with urea. After the immediate loss of turbidity upon addition of TBABr, the solution was chilled in an ice bath and dialyzed against cold phosphate bromide buffer. The resulting protein solution exhibited temperature-reversible turbidity attributable to ordered rodlike particles as demonstrated electron microscopically.

Preliminary observations on the temperature dependence of turbidity development in the presence of 0.03 M TBABr (pH 6.5, 0.1 μ) indicate, as with urea, that the "initiation" temperature increases. In this case, it is about 3° higher than either the KBr or phosphate buffer controls.

B. The effect of TMABr. Comparable experiments at the same concentrations carried out with TMABr showed little, if any, effect on reversible polymerization.

IV. The Effect of Dioxane. The effect of 1,4-dioxane on TMV protein in 0.10 μ phosphate buffer at pH 6.5 was tested. At 25° at a dioxane concentration of $^1/_{15}$ ml/ml very slight turbidity developed which was almost completely reversed at ice-bath temperature. At a dioxane concentration of one-tenth appreciable turbidity developed which was not reversible at lower temperatures. At a dioxane concentration of $^1/_{30}$ turbidity developed which was smaller than that of the control but was temperature reversible. These observations indicate that dioxane inhibits the polymerization and promotes denaturation of TMV protein.

Discussion

The polymerization of tobacco mosaic virus protein as a function of time has been followed by turbidimetry and electron microscopy (Lauffer *et al.*, 1958; Ansevin and Lauffer, 1963). These kinetic studies indicate that the mechanism of polymerization can be approximated by the mathematics of linear condensation polymerization. The turbidity measurements here can be similarly analyzed.

The theory of linear condensation polymerization (Flory, 1936, 1953) derives from the single assumption that the reactivities of the functional groups are independent of the size of the molecule to which they are attached. No matter what model is chosen for TMV A protein of mol wt 52,500, the reactive surfaces must be of the order of magnitude of 50 A apart. Thus, there is every reason to believe that a "bond" on one end of an A protein unit will not affect the reactivity of the other end. Therefore, linear condensation polymerization theory is a reasonable choice on an *a priori* basis. In this theory, the extent of polymerization is given by p, the fraction reacted of the functional groups initially present in monomers. Statistical considerations then show that the weight-average molecular weight of the polymer distribution is given by $\bar{M}_w = M_0 (1 + p)/(1 - p)$, where M_0 is the monomer molecular weight (Flory, 1936, 1953). In dilute solutions of small molecules, the turbidity (τ) is given by $\tau = Hc\bar{M}_w$, where H is an optical factor (see below) and c is concentration in grams per milliliter (Debye, 1944; Zimm and Doty, 1944; Doty *et al.*, 1945). Thus, the extent of reaction is related to the turbidity by

$$\tau = HcM_0(1 + p)/(1 - p) = \tau_0(1 + p)/(1 - p) \quad (1)$$

where $\tau_0 = HcM_0$ is the initial turbidity when p is 0.

This formulation is valid only for solutions dilute enough to be ideal and for particles considerably smaller than the wavelength of the light used. Osmotic coefficients for hydrated proteins with molecular weights less than 100,000 are usually between 1.00 and 1.01 at concentrations of 1 g/l. Thus, it is reasonable to assume that the activity coefficient (defined to exclude its tendency to polymerize) of hydrated A protein at a concentration of 0.88 g/l. does not deviate from unity by more than 1%. The ultimate polymer obtained is a rod somewhat like the TMV particle. Oster *et al.* (1947) showed by light scattering that TMV does not depart from ideality at concentrations up to 0.3 g/l. and Banerjee and Lauffer (1966) showed by osmotic pressure measurements that TMV does not depart from ideality at concentrations up to 10 g/l. Since all polymeric species in our studies are probably less concentrated than the TMV in the solutions of Oster *et al.*, it is highly unlikely that any of the polymerized particles deviate significantly from ideality.

The second requirement for the validity of eq 1 is that the maximum dimension of the largest particles studied must be considerably less than the wavelength of the light used. The maximum values of $(OD - OD_0)$ encountered in the present study are less than 0.1. This corresponds to maximum values of $\tau - \tau_0$ of less than 0.23, Since, as is shown below, τ_0 is approximately 0.002, the maximum values of τ/τ_0, and, therefore, of $\bar{M}_w/M_0$, is less than 116. Since M_0 is 52,500, this corresponds to maximum values of M_w of less than 6.1×10^6. If the molecular weight of TMV is taken to be 40×10^6 and if 95% of this is protein, then this maximum value of $\bar{M}_w$ corresponds to 0.16 of the protein in a TMV particle. If the length of a TMV particle is regarded as 3000 A, this corresponds to 480 A. Since the wavelength of the light used is 3200 A, this corresponds to 0.15 λ. Because most values of $(OD - OD_0)$ are much lower than 0.1, most of the particles encountered are much smaller than 0.15 λ. Thus, it seems reasonable to assume that eq 1 is valid for the present study.

Ansevin and Lauffer (1963) have demonstrated that the polymerization of tobacco mosaic virus protein is controlled by a second-order process, and point out that other data (Lauffer and Price, 1940; Lauffer and Dow, 1941; Lauffer, 1943) indicate that depolymerization may reasonably be considered a first-order process. They have suggested, therefore, that the equilibrium constant (K) for the polymerization of TMV-P can be expressed as the ratio of a second-order rate constant (k_1) for polymerization to a first-order rate constant (k_2) for dissociation. Thus, for the equilibrium characterized by extent of reaction (p)

the concentration of reacted groups is pm and the concentration of unreacted groups is $z = m(1 - p)$, where m is the initial molar concentration of monomers. The monomer for present purposes is a trimer of the chemical repeat unit (Banerjee and Lauffer, 1966). At equilibrium $(dz/dt)_{net} = 0 = -k_1m^2(1 - p)^2 + k_2mp$ from which

$$K = \frac{k_1}{k_2} = \frac{p}{m(1 - p)^2} \quad (2)$$

In terms of turbidity,[3] K is given by

$$K = \frac{(\tau/\tau_0)^2 - 1}{4m} \quad (3)$$

The relation between turbidity and the observed optical density attributable to scattering (OD) is $\tau = (1/d)$-$(\ln I_0/I) = 2.303(OD)/d$, where I_0 is the incident light intensity and I is the light intensity after passing through d centimeters of solution. Here, d is 1 cm so that

$$(\tau - \tau_0) = 2.303(OD - OD_0) \quad (4)$$

or

$$\tau = \tau_0 + 2.303(OD - OD_0)$$

From the familiar relation

$$-RT \ln K = \Delta F^\circ = \Delta H^\circ - T\Delta S^\circ \quad (5)$$

and eq 3

$$\ln (\tau^2 - \tau_0^2) = \ln 4m\tau_0^2 + \Delta S^\circ/R - (\Delta H^\circ/R)1/T \quad (6)$$

Substitution of eq 4 gives

$$\log [2\tau_0 + 2.303(OD - OD_0)] + \log [2.303(OD - OD_0)] = (\Delta S^\circ/2.303R + \log 4m\tau_0^2) - \Delta H^\circ/2.303RT \quad (7)$$

If $\log [2.303(OD - OD_0)] + \log [2\tau_0 + 2.303(OD - OD_0)]$ is plotted *vs.* $1/T$ a straight line $Y = a + bX$ should result in which $a = \log (4m\tau_0^2) + (\Delta S^\circ/2.303R)$ and $b = -\Delta H^\circ/2.303R$.

There is evidence that analysis in terms of Flory's mathematics may be valid. Banerjee and Lauffer (1966) showed that the osmotic pressure for solutions of TMV protein in 0.1 ionic strength phosphate buffer at pH 6.5 and at constant temperatures between about 4 and 12° varied with initial protein concentration in a manner entirely consistent with the equilibrium formulation shown in eq 2. Furthermore, Banerjee and Lauffer published optical density *vs.* temperature for TMV protein solutions ranging in concentration from 2 to 7 mg/ml in steps of 1 mg/ml. At 14° the data at all concentrations can be fitted to eq 3 with a single value of K. The same is true at 16° if one excludes data in which the optical density exceeds 0.05. Furthermore, eq 6 and 7 fit all of the data at low values of optical density described here and in Shalaby and Lauffer (1967) and Khalil and Lauffer (1967). Equations 8 and 9 fit over the entire range of all the data reported herein and in Shalaby and Lauffer (1967) and Khalil and Lauffer (1967).

$$\ln (\tau^2 - \tau_0^2) - 2 \ln (\tau_m - \tau) = \ln 4m\tau_0^2 + \Delta S^\circ/R - 2 \ln \tau_m - \Delta H^\circ/R1/T \quad (8)$$

$$\log [2\tau_0 + 2.303(OD - OD_0)] + \log [2.303(OD - OD_0)] - 2 \log [2.303(OD_m - OD)] = (\Delta S^\circ/2.303R + \log 4m\tau_0^2 - 2 \log [\tau_0 + 2.303 \times (OD_m - OD_0)]) - \Delta H^\circ/2.303RT \quad (9)$$

From eq 8, one obtains $\tau_i = \tau_m/2$, where $\tau_m \gg \tau_0$ and τ_i is the turbidity at the inflection point of the graph of τ *vs.* $1/T$.

A variety of assumptions will lead to equations which reduce to 8 when τ_m is much greater than τ_0. In each case, the effect of the assumption is to modify eq 1 or 2, or both, and then a procedure comparable to the development of eq 6 leads to eq 8. From an unknown number of possibilities, three assumptions have been shown to lead to this result. One is that some of the monomeric units are defective on one face so that they are incapable of forming a bond at that face, and that as many are similarly defective on the other face. A second, initially made by Caspar (1963), is that there is a probability of fracture of each polymer rod which is proportional to the length of the rod and, therefore, to p. Still another assumption which leads to the same result is that, while the rate of depolymerization is k_2mp, the rate of polymerization is $k_1(1 - \beta\bar{M}_w)^2m^2(1 - p)^2$, where β is a constant with dimensions of $1/M$. If any of these assumptions is valid, τ_m should be proportional to m. Data of Banerjee and Lauffer (1966) indicate no dependence of τ_m on m but inverse dependence of inflection temperature on m. All in all, therefore, eq 8 and 9 are empirical; no interpretation of τ_m will be made at present.

A different approach follows from the work of Brandts (1964), who has shown that the unitary free energy of transfer of all hydrophobic amino acid residues except tyrosyl from an organic environment to an aqueous environment are positive and are described by $AT + BT^2 + CT^3$, where A, B, and C are arbitrary constants, and T is absolute temperature. The relationships are practically indistinguishable from straight lines between 0 and 30°, but show maxima at 60–80°, depending on the type of residue. The model described by Lauffer (1966b) suggests that polymerization of TMV protein is analogous to the transfer of hydro-

[3] From eq 1 $\tau/\tau_0 = (1 + p)/(1 - p)$, $p = [(\tau/\tau_0) - 1]/(\tau/\tau_0) + 1)]$, and $(1 - p) = 2/((\tau/\tau_0) + 1)$. Then, $K = (p/m)$-$(1/(1 - p)^2) = [((\tau/\tau_0) - 1)/m((\tau/\tau_0) + 1)][((\tau/\tau_0) + 1)^2/4] = ((\tau/\tau_0)^2 - 1)/4m$.

phobic residues from an aqueous to an organic environment. If one writes for the unitary free energy of transfer of a water-interacting center from aqueous to nonaqueous environment, $n(\Delta h - T\Delta s)$, where Δh and Δs are constants independent of temperature but $n = n_0 + b\Delta T + c(\Delta T)^2$, one can derive eq 10. This treatment is consistent with Brandts. ΔT is defined as $(T - T_e)$ and T_e as $\Delta h/\Delta s$, while n_0, b, and c are constants.

$$\ln K = \Delta S^\circ/R - \Delta H^\circ/RT + (b\Delta s/RT)(\Delta T)^2 + (c\Delta s/RT)(\Delta T)^3 \quad (10)$$

Here, ΔS° and ΔH° are $n_0\Delta s$ and $n_0\Delta h$. When ΔT is 0, all except the first two terms on the right vanish. Furthermore, when ΔT is 0, d ln K/d(1/T) is $-\Delta H^\circ/R$. Thus, when K is given by eq 3, eq 8 and 10 become identical in the limit if ΔT approaches 0 as τ approaches τ_0. Furthermore, from eq 3, 8, and 10, one obtains eq 11.

$$2RT \ln (\tau_m - \tau)/(\tau_m + \tau_0) = b\Delta s(T - T_e)^2 + c\Delta s(T - T_e)^3 \quad (11)$$

Thus, when τ_m and τ_0 are known, one can evaluate $b\Delta s$, $c\Delta s$, and T_e from three pairs of values of τ and T.

This analysis in terms of Brandts' ideas is instructive in that it shows that d ln K/d(1/K) can contain terms other than $-\Delta H^\circ/R$, which under some conditions might be large enough to be significant. However, as an explanation for the sigmoidal shape of experimental graphs such as those shown in Figures 1 and 2, this approach might be questionable. In contrast with the residues discussed by Brandts (1964), departure from simple theory in our experiments generally occurs at temperatures which are well below 30° and which vary not only with pH, ionic strength, and added solutes, but also with protein concentration.

The value of $\tau_0 = HcM_0$ was calculated by first computing the value of the optical factor H

$$H = \frac{32\pi^3}{3\lambda^4 L} n_0^2 \left(\frac{dn}{dc}\right)^2$$

where λ = wavelength (in centimeters) in vacuum, L = Avogadro's number, n_0 = refractive index of buffer solvent, and dn/dc = specific refractive increment of protein.

Both n_0 and dn/dc are functions of wavelength. Their values at 320 mμ were obtained as follows. From graphical interpolation of data in the International Critical Tables (1930), the refractive index of water at 320 mμ (and 20°) was found to be 1.3543. To this should be added the contributions from the buffer salts. Although these contributions vary with the ionic strength, they are small, and a single value of n_0, that applying to the 0.1 μ buffer, has been applied to all cases. The value of n_0 at 320 mμ (and 20°) has been approximated by measuring the refractive index at 589.3 mμ (20°) of water and buffer and applying this measured increment (0.0013) to the International Critical Tables' (1930) value for water at 320 mμ given above. This gives 1.3556 for n_0 at 320 mμ.[4]

Perlmann and Longsworth (1948) have shown that the specific refractive increment of a number of proteins follows a two-term Cauchy dispersion relation in the visible region of the spectrum (Perlmann and Longsworth, 1948; Stacey, 1956)

$$\left(\frac{dn}{dc}\right) = \left(\frac{dn}{dc}\right)_{5780A} \left(0.940 + \frac{2.00 \times 10^6}{\lambda_2}\right)$$

where λ is in Angstroms. Although the lowest wavelength for which this relation was shown to hold was about 436 mμ, it has been extrapolated here to 320 mμ. Such extrapolation is probably allowable since the absorption band of TMV-P does not start until about 305 mμ. The value of $(dn/dc)_{5780A}$ was computed from this equation by using the measured value of $(dn/dc)_\lambda$ for TMV-P of Stevens and Lauffer (1965) at 546 mμ. The value of Stevens and Lauffer (0.1856 g/g) was first changed to grams per milliliter (20°) giving 0.1859 $(g/ml)^{-1}$. The Perlmann–Longsworth relation then gives $(dn/dc)_{320} = 0.2096$ $(g/ml)^{-1}$. H_{320} can then be calculated as 4.23×10^{-5} $(cm/g)^2$.

In the ionic strength experiments of Figure 1, the concentration (c) of protein was 0.88×10^{-3} g/ml. Detailed osmotic pressure studies on TMV-P have been carried out (Banerjee and Lauffer, 1966). These results show that in 0.1 ionic strength phosphate buffer at pH 6.5 and at TMV protein concentrations of 1 mg/ml or less, the molecular weight is 52,500. It is, therefore, clear that M_0 for initial stages of the polymerization represented by the data of Figure 1 must be 52,500. The value of $\tau_0 = HcM_0$ can now be computed as $\tau_0 = 4.23 \times 10^{-5} \times 0.88 \times 10^{-3} \times 52.5 \times 10^{-3} = 0.00195$, and $2\tau_0 = 0.00390$. The molar concentration of protein in these experiments is $m = 1000$, $c/M_0 = 1.676 \times 10^{-5}$ moles/l. The left member of eq 9 then becomes

$$\log [2.303(OD - OD_0)] + \log [0.00390 + 2.303(OD - OD_0)] - 2 \log [2.303(OD_m - OD)]$$

in which OD_0 and OD_m are, respectively, the lowest value of optical density observed at 320 mμ and the maximum approached. It may be noted that this expression allows the data to be analyzed in terms of observed differences in optical density $(OD - OD_0)$

[4] This determination, while accounting for the dispersion of water, neglects that of the specific refractive increment of the buffer salts themselves. The 0.1 μ buffer contains 9.141 g/l. of potassium phosphate salts. If the measured value of the average specific refractive increment at 589.3 mμ, 0.0013/9.141 = 1.422×10^{-4} $g/l.^{-1}$, is combined with any reasonable amount of dispersion, the value of n_0 will be changed only slightly, most probably only in the fourth decimal place. This calculation also neglects the small, continuous change of refractive index with temperature.

in combination with the theoretical value of τ_0. This eliminates difficulty problems of dirt and slight denaturation affecting the experimental value of OD_0, while retaining the sensitivity of measuring differences in scattering optical densities.

The left members of eq 6 or 7 plotted against $1/T$ for the data of Figure 1 give straight lines except at high values. The left members of eq 8 or 9 similarly plotted give straight lines over the entire range. These results are consistent with the assumption that $\Delta H°$ is constant over the temperature range studied. Figure 3 illustrates this for polymerization of TMV protein in 0.1 ionic strength buffer. The data were fitted to the equation by use of a computer, in the manner described by Shalaby and Lauffer (1967). Values for the parameters are shown in Table I. Similarly evaluated parameters for systems containing urea are shown in Table II. The meaning of T^* and of figures in parentheses is explained by Shalaby and Lauffer. For reasons discussed by Shalaby and Lauffer, data corresponding to values for (OD − OD_0) of less than 0.005 were omitted in the curve-fitting operation.

TABLE I: Parameters for Polymerization of TMV Protein at pH 6.5.

μ	$\Delta H°$ (kcal)	$\Delta S°$ (eu)	τ_m	T^* (°C)
0.05	209	740	0.157	(25)
0.08	158	574	0.283	22.5
0.10	197	708	0.243	20.8

TABLE II: Parameters for Polymerization of TMV Protein at pH 6.5, 0.1 μ in the Presence of Urea.

Urea Concn	$\Delta H°$	$\Delta S°$	τ_m	T^* (°C)
0.00	193	695	0.288	20.2
0.20	175	631	0.301	22.1
0.40	137	498	(0.719)	(25)

One must consider the additional possibility that a stable intermediate with values of M_0 and τ_0 greater than 52,500 and 0.00195, respectively, is the polymerizing unit at the higher degrees of polymerization. The next larger stable unit for which conclusive evidence exists is a double disk with mol wt 560,000 and τ_0 0.021 (Lauffer, 1966a,b). The evidence for the existence of this intermediate in the solid state is conclusive; that for its existence in solution is merely suggestive. The difference between the higher and the lower τ_0 values corresponds to an optical density difference

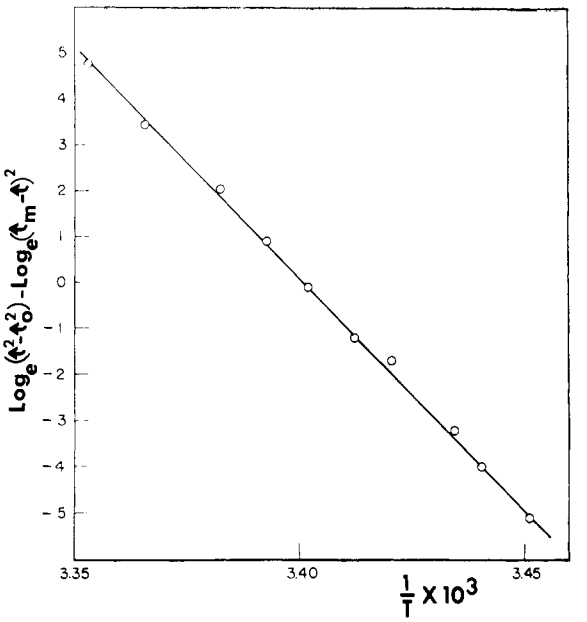

FIGURE 3: Evaluation of τ_m, $\Delta H°$, and $\Delta S°$. Fit of 0.1 μ data of Figure 1 to eq 8.

of 0.0083. Up to this value of (OD − OD_0), the polymerization reaction must involve units smaller than the double disk. Because the data presented here and by Shalaby and Lauffer (1967) and Khalil and Lauffer (1967) all fit eq 9 down to (OD − OD_0) values of 0.005, there is no evidence in these results for the existence of different polymerization processes at (OD − OD_0) values below and above 0.0083. Furthermore, the data of Banerjee and Lauffer (1966) for various protein concentrations up to 8 mg/ml all fit eq 8 with $\Delta H°$ values comparable to those reported here, even though at the higher concentration most of the data correspond to optical density values less than those corresponding to pure double disks. But in any case, recalculation of the data in Figure 3 in terms of M_0 = 560,000 and τ_0 = 0.021 would result in a decrease in $\Delta H°$ of the order of magnitude of 10%.

Additional measurements of $\Delta H°$ and $\Delta S°$ in 0.1 μ phosphate buffer at pH 6.5 are reported by Shalaby and Lauffer (1967). The mean of the values reported in Tables I and II and those reported by Shalaby and Lauffer, with their standard errors, are (206 ± 6.7) × 10^3 cal/mole and 738.7 ± 22.5 eu for the enthalpy and the entropy, respectively.

The large increases in enthalpy and entropy can be interpreted according to the hypothesis of Lauffer *et al.* (1958). They showed that $\Delta H°$ and $\Delta S°$ were positive, and postulated that water was released from the protein upon polymerization. The measured value of $\Delta S°$ of +739 eu at pH 6.5, 0.1 μ is the net value. There must be a contribution from the joining together of protein fragments. This was estimated by Lauffer (1966b) to be approximately −100 eu/bond. There is an additional contribution from the fact that

possibly as many as 3 moles of hydrogen ion are bound/mole of A protein (trimer) during polymerization (Scheele and Lauffer, 1967). Since this is coupled with the conversion of three monovalent phosphate ions to three divalent phosphate ions, the standard entropy change at pH 6.5 is 3×-8.5 or -25.5 eu/mole of bond (Ansevin *et al.*, 1964). If the only other contribution to the over-all entropy change is from the release of water molecules, the entropy change attributable to this process alone must be $+865$ eu to give the net value of $+739$. If one uses the entropy of melting of ice at 0°, 5.26 cal/deg per mole, as a first approximation to the entropy of releasing this "bound" water, then the entropy increases correspond to 164 moles of water released/mole of TMV-P polymerizing at pH 6.5 in 0.1 ionic strength buffer. This may be compared with the measured release of water (at about 0.1 μ) obtained by Stevens and Lauffer (1965). Their experiment, as reinterpreted by Lauffer and Stevens (1967), indicates that approximately 108 moles of water are released/mole of bond formed. The agreement with the present value is better than one has reason to expect in view of the uncertainties involved. It does seem, however, that one can conclude at least that the two approaches give values of the same order of magnitude.

The results obtained in the present study show that urea has two effects on TMV protein. One is to denature irreversibly and the other is a reversible depolymerization of polymerized protein. This depolymerization is endothermic in that it proceeds more readily at low than at high temperatures. This result illuminates the earlier finding of Lauffer (1943) that the specific reaction velocity for the disintegration of TMV nucleoprotein in urea had a minimum rate at room temperature and higher rates at both lower and higher temperatures. The interpretation was that the disintegration was accomplished by two parallel reactions, one with a negative temperature coefficient which dominates below, and one with a positive temperature coefficient which dominates above room temperature. The exothermic depolymerization observed in the present study thus might correspond to the low-temperature reaction observed earlier by Lauffer.

When TMV A protein polymerizes, surfaces initially in contact with water come into contact with the essentially organic environment afforded by adjacent protein material. Kauzmann (1959) cites data showing that when 1 mole of methane, for example, is transferred from a dilute ideal aqueous solution to a solution of the same mole fraction in ether at 25°, there is a decrease in free energy of -3300 cal, an increase in enthalpy of 2400 cal, and a unitary entropy increase of 19 eu. Thus, the polymerization of TMV A protein, which, under the conditions of the present experiments has a negative standard free-energy change and positive enthalpy and standard entropy changes at room temperature, correlates with the transfer of organic residues from an aqueous to an organic environment.

Since electrolytes have a salting-out effect on organic molecules in aqueous solution, the increased ease of polymerization of TMV A protein in the presence of higher values of ionic strength also correlates with the behavior of simple organic molecules. This is discussed more fully by Khalil and Lauffer (1967).

Kauzmann applied the name "hydrophobic bond" to the type of interaction exhibited here between protein units within the polymerized particles. However, Lauffer (1966b) pointed out that a better name would be "entropic union" because bond is misleading inasmuch as the stability of the polymer derives at least in large measure from the circumstances that certain surfaces of the protein find contact with water thermodynamically unfavorable.

References

Ansevin, A. T. (1958), M.S. Thesis, University of Pittsburgh, Pittsburgh, Pa.

Ansevin, A. T., and Lauffer, M. A. (1963), *Biophys. J. 3*, 239.

Ansevin, A. T., Stevens, C. L., and Lauffer, M. A. (1964), *Biochemistry 3*, 1512.

Banerjee, K., and Lauffer, M. A. (1966), *Biochemistry 5*, 1957.

Brandts, J. F. (1964), *J. Am. Chem. Soc. 86*, 4302.

Caspar, D. L. D. (1963), *Advan. Protein Chem. 18*, 37.

Debye, P. (1944), *J. Appl. Phys. 15*, 456.

Doty, P. M., Zimm, B. H., and Mark, H. (1945), *J. Chem. Phys. 13*, 159.

Englander, S. W., and Epstein, H. T. (1957), *Arch. Biochem. Biophys. 68*, 144.

Flory, P. J. (1936), *J. Am. Chem. Soc. 58*, 1877.

Flory, P. J. (1953), Principles of Polymer Chemistry, Ithaca, N. Y., Cornell University.

Fraenkel-Conrat, H. (1957), *Virology 4*, 1.

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.

Ginoza, W., Atkinson, D., and Wildman, S. (1954), *Science 119*, 269.

International Critical Tables (1930), Vol. 7, New York, N. Y., McGraw-Hill, p 12.

Kauzmann, W. (1959), *Advan. Protein Chem. 14*, 1.

Khalil, M. T., and Lauffer, M. A. (1967), *Biochemistry 6*, 2474 (this issue; paper after the following paper).

Lauffer, M. A. (1943), *J. Am. Chem. Soc. 65*, 1793.

Lauffer, M. A. (1962), *in* The Molecular Basis of Neoplasia, Austin, Texas, The University of Texas M. D. Anderson Hospital and Tumor Institute, pp 180–206.

Lauffer, M. A. (1964a), *Biochemistry 3*, 731.

Lauffer, M. A. (1964b), *in* Symposium on Foods, Proteins and Their Reactions, Schultz, H. W., and Anglemier, A. F., Ed., Westport, Conn., Avi, Chapter 5.

Lauffer, M. A. (1966a), *Biochemistry 5*, 1952.

Lauffer, M. A. (1966b), *Biochemistry 5*, 2440.

Lauffer, M. A., Ansevin, A. T., Cartwright, T. E., and Brinton, C. C. (1958), *Nature 181*, 1338.

Lauffer, M. A., and Dow, R. B. (1941), *J. Biol. Chem. 140*, 509.
Lauffer, M. A., and Price, J. C. (1940), *J. Biol. Chem. 133*, 1.
Lauffer, M. A., and Stevens, C. L. (1967), *Advan. Virus Res.* (in press).
Oster, G., Doty, P. M., and Zimm, B. H. (1947), *J. Am. Chem. Soc. 69*, 1193.
Perlmann, G. E., and Longsworth, L. G. (1948), *J. Am. Chem. Soc. 70*, 2719.
Scheele, R. B., and Lauffer, M. A. (1967), *Biochemistry* (in press).
Schramm, G., Zillig, W. (1955), *Z. Naturforsch. 106*, 493
Shalaby, R. A., and Lauffer, M. A. (1967), *Biochemistry 6*, 2465 (this issue, following paper).
Stacey, K. A. (1956), Light Scattering in Physical Chemistry, New York, N. Y., Academic, p 144.
Stevens, C. L., and Lauffer, M. A. (1965), *Biochemistry 4*, 31.
Zimm, B. H., and Doty, P. M. (1944), *J. Chem. Phys. 12*, 203.

Polymerization–Depolymerization of Tobacco Mosaic Virus Protein. IX. Effect of Various Chemicals*

Ragaa A. F. Shalaby† and Max A. Lauffer

ABSTRACT: The effect of KSCN, thiourea, acetamide, EDTA, prolylalanylthreonine, and sucrose on the reversible endothermic polymerization of tobacco mosaic virus (TMV) protein was studied. The polymerization followed by turbidity measurements at a wavelength of 320 mμ was carried out at several pH values between 6.0 and 6.75 and at different ionic strengths and different concentrations of the added chemicals. KSCN, thiourea, acetamide, and EDTA were all found to shift the polymerization toward higher temperatures with the exception of KSCN at pH 6.0. Characteristic temperatures, T^*, were found to increase linearly with increasing concentration of KSCN, thiourea, and acetamide at all the pH and ionic strength values investigated except pH 6.0 in KSCN. On a molar basis, it was found that KSCN is the most effective in retarding the polymerization. Both sucrose and prolylalanylthreonine lowered the polymerization temperatures. On the assumption that polymerization follows condensation polymerization mathematics, thermodynamic parameters were calculated under the different conditions. The values of ΔH° and ΔS° decreased markedly with increasing pH, whether in the presence or absence of the different compounds. It was also found that the added chemicals have a pronounced effect on these parameters.

The increases in enthalpy and entropy which accompany polymerization of tobacco mosaic virus (TMV) protein into high molecular weight particles were assumed by Lauffer *et al.* (1958) to be caused by release of water molecules during polymerization. Stevens and Lauffer (1965) substantiated this hypothesis by directly measuring water release on polymerization. Since new work on water structure is appearing and relevant new information about the effect of solutes on this structure is at hand, this research was undertaken to see how changing the water structure by adding different simple compounds affects the polymerization and the thermodynamic parameters.

Materials and Methods

Preparation of Virus. The common strain of TMV was purified by differential centrifugation with a depigmentation step (Ginoza *et al.*, 1954) or by the method of Boedtker and Simmons (1958).

Preparation of Protein. "A" protein was prepared by acetic acid extraction of the virus (Fraenkel-Conrat, 1957).

Concentration Determination. Concentrations of TMV and TMV protein were determined spectrophotometrically (Smith and Lauffer, 1967) by using a Cary spectrophotometer.

* From the Department of Biophysics and Microbiology, University of Pittsburgh, Pittsburgh, Pennsylvania 15213. *Received February 17, 1967.* Publications I–VIII of this series are Ansevin and Lauffer (1963), Lauffer (1964), Ansevin *et al.* (1964), Stevens and Lauffer (1965), Lauffer (1966a), Banerjee and Lauffer (1966), Lauffer (1966b), and Smith and Lauffer (1967), respectively. This is publication No. 130 of the Department of Biophysics and Microbiology, University of Pittsburgh. Work was supported in part by a U. S. Public Health Service grant (GM 10403).

† The data presented in this paper are taken from the dissertation of R. A. F. S. submitted to the University of Pittsburgh in partial fulfillment of the requirements of the degree of Doctor of Philosophy, July 6, 1966.