Polymerization–Depolymerization of Tobacco Mosaic Virus Protein. VI. Osmotic Pressure Studies of Early Stages of Polymerization*

Krishnadas Banerjee† and Max A. Lauffer

ABSTRACT: The molecular weight of tobacco mosaic virus (TMV) "A" protein under different conditions of pH, ionic strength, temperature, and concentrations was studied with a high-speed membrane osmometer. The number-average molecular weight of the TMV protein in 67% acetic acid was found to be 18,200, in close agreement with other values reported for a monomer subunit.

Experimental evidence was found indicating that a trimer with molecular weight of *ca.* 52,500 is a stable intermediate in "A" protein polymerization. It has been shown that the number-average molecular weight of "A" protein increases with increasing concentration and with increasing temperature. The enthalpy and entropy for reversible polymerization of TMV protein in phosphate buffer were calculated from equilibrium constants at different temperatures obtained on the basis of condensation polymerization theory to be 30,000 cal/mole and 124 eu, respectively. Comparison of light scattering and osmotic pressure data are reported. Experiments done with TMV showed negligible Donnan effect.

After Schramm *et al.* (1955) separated tobacco mosaic virus (TMV) protein from nucleic acid and named it "A" protein, its physicochemical properties were studied extensively in different laboratories. The molecular weight was determined under different conditions of pH, ionic strength, etc., by various methods, including centrifugation, diffusion, viscosity measurement, light scattering, etc. The values in the literature vary from 80,000 to 105,000. The molecular weight of the smallest subunit was found by end group analysis and by centrifugation after detergent treatment and when diluted to a concentration <0.1%. All these procedures yielded a molecular weight of *ca.* 18,000. A detailed review of the structure of TMV has been published (Anderer, 1963).

The purpose of the present investigation was to find the molecular weight of "A" protein under different experimental conditions, to look for possible stable intermediates in "A" protein polymerization, and to examine certain aspects of the early stages of reversible polymerization of TMV protein. This reaction is endothermic and involves the release of water molecules during the polymerization process as first reported by Lauffer *et al.* (1958) and described in greater detail in subsequent publications (Ansevin and Lauffer, 1963; Lauffer, 1964; Ansevin *et al.*, 1964; Stevens and Lauffer, 1965).

Since the osmometer is ideally suited for the study of early stages of polymerization, where molecular weights are of the order of magnitude of 100,000, it was the instrument chosen for this study. Since the polymerization reaction seems to involve changes in hydration, it is necessary to understand the theory of the osmotic behavior of hydrated protein. In the preceding paper (Lauffer, 1966), it was shown that from the osmotic pressure at infinite dilution of a hydrated protein one obtains the anhydrous molecular weight. The second virial coefficient includes three terms, the Donnan term related to protein charge, a β_{22}^0 term related to excluded volume and the interaction of hydrated protein, and a term, usually even $<\beta_{22}^0$, proportional to the degree of hydration. The findings in this study were interpreted in the light of this theoretical analysis. Part of the work reported here was presented in abbreviated form (TD3) Banerjee and Lauffer (1965).

Materials and Methods

Preparation of TMV. TMV was isolated from Turkish tobacco plants infected with the common strain of the virus by a procedure involving alternate high- and low-speed centrifugation (Boedtker and Simmons, 1958) in the presence of Versene.

Preparation of TMV Protein. TMV protein was prepared by the acetic acid extraction of virus (Fraenkel-Conrat, 1957), except in one case when the protein was prepared by the alkaline degradation method of

* From the Department of Biophysics, University of Pittsburgh, Pittsburgh, Pennsylvania. *Received October 26, 1965; revised March 29, 1966.* Publications I–V of this series are Ansevin and Lauffer (1963), Lauffer (1964a), Ansevin *et al.* (1964a), Stevens and Lauffer (1965), and Lauffer (1966), respectively. This is publication No. 117 of the Department of Biophysics, University of Pittsburgh. Work was supported by a U. S. Public Health Service grant (GM 10403-04).

† The data presented in this paper are taken in part from the dissertation presented by K. Banerjee to the University of Pittsburgh in partial fulfillment of the requirements for the degree of Doctor of Philosophy, May 6, 1965.

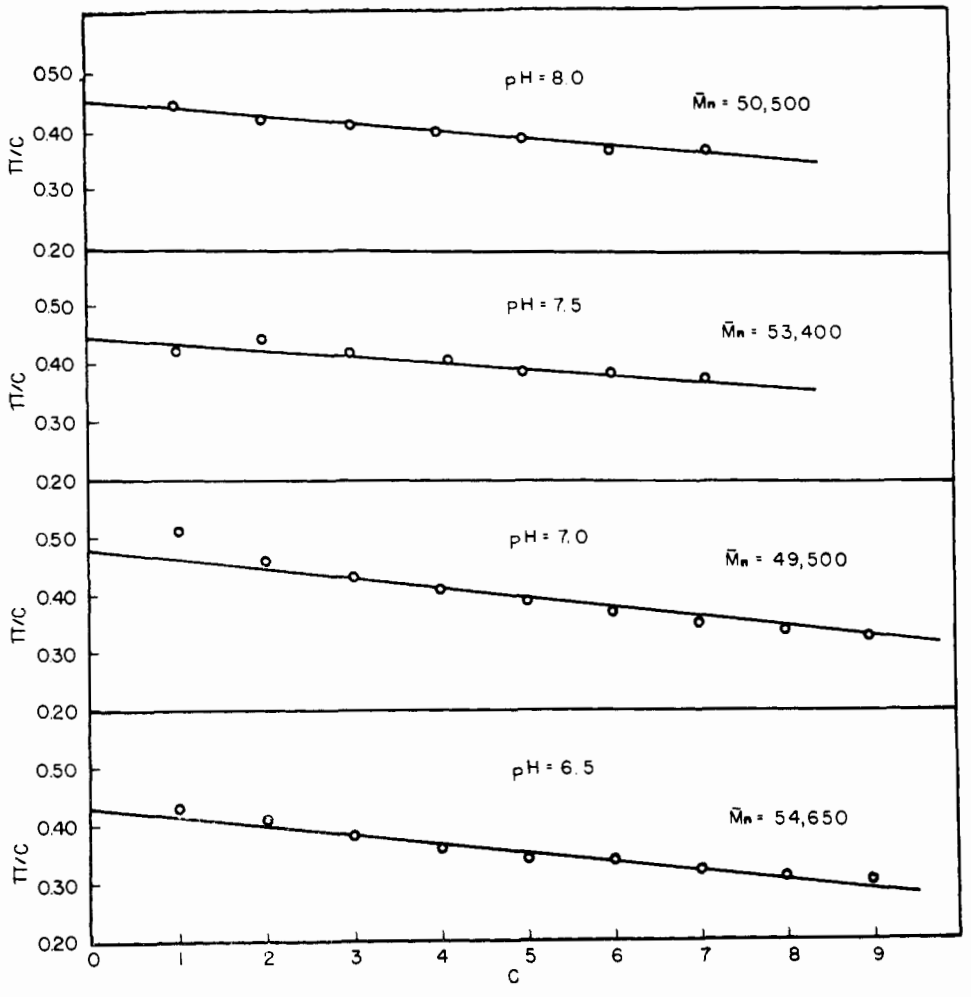

FIGURE 1: Effect of pH on osmotic pressure of TMV protein in phosphate buffer, ionic strength 0.1; π is the osmotic pressure in centimeters of water and c, the protein concentration in milligrams per milliliter.

Schramm (1943). The concentration was determined with a Cary spectrophotometer.

Osmometer. The instrument used in the study was a high-speed membrane osmometer.[1] There were two main reasons for choosing osmotic pressure. (1) The number-average molecular weight can be determined directly without using any other subsidiary experiment, and the instrument is most sensitive when the molecular weight is <100,000. (2) Any small amount of insoluble denatured protein (TMV protein is very susceptible to denaturation), if present, will not affect the determined molecular weight appreciably.

During experimentation, after temperature equilibration, which usually takes 6–7 hr, solvent is first placed on both sides of the membrane, and a stable reading is obtained after 15 or 20 min. The solvent on top of the membrane is then replaced with solution and a new reading is obtained in another 20 min. The difference between the two readings, corrected for density of the solvent, is the osmotic pressure of the solution expressed in centimeters of water.

Membrane. The membranes used for most of the experiments were obtained from Carl Schliecher and Schuell Co., Keene, N. H. The trade name is "Bac-T-Flex B-19."

In those experiments which utilized acetic acid, Visking cellulose tubing of regenerated cellulose made by the viscose process was used as the membrane. It was conditioned by washing in distilled water, soaking in a molar solution of sodium chloride for 0.5 hr, washing in distilled water, and soaking in 67% acetic acid for 24 hr.

Light-Scattering Apparatus. A Beckman DU spectrophotometer was used for all optical density measurements. The desired temperature was maintained by circulating water from a precooled water bath through a jacket around the specimen holder. The wavelength for measuring scattering was 320 mμ.

[1] Manufactured by Mechrolab, Inc., Mountain View, Calif.

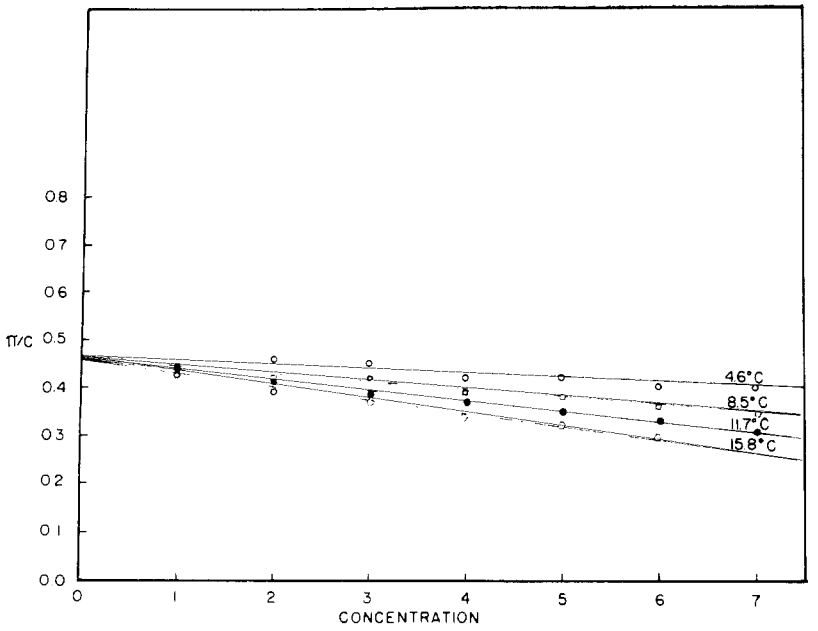

FIGURE 2: Effect of temperature on osmotic pressure of TMV protein in phosphate buffer, pH 7.5, ionic strength 0.067. π is the osmotic pressure in centimeters of water and c, the protein concentration in milligrams per milliliter.

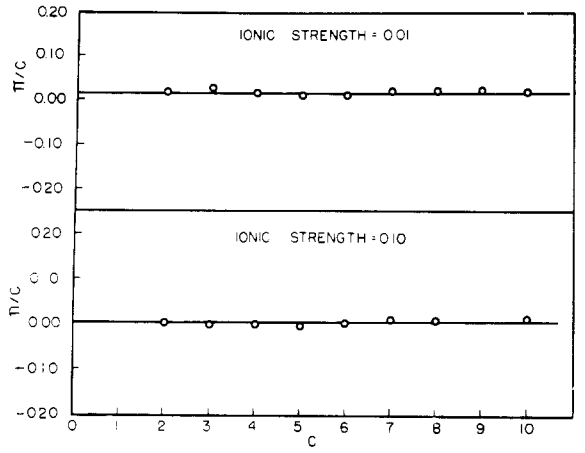

FIGURE 3: Plot of π/c *vs.* c for TMV in phosphate buffer at pH 7.5. π is the osmotic pressure in centimeters of water and c, the protein concentration in milligrams per milliliter.

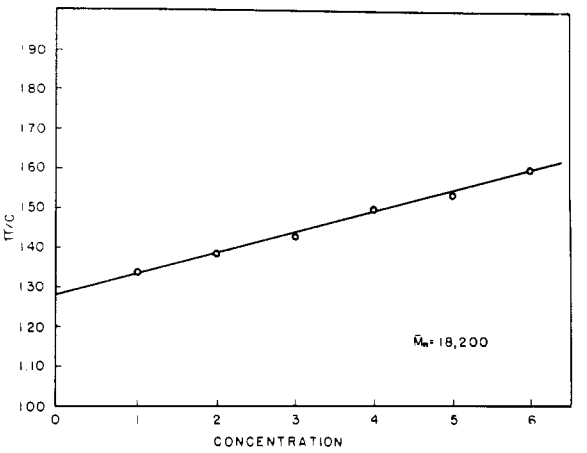

FIGURE 4: Plot of π/c *vs.* c for TMV protein in 67% acetic acid. π is the osmotic pressure in centimeters of water and c, the protein concentration in milligrams per milliliter.

Experimental Results

A variable temperature controller supplied with the osmometer was used to control the temperature of the membrane compartment. Almost all experiments were done around 5°, because TMV protein is very stable at low temperatures. While studying the reversible polymerization of "A" protein, the temperature was varied from 4 to 13°.

In order to check the functioning of the instrument, the molecular weight of bovine serum albumin in phosphate buffer at pH 7.5 was determined. The value of the molecular weight obtained by extrapolation to zero concentration was 68,600, in close agreement with the value obtained by Scatchard *et al.* (1946).

Osmotic pressure of TMV protein as a function of pH is shown in Figure 1. The temperature of the experiments was 5°. Two features of these curves are worth mentioning. (1) The value of the molecular weight obtained by extrapolation to zero concentration in all the experiments was nearly 53,000. (2) The slopes of

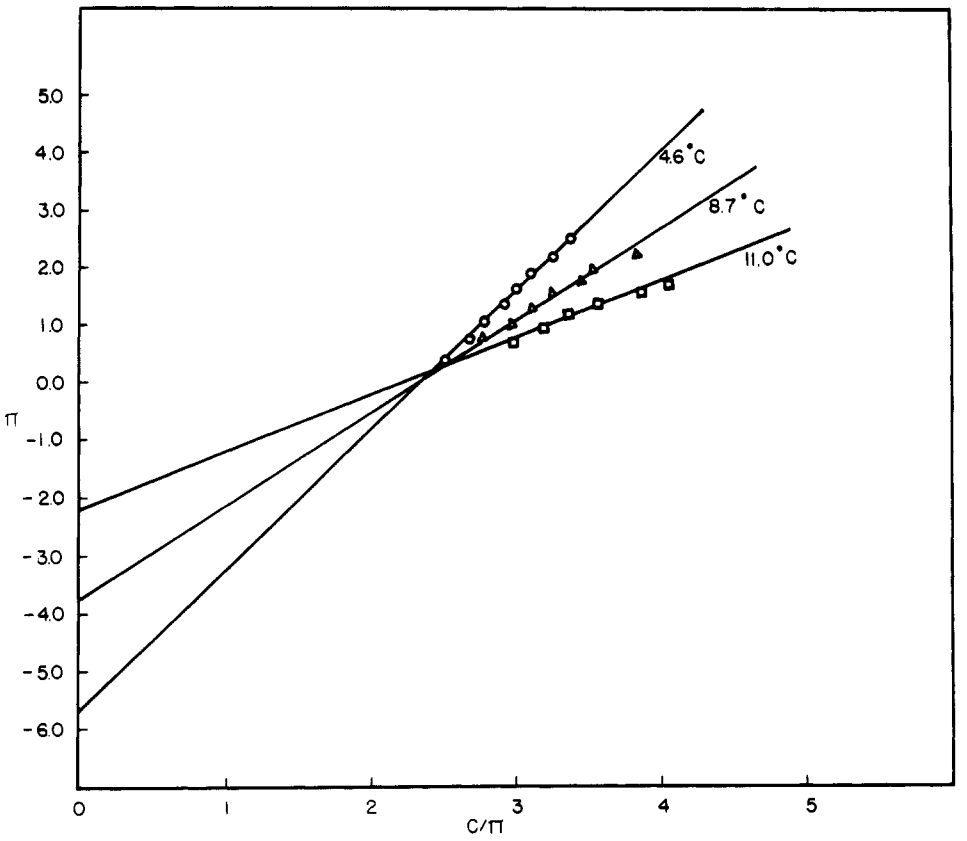

FIGURE 5: Plot of π *vs.* c/π for TMV protein in phosphate buffer, pH 6.5, ionic strength 0.1, as a function of temperature. π is the osmotic pressure in centimeters of water and c, protein concentration in milligrams per milliliter.

all the lines in Figure 1 are negative and there is almost no change in slope from one pH value to another.

In Figure 2 is shown the results of experiments in which the pH and the ionic strength were maintained constant but the temperature was varied from 4.6 to 15.8°. It can be seen from the graph that as the temperature is increased the slopes become steeper and steeper in the negative sense, but they all extrapolate almost to the same point on the ordinate.

Osmotic pressure data for TMV are shown in Figure 3. The points lie on a straight line parallel to the abscissa.

Figure 4 shows the osmotic pressure data of TMV protein in 67% acetic acid. The protein was prepared by adding 1 volume of cold virus solution to 2 volumes of cold glacial acetic acid. The nucleic acid precipitate was centrifuged in the cold and the clear supernatant solution was dialyzed *vs.* 1 l. of 67% acetic acid for 24 hr. The value of the molecular weight obtained by extrapolation to zero concentration was 18,200.

A plot of c/π *vs.* π for TMV protein at different temperatures is shown in Figure 5.

Figure 6 shows the plot of increase in optical density *vs.* temperature for different concentrations of TMV protein. In this particular experiment an aliquot corresponding to each concentration of the sample was used for optical density measurement and the rest of the sample was used for osmotic pressure measurement.

Discussion

When experiments were done with TMV protein, attempts to measure osmotic pressure below 0.1% concentration failed because the data in this range were not reproducible. Therefore, in all graphs osmotic pressure data corresponding to concentrations of $\geq 0.1\%$ are shown.

Extrapolation to zero concentration with data only for concentrations above 0.1% would occasion no comment if there were no dissociation of the protein molecules below 0.1% concentration. However, since the TMV protein is known to dissociate into smaller subunits upon dilution (Ansevin and Lauffer, 1959) when the concentration becomes $<0.1\%$, the extrapolated intercepts shown in Figures 1 and 2 are called virtual intercepts and the value of the virtual intercept is called the apparent molecular weight.

Under the experimental conditions of pH, ionic strength, and temperature described for expt 1 and 2, TMV protein in the concentration range above 0.1% behaves as if the starting material had a molecular weight of 53,000. In one particular case (*i.e.*, for TMV protein in phosphate buffer pH 6.5, ionic strength 0.1) the

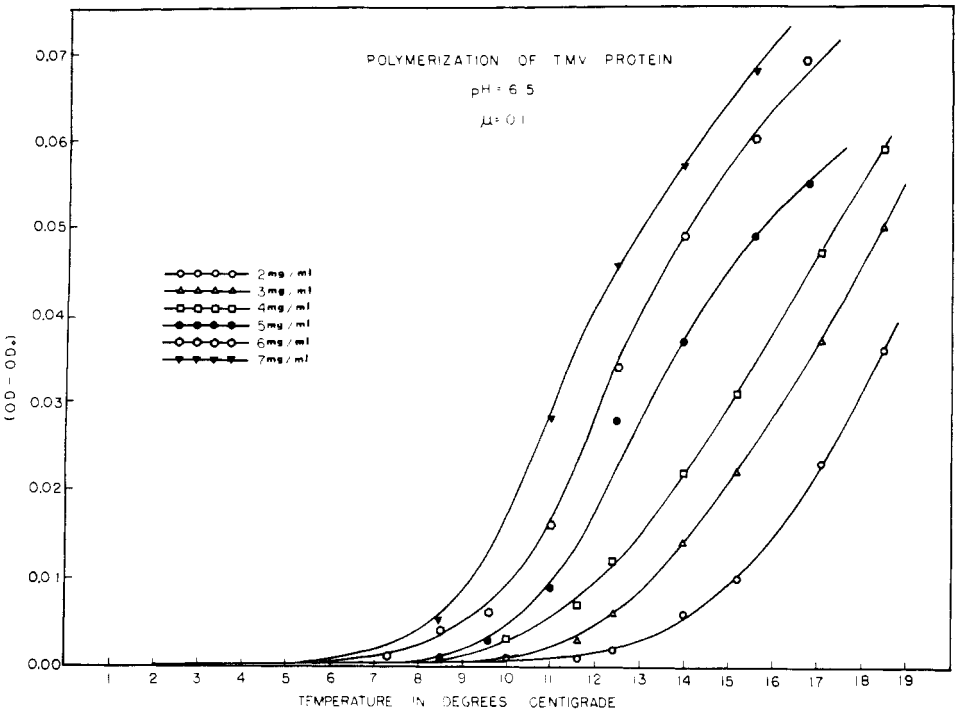

FIGURE 6: Increase in optical density at 320 mμ with temperature for different concentrations of TMV protein solution in phosphate buffer.

same value for the molecular weight of the starting material was obtained from an entirely different consideration, as described later. Since the molecular weight of the smallest subunit is known to be 17,500, the value obtained in this case corresponds to a trimer. Caspar (1963), on theoretical grounds, proposed trimer as the first stable intermediate in "A" protein polymerization. Recently Haggis (1965) reported studies on electron micrographs of TMV taken at various stages of drying and suggested the formation of clusters of three subunits.

The negative slopes as shown in Figures 1 and 2 must be explained. From electrophoretic mobility measurements of Kramer and Witmann (1958) it is known that TMV protein is negatively charged at pH 6.5 and above. With either positive or negative charge there should be a positive slope due to Donnan pressure. Furthermore, if the net charge is directly related to base binding, the slope should increase as the pH is raised from 6.5 to 8.0, because titration experiments of Ansevin *et al.* (1964) show an increase in base binding in this range. Assuming only Donnan effect to be present, and no other protein–protein interaction, a plot of π/c *vs.* c would give a positive slope. But if there is association of protein molecules, *i.e.*, increase in molecular weight with increase in concentration and no Donnan effect, the same plot would give a negative slope. When both these effects are present the resultant slope will be positive if Donnan effect is greater, and negative if the reverse is true. Since in this case a negative slope is obtained, it is evident that the effect due to association of protein molecules is greater than the Donnan effect.

TMV protein polymerizes into rodlike structures and Ansevin *et al.* (1964) have shown that titration curves for "A" protein and virus intersect near pH 7.5. So a simple experiment was done with TMV at this pH 7.5 to see whether Donnan effect is at all observable. The results have already been shown in Figure 3. To intensify the Donnan effect, the ionic strength was decreased 10-fold, but as shown in Figure 3, the plot of π/c *vs.* c was still parallel to the abscissa. In one case the phosphate buffer was replaced with 0.01 M potassium chloride solution, but again the plot of π/c *vs.* c was a straight line parallel to the abscissa and almost passing through the origin.

Thermodynamic analysis (Lauffer, 1966) leads to the conclusion that the osmotic pressure of a hydrated charged macromolecule is proportional to the sum of four kinds of terms: (1) concentration divided by molecular weight; (2) hydration terms, usually very small; (3) protein–protein interaction, excluded volume, etc., which can be expressed as a power series in concentration beginning with the square; and (4) a Donnan term which can also be expressed as a power series in concentration beginning with the square. The Donnan term must always be positive, *i.e.*, add to the pressure. The protein–protein interaction term can be either positive or negative. However, the absolute magnitude of the interaction effect, when negative, can never be greater than the positive contribution proportional to concentration divided by molecular weight (Lauffer, 1966). Because of its extremely high molecular weight, the osmotic pressure attributable to concentration divided by molecular weight is immeasurably small for

tobacco mosaic virus. Therefore, the interaction term, if negative, is also immeasurably small. As a result, any measurable osmotic pressure must be approximately equal to or greater than (if the interaction term is positive) the contribution from the Donnan effect. Thus, the only conclusion that can be drawn from this experiment is that the contribution due to Donnan effect is negligible in this case. However, the Donnan effect calculated on the assumption that net charge is directly obtainable from base neutralization is appreciable. Therefore, the net charge responsible for Donnan effect is smaller than the charge calculated from the titration experiments. One way of explaining this is to assume that the charges are hidden within the structure or otherwise shielded or neutralized by positive ions, through binding, for example.

This unexpected result forces one to reinterpret slightly the experiment of Stevens and Lauffer (1965). In this experiment TMV was used as a control to cancel out the Donnan contribution to the "weight" of a protein solution in equilibrium with the low molecular components of the system. It was assumed that changing pH from 7.5 to 5.5 involved equal changes in the Donnan term for TMV and for TMV protein because they neutralize equal amounts of acid over this pH range. However, since the Donnan term for TMV is much less than expected and, since it might have more nearly the expected value for TMV protein, this assumption is not valid. The ideal control for the experiment of Stevens and Lauffer would be a hypothetical polymerized TMV protein which remains polymerized when titrated from the lower to the higher pH used. To the extent that TMV is a useful substitute for the ideal control, the experiment of Stevens and Lauffer can be interpreted to mean that the polymerization of 100,000 g of TMV protein involves releasing 150 moles of water and attracting by all mechanisms, the Donnan effect included, 7 moles of sodium chloride.

The next experiment was done with two purposes in mind. The first was to determine the molecular weight. In 67% acetic acid the TMV protein is known to exist as the smallest subunit and, therefore, the molecular weight obtained in this case should be nearly 17,500, the molecular weight of the protein subunit. The second was to see whether the Donnan effect can be observed when the protein exists in the monomeric form. As can be seen from Figure 4, the osmotic pressure data yielded mol wt 18,200, very close to other values reported from end group analysis, sedimentations, etc. A similar value for M was found by the method of low-angle X-ray scattering by Anderer *et al.* (1964). The slope of the plot of π/c *vs.* c in this case was positive, indicating a Donnan effect of appreciable magnitude, but without knowledge of the charge of the protein at this pH value (1.02), it was not possible to calculate the Donnan pressure to compare quantitatively with the slope.

The reversible polymerization and depolymerization was studied in more detail. This endothermic reaction has been explained before by Lauffer (1962, 1964b) assuming condensation polymerization. In order to treat the osmotic pressure data on the same assumption, the following relationship was derived.[2]

$$\pi = \frac{(RT)^2}{KM_n}\left(\frac{c}{\pi}\right) - \frac{RT}{K}$$

K is the equilibrium constant at temperature T, M_n is the molecular weight of the polymerizng unit, and other symbols have their conventional meanings. To apply the condensation polymerization theory in this case, it is assumed that for the small concentration range of protein solution the solution behaves ideally in terms of molalities.

The experimental data were plotted fitting this equation as shown in Figure 5. A straight line was obtained when π was plotted *vs.* c/π. As can be seen from the above equation, the molecular weight and the equilibrium constant at different temperatures could be calculated knowing the slope and the intercept of the line. The calculated values are shown in Table I. The

TABLE I

T (°C)	K	M_n
4.6	4.21×10^3	54,000
8.7	6.99×10^3	53,000
11.0	12.93×10^3	50,500

value of the equilibrium constant increased in magnitude as the temperature increased, but for all temperatures

[2] For condensation polymerization of an ideal solution, $K = p/(1-p)^2[A_0]$, where K is the equilibrium constant, p is the fraction of ends of A protein units which are no longer free in a particular polymer solution (Flory, 1936), and $[A_0]$ is the total concentration expressed as moles of polymerizing unit/1000 g of solvent (Lauffer, 1964b; Smith, 1961). For an ideal polymer solution, $\pi = RT[A_0](1-p)$. When p is 0, $\pi_0 = RT[A_0]$.

$$\frac{\pi}{\pi^0} = (1-p) = \frac{\pi}{RT[A_0]}$$

$$K = \frac{1 - \dfrac{\pi}{RT[A_0]}}{\dfrac{\pi}{(RT)^2[A_0]}}$$

$$\pi = \frac{(RT)^2}{K}\frac{[A_0]}{\pi} - \frac{RT}{K}, \text{ or}$$

$$\pi = \frac{(RT)^2}{KM_n}\frac{c}{\pi} - \frac{RT}{K}$$

where c is total concentration in g/1000 g of solvent and M_n is molecular weight of the polymerizing unit.

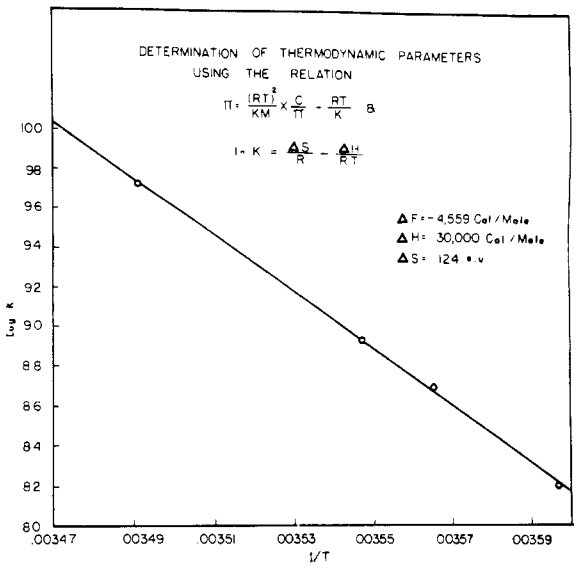

FIGURE 7: Plot of log K *vs.* $1/T$, where K is the equilibrium constant and T, absolute temperature.

the value of the molecular weight of the starting material (polymerizing unit) was again nearly that of a trimer.

Knowing the equilibrium constant at different temperatures the thermodynamic parameters can be calculated from the relation

$$\ln K = \frac{\Delta S}{R} - \frac{\Delta H}{RT}$$

An example of plot of ln K *vs.* $1/T$ is shown in Figure 7. The thermodynamic parameters calculated from this graph are: $\Delta F = -4559$ cal/mole; $\Delta H = 30{,}000$ cal/mole; $\Delta S = 124$ eu. The increases in enthalpy and entropy in the temperature range 4–11° are substantially less than those obtained previously for the temperature range 12–19° (Smith, 1961; Ansevin and Lauffer, 1963). To account for the enthalpy and entropy increases, Lauffer *et al.*, (1958) postulated that structured water is released from the protein upon polymerization. As a first approximation the number of water molecules released per linkage formed between protein molecules can be calculated using the entropy of melting of water at 0°, 5.26 cal/deg per mole, or the enthalpy of melting of ice, 1440 cal/mole, to be *ca.* 23. The result is then consistent with the water dissociation hypothesis of Lauffer *et al.* (1958).

The last experiment was done to correlate, in a preliminary way, light-scattering data and osmotic pressure data. Assuming condensation polymerization, the following relationship was obtained[3]

$$\frac{OD - OD_0}{c} = 20.99\,\frac{Hc}{\pi} - \frac{2 \times 10^{-3}}{2.3}\,HM_n$$

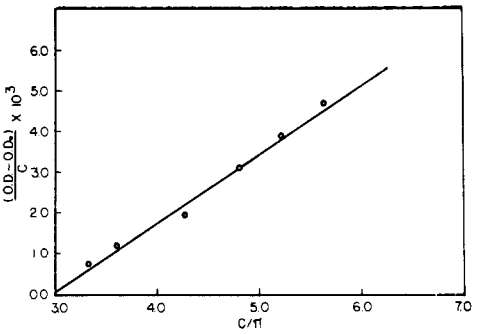

FIGURE 8: Correlation between optical density and osmotic pressure data of TMV protein in phosphate buffer, pH 6.5, ionic strength 0.1; π is in centimeters of water and c is the protein concentration in milligrams per milliliter.

The experimental data were plotted fitting this equation to obtain additional information for the condensation polymerization hypothesis. An inherent difficulty was faced. The light-scattering data were good only above 14° for the lowest concentration used. But in the osmometer no reliable data were obtainable at this temperature. So a compromise was made. An optimum temperature of 11.8° was chosen where data could be compared from both sets of experiments. The concentration range was chosen above 3 mg/ml (OD − OD_0/c) was plotted *vs.* c/π and is shown in Figure 8. The value of H and M_n were calculated and found to be 8.13×10^{-5} and 71,100, respectively. When the theoretical value of H, 4.27×10^{-5} (Smith, 1961), and a molecular weight of 52,500 are used with the theoretical equations, the data obtained at the three lowest concentrations are in fair agreement with the calculated values. However, this is the region where the data are least precise.

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[3] For condensation polymerization in an ideal solution $\tau/\tau_0 = (1 + p)/(1 - p)$, where τ and τ_0 are the turbidity of solution of polymers and of unpolymerized material, respectively (Lauffer, 1962; Smith, 1961). Since $\pi/\pi_0 = (1 - p)$

$$\frac{\tau}{\tau_0} = \frac{2\pi_0}{\pi} - 1 \text{ and } \frac{\tau - \tau_0}{\tau_0} = 2\left(\frac{\pi_0}{\pi} - 1\right)$$

But $\tau = 2.3$ OD, $\tau_0 = 10^{-3}HcM_n$, and $\pi_0 = RT[A_0] = RTc/M_n$ for ideal solutions, where OD means optical density and H is the usual light-scattering factor.

When $T = 284.9$°K and π is expressed in centimeters of water,

$$\frac{OD - OD_0}{c} = \frac{2 \times 10^{-3}RTH}{2.3}\,\frac{c}{\pi} - \frac{2 \times 10^{-3}}{2.3}\,HM_n$$

the first term on the right becomes 20.99 Hc.

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The Alteration in the Reactivity of the Tyrosine and Tryptophan Groups of Trypsin upon Combination with Protein Inhibitors*

Robert F. Steiner

ABSTRACT: When trypsin combines with any of several protein inhibitors, one to two tryptophan groups and two to three tyrosines are shielded from reaction with *N*-bromosuccinimide and iodine, respectively. This may reflect their presence in the zone of contact of trypsin and inhibitor, presumably in the active site vicinity.

The combination of trypsin with several protein inhibitors has been shown to result in distinct changes in a number of physical properties. In particular, an ultraviolet difference spectrum is developed, which has similar features for all inhibitors, although quantitative differences exist (Edelhoch and Steiner, 1965). The difference spectrum is developed at wavelengths characteristic of both tyrosine and tryptophan residues, suggesting that the environments of both groups are altered as a consequence of complex formation. The positive sign of the change in absorbancy is that usually associated with the shift of a chromophore from a polar to a nonpolar environment (Edelhoch and Steiner, 1965; Wetlaufer, 1962).

In the present investigation the change in reactivity of the tryptophan and tyrosine groups of trypsin accompanying the combination of the enzyme with several protein inhibitors has been examined. Of the four inhibitors studied [ovomucoid, lima bean (LBI),[1] pancreatic (PTI), and soy bean (STI)] only STI contains tryptophan groups. Thus, with the exception of STI, any change in tryptophan reactivity resulting from trypsin–inhibitor interaction must reflect changes in the environment of one or more of the four tryptophans (Walsh *et al.*, 1964) of trypsin alone. *N*-Bromosuccinimide (NBS), which has been shown to oxidize tryptophan groups selectively (Witkop, 1961; Green and Witkop, 1964), has been used as a specific reagent for this residue.

Iodination has been used to assess the reactivity of the tyrosine groups of trypsin. Trypsin contains 10 tyrosines (Walsh *et al.*, 1964). As tyrosines are also present in all four of the protein inhibitors (Laskowski and Laskowski, 1954), a change in tyrosine reactivity

* From the Bureau of Medicine and Surgery, Navy Department, Naval Medical Research Institute, Bethesda, Maryland. Research Task MR 005.06-0001.01. *Received February 7, 1966.* The opinions or assertions contained herein are the private ones of the author and are not to be construed as official or reflecting the views of the Navy Department or the Naval service at large.

[1] Abbreviations used: LBI, lima bean inhibitor; PTI, pancreatic inhibitor; STI, soy bean inhibitor; NBS, *N*-bromosuccinimide; TAME, toluenesulfonylarginine methyl ester.