Polymerization–Depolymerization of Tobacco Mosaic Virus Protein. X. Effect of D_2O*

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ABSTRACT: The endothermic reversible polymerization of tobacco mosaic virus (TMV) protein was examined in 99.8% D_2O as well as in H_2O with phosphate buffer at different pH or pD values and different ionic strengths. The reaction was followed by turbidity determined from optical density measurements at 320 mμ with a Beckman DU spectrophotometer. Sigmoidal graphs of optical density *vs.* temperature were obtained. The D_2O data are qualitatively the same as the H_2O data under identical conditions of ionic strength and pH; however, the characteristic temperature (T^*) for deuterated protein is always lower than that for the protonated one. T^* increases linearly with pH or pD and decreases with increasing ionic strength. The calculated thermodynamic parameters ($\Delta H°$ and $\Delta S°$) decreased on the average as ionic strength increased. The values in D_2O are higher if compared with those in water at the same pH and the same ionic strength. Moreover, $\Delta H°$ and $\Delta S°$ increase with decrease in pH or pD.

In the preceding two publications (Smith and Lauffer, 1967; Shalaby and Lauffer, 1967), it was shown that the endothermic polymerization of TMV protein can be followed by estimating turbidity through measurements of optical density at a wavelength of 320 mμ with a Beckman DU spectrophotometer. It was shown further that this reaction can be carried out reversibly and that it can be interpreted in terms of the mathematics applicable to condensation polymerization. Because it had been demonstrated earlier (Stevens and Lauffer, 1965) that water is released during polymerization, the effect on the polymerization reaction of various chemicals which alter the structure of water was investigated (Shalaby and Lauffer, 1967). A logical extension of this investigation is to study the effect of substitution of heavy water for water. Accordingly, in the present study, polymerization of TMV protein in D_2O as well as in H_2O was investigated by the same method at various pH or pD values and at various values of ionic strength.

Materials and Methods

Virus and Protein. The common strain of TMV was purified by differential centrifugation followed by further purification by fluorocarbon (Paster, 1956). The resulting virus solution is colorless at very high concentration, but it changes to iridescent yellowish-green or iridescent blue on dilution.

The protein was prepared from the virus by the acetic acid method of Fraenkel-Conrat (1957). Part of the protein was suspended in H_2O, the rest in 99.8% D_2O. A few drops of NaOH solution at pH 11 were added to the protein in H_2O and the same amount of NaOD at pD 11 was added to the protein in D_2O. After 12 hr, both solutions were adjusted to pH or pD 8, then centrifuged at 40,000 rpm.

Preparation of the Buffer. Stock solutions of potassium phosphate buffers of ionic strength 0.4 were prepared. The pH and pD values were determined with a Beckman pH meter. To obtain pD, 0.4 was added to the reading of the pH meter (Glasoe and Long, 1960). All solutions were prepared in glass volumetric flasks and then transferred to plastic bottles in the case of aqueous buffers and deuterium-equilibrated bottles in the case of deuterated buffers or proteins.

Experimental Procedure. Buffered TMV protein at the required ionic strength was exposed to sudden heating (25°) followed by cooling (4°), then the solution was centrifuged at 4° at 40,000 rpm for 2 hr to remove any denatured or irreversibly aggregated protein. For further purification, protein and buffer solutions were filtered through a Millipore filter of pore size 0.45 μ with the use of a Swinny adapter. The concentrations of the protein solutions were determined as described previously (Smith and Lauffer, 1967) and were adjusted to 1 mg/ml.

The procedure reported by Smith and Lauffer (1967) was followed for measuring turbidity. The temperature was measured by a Tel-Thermometer equipped with a probe immersed in one of the cells through a hole in the

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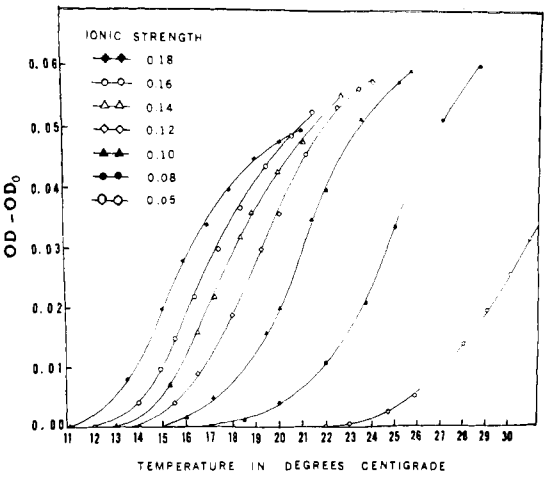

FIGURE 1: Polymerization of TMV protein in H_2O.

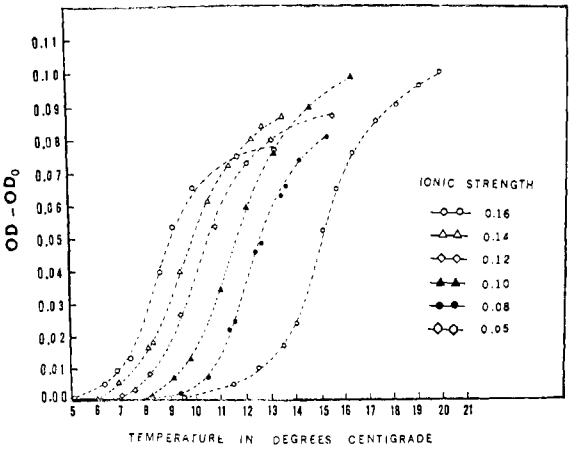

FIGURE 2: Polymerization of TMV protein in D_2O.

cover of the cell compartment.

Results

A quantitative study of the polymerization reaction as a function of ionic strength (0.18, 0.16, 0.14, 0.12, 0.1, 0.08, and 0.05) and pH (6, 6.2, 6.4, 6.6, 6.8, and 7) was carried out in the manner described previously. Figures 1 and 2 show an example for pH and pD 6.6. The characteristic temperature (T^*) defined by Shalaby and Lauffer (1967) was chosen to describe the results. The relation between T^* and ionic strength for various pH and pD values, except at pH 6.6, is linear. When T^* is plotted against pH or pD for different ionic strengths, the D_2O data all fit straight lines essentially parallel to each other, as do those of H_2O, except at $\mu = 0.05$, but $dT^*/\mathrm{dpD} > \mathrm{d}T^*/\mathrm{dpH}$. At pD 6.2, there was depolymerization at very low ionic strengths only (0.05 and 0.025).

The data, when sufficient were available, were analyzed in terms of eq 8 and 9 or, when more limited, in terms of eq 6 and 7 of Smith and Lauffer (1967). These equations were derived by combining simple light-scatter theory with the mathematics developed by Flory (1953) for linear condensation polymerization. The index of refraction of D_2O (1.338) is nearly the same as that of H_2O (1.333). It was assumed, therefore, that the specific refractive increment of protein in D_2O is the same as in H_2O. Thus, the same light-scatter factor (H) was used for D_2O and H_2O. The data were fitted to the equations in the manner described by Shalaby and Lauffer (1967). The constants obtained by the curve-fitting procedures are shown in Table I.

Discussion

Effect of pH and Ionic Strength. In the present study, as in those of Shalaby and Lauffer (1967) and Smith and Lauffer (1967), the most dependable measure of change is the characteristic temperature (T^*). With the exception of data obtained at high pH and low ionic strength, T^* increases regularly with pH or pD and decreases regularly with ionic strength. For most of the data, $\mathrm{d}T^*/\mathrm{dpH} = 8.66$ and $\mathrm{d}T^*/\mathrm{dpD} = 24$. Except as noted before, these values are essentially independent of ionic strength. $\mathrm{d}T^*/\mathrm{d}\mu$ is -59 in water, -54 in heavy water and is approximately independent of pH or pD. Measurable polymerization–depolymerization in water occurs in the pH range 6.0–6.6 and in heavy water in the pD range 6.4–7.0.

In two respects, at least, the effect of increasing electrolyte concentration resembles salting out of proteins. First, the effect is essentially independent of pH (or pD). Second, as shown by Shalaby and Lauffer (1967), substitution of thiocyanate for phosphate results in suppression of polymerization. The thiocyanate ion falls far below the phosphate ion in the Hofmeister series; it is a much less effective salting-out ion than phosphate.

In contrast with T^*, ΔH° and ΔS° vary erratically. As was pointed out by Shalaby and Lauffer (1967) individual measurements can deviate as much as 15 or 20% from mean values. The data of Table I exhibit numerous irregularities in ΔH° and ΔS°. Nevertheless, definite trends are discernible. On the average, increase in pH or pD and ionic strength reduce both ΔH° and ΔS°. When data at all ionic strengths are averaged, $\mathrm{d}\ln \Delta H^\circ/\mathrm{dpH} = -0.767$ and $\mathrm{d}\ln \Delta S^\circ/\mathrm{dpH} = -0.748$. Qualitatively at least, the data of Shalaby and Lauffer agree, but slopes are steeper. When calculated in a similar manner, $\mathrm{d}\ln \Delta H^\circ/\mathrm{dpD} = -1.554$ and $\mathrm{d}\ln \Delta S^\circ/\mathrm{dpD} = -1.480$. When data at all pH values are averaged, $\mathrm{d}\Delta H^\circ/\mathrm{d}\mu = -980{,}000$, $\mathrm{d}\Delta S^\circ/\mathrm{d}\mu = -3000$ for studies carried out in water. The data of Shalaby and Lauffer are in reasonable agreement quantitatively. For results obtained in heavy water, $\mathrm{d}\Delta H^\circ/\mathrm{d}\mu = -635{,}000$ and $\mathrm{d}\Delta S^\circ/\mathrm{d}\mu = -1180$. In general, the data from which these slopes were derived fit the graphs reasonably well except at ionic strength 0.075 or 0.08. In case of $\mathrm{d}\ln \Delta H^\circ/\mathrm{dpD}$ and $\mathrm{d}\ln \Delta S^\circ/\mathrm{dpD}$, the fit is good except at pD 6.4. Furthermore, sufficient data have

TABLE I: Polymerization of TMV Protein in H_2O and D_2O.

pH	ΔH (kcal)	ΔS (eu)	τ_m	T^*
		$\mu = 0.18$		
6.6	180	667	1.57	15.8
6.8	77.3	295	1.39	
pD				
6.8	190	701	0.155	12
7.0	103	388	0.146	17
		$\mu = 0.16$		
pH				
6	215	789	0.272	11.65
6.2	202	738	0.269	14.0
6.4	211	765	0.244	15.1
6.6	199	724	0.123	16.5
6.8	98.8	368	0.099	
pD				
6.6	347	127	0.183	8
6.8	198	726	0.161	13.3
7.0	164	599	0.125	17.5
		$\mu = 0.14$		
pH				
6	294	1065	0.264	12.4
6.2	205	744	0.238	15.3
6.4	243	873	0.233	16.1
6.6	200	723	0.134	17.6
6.8	215	764	0.052	
pD				
6.4				
6.6	283	1039	0.214	8.8
6.8	231	839	0.198	13.8
7.0	175	636	0.094	17.9
		$\mu = 0.12$		
pH				
6	312	1121	0.246	14
6.2	312	1120	0.246	16.1
6.4	182	661	0.226	16.9
6.6	210	756	0.143	18.85
pD				
6.4	219	814	0.181	7.5
6.6	280	1026	0.210	9.4
6.8	303	1085	0.181	14.65
7.0	170	616	0.090	19.3
		$\mu = 0.1$		
pH				
6	391	1388	0.246	15.1
6.2	231	827	0.157	17.5
6.4	280	994	0.204	18.2
6.6	212	755	0.148	20.6
pD				
6.4	258	950	0.180	8.3
6.6	299.5	1089	0.236	10.7
6.8	202	733	0.158	15.3
		$\mu = 0.08$		
pH				
6	260	933	0.27	16
6.2	228	812	0.203	19
6.4	222	791	0.187	19.5
6.6	183	650	0.167	24.3
pD				
6.4	242	893	0.215	9
6.6	361	1302	0.199	11.7
6.8	242	871	0.165	16
7.0	157	566	0.115	21.8
		$\mu = 0.05$		
pH				
6	400	1397	0.20	20
6.2	399	1390	0.256	20.8
6.4	278	975	0.196	12.8
6.6	203	705	0.104	29.4
pD				
6.4	253	927	0.255	10.6
6.6	256	927	0.246	13.6
6.8	233	830	0.187	19

been obtained for polymerization in 0.1 μ, pH 6.5 phosphate buffer to provide reliable values of ΔH° and ΔS° (Smith and Lauffer, 1967; Shalaby and Lauffer, 1967). They are $\Delta H^\circ = 206{,}000 \pm 6000$ cal/mole and $\Delta S^\circ = 739 \pm 22$ eu/mole.

It is instructive to attempt interpretation of the present findings in terms of the model proposed by Lauffer (1966b), already shown to be rather successful in mimicking many aspects of the polymerization of TMV A protein. The model is completely inert except that it has on its surface certain centers capable of interacting with water and binding hydrogen ions. These centers are assumed to be composed of aliphatic or aromatic side chains and carboxyl and amino groups, such as found in TMV protein. Because simple organic molecules are susceptible to salting out, the centers on the model should also be. When the model polymerizes, these water-interacting centers are transferred from an aqueous to an organic environment where there is no longer interaction with water. The ΔH° per "bond" formed in this manner is assumed to result in the model entirely from the release of water

and to equal $n\Delta H^\circ_w$, where n is the number of water molecules released when a bond is formed and ΔS°_w is the entropy per mole associated with release of water. This will be essentially true even though the model binds hydrogen ions on polymerization, because ΔH° for hydrogen ion binding by carboxylate ions in equilibrium with phosphate buffer is very low (Ansevin *et al.*, 1964). For polymerization of the model, we assume that

$$\Delta S^\circ = n\Delta S^\circ_w + x\Delta S^\circ_H + \Delta S^\circ_S \quad (1)$$

ΔS°_S is what might be called the Sackur–Tetrode entropy, ΔS°_w is the entropy per mole associated with release of water, ΔS°_H is the entropy per mole for binding hydrogen ions, and x is the number of hydrogen ions bound per mole of bond formed. In phosphate buffer, ΔS°_H is approximated by $-11.7 + 4.6(7.2 - \text{pH})$.

Salting out of small organic molecules depends on the effect of the solute on the dielectric constant of the solution and on the radii and hydration of the salt ions. It is conventional to interpret salting out as an effect on f, the activity coefficient of the solute.

$$RT \ln f = A\beta'\mu \quad (2)$$

In eq 2, A is a constant characteristic of the salt, β' is a constant characteristic of the solute, and μ is the ionic strength. If ions affect the activity coefficient of exposed but not of bonded centers on the model, then, since the forward reaction in polymerization is bimolecular, $K_a f^2$ replaces K in eq 2 or 2a of Smith and Lauffer (1967). K_a is the equilibrium constant in terms of activity. From eq 2, 3, and 4, eq 5 and 6 can be derived.

$$-RT \ln K_a = \Delta F^\circ = \Delta H^{\circ\circ} - T\Delta S^\circ \quad (3)$$

$$K = K_a f^2 \quad (4)$$

$$T^* = (\Delta H^{\circ\circ} - 2A\beta'\mu)/(\Delta S^\circ - R \ln K^*) \quad (5)$$

$$dT^* = \frac{[(\Delta S^\circ - R \ln K^*)d(\Delta H^{\circ\circ} - 2A\beta'\mu) - (\Delta H^{\circ\circ} - 2A\beta'\mu)d\Delta S^\circ]}{(\Delta S^\circ - R \ln K^*)^2} \quad (6)$$

In these equations, K^* is that constant value of the equilibrium constant (K) at which the characteristic temperature (T^*) is evaluated, and $\Delta H^{\circ\circ}$ is the "true" enthalpy and is related to the enthalpy in eq 8 of Smith and Lauffer (1967) by the relationship $\Delta H^\circ = \Delta H^{\circ\circ} - 2A\beta'\mu$. It should be noted that the entries in Table I are ΔH°.

Consider polymerization of the model in 0.1 ionic strength phosphate buffer at pH 6.5. Let ΔH° and ΔS° be 206,000 cal and 739 eu, respectively, and assign a value of 17 for ln K^*, the value corresponding to T^*. Let x have the value of 3 at pH 6.5 (Scheele and Lauffer, 1967). It follows, then, from eq 6 that

$$\partial T^*/\partial\mu = -2A\beta'/705 \quad (7)$$

and

$$\partial T^*/\partial \text{pH} = \frac{\left(705 - 206{,}000\,\frac{\Delta S^\circ_w}{\Delta H^\circ_w}\right)\frac{\partial \Delta H^\circ}{\partial \text{pH}} - 206{,}000\left(-8.5\frac{dx}{d\text{pH}} - 4.6x\right)}{(705)^2} \quad (8)$$

Different constants will obtain under other conditions of polymerization.

In accordance with the postulates made concerning the model, $n = \Delta H^{\circ\circ}/\Delta H^\circ_w$. Lowering of pH or pD and increase of ionic strength both have the effect of lowering the characteristic temperature. However, the first has the effect of increasing n and the second of decreasing n. If the major factor involved in the effect of reducing pH and increasing ionic strength were simple electrostatic repulsion, then one ought not to observe this difference. Therefore, we conclude that alteration of potential is not the dominant factor involved. The equations of the preceding paragraph permit one to examine the effect of ionic strength in terms of salting out. Since $\partial T^*/\partial\mu = -59$, one can readily calculate with eq 7 that $A\beta' = 2.1 \times 10^4$. Since $A\beta'/2.3RT$ is equal to the salting-out constant (K_S') one obtains a value of 15 for it. This is an order of magnitude greater than salting-out constants for most proteins, but Ho and Waugh (1965) obtained a value twice this large for α_s-casein.

The high value for K_S' is not necessarily unreasonable for our system. The molar increase in entropy upon polymerization of TMV A protein attributable to release of water molecules is 40–50 times the entropy change for transferring 1 mole of simple hydrocarbon from water to an organic environment. Thus, for each mole of half-bond, one might expect an effect 20–25 times that of a simple organic molecule. The reasoning behind this is that the salting-out constant depends upon the effect per mole of solute on the dielectric constant of the solution. If one transfers 20–25 moles of organic residue/mole of face during polymerization, then one would expect the dielectric constant effect per mole of face to be 20–25 times that for a simple molecule. Simple organic molecules have salting-out constants in sodium chloride between 0.1 and 0.2. In phosphate they would be expected to have values perhaps three times as high. Thus, one can rationalize a salting-out constant for our system as great as 15. Therefore, the idea that the effect of ionic strength on polymerization of TMV A protein is an aspect of salting out is tenable.

Von Hippel and Wong (1964) reported that various ions have different effects on the conformation of macromolecules. The following sequence was obtained: potassium phosphate (at pH 6.6) $(NH_4)_2SO_4$, NaCl, KCl, LiCl, NaBr, $CaCl_2$, and KSCN. Salts to the left

of NaCl stabilize, but those to the right destabilize the native conformation. In a sense, stabilizing the normal conformation of a macromolecule is analogous to favoring polymerization in our system, because both during depolymerization of TMV protein and during unfolding of the usual configuration of a macromolecule, new surface elements are exposed to the aqueous solvent. Thus, the findings of Von Hippel and Wong (1964) correlate with those reported here and with the effect of KSCN reported by Shalaby and Lauffer (1967).

Ansevin *et al.* (1964) and Scheele and Lauffer (1967) showed that hydrogen ions are bound when TMV A protein polymerizes. Apparently, one hydrogen ion is bound for each mole of chemical unit polymerized (Scheele and Lauffer, 1967) when the reaction is carried out at pH 6.5. The polymerizing unit is a trimer (Banerjee and Lauffer, 1966). This is the reason for assuming that $x = 3$ for our model for polymerization at pH 6.5. The results of Scheele and Lauffer also show that the number of hydrogen ions bound decreases as pH is increased. At pH 6.5 dx/dpH has a value of -2.4. We assign the same value to our model. When one substitutes 8.66 for dT^*/dpH ($\Delta H^\circ d \ln \Delta H^\circ/dpH$) or $-158{,}000$ for $d\Delta H^\circ/dpH$ (-2.4 for dx/dpH), and 3 for x in eq 8, one obtains a value of 278 for ΔH°_w ΔS°_w. This corresponds to the "melting temperature" for interaction with water in the model (Lauffer, 1966b). It is reasonably close to the melting temperature of type III centers, 287°. With this value for $\Delta H^\circ_w/\Delta S^\circ_w$ and the assumptions stated above, one can calculate $d\Delta S^\circ/dpH$ from $d\Delta H^\circ/dpH$. A value of -561 eu/mole of bond is obtained. The value calculated from ΔS°-($d \ln \Delta S^\circ/dpH$) for TMV A protein is -552 eu.

The ratio, $\Delta H^\circ_w/\Delta S^\circ_w$, can be evaluated in another manner, from ΔH° and ΔS° at pH 6.5 in 0.1 μ phosphate buffer. The values are 206,000 cal/mole and 739 eu, respectively. $\Delta H^\circ = n\Delta H^\circ_w - 2A\beta'\mu$, where $A\beta'$ has a value of 21,000 cal/mole. ΔS° is given by eq 1. $x\Delta S^\circ_H$ has a value of 3×-8.5 under the specified conditions, and ΔS°_S is assumed to have a value of -100 (Lauffer, 1966b). In this manner, one obtains a value of 243 for $\Delta H^\circ_w/\Delta S^\circ_w$. This number is considerably different from the 278 calculated above. The difference, however, does not indicate nonvalidity of the model. The total ΔH° and ΔS°, from which the 243 was calculated, are assumed to be the sum of values for type II and type III centers. If ΔH° and ΔS° on type III centers change with pH but those on type II centers do not, then this would be the expected result. In the model the polymerization achieved by increasing concentration at 4° is attributed to type II centers; that obtained by raising the temperature from 4 to 20° is attributed to the sum of type II and type III centers. Scheele and Lauffer (1967) have shown that there is no change in charge during low-temperature polymerization, but Ansevin *et al.* (1964) have shown that there is a charge change during the polymerization achieved by raising the temperature. Thus, one would expect only type III centers to be sensitive to pH and changes in ΔH° and ΔS° with change in pH should reflect properties of type III centers only. The results of Banerjee and Lauffer (1966) bear this out; low-temperature polymerization is essentially independent of pH.

In a general sort of way at least, the detailed results obtained by studying polymerization as a function of pH and of ionic strength can be rationalized in terms of the model proposed by Lauffer (1966b).

TMV protein contains relatively high proportions of amino acids with nonpolar side chains. The tendency of nonpolar groups to adhere to one another in aqueous environments has been attributed to "hydrophobic bonds" by Kauzmann (1959). The word hydrophobic bond is misleading, because under some circumstances at least, no true bonds occur. Lauffer (1966b) suggested that "entropic union" would describe the actual situation better than hydrophobic bond. The probable origin of the large negative entropy change when a protein surface is transferred from an organic to an aqueous environment was discussed by Frank and Evans (1945). When a nonpolar molecule is present in water, the water molecules in the immediate vicinity arrange themselves into quasi-crystalline structures or "icebergs." Their formation leads to a large decrease in entropy. Therefore, the large loss of entropy when nonpolar groups are exposed to water can be attributed to a change in the structure of water. This makes ($-T\Delta S^\circ$) positive and, at sufficiently high temperatures, results in a positive ΔF°. Thus, when two apolar groups in an aqueous environment come out of water and come into contact with each other, some of the ordered water will be released, which increases ΔS° and makes ΔF° negative. On the other hand, at lower temperatures, when $\Delta H^\circ - T\Delta S^\circ$ is negative for water binding, water will be a thermodynamically favorable environment and depolymerization is favored.

Effect of Substitution of H_2O by D_2O. Water and heavy water are closely matched in most properties. Therefore, it was expected that D_2O should have more or less the same effect on TMV protein polymerization as H_2O. Liquid water exists in the form of monomers and clusters, according to Frank and Wen (1957). Chemical and physical considerations led Némethy and Scheraga (1964) to suggest that there is more structured order in D_2O than in H_2O, and the size of the clusters is greater for D_2O than for H_2O at any given temperatures. However, the D_2O clusters decrease more rapidly with increasing temperature than do those of H_2O. Our present results show that TMV protein polymerizes more readily in D_2O than in H_2O at the same condition of pH and ionic strength. Moreover, the calculated thermodynamic parameters for D_2O showed greater values than those for H_2O at the same pH and ionic strength. If deuterium is substituted for hydrogen and the latter is involved in entropic union, then one would expect a deuterated macromolecule to have a different stability from the protonated one. This alteration in stability should manifest itself in a change in the characteristic temperature (T^*) and the entropy (ΔS°). Krescheck *et al.* (1965) have shown that when one transfers the non-

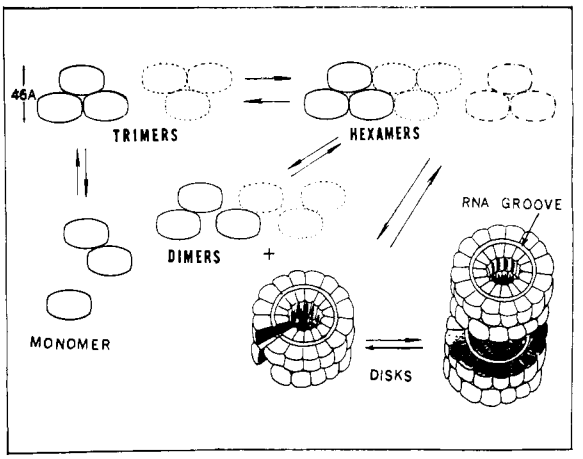

FIGURE 3: Disk formation during polymerization.

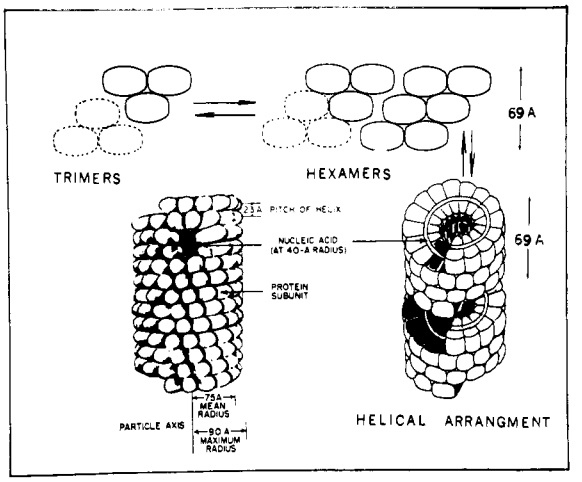

FIGURE 4: Helical arrangement during the process of polymerization.

polar residues of amino acids and alcohols from D_2O to an organic medium, $\Delta F°$ is more negative and $\Delta H°$ and $S\Delta°$ are more positive than when the same residues are transferred from water to an organic medium. Thus, if polymerization of TMV protein involves the transfer of nonpolar residues from an aqueous to an organic environment, then the results of the substitution of H_2O by D_2O on the polymerization of TMV protein are in accord with the observations made on simple model systems.

Our results obtained by substituting heavy water for water correlate with the findings of Shalaby and Lauffer (1967) on the effect of uncharged chemicals on the polymerization process. Those molecules which are reputed to increase the structure of water cause polymerization of TMV A protein to occur at lower temperatures, that is, they favor polymerization. This is comparable to the result obtained by substituting heavy water, which is reputed to have greater structure, for water. On the other hand, those chemicals studied by Shalaby and Lauffer which are reputed to reduce the structure of water have the opposite effect, that is, they favor depolymerization and raise the temperature at which polymerization takes place. One must conclude, therefore, that introduction of an organic protein surface, or for that matter, of aliphatic residues, into a medium with relatively high structure-forming tendency results in greater change in solvent structure than the introduction of the same organic residues into solvents with lower structure-forming tendency.

Substitution of deuterium for hydrogen increases the pK of acetic acid about 0.5 pH unit (Korman and Lamer, 1936). If carboxyl groups on proteins behave similarly, then one would expect protein in D_2O to behave somewhat like protein in H_2O at pH values about 0.5 unit lower. The range of pH values over which one can study polymerization of TMV A protein is about 0.4 unit lower than the range of pD values. Nevertheless, the slopes of the T^* *vs.* pD curves are two to three times greater than those of T^* *vs.* pH.

When one supplies the numerical values in eq 8, it is clear that the dominant factor influencing dT/dpH is d$\Delta H°$/dpH. It is equally evident that the dominant known factor accounting for dT/dpD being greater than dT/dpH is that dΔH/dpD is greater than dΔH/dpH. In the model (Lauffer, 1966b) d$\Delta H°$/dpH = $(1/\Delta H°_w)$ (dn/dpH). The number of water molecules released when a bond is formed (n) is greater at pH 6 that at pH 6.6. A simple assumption will account for this—at pH 6 the structure of the polymer is tight and at pH 6.6, loose. Thus, if a water-interacting center on the model is completely removed from an aqueous environment when polymerization takes place at pH 6.0, it is only partially removed when polymerization takes place at pH 6.6. Therefore, n is smaller at the high pH. The same assumption can account for the fact that polymerized protein binds hydrogen ions between pH 6 and 7, far removed from the pK of any group present in TMV protein (Scheele and Lauffer, 1967). One would infer that in D_2O the tightening up of polymerized protein should occur over a pD range narrower than the pH range in H_2O.

Speculation Concerning Protein Assembly during Polymerization. The osmotic pressure studies of Banerjee and Lauffer (1966) showed that a trimer of the TMV chemical subunit is a stable intermediate in the polymerization process. Caspar (1963) had predicted this earlier on theoretical grounds. It is assumed, therefore, that the formation of rodlike polymers involves the polymerization of trimers. Both electron microscopy (Markham *et al.*, 1963) and X-ray diffraction (Franklin and Commoner, 1955) show that polymerized TMV protein can exist in two forms, stacked double disks and helices resembling TMV itself. The specific conditions which favor the formation of one or the other have not been determined. However, there is some basis for speculating that the helical form is obtained at low

ionic strength while, at high ionic strength, polymerization leads to double disks which stack to form rodlike particles (Caspar, 1965). One cannot exclude the possibility that stacked double disks rearrange to form helical rods, but for the time being, the possibility that the two forms arise as a result of different polymerization processes will be discussed. The present study showed that the maximum extent of polymerization is greater at low pH or pD values and high ionic strength and vice versa. This might be an indication of differences in structure of the rods formed under the different conditions of polymerization.

Purely geometric consideration leads to an intriguing possibility. The first step in polymerization is the combination of two trimers to form one hexamer, as shown in Figure 3. This step is in agreement with the model produced from thermodynamic consideration (Lauffer, 1966b). The growth of hexamers can follow either the diagonal or the horizontal path shown in Figures 3 and 4. If the growth occurs horizontally, the result is a stable intermediate in the form of a double disk having 32 or 30 units, with 16 or 15 units in each layer (Figure 3).

Our speculation concerning the disk formation agrees very well with the model suggested by Lauffer (1966b). The last part in this reaction is the fitting of these disks over each other. The final result is a cylindrical rod of the length of the virus.

The second way of polymerization is diagonal growth, that is, growth along the short axis of the hexamer. In this case, two forms could be obtained, a triply stacked disk of 48 subunits or a helical arrangement as shown in Figure 4. Whether one obtains a triple disk or a helix depends on the shape of the hexamer; if the hexamer is arranged in a slightly strained pattern, one will get a helix. A condition of high electrostatic repulsion at high pH and low ionic strength could cause such a strain. Together with the geometrical argument this suggests that there may be another intermediate of the type shown in Figure 4, consisting of 48 subunits and of thickness of 69 A. The helix will fit on another only when the position is that shown in Figure 4, which might be difficult to realize. This should shift the equilibrium toward lower degrees of polymerization. The experimental results showed low maximum degrees of polymerization at high pH values and low ionic strength, consistent with this speculation.

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