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Polymerization–Depolymerization of Tobacco Mosaic Virus Protein. XI. Osmotic Pressure Studies of Solutions in Water and in Deuterium*

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ABSTRACT: Polymerization of tobacco mosaic virus (TMV) protein as a function of concentration has been studied by the method of osmometry at various temperatures between 4 and 25°. The solvents were usually tenth ionic strength phosphate or barbital buffer in water at pH values of 6.0, 6.5, and 7.5, and tenth ionic strength phosphate buffer in D_2O at pD values of 7.0 and 8.0. The theory of linear condensation polymerization was extended to take into account departures from ideality at high concentration. It is assumed that such departures were the sum of a calculated excluded volume effect and a calculated Donnan effect. The theory could be fitted reasonably satisfactorily to the data obtained in aqueous solvents up to concentrations of 30 mg/ml. The theory permits evaluation of M_0, the molecular weight of the smallest polymerizing unit and K, the equilibrium constant for polymerization. The values of M_0 usually turned out to be close to 50,000. Since the molecular weight of a stable trimer of the basic chemical unit of TMV protein is 52,500, all of the data were analyzed on the assumption that M_0 actually is 52,500. The values of K thus obtained indicate that in aqueous solutions the polymerization at a temperature of 4° is not appreciably affected by pH or specific buffer ions. Values of $\Delta H°$ and $\Delta S°$ were evaluated for polymerization at pH 6.5 and at 7.5 in tenth ionic strength phosphate buffer and were found to be of the same order of magnitude as those previously reported by Banerjee and Lauffer (Banerjee, K., and Lauffer, M. A. (1966), *Biochemistry 5*, 1957), namely, +30,000 cal/mole for $\Delta H°$ and +124 entropy units for $\Delta S°$. Polymerization in D_2O was more complex. Only the data at 4° at pD 7.0 and 8.0 could be analyzed in terms of the theory presented here. In contrast with the results obtained in aqueous solvent, the equilibrium constant was much higher at pD 7.0 than at pD 8.0. This indicates that the reaction in D_2O, particularly at pD 7.0, resembles more the high-temperature than the low-temperature polymerization process. At pD 8.0 and 20°, π/c increased with protein concentration. This effect can be interpreted quantitatively as being due to the Donnan term, with the protein in the double-disk state over a wide range of concentrations. The measured values of π/c at pD 7.0 at 8 and 11.7° were very much lower than the calculated Donnan contribution. This is evidence that the polymerization process at pD 7.0 is different from that at pD 8.0 and that it leads to a product which does not exhibit the Donnan effect. Previous studies have shown that TMV does not exhibit the Donnan effect in the osmometer and that high-temperature polymerization leads to a polymer with a structure resembling the arrangement of protein in TMV. Experiments designed to correlate light scatter and osmotic measures of polymerization in terms of condensation polymerization theory yielded the correct value of M_0 but a value of H 70% higher than that calculated from the appropriate constants of the solutions.

Tobacco mosaic virus protein (TMV) is one of the best investigated proteins and many of its biophysical properties are known. Of these, its polymerization–depolymerization (Lauffer *et al.*, 1958) has been studied in detail (Ansevin and Lauffer, 1963; Lauffer, 1964, 1966a,b; Ansevin *et al.*, 1964; Stevens and Lauffer, 1965; Banerjee and Lauffer, 1966; Smith and Lauffer, 1967; Shalaby and Lauffer, 1967; Khalil and Lauffer, 1967).

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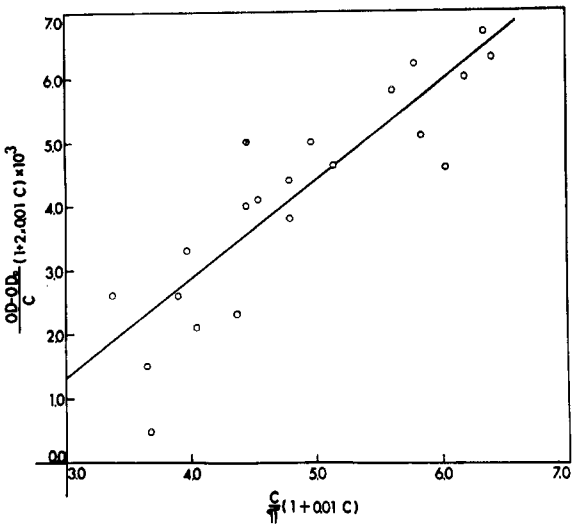

FIGURE 1: A least-squares plot of optical density *vs.* c/π, both modified for nonideal solutions. π is in centimeters of water and c is the concentration of TMV protein in milligrams per milliliter. Phosphate buffer solution, pH 6.5, 0.1 μ. The temperature is 11.7°.

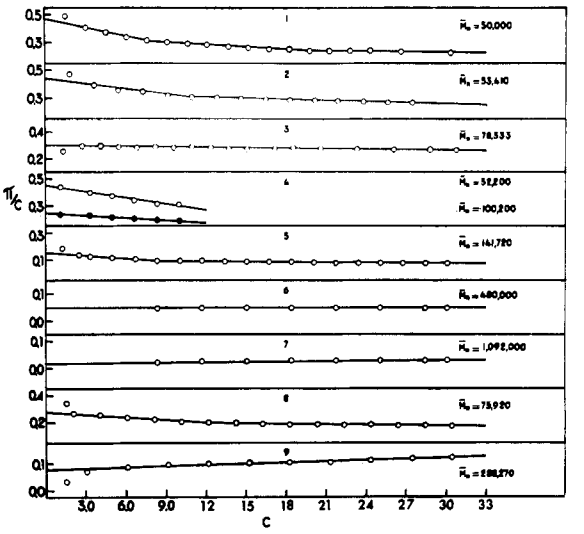

FIGURE 2: Plot of π/c *vs.* c. π is in centimeters of water and c is the concentration of TMV protein in milligrams per milliliter in: expt 1: phosphate buffer, pH 6.5, 0.1 μ, at 4°; expt 2: phosphate buffer, pH 7.5, 0.1 μ, at 4° (dilutions made 24 hr before); expt 3: phosphate buffer, pH 7.5, 0.1 μ, at 4° (dilutions made 2 hr before); expt 4a: phosphate buffer, pH 7.5, 0.1 μ, at 4°; expt 4b: phosphate buffer, pH 7.5, 0.1 μ at 20°; expt 5: phosphate buffer in D_2O, pD 7.0, 0.1 μ at 4°; expt 6: phosphate buffer in D_2O, pD 7.0, 0.1 μ at 8°; expt 7: phosphate buffer in D_2O, pD 7.0, 0.1 μ at 11.7°; expt 8: phosphate buffer in D_2O, pD 8.0, 0.1 μ at 4°; and expt 9: phosphate buffer in D_2O, pD 8.0, 0.1 μ at 20°.

Banerjee and Lauffer (1966) used osmotic pressure to study the early stage of polymerization under different conditions of pH, ionic strength, and temperature. In their experiments, the concentration range studied was between about 1 and 9 mg/ml of TMV protein. They showed that the molecular weight in 67% acetic acid corresponded to a monomer with $\bar{M}_n$ of 18,200, and they also provided experimental evidence that a trimer with molecular weight 52,500 is a stable intermediate in "A" protein polymerization. Thermodynamic parameters for the polymerization reaction were calculated from their experiments. Preliminary experiments to correlate light-scattering data and osmotic pressure data and to calculate H (the optical factor) and $\bar{M}_n$ were also performed. The values obtained were 8.13×10^{-5} and 71,100, respectively.

The experiments described herein were designed with several purposes in mind: (1) to try to improve the values of H and $\bar{M}_n$ by correlating light-scattering data and osmotic pressure data; (2) to study the polymerization of TMV protein by osmometry at concentration and temperature ranges above those used by Banerjee and Lauffer; (3) to study the polymerization reaction in other buffer solutions; and (4) to determine the effect of D_2O on TMV protein polymerization under conditions amenable to study with the osmometer, to supplement the observations of Khalil and Lauffer (1967).

Materials and Methods

Purification of TMV. TMV was isolated by squeezing the juice from Turkish tobacco plants infected with the common strain of the virus, adding 30 g of K_2HPO_4/l. of juice and centrifuging alternately at high and low speeds (Boedtker and Simmons, 1958). After the first high-speed centrifugation, the virus was resuspended and incubated for about 4 hr at 40° in the presence of 0.01 Versene–NaOH buffer of pH 7.0–7.5 to remove pigments. The purified stock virus solution was stored at 4°.

Preparation of TMV Protein. TMV protein was prepared by the acetic acid method of Fraenkel-Conrat (1957) with slight modifications. Dialysis against distilled water was performed with two water changes the first day and one water change on the second, third, and fourth days. The milky suspension of TMV protein was centrifugated at 20,000 rpm for 40 min at 4° in the no. 21 rotor in the Spinco Model L ultracentrifuge.

In the D_2O experiments, the tubes were completely drained and dried with "Kimwipes" after centrifugation. The pellet was suspended in D_2O and then dissolved by drop-by-drop addition of 0.1 M KOH in D_2O.

In some instances, after the RNA was precipitated, the supernatant fluid was passed through a Sephadex G-25 column equilibrated with 0.1% of acetic acid. The same concentration of acetic acid was used for elution of the protein. Fractions of 10 ml each were collected and mixed with 1 ml of phosphate buffer solution at pH 7.5, ionic strength 0.1. Fractions containing TMV protein showed a slight precipitate. To remove this, the TMV protein suspension was then centrifuged at 3000 rpm for 10 min. All operations were performed at 4° (C. L. Stevens, unpublished data).

Protein solutions were dialyzed against the proper solvent for 48–72 hr and then centrifuged at 4° for 3 hr at 40,000 rpm in the Spinco Model L ultracentrifuge with no. 40 rotor. When using D_2O, the original D_2O

TABLE I

Expt No.	Buffer In H_2O	pH	Temp (°C)	Ionic Strength	$\bar{M}_n$ (g)	K (moles/l.)
10	PO_4	6.0	4	0.1	152,400	
1	PO_4	6.5	4	0.1	50,000	5.56×10^3
15	PO_4	6.5	11.7	0.1	64,155	29.2×10^3
2	PO_4	7.5	4	0.1	53,410	4.14×10^3
3	PO_4[a]	7.5	4	0.1	78,333	
4b	PO_4	7.5	20	0.1	100,200	27.2×10^3
16	PO_4	7.5	25	0.1	101,104	33.3×10^3
11	Barbital	6.5	4	0.1	50,865	3.26×10^3
13	Barbital	6.5	4	0.055	48,755	2.06×10^3
12	Barbital	7.5	4	0.1	50,213	3.04×10^3
14	P_2O_7	7.3	25	0.1	87,160	
	In D_2O	pD				
5	PO_4	7.0	4	0.1	141,720	75.7×10^3
6	PO_4	7.0	8	0.1	480,000	
7	PO_4	7.0	11.7	0.1	1,092,000	
8	PO_4	8.0	4	0.1	75,920	11.2×10^3
9	PO_4	8.0	20	0.1	288,270	

[a] Dilutions were made between 1 and 2 hr before beginning the experiment.

bottle was used as a container for performing the dialysis, since it was preequilibrated with D_2O. The concentration of TMV protein was determined with a Cary Model 14M spectrophotometer, using 0.13 as the extinction coefficient for TMV protein (Stevens and Lauffer, 1965).

Light-Scattering Experiments. A Beckman DU spectrophotometer was used to measure the turbidity of the protein solutions. The desired temperature was maintained by circulating water from a bath at constant temperature through a jacket around the specimen holder. The wavelength chosen for the measurement was 320 (Smith and Lauffer, 1967).

Osmotic Pressure Experiments. A high-speed membrane osmometer[1] was used for these experiments following the experimental procedure described by Banerjee and Lauffer (1966) and modified by Paglini (1968). The membranes used were the cellulose acetate membrane filters type B-20 obtained from Carl Schleicher & Schuell Co., Keene, N. H.

Experimental Results

The temperature chosen for correlating light-scatter data and osmotic pressure data was 11.7°. The experimental value for $OD - OD_0$ obtained at that temperature in the light-scatter experiments was used instead of the value one can get from a plot of $OD - OD_0$ *vs.* T (temperature in degrees centigrade).

A plot of $(OD - OD_0)(1 + 0.02c)/c$ *vs.* $c(1 + 0.01c)/\pi$ is shown in Figure 1. The reasons for this type of plot are found in the Discussion.

Osmotic pressure data, presented as conventional π/c *vs.* c plots, for TMV protein in several buffer solutions in water and D_2O at various pH values and temperatures are shown in Figure 2 and in Table I. Values of $\bar{M}_n$ recorded in Figure 2 and Table I were determined by extrapolation. At pH 7.5, expt 3 was done by making dilutions from the starting concentration about 2 hr before beginning the experiment; in expt 2 dilutions were made 24 hr before. The value of $\bar{M}_n$ obtained by extrapolation to zero concentration was 78,333 for the first and 53,410 for the second, showing a time dependence of the equilibrium state. For this reason, dilutions in the other experiments were ordinarily made 24 hr in advance of osmotic pressure measurements.

In most experiments, osmotic pressure equilibrium was reached in between 10 and 40 min after the protein solution was placed in the osmometer. However, in an experiment involving TMV protein in 0.1 ionic strength phosphate buffer at pD 8.0 and at 25°, osmotic pressure equilibrium was reached only for the lowest concentrations; above 8 mg/ml, osmotic equilibrium was not reached even after 60 min. The evidence for this is that the osmotic pressure readings dropped continuously and did not stop even after 60 min.

To find an explanation for this behavior, a turbidity experiment was performed at pD 8.0 and a protein concentration of 29 mg/ml. The results are shown in Table II. Optical density remained constant from 8 to 20° for a period of 150 min. When the temperature was increased to 25°, there was an increase in optical

[1] Mechrolab Model 503 (Hewlett Packard Co., Avondale, Pa.).

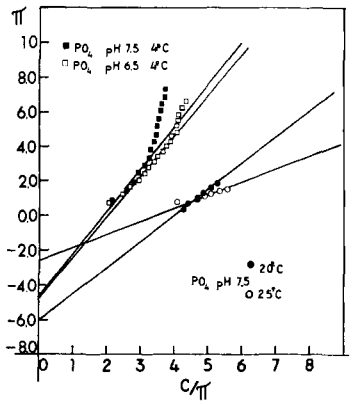

FIGURE 3: π *vs.* c/π as a function of pH, concentration, and temperature. π is in centimeters of water, TMV protein concentration is in milligrams per milliliter.

density indicative of polymerization. However, a period of 90 min was required before the maximum optical density was reached. When the temperature was subsequently dropped to 20°, a greater reading was obtained than that previously obtained at the same temperature. When the temperature was then dropped to 11°, a reading roughly equal to that obtained initially was found. When the temperature was again increased to 20°, no change in optical density was observed. This experiment demonstrates that at high protein concentrations and at 25°, polymerization equilibrium is approached slowly, requiring approximately 90 min. This explains, therefore, why osmotic pressure equilibrium was not achieved at the higher concentrations in this experiment. It is evident, therefore, that the data obtained in this experiment are not as reliable as those obtained in the other experiments.

TMV protein dissolved in barbital buffer solutions at pH 6.5 behaved in the osmometer at low temperatures approximately the same as TMV protein in phosphate buffer. However, it was observed that at room temperature, the solutions in barbital buffer were not as turbid as in phosphate buffer. For this reason, preliminary light-scatter experiments were carried out in which samples of TMV protein in phosphate buffer at pH 6.5, ionic strength 0.1, and in a barbital sodium–barbital buffer at pH 6.5, ionic strength 0.01, adjusted to 0.1 with KCl, were run simultaneously. Protein concentrations ranged from 1 to 6 mg/ml. The polymerization in barbital for all concentrations started at higher temperatures than polymerization in phosphate buffer solutions. Furthermore, at a given temperature, the optical density in barbital was considerably less than phosphate, indicating a smaller degree of polymerization. Polymerization was completely reversible in both barbital and phosphate buffer solutions. The optical density–temperature curves obtained at a concentration of 1 mg/ml with barbital buffer in this case resembled the curves obtained by Shalaby and Lauffer (1967) with 1.0 M acetamide and with 0.25 M thiourea. The effects of specific ions as studied by Shalaby and Lauffer on polymerization of TMV could be arranged in a manner consistent with the Hofmeister series.

TABLE II

Temp (°C)	Optical Density	Time (min)
8.00	0.180	0
20.0	0.185	150
25.0	0.330	240
20.0	0.275	310
15.0	0.240	355
13.5	0.200	375
11.0	0.185	405
20.0	0.185	480

Discussion

It is now well established that there are two distinct modes of polymerization of TMV A protein (Lauffer and Stevens, 1967; Lauffer *et al.*, 1967). Low-temperature polymerization proceeds without binding hydrogen ions (Scheele and Lauffer, 1967), is essentially independent of pH (Banerjee and Lauffer, 1966), is associated with only a small volume change (Lauffer and Stevens, 1967), has positive enthalpy and entropy of approximately 30,000 cal/mole and 124 eu/mole of bond formed (Banerjee and Lauffer, 1966), and leads ultimately to the formation of disks and stacked disks (Lauffer *et al.*, 1967). High-temperature polymerization is accompanied by the binding of hydrogen ions (Scheele and Lauffer, 1967), is strongly dependent on pH (Lauffer *et al.*, 1958), is associated with a large increase in volume (Stevens and Lauffer, 1964) has positive enthalpies and entropies of several 100 kcal/mole and of 500–1000 eu/mole of bond formed, depending upon the conditions (Smith and Lauffer, 1967; Khalil and Lauffer, 1967; Shalaby and Lauffer, 1967), and leads ultimately to the formation of "open" helices which later aggregate side by side to form complete helical rods in which the arrangement of protein subunits resembles that in TMV (Lauffer *et al.*, 1967).

Banerjee and Lauffer (1966) proposed that in what we now call low-temperature polymerization, the polymerizing unit is a cyclical trimer of the ultimate chemical unit and the polymerization follows the mathematical formulation for condensation polymerization (Flory, 1936). On the basis of these assumptions, and for solutions sufficiently dilute to be essentially ideal in all respects except their tendency to polymerize, Banerjee and Lauffer derived eq 1 and 2. In these

$$\pi = [(RT)^2/KM_0]c/\pi - RT/K \quad (1)$$

$$\frac{OD - OD_0}{c} = \frac{2 \times 10^{-3}}{2.3} RTH \frac{c}{\pi} - \frac{2 \times 10^{-3}}{2.3} HM_0 \quad (2)$$

equations, π is the osmotic pressure in centimeters of H_2O, c is the concentration of protein in g/1000 g of solvent, K is the equilibrium constant, R is the gas constant which has a value of 84.775 when the units

of pressure are in centimeters of water, T is the absolute temperature, M_0 is the molecular weight of the polymerizing unit, H is the light-scatter factor, and $OD - OD_0$ is the difference between the optical density attributable to light scatter of polymerized and that of unpolymerized protein.

Since the mathematical formulation for condensation polymerization is based on the assumption that the polymerization process is linear and open ended, it is obvious that this theory can be strictly valid only for the initial stages of the polymerization process when formation of double disks is a result. Double disks, D, have been reported with 32 and also with 34 chemical subunits (Markham *et al.*, 1963; Finch *et al.*, 1966). Thus, on the average, one can write 11A = D. The condensation polymerization mechanism is based on the assumption that $-\Delta F^\circ$ is the same for adding to a chain regardless of its length. If, when the unit which closes a ring is added, $-\Delta F^\circ$ is twice that for adding to any shorter chain, then disks should form in sufficient number to distort significantly the number-average weight distribution when π/π_0 (see eq 3 for definition of π_0) is 0.7. The result would be to make the values of π smaller than predicted by simple condensation polymerization mathematics, and this would lead at relatively low values of π to significant deviations from eq 1 in the direction opposite that of the deviations shown in Figures 3 and 4. We are led to the conclusion, therefore, that ring closing is inhibited, possibly because it is accompanied by strain. Thus, when followed by osmotic pressure measurements, the early stages of low-temperature polymerization can be analyzed successfully in terms of condensation polymerization mathematics over a considerable range. However, a concentration of double disks too low to change significantly the number-average weight distribution and, therefore, the osmotic pressure, can increase quite appreciably the light scatter, which depends on the weight-average molecular weight.

One of the purposes of the present investigation is to determine the applicability of the mathematical formulation for condensation polymerization to solutions at concentrations too high to be considered ideal. As a first approximation, one can write

$$\pi_0 = \pi_{0i}(1 + M_0Bc) \tag{3}$$

and

$$OD_0 = OD_{0i}/(1 + 2M_0Bc) \tag{4}$$

where π_0 is the osmotic pressure of unpolymerized protein (trimer) and the subscript i refers to the appropriate value for an ideal solution and where B is the second virial coefficient defined in the manner of Tanford (1961). According to eq 24 of Lauffer (1966), for a hydrated protein, B is $\zeta_0/M_0^2 + \beta_{22}^0/2M_0^2 + x^2/4M_0^2m_3'$. The term, 1000, is omitted because we here express concentration in g/1000 g of solvent instead of grams per gram of solvent. The term, ζ_0, is a hydration factor equal to the weight of solvent bound

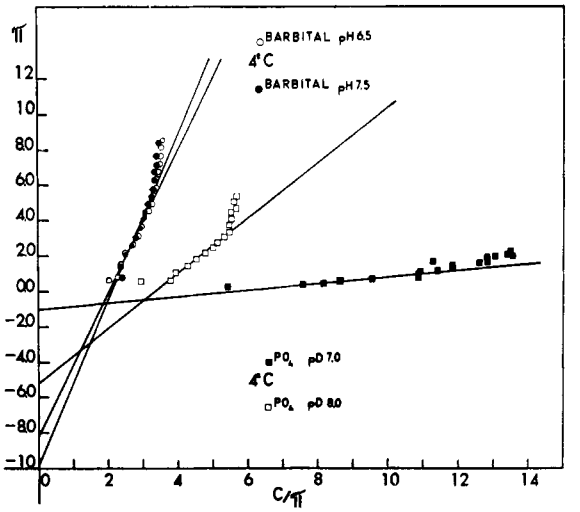

FIGURE 4: π *vs.* c/π for different buffers in water and heavy water. π is in centimeters of water and TMV protein concentration is in milligrams per milliliter. This was done at 4° with pH 7.5 barbital.

by 1 mole of protein, divided by 1000. It is usually small compared with the other terms and will therefore be neglected. $\beta_{22}^0/2$ for spherical particles with no interaction is attributable to excluded volume and is equal to the hydrated volume of a mole of protein divided by 250 times the specific volume of the solvent. If the specific volume of solvent is 1 ml/g, the molecular weight of the protein 52,500 and the hydrated specific volume of the protein 1/1.25, $\beta_{22}^0/2$ has a value of 168 and $M_0\beta_{22}^0/2M_0^2$ has a value of 0.0032. The value of 1/1.25 for the specific volume of protein was derived from the fact that TMV in a sucrose solution of density 1.27 has a hydrated specific volume of 1/1.27 (Schachman and Lauffer, 1949). Since protein is somewhat more hydrated than polymerized protein and since nucleic acid is absent, the hydrated specific volume of protein must be somewhat greater. It is appropriate in the present case to relate β_{22}^0 to the excluded volume term only, neglecting the interaction between protein particles because our treatment deals with interaction in terms of extent of polymerization. The final term is the Donnan contribution. If m_3' is 0.1 and x is 12 (Scheele and Lauffer, 1967), then $x^2/4m_3'M_0^2$ has a value of 13.1×10^{-8} and M_0 times this quantity has a value of 0.00687. Thus, M_0B has a value of 0.01 for unpolymerized protein with a molecular weight of 52,500. When the protein polymerizes, the excluded volume term should change, being more or less inversely proportional to M. The Donnan term should not change when the protein polymerizes because there is no change in charge per unit mass associated with a low-temperature polymerization of TMV protein (Scheele and Lauffer, 1967). Since the Donnan contribution is more than twice the excluded volume contribution and since the excluded volume term should decrease as average molecular weight increases, the over-all value of M_0B should not vary greatly when polymerization takes place. Therefore, we assume that the factor $(1 + 0.01c)$ is appropriate for partially

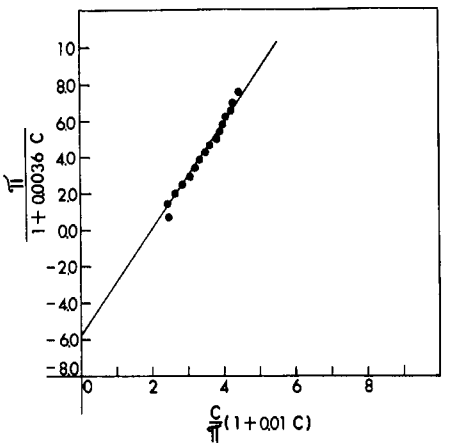

FIGURE 5: A least-squares plot of π *vs.* c/π, both modified for nonideal solutions.

polymerized protein as well as for the unpolymerized material.

By the same line of reasoning, the appropriate factor for the turbidity or optical density measurements is $(1 + 0.02c)$. Thus, eq 1 and 2 must be corrected by substituting $\pi/(1 + 0.01c)$ for π and $(OD - OD_0)(1 + 0.02c)$ for $OD - OD_0$. Equation 2 requires no further change. Figure 1 shows a plot of data corrected in this manner. The straight line fitting the data was determined by the method of least squares. From the slope and the intercept, one should be able to evaluate the light-scatter factor, H, and the molecular weight, M_0, of the polymerizing unit. A value of 55,000 is obtained for M_0, in good agreement with our assumption that the trimer is the polymerizing unit. The light-scatter factor, H, derived from these results, has a value of 7.29×10^{-5}. This must be compared with a value of 8.13×10^{-5} obtained in a similar manner by Banerjee and Lauffer (1966) but with data uncorrected for nonideality. The value of H calculated from the refractive index and the refractive index gradient has a value of 4.27×10^{-5} (Smith and Lauffer, 1967).

It is thus apparent that the turbidity values are higher than those calculated from osmotic pressure using modified eq 2 and 4.27×10^{-5} as H. This can mean that the distribution of polymer length departs from that characteristic of condensation polymerization mathematics. For example, since osmotic pressure depends on number-average molecular weight and turbidity on weight-average molecular weight, a concentration of double disks much greater than that calculated for an open-ended polymer of the same size could be too small to cause a large decrease in osmotic pressure but large enough to cause an appreciable increase in turbidity from those characteristic of condensation polymerization.

Equation 1 requires further modification because of the effect of nonideality on the equilibrium between various polymeric species. Flory (1936) showed that eq 5 derived from probability theory obtained in condensation polymerization. In this equation, N_x is the number of x-mers, N_0 is the initial number of monomers before there was any polymerization, p is the probability that an end of a monomeric unit is combined, $[A_x]$ and $[A]_0$ are the molar concentration of x-mer and the initial molar concentration of monomer. When x is 1

$$N_x/N_0 = p^{x-1}(1 - p)^2 = [A_x]/[A]_0 \quad (5)$$

$$[A]/[A]_0 = (1 - p)^2 \quad (6)$$

Also

$$[A_x]/[A_{x-n}] = p^n \quad (7)$$

where p is independent of x and n.

Consider the reaction, $[A_{x-n}] + [A_n] = [A_x]$. Consistent with the assumptions of condensation polymerization, K is a constant independent of x and n. From eq 7 and 8

$$[A_x]/[A_{x-n}] = (\gamma_{x-n}/\gamma_x)K\gamma_n(A_n) = (\gamma_{x-n}/\gamma_x)(K\gamma[A])^n \quad (8)$$

$$p = (\gamma_{x-n}/\gamma_x)^{1/n}K\gamma[A] \quad (9)$$

Now, $(\gamma_{x-n}/\gamma_x)^{1/n} = \gamma_{x-1}/\gamma_x$, if γ_{x-1}/γ_x is independent of x. By making this substitution and introducing eq 6, one obtains $p = (\gamma_{x-1}/\gamma_x)\gamma K[A] = (\gamma_{x-1}/\gamma_x)\gamma K[A]_0(1 - p)^2$ or

$$(\gamma_{x-1}/\gamma_x)\gamma K = p/(1 - p)^2[A]_0 \quad (10)$$

Equation 10 is the equilibrium expression for condensation polymerization involving nonideal solute species. The equation used by Banerjee and Lauffer to derive eq 1 was the same except that all activity coefficients were equal to one. Thus, eq 1 must be further modified by replacing K by $(\gamma_{x-1}/\gamma_x)\gamma K$.

It is probable that γ_{x-1}/γ_x does not differ greatly from 1 and, therefore, we will make the assumption that it is 1. It is possible to calculate the value of γ, the activity coefficient of the monomeric species, before any polymerization takes place, from the excluded volume effect. While the value of γ probably changes somewhat after some polymerization occurs, it is difficult to calculate this change. It is assumed that the change is small and that, therefore, as an approximation, γ remains constant (eq 11). It is apparent, therefore, that K in eq 1 must be multiplied by $1 + 0.0064c$. Thus, the total correction of eq 1 for nonideality must be to divide π on the left side of the equality by $1 + 0.0036c$ and to multiply c/π on the right by $1 + 0.01c$.

$$\gamma = 1 + \beta_{22}^0[A]_0 = 1 + \beta_{22}^0 c/M_0 = 1 + 2 \times 168c/52{,}500 = 1 + 0.0064c \quad (11)$$

When the data for the polymerization in aqueous solutions of barbital buffer at pH 7.5 and at 4° (expt 12) are corrected in this manner, the results are as shown in Figure 5. These are the data initially shown in the

uncorrected form on Figure 4. The straight line was fitted by the method of least squares. The value of M_0 corresponding to this analysis is 46,000 and the value of K is 4.11×10^3. Somewhat more serious than the small deviations of the data from a straight line is the fact that the calculated molecular weight for the polymerizing unit is somewhat too low. The molecular weight must be some integral multiple of 17,500; these data and many others suggest that multiple is 3. Thus, the value of 46,000 obtained from this analysis must be compared with a probable true value of 52,500.

There is no reason why the experimental points should actually have equal reliability over the entire range of concentration. The data at high concentrations and, therefore, high osmotic pressures, have been altered most by our procedure for correcting for departure from ideality and are therefore suspect because our procedure might introduce inaccuracies. The data at the lower concentrations and therefore lower osmotic pressures are suspect on three counts. First, it is common experience that the fractional error in measuring osmotic pressures in the neighborhood of 1 cm of water is higher than for higher pressures. Second, we know that at very low concentrations, trimer dissociates to monomer (Ansevin and Lauffer, 1959); at concentrations of 1 mg/ml there is evidence for a little dissociation. Even a small degree of dissociation will contribute a considerable error in π. Third, as illustrated by a comparison of the second and third graphs from the top in Figure 2, when concentrated solutions of TMV protein are diluted, dissociation to the equilibrium condition apparently takes place slowly. Especially at lower concentrations, the osmotic pressure can be too low if insufficient time for equilibration was allowed. It was our standard practice, except when otherwise indicated, to allow 24 hr for equilibrium to be attained after dilution was made. It is our belief that this usually was a sufficient time. Nevertheless, if in some instances equilibrium was not yet attained, the effect would be to make the measured values of π at lower concentrations too low. Errors of all sorts in π contribute to errors in calculated c/π. Thus, the data in the middle of the range are the most reliable. One can fit a straight line to the middle data with the arbitrary restriction that M_0 is 52,500. In the case of the data shown in Figure 5, this line also fits the data at the highest concentrations (in the neighborhood of 30 mg/ml) but three of the four data at lowest concentrations lie somewhat above the line. This deviation, however, is in the direction expected if there is some dissociation to monomer at the lower concentrations.

We have adopted the practice of correcting data in the manner illustrated and then calculating K from the data in the middle of the range when M_0 is assigned the arbitrary value of 52,500. The values of K listed in Table I were obtained in this manner. For most of our data, this type of forcing to a single mechanism seems to be acceptable; in three instances—0.1 ionic strength PO_4 buffer, pH 7.5, 20° (expt 4b); 0.1 ionic strength PO_4 buffer in D_2O, pD 8.00, 4° (expt 8); and 0.1 ionic strength PO_4 buffer, pH 7.5, 25° (expt 16)—the forced fit is not good and the data, after correction for nonideality, fit eq 1 better when a value of M_0 significantly higher than 52,500 is assigned. Paired experiments (4a and b) in phosphate buffer at pH 7.5 but at temperatures of 4 and 20° are thus worthy of note because they could indicate that with the same solution of TMV protein in the same solvent, two different equilibria can be obtained by just changing the temperature from 4 to 20°. At 4°, TMV protein has an equilibrium in which trimer is predominant; while at 20°, a new equilibrium is suggested in which a hexamer predominates. There might, in fact, be such, but the data which suggest it were obtained at adversely low values of π. It is more conservative, therefore, to retain the simpler hypothesis, namely, that a single polymerization process is involved in all of the studies herein reported and that deviations are the result of error, until conclusive evidence demonstrates the existence of additional stable intermediates.

It was not possible to analyze certain of our data in terms of the above mechanism. Two of the cases in point were the experiment in aqueous phosphate buffer at pH 7.5 in which the measurements were made only 2 hr after dilution (expt 3) and also the experiment in pyrophosphate buffer at pH 7.3 and 25° where measurements were also made very shortly after dilution (expt 14). In both of these cases the probable reason is that the equilibrium values of the molecular weight had not been achieved. Neither was it possible to apply this type of analysis to those experiments involving phosphate buffer in D_2O at temperatures of 8° and higher (expt 6, 7, and 9). Inspection of graphs 6, 7, and 9 of Figure 2 will show that in all of these cases the molecular weight was unusually high and there was no evidence at all of dissociation upon dilution down to the lowest concentration studied. In two of these three cases, the number-average molecular weight obtained by extrapolating π/c to infinite dilution was so high that a formulation based on Flory's mathematical treatment of condensation polymerization could not possibly apply.

The values of $\bar{M}_n$ found in Table I and in the legend of Figure 2 were obtained by extrapolating π/c to infinite dilution. This procedure reflects strongly the actual values of π/c at the lowest concentrations studied. For the reasons presented below, such extrapolated values have descriptive value only and are of no theroetical significance. There are two recognized difficulties with this extrapolation for our system. First, the simplified form of eq 1, applicable to solutions which are ideal except for their tendency to polymerize, can be written as

$$(\pi_i + RT/K) = (RT/K)(RT/M_0)(c/\pi_i) \qquad (12)$$

From this, one obtains

$$(\pi_i/c) = (RT/M_0)(1 - \pi_i/(RT/K)) + (\pi_i/(RT/K) - \text{etc.}) \qquad (13)$$

When RT/K is much greater than π_i, the squared and higher order terms can be dropped from eq 12

and also RTc/M_0 can be substituted for π_i. Under these circumstances one obtains a linear relationship between π_i/c and c (eq 14). When eq 3 is introduced,

$$\pi_i/c = (RT/M_0)(1 - kc/M_0) \quad (14)$$

one obtains eq 15.

$$\pi/c = (RT/M_0)(1 + (M_0B - K/M_0)c) \quad (15)$$

Correcting K for nonideality makes no difference here because we are dropping all terms in c with powers higher than 1. Thus, when K is considerably smaller than RT, π/c will be a linear function of c at all concentrations (2–3 mg/ml) equal to and less than those at which π is 1. However, at higher values of K, the relationship becomes linear only at very much lower values of π and, therefore, of c. It is thus evident that when K is large, the linear extrapolation of π/c *vs.* c is not justified on theoretical grounds and there is no reason to expect a value of $\bar{M}_n$ obtained by such an extrapolation to equal M_0. The value of $\bar{M}_n$ found in Table I and Figure 2 are in reasonable agreement with an M_0 of 52,500 when K is 5×10^3 or less, and much higher when K is twice that value or more. This is quite in accord with our theoretical treatment. The second difficulty inherent in linear extrapolation was pointed out by Banerjee and Lauffer (1966). At exceedingly low concentrations well below 1 mg/ml in the temperature range 0–6°, TMV A protein dissociates almost completely from trimer to monomer. If it were possible to obtain accurate osmotic pressure measurements in this range, the actual data would not even follow the extrapolation based on eq 14.

Except for those experiments for which there is a reason to believe that equilibrium was not attained, our data obtained in aqueous systems up to concentrations of about 30 mg/ml can be interpreted quite successfully in terms of the theory of condensation polymerization modified in the manner described to account for departure from ideality. The values of K shown in Table I do not change greatly when the pH is changed at constant temperature, both in phosphate buffer and in barbital buffer. Furthermore, the various values of K at 4° are close to the value found by Banerjee and Lauffer (1966) for comparable conditions of temperature and pH but for data obtained with solutions no more concentrated than 9 mg/ml and analyzed in terms of the theory for ideal solutions.

While the accuracy attainable in computations with so few data is limited, it is possible to calculate the values of $\Delta H°$ and $\Delta S°$ for polymerization in 0.1 ionic strength phosphate buffer at pH 6.5 from expt 1 and 15 and for polymerization 0.1 ionic strength phosphate buffer at pH 7.5 from expt 2 and 4b. Values for $\Delta H°$ and $\Delta S°$ at pH 6.5 are +34,000 cal/mole and +139 eu for pH 6.5 and +19,000 cal/mole and +86 eu for pH 7.5. Banerjee and Lauffer (1966) obtained 30,000 cal/mole for $\Delta H°$ and 124 eu for polymerization in 0.1 ionic strength phosphate buffer at pH 6.5. It can thus be seen that the essential features of low-temperature polymerization of TMV protein have been confirmed.

The results obtained with D_2O are more difficult to interpret. However, a few conclusions can be drawn. For the two experiments done at 4° in buffers at pD 7.0 and 8.0, the only ones which can be analyzed by our method, it can be observed by inspecting the right-hand column of Table I that polymerization in D_2O is strongly dependent upon pD in contrast to the results at 4° for polymerization in water. Furthermore, at pD 8, the equilibrium constant at 4° is higher than that at pH 7.5 and 4°. This shows that polymerization takes place more readily in D_2O than in H_2O. Khalil and Lauffer (1967) had arrived at the same general conclusion for high-temperature polymerization. The fact that even at the low temperature of 4°, polymerization in D_2O is strongly sensitive to pD suggests that at least at pD 7.0 the reaction is more akin to high-temperature than to low-temperature polymerization.

Further analysis lends support to this conclusion. Inspection of expt 9 (Figure 2) shows that π/c increases with concentration. One can interpret this by assuming that in a buffer at pD 8.0 at 20° the state of polymerization is essentially constant with double disks predominating at all values of concentration studied and that the increase in π/c as concentration is increased is attributable to the Donnan effect. The excluded volume term should be negligible because of the high molecular weight. If the negative charge per monomer (mol wt 17,500) is 4 at pD 8, the contribution of the Donnan effect in 0.1 μ buffer to π/c, when c is 30 mg/ml should be 0.097. The contribution of RT/M should be 0.044 if the protein is assumed to be in the form of double disks with a molecular weight of 560,000. The sum of these two terms is in quite good agreement with the actual value of π/c obtained at a concentration of 30 mg/ml. The assumption that the protein is in the form of double disks at pD 8 is reasonable in view of the fact that it was demonstrated by Lauffer *et al.* (1967) that at pH 7.5, double disks are formed during polymerization in an aqueous medium. At pD 7, however, at temperatures of 8 and 11.7° the observed values of π/c at 30 mg/ml are significantly lower than the calculated Donnan term alone. Banerjee and Lauffer (1966) reported that the Donnan term could not be observed in osmotic pressure experiments on TMV, in which the protein is arranged in a helical fashion comparable with that of the end product of high-temperature polymerization (Lauffer *et al.*, 1967). This result, therefore, indicates that the reaction at pD 7 is high-temperature polymerization.

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Human Serum Albumin. Tyrosyl Residues and Strongly Binding Sites*

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ABSTRACT: The binding of dodecanoate and of other fatty acids was found to affect the properties of some of the tyrosyl residues in human serum albumin. The binding gave rise to a long-wavelength shift of the tyrosyl absorption spectrum; no involvement of the tryptophyl residue was detected. The magnitude of the 290-mμ difference absorbancy increased with increasing binding ratio. Concurrently, the Moffit a_0 parameter was less negative but the b_0 parameter remained invariant. The accessibility of tyrosyl residues to methanol perturbation decreased linearly with increasing binding ratios. The saturation of all seven strongly binding sites resulted in the masking of approximately five tyrosyl residues. The ionization of tyrosyl residues could be hindered as well as reverted by the binding of long-chain fatty acids; the magnitude of this effect depended also on the binding ratio. Difference titration analysis showed that a total of about six tyrosyl residues are affected in the alkaline solution by the binding of dodecanoate. The results are interpreted in terms of two sets of strongly binding sites, consisting of two and of five sites each. It is concluded that three tyrosyl residues are located in, or closely associated with, each set of the strongly binding sites in human serum albumin.

A wealth of information has been accumulated in the past concerning the binding properties of serum albumins. Since the matter has been comprehensively reviewed in recent years (Foster, 1960; Putnam, 1965), no attempt will be made here to treat it in detail. Based mainly on studies with detergents, it is considered that there are two major sets of the binding sites: a small number of preexisting and strongly binding sites, and a much larger number of weaker sites which are formed as a result of a cooperative alteration of albumin conformation following the saturation of the strongly binding sites. Thus the interactions taking place at the preexisting sites appear to prepare the molecule of serum albumin for the subsequent conformational transition.

Estimates of the number of strongly binding sites are somewhat variable, possibly depending on the nature of the albumin preparation and/or of the ligand employed. For anionic detergents there are 10–12 such sites (Yang and Foster, 1953; Pallansch and Briggs 1954; Decker and Foster, 1966); they appear to be essentially equivalent, although this has been questioned recently by Reynolds *et al.* (1967). For the long-chain fatty acids the heterogeneity of the strongly binding sites has been rather convincingly documented. Goodman (1958), who was the first to study the binding properties of human serum albumin, carefully freed of residual fatty acids, found that his experimental data could be best accounted for by assuming the existence of three classes of binding sites. The strongest class consisted of two sites, and the intermediate class of five sites; in addition there were more than twenty weaker sites. In an earlier study, Teresi and Luck (1952) found five strongly bind-

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