Scanning Calorimetric Investigation of the Polymerization of the Coat Protein of Tobacco Mosaic Virus†

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ABSTRACT: The endothermic polymerization of the coat protein of tobacco mosaic virus has been studied by high-sensitivity differential scanning calorimetry, with control experiments involving turbidimetry and sedimentation velocity measurements. The variation of the apparent extent of polymerization under conditions close to equilibrium as the temperature is raised follows a course which is difficult to duplicate on the basis of simple models for the process. The enthalpy of polymerization at low protein concentration varies from 12.5 kcal (mol of monomer)$^{-1}$ (17 500 daltons) under conditions where the product is largely a mixture of short helical rods to 6.0 kcal mol^{-1} for the formation of double disks containing 34 monomer units. In the former case, the polymerization is accompanied by a decrease in apparent heat capacity of 350 cal K^{-1} mol^{-1} while in the latter there is an increase of 150 cal K^{-1} mol^{-1}. These results constitute evidence that these two types of polymerization involve intersubunit bonds of quite different chemical character.

The self-assembly of tobacco mosaic virus (TMV)[1] and the simpler related process of reversible polymerization of its coat protein (TMV-P) have been extensively studied in many laboratories. Some of the more recent reviews of this work are those by Butler & Durham (1977), Richards & Williams (1976), Lauffer (1975), and Lebeurier & Hirth (1975). Despite the variety of aggregates which can be formed from TMV-P (Durham et al., 1971; Lauffer, 1975; Bloomer et al., 1978; Schuster et al., 1979; Shire et al., 1979; Vogel et al., 1979), the occasional appearance of irreversible aggregation (Durham, 1972), and kinetic complications (Scheele & Schuster, 1971; Schuster et al., 1979), the polymerization of TMV-P remains, perhaps, that protein polymerization which is best suited for quantitative study.

Under appropriate conditions of concentration, pH, and ionic strength, TMV-P is caused to polymerize by an increase in temperature and to recover its original state of nearly complete depolymerization, the so-called A protein, when the temperature is lowered. This behavior suggests that the thermodynamics of the process can be profitably studied by means of differential scanning calorimetry (DSC). In this paper, we report the results of such study in 0.1 M ionic strength phosphate buffer at various values of the pH and the protein concentration. It will be shown that the calorimetric experiments give useful information concerning the nature of the chemical polymerization in addition to yielding thermodynamic data.

There have been two previous applications of calorimetry to the study of TMV-P polymerization (Stauffer et al., 1970; Srinivasan & Lauffer, 1970), both of them employing isothermal as contrasted with scanning calorimetry. These studies have yielded enthalpy data for a restricted range of conditions in good agreement with those reported here.

The application of high-sensitivity DSC to biochemical systems has been discussed by Mabrey & Sturtevant (1978).

Materials and Methods

TMV grown in *Nicotiana tabacum* (var. Samsun) was isolated according to Paglini & Lauffer's (1968) adaptation of the method of Boedtker & Simmons (1958), and by the method of Leberman (1966). The stock solutions in 10 mM EDTA (pH 7.5) were stored at 0 °C. TMV-P was extracted from the virus by the acetic acid method (Fraenkel-Conrat, 1957). The isoelectric precipitate was dissolved in 0.15 M Tris buffer, pH 8.0, and clarified by centrifugation at 100000*g*. For achievement of high concentrations (>10 mg mL^{-1}), ultrafiltration was applied with Diaflo or Minicon concentrators (Amicon, Lexington, MA). Equilibration at the desired pH values (phosphate buffer, pH 6.4–7.5, ionic strength I = 0.1 M) was provided by dialysis over a period of 36 h or more at 0 °C. The concentration of the protein was determined spectrophotometrically at pH 7.5 from spectra in the range between 450 and 230 nm [corrected for scattering according to Englander & Epstein (1957)], using the extinction coefficient $A^{1\%}_{281\,nm}$ = 1.30 (Fraenkel-Conrat & Williams, 1955). Cary 118 and Zeiss DMR 10 spectrophotometers were employed. The ratio $A_{281}/A_{251} \geq 2.40$ was used as a criterion for purity. The various preparations showed no significant differences in their polymerization behavior as observed by DSC, turbidimetric analysis, and ultracentrifugation. All chemicals were of analytical reagent grade. Quartz doubly distilled water was used throughout.

Turbidimetric experiments were performed in a Gilford Model 2400 spectrophotometer equipped with a Thermoprogrammer 2527, using light of wavelength 320 nm. Repetitive experiments in the temperature range from −1 to ≤30 °C were performed with the same heating rates as used in the calorimetric measurements. The range of thermal stability was determined by extending the experiments to 58 °C.

Sedimentation velocity experiments were performed in an analytical ultracentrifuge (Beckman, Spinco E) to characterize the initial and final states of polymerization. AnD, AnE, and AnG rotors with double-sector cells (1.2–30.0-mm path length)

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[1] Abbreviations used: DSC, differential scanning calorimetry (or calorimeter); TMV, tobacco mosaic virus; TMV-P, coat protein of TMV.

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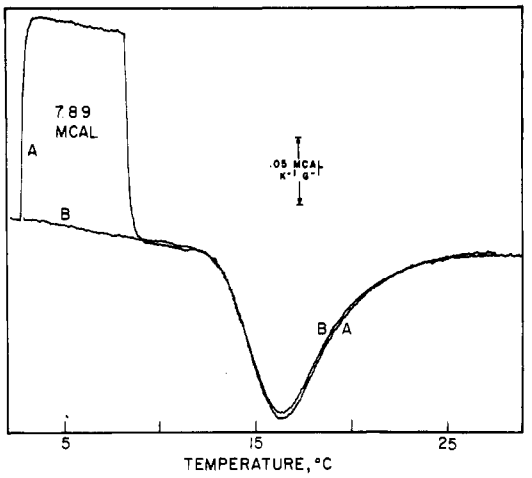

FIGURE 1: DSC traces for a 5.6 mg mL^{-1} solution of TMV-P heated at a rate of 0.5 K min^{-1} at pH 7.0. (A) First heating after cooling to −1.8 °C; (B) second heating after cooling to 0.5 °C. A calibration signal of 50 μW was introduced into the sample cell in the early part of the first scan. In these and all subsequent DSC traces (inverted relative to these) in this paper, the excess heat capacity is shown as a function of temperature.

and either normal quartz/sapphire or 1° positive wedge windows were used. To provide comparable conditions, we treated solutions the same way as in the calorimetric and turbidimetric experiments; for example, the final state of polymerization at high temperature was analyzed within the heating period of the DSC. Schlieren patterns were analyzed with 20-fold magnifications. Sedimentation coefficients were calculated from log r vs. t plots and corrected to standard conditions (water and 20 °C). The relative amounts of the various types of aggregates of TMV-P were calculated from the areas under the Schlieren profile, using Simpson's formula or a planimeter (Ott, Kempten); dilution effects were corrected according to Svedberg & Rinde (1924).

The calorimetric experiments were performed in a high-sensitivity differential scanning calorimeter, DASM-1M (Privalov et al., 1975), purchased from Mashpriborintorg, Moscow, USSR. The instrument employed had been somewhat modified with respect to the feedback systems controlling the supply of electrical energy to the sample and reference cells (1-mL volume) and to the two adiabatic shields. The essential features of the operation and sensitivity of the instrument were not altered by these modifications.

Results

A pair of typical DSC traces is shown in Figure 1. The protein concentration was 5.6 mg mL^{-1}, and the pH was 7.0. The negative-going peaks in the figure indicate heat absorption. In the other figures in this paper, the experimentally observed peaks have been smoothed and inverted.

Trace A in Figure 1 was obtained after cooling the protein solution to −1.8 °C. Shortly after this scan was started, a calibrating signal of 50 μW was introduced into the sample cell for 11 min. The heating, at 0.5 K min^{-1}, was interrupted at 30 °C, and the solution was cooled in the calorimeter to 0.5 °C over a period of about 1.5 h. The second scan, B in the figure, indicates essentially complete reversibility. Turbidimetric experiments also indicated essentially complete and rapid reversibility, with the original apparent absorbance regained even after cooling through the entire polymerization range within a period of 10 min.

It has been reported (Shire et al., 1979), in a paper appearing after the experimental work reported here was completed, that at pH 6.5 and 6.5 °C the depolymerization of

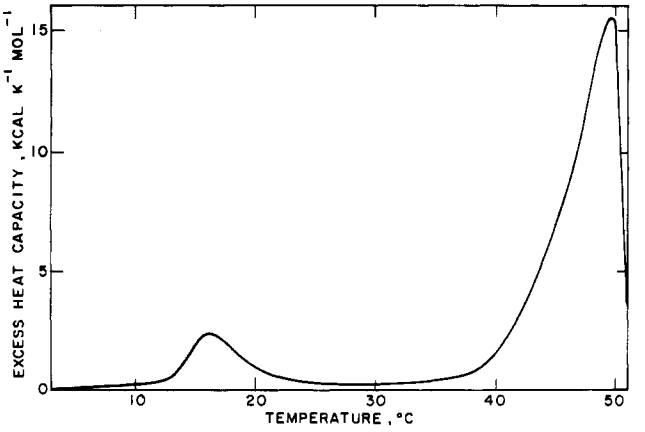

FIGURE 2: Scan of TMV-P at pH 6.75, 10.45 mg mL^{-1}. The peak at 16 °C is due to the polymerization of the protein, and that at 50 °C to irreversible denaturation.

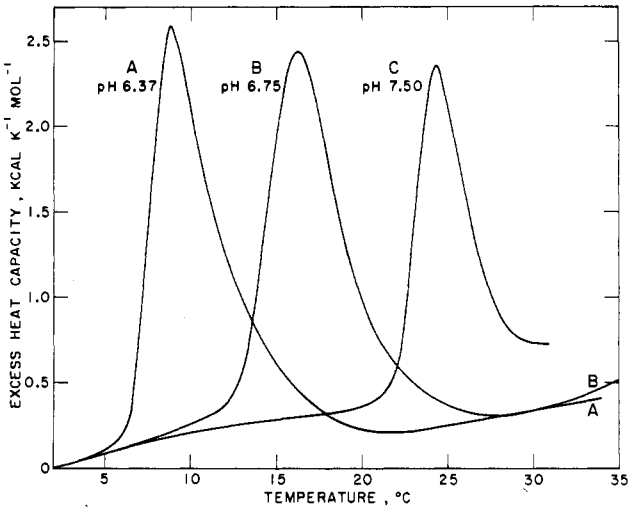

FIGURE 3: DSC scans at 0.5 K min^{-1} illustrating the effect of pH on the temperature range of polymerization. The experimental conditions were as follows: (A) pH 6.37, protein concentration 4.48 mg mL^{-1}; (B) pH 6.75, protein concentration 9.41 mg mL^{-1}; (C) pH 7.50, protein concentration 5.45 mg mL^{-1}.

TMV-P rods is very slow. Evidently, the depolymerization is much more rapid at 0–1 °C, as indicated by our DSC, turbidimetric, and sedimentation experiments at this pH and also at higher pH. This effect of temperature has also been observed by Schuster et al. (1979).

Base Lines. A ubiquitous problem in scanning calorimetry, as in many other experimental procedures, is the establishment of a base line. The low-temperature base line in Figure 1, though well defined, is slightly curved so that its extrapolation into the reaction zone is quite uncertain. As shown in Figure 2 for an experiment at pH 6.75, irreversible thermal denaturation with a relatively enormous absorption of heat sets in somewhat above 30 °C so that a high-temperature base line cannot be established at this pH after completion of the polymerization. The temperature, T_m, of maximal excess heat capacity is strongly dependent on both concentration and pH; with a scan rate of 0.5 K min^{-1}, it is within a range centered at about 11 °C at pH 6.4 and 23 °C at pH 7.5. Thus, as illustrated in Figure 3, at the lower pH it is possible to observe 10 °C or more of base line between the completion of polymerization and the onset of denaturation, and at the higher pH to observe 15 °C or more of base line before the start of polymerization. A careful comparison of all the recorded base lines suggests that we may adopt pre- and postpolymerization base lines which are independent of pH and concentration, the

Table I: Effect of Scan Rate on the Polymerization of TMV-P[a] at pH 6.75 As Observed by DSC

scan rate (K min^{-1})	ΔH_{cal} (kcal mol^{-1})	A^b (cal K^{-1} mol^{-1})	C^b (cal K^{-1} mol^{-1})	B^b (cal K^{-2} mol^{-1})	D^b (cal K^{-2} mol^{-1})	$T_{1/2}{}^c$ (°C)	$\Delta H_{vH}{}^c$ (kcal mol^{-1})
0.25	11.1	390	13	31	21	15.5	183
0.50	11.4	323	18	26	22	16.2	172
1.00	11.3	380	30	28	21	17.3	160

[a] Protein concentration 10.45 mg mL^{-1}. [b] Coefficients in the expressions for the apparent heat capacities of unpolymerized [$C_1 = A + B(T - T_{1/2})$] and polymerized [$C_2 = C + D(T - T_{1/2})$] protein, relative to the value 0 assigned to unpolymerized protein at 3 °C. [c] Parameters in the best fit of the experimental heat capacity curves (Figure 4) to the isodesmic model (see text).

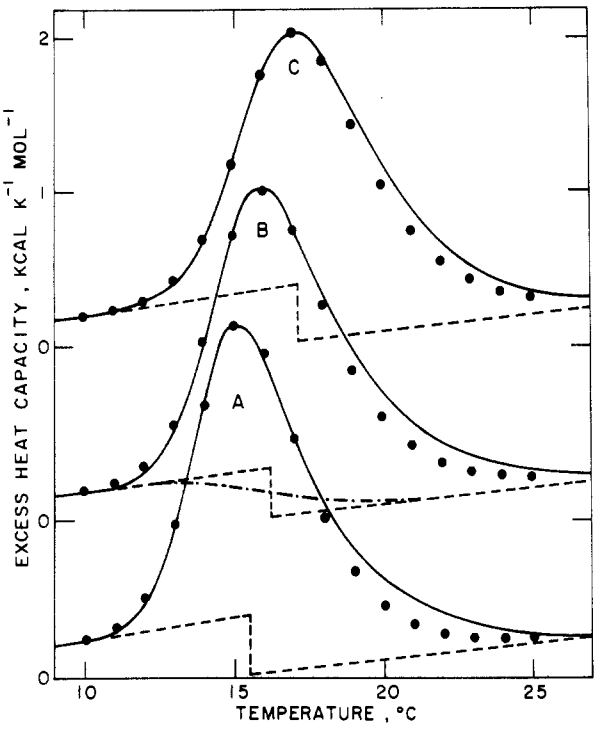

FIGURE 4: DSC traces illustrating the effect of scan rate: (A) 0.25 K min^{-1}; (B) 0.5 K min^{-1}; (C) 1.0 K min^{-1}, pH 6.75, protein concentration 10.45 mg mL^{-1}. The filled circles are values calculated according to the isodesmic model (see text) for the polymerization by using the parameters listed in Table I.

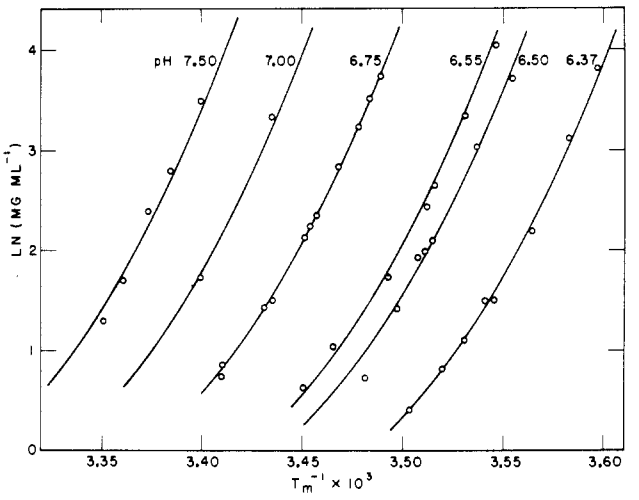

FIGURE 5: Variation of T_m, the temperature of maximal excess heat capacity, with protein concentration and pH. $1/T_m$ is plotted against the logarithm of the protein concentration at six values of the pH.

former starting at 31 cal K^{-2} mol^{-1} at 3–5 °C, decreasing to 22 cal K^{-2} mol^{-1} by 12–15 °C, and to 10 cal K^{-2} mol^{-1} at 20 °C and above, and the latter being equal to 22 cal K^{-2} mol^{-1} at all realizable temperatures. These variations of apparent heat capacity with temperature are comparable to those which have been observed with a number of globular proteins (Privalov, 1979).

Effect of Scan Rate. Scans A, B, and C in Figure 4 were observed at 0.25, 0.5, and 1.0 K min^{-1}, respectively, at pH 6.75. It is evident that T_m increases with increasing scan rate, showing that the polymerization is to some extent kinetically limited at these scan rates. However, as shown by the summary of data in Table I, variation of the scan rate within this range has no significant effect on the value of ΔH_{cal} obtained by evaluating the area under the polymerization curve, or on the shape of the curve. In experiments at pH 6.37 at a concentration of 22.4 mg mL^{-1}, T_m was 4.6 °C at a scan rate of 0.1 K min^{-1} and 5.9 °C at 0.5 K min^{-1}, again indicating a degree of kinetic limitation. We have arbitrarily selected 0.5 K min^{-1} as our standard scan rate, and the data considered in subsequent discussions were all obtained at this scan rate.

Polymerization Enthalpies. Values of ΔH_{cal}, the enthalpy of polymerization obtained by planimeter integration of the DSC traces, are listed in Table II. Experiments were performed at six values of the pH. Correction of the enthalpies for buffer ionization heats will be discussed later. Also listed in the table are the changes in apparent heat capacity which result from polymerization.

Polymerization Temperatures. The variation of T_m, the temperature of maximal excess heat capacity, with concentration and pH is shown in Figure 5. The reason for plotting ln (concentration) and $1/T_m$ will be explained later. As expected for an equilibrium process in which there is a decrease in mole number, T_m increases with decreasing protein concentration. The variation with concentration is the same at each pH; that is, all the curves in Figure 5 are parallel.

Discussion

Kinetic Factors. As indicated by the results in Figures 1 and 3, the polymerization of TMV-P is reversible and to some degree kinetically limited when brought about by heating at 0.1–1.0 K min^{-1}. Two aspects of kinetic limitation need consideration; namely, the possibility should be ruled out that the polymerization under the experimental conditions employed by us is so strongly kinetically limited as to invalidate any interpretation of our data in terms of reversible thermodynamics, and the possible effects of the kinetic overshoot reported by Schuster and his co-workers [Schuster et al. (1979), Shire et al. (1979), and earlier papers referenced therein].

The effect of scan rate shown in Figure 4 and similar effects observed in other experiments indicate that the polymerization as observed in DSC at a scan rate of 0.5 K min^{-1} is not very strongly affected by kinetic limitation. This view is further substantiated by the turbidimetric experiment shown in Figure 6. Protein at a concentration of 20.6 mg mL^{-1} at pH 6.50 was heated in successive 2-min periods at a rate of 1 K min^{-1} for 1 min, and the approach to the new equilibrium was recorded for the remaining minute. It is evident that in the early stages of the reaction the new equilibrium was not reached before the next heating period was started. However, a rough estimate of the worst deviation from equilibrium at this average

Table II: DSC Data for the Polymerization of TMV-P

pH	protein concn (mg mL^{-1})	T_m (°C)	ΔH_{cal} (kcal mol^{-1})	$\Delta H_{cal(corr)}$ (kcal/mol^{-1})	ΔC (cal K^{-1} mol^{-1})	mean ΔC (cal K^{-1} mol^{-1})
6.37	1.49	12.25	12.35	11.23	−181	
	2.24	10.95	14.28	13.13	−276	
	2.99	10.05	15.99	14.80	−382	
	4.48	8.90	14.93	13.41	−173	
	4.48	9.25	14.60	13.08	−357	
	8.96	7.40	13.79	12.11	−283	
	22.4	5.95	10.68	9.48	−173	
	44.8	4.90	8.38	7.19	−105	−241 ± 37[a]
6.50	2.06	14.10	14.04	12.81	−413	
	4.12	12.75	13.86	12.69	−340	
	6.86	11.95	13.13	12.29	−279	
	8.15	11.35	14.11	13.25	−294	
	20.6	9.55	12.73	11.83	−325	
	40.8	8.15	11.47	10.37	−285	−323 ± 22
6.55	1.88	16.65	12.05	11.20	−478	
	2.82	15.40	12.94	11.74	−350	
	5.64	13.15	12.64	11.41	−386	
	11.3	11.55	12.55	11.69	−560	
	14.1	11.25	10.75	9.89	−409	
	28.2	10.05	8.19	7.00	−288	
	56.4	8.80	7.39	6.20	−343	−402 ± 37
6.75	2.09	20.10	(17.85)	(16.78)	(−1068)	
	2.35	20.10	(16.84)	(15.66)	(−375)	
	4.18	18.25	11.85	10.85	−478	
	4.71	17.90	13.69	12.68	−338	
	8.36	16.55	12.20	11.28	−154	
	9.41	16.35	13.04	12.06	−266	
	9.41	16.30	13.16	12.18	−351	
	9.41	16.30	13.13	12.15	−334	
	10.4	16.05	11.40	10.47	−300	
	16.7	15;15	11.80	10.91	−374	
	25.1	14.30	11.75	10.83	−424	
	33.4	13.90	11.40	10.60	−381	
	41.8	13.40	10.85	10.04	−384	−344 ± 27
7.00	5.60	21.00	10.41	9.99	−69	
	5.60	21.00	10.11	9.69	−69	
	28.0	17.95	11.07	10.81	−104	−81 ± 12
7.50	3.63	25.30	5.99	5.90	+209	
	5.45	24.40	6.46	6.33	220	
	10.9	23.30	6.87	6.77	102	
	16.3	22.35	6.91	6.81	97	
	32.7	21.00	7.43	7.33	137	+153 ± 29

[a] Standard error.

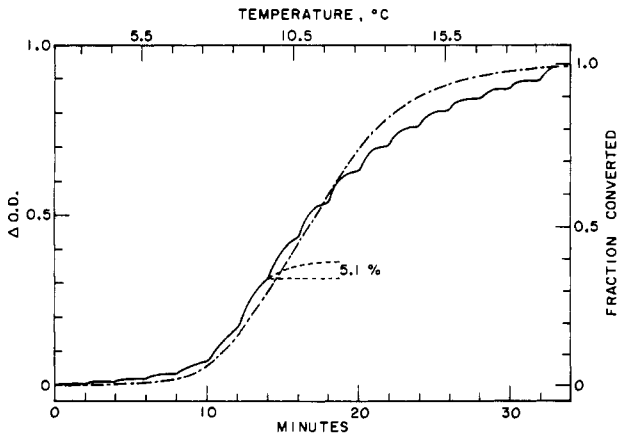

FIGURE 6: Polymerization of TMV-P as monitored by turbidimetry (solid curve) and DSC (dot–dash curve). In the turbidimetric experiment, the temperature of the solution was increased by 1 °C during the first minute of each 2-min interval, and the approach to the new equilibrium was recorded during the second minute. An estimate of the deviation from equilibrium at the end of the seventh interval is indicated by the dashed lines. The DSC experiment was run at 0.5 K min^{-1} on another sample of the same protein solution, and the output curve was integrated stepwise to obtain the curve shown in the figure. Protein concentration 20.6 mg mL^{-1}, pH 6.50.

heating rate of 0.5 K min^{-1} can be made as indicated in Figure 6, giving a value of about 5% of complete reaction.

Schuster and his co-workers have observed at pH values below 6.8 kinetic overshoot of the equilibrium condition of polymerization, with the formation of very long rods, and extremely slow decay to the equilibrium distribution of shorter rods. Our calorimetric, sedimentation, and turbidimetric observations all indicate adequately rapid reversal to A protein when material polymerized under any conditions employed by us was cooled to 0 °C. It is probable that kinetic overshoot would not be detectable in calorimetric experiments since the enthalpy change on addition of each successive unit to a growing polymer is presumably independent of polymer size above some low level of polymerization. Schuster et al. (1979) conclude that the true equilibrium polymer formed at pH 6.5 and 20 °C at very low heating rates has sedimentation coefficients in the range 24–34 S rather than 100–200 S as observed with very high heating rates.

The comparisons shown in Figures 6 and 7 between DSC and turbidimetric experiments indicate that these methods provide nearly equal measures of the extent of polymerization, at least at pH 6.50 (Figure 6) and at pH 6.75 (Figure 7). This being the case, it is reasonable to assume that both measures are approximately linear. The dot–dash curve in Figure 6 was calculated by stepwise integration of the DSC curve obtained with a sample of the same protein solution as used in the turbidimetric experiment in that figure. The solid curve in

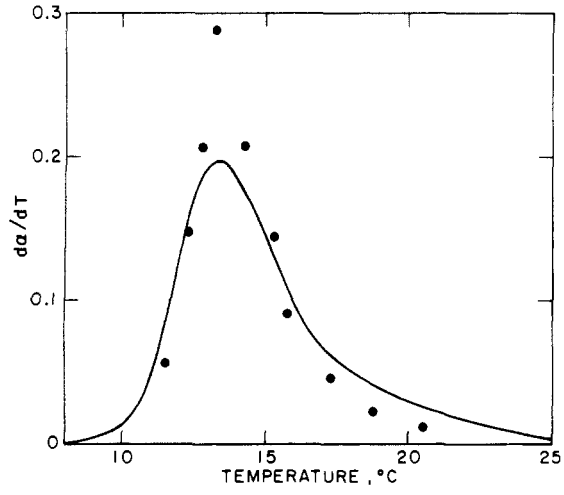

FIGURE 7: Comparison of calorimetry and turbidimetry as methods for monitoring the extent of polymerization of TMV-P as a function of temperature. The rate of change with temperature of the fraction converted, $d\alpha/(dT)$, is plotted against temperature, assuming each type of measurement to give a linear measure of the extent of reaction. The curve was obtained by equating $d\alpha/(dT)$ to $C_{ex}'/\Delta H_{cal}$, where C_{ex}' is the excess heat capacity above the base line, and the filled circles by graphical differentiation of the turbidity curve. Protein concentration 41.8 mg mL^{-1}, pH 6.75.

Figure 7 was calculated by means of the relation $d\alpha/(dT) = C_{ex}'/\Delta H_{cal}$, where α is the extent of conversion and C_{ex}' is the excess heat capacity above the base line, and the filled circles were estimated by graphical differentiation of the turbidimetric curve.

In further discussion, we shall assume that the DSC curves can be interpreted in terms of equilibrium polymerization. We must also assume ideal behavior since there is no experimental or theoretical basis for attempts to include the effects of excluded volumes or other factors leading to nonideal behavior.

Mechanism of Polymerization. The unsymmetrical shape of the DSC traces shows immediately that, as expected, the polymerization is not a two-state process. Evaluation of thermodynamic parameters from the experimental data is therefore to some extent model dependent. This applies to a slight degree even to ΔH_{cal}, the calorimetric enthalpy, obtained by integration of the DSC curves, since detailed delineation of the base line depends on the model; for example, in Figure 4, the vertical segments are drawn at the respective values of $t^{1/2}$ according to the isodesmic model (see below). This procedure was followed for all the values listed in Table II.

It has been reported by several authors (Banerjee & Lauffer, 1966; Smith & Lauffer, 1967; Paglini & Lauffer, 1968; Westover, 1971), employing either turbidimetry or osmometry to follow the reaction, that the polymerization of TMV-P follows closely the course predicted on the basis of the simple isodesmic model, although this model does not take into account the structures known to be formed by this polymerization [see Bloomer et al. (1978)]. We have found that the DSC polymerization curves at all the pH values employed can be fitted reasonably well to the formalism of this type of process, provided a value is selected for the van't Hoff enthalpy, ΔH_{vH}, which controls the variation with temperature of the equilibrium constant for each step in the polymerization, which is considerably in excess of the observed calorimetric enthalpy.

The isodesmic model can be expressed in the form

$$A_1 + A_1 \rightleftarrows A_2 \quad (K_p, \Delta H_p, \Delta H_{vH}) \tag{1}$$

$$A_2 + A_1 \rightleftarrows A_3 \quad (K_p, \Delta H_p, \Delta H_{vH}) \tag{2}$$

. . .

where A_1, A_2, A_3, ... represent monomer, dimer, trimer, ... species and also their concentrations. Each step is assumed to have the same equilibrium constant, K_p, polymerization enthalpy, ΔH_p, and van't Hoff enthalpy, ΔH_{vH}, the latter to be used in the van't Hoff equation[2]

$$\frac{d \ln K_p}{dT} = \frac{\Delta H_{vH}}{RT^2} \tag{3}$$

ΔH_p and ΔH_{vH} in most cases have to be taken as having considerable temperature dependence because of the difference in apparent heat capacities between unpolymerized and polymerized protein, as seen in Figure 4 and Table II.

The agreement of the experimental data with this model is illustrated by the filled circles plotted in Figure 4, which were calculated by employing the parameters given in Table I. In each case, ΔH_p was set equal to ΔH_{cal}, the enthalpy obtained by integration by using the indicated base line with the vertical line drawn at the value of $T^{1/2}$, the temperature at which $A_1 = A_0/2$, A_0 being the total protein concentration, and $T_{1/2}$ and ΔH_{vH} were selected to duplicate the observed values for the temperature of maximal apparent heat capacity, T_m, and the maximal apparent heat capacity, C_{max}. One should in principle use a calculated base line as shown for curve B, obtained with a suitable weighting of the pre- and post-polymerization base lines; however, negligible errors generally arise from using instead the base line composed of straight line segments. Despite the reasonably good agreement between the observed and calculated curves, the model cannot be considered to be acceptable because ΔH_{vH} is very much larger than ΔH_p, whereas they should, of course, be equal. The previous authors who have applied this model to the polymerization of TMV-P have also found values for ΔH_{vH} in excess of the calorimetric enthalpies reported here.

There is much evidence that the low-temperature form of TMV-P, the A protein, is composed on the average of three or more monomer units (Banerjee & Lauffer, 1966; Paglini & Lauffer, 1968; Durham & Klug, 1972; Vogel et al., 1979) and that the average degree of polymerization of the A protein increases with increasing protein concentration. If the actual polymerizing unit contains n monomer molecules, the calorimetric enthalpy with which ΔH_{vH} is to be compared is n times the value expressed in terms of the 17 500-dalton molecules called monomer molecules in this paper. On this basis, the van't Hoff enthalpy may be as little as twice the calorimetric enthalpy, but this is still an unacceptably large factor.

Durham & Klug (1972) proposed a model for the polymerization of TMV-P on the basis of their extensive equilibrium sedimentation data. These authors interpreted their results in terms of the formation of trimers and higher polymers, with or without the formation of the well-substantiated double disks containing 34 monomer units (Bloomer et al., 1978). They listed free energies and enthalpies, the latter assumed to be independent of temperature, at pH 7.0 for trimer formation, for addition of monomer units to trimers and higher polymers, and for formation of double disks from the 34-mer. Given these parameters, one may calculate the excess apparent heat capacities given in Figure 8. Curve A was calculated excluding, and curve B including, disk formation. As a check on this calculation, it may be mentioned that we duplicate the

[2] ΔH_{vH} is a standard-state quantity. We make here the usual and, in the absence of information concerning deviations from ideality, unavoidable assumption that significant errors are not involved in the comparison of standard-state enthalpies with calorimetrically measured enthalpies, regardless of the choice of standard states.

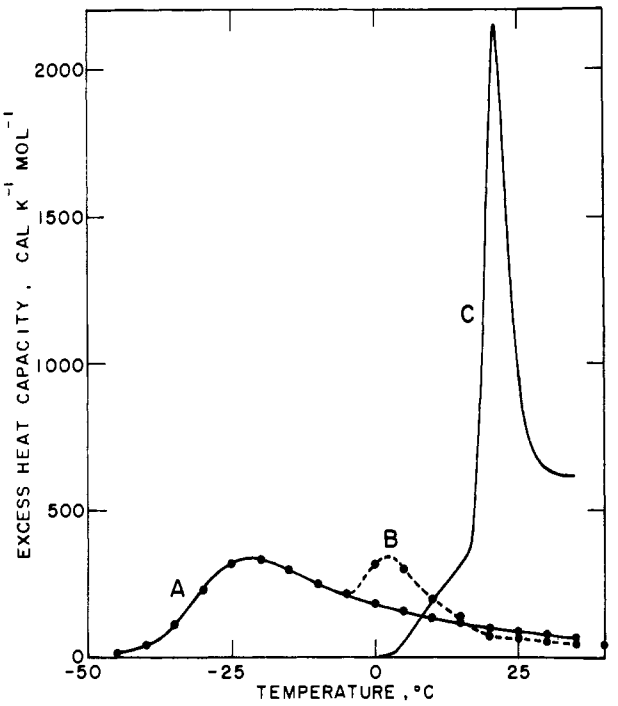

FIGURE 8: Comparison of excess heat capacities predicted according to the model proposed by Durham & Klug (1972) for the polymerization of TMV-P (A and B) with those observed by DSC (C). Protein concentration 5.6 mg mL^{-1}, pH 7.0. For (A), the parameters used were those given by Durham and Klug: $\Delta G_3° = -14578$ cal mol^{-1}, $\Delta G_a° = -7344$ cal mol^{-1}, $\Delta S_3° = 153$ cal K^{-1} mol^{-1}, and $\Delta S_a° = 80.4$ cal K^{-1} mol^{-1}, all at 0 °C and $K_c = 0$. For (B), these same parameters were used, and in addition the parameters for closure to form disks, $\Delta G_c°$ and $\Delta S_c°$, were set equal to $\Delta G_a°$ and $\Delta S_a°$, respectively; i.e., the strain energy on disk closure was set equal to zero.

histogram given by Durham & Klug (1972, p 324) showing the equilibrium distribution of polymeric species at pH 7.0, 5 °C, for a protein concentration of 5 mg mL^{-1}. In view of the fact that the enthalpies derived by Durham and Klug are not very different from our calorimetric values, it is not surprising that they predict excess heat capacity curves which are much broader than those observed by DSC (cf. curve C in Figure 8).

We have attempted to fit the data to various forms of cooperative, or nucleated, polymerization. Although these models in general require somewhat smaller values for the ratio $\Delta H_{vH}/\Delta H_{cal}$ than those required by the isodesmic model, these values are still considerably larger than unity, and, furthermore, these models predict excess heat capacity curves which are steeper on the low-temperature side and more gradual on the high-temperature side than the observed curves.

Variation of T_m with Protein Concentration. As seen in Figure 5, T_m is a strong function of the protein concentration. The expected dependence of T_m on concentration according to the isodesmic model may be obtained as follows. It can be shown that

$$K_p A_0 = x/(1 - x)^2 \qquad (4)$$

where $x = K_p A_1$. Differentiation of this expression twice with respect to temperature, making use of the van't Hoff equation, and setting the second derivative equal to zero give

$$\frac{1 - 2x_m - x_m^2}{(1 + x_m)^2} \frac{\Delta H_{vH}}{RT_m} = 2 \qquad (5)$$

Numerical solution of this equation shows that x_m is 0.40 ± 0.01 for $\Delta H_{vH}/RT$ in the range 10–10^4. Therefore, according to eq 4, $K_{p(m)}A_0$ is very nearly constant, and since $nK_{p(m)} = -\Delta H_{vH}/(RT_m)$ + constant, it follows that

$$nA_0 = \frac{\Delta H_{vH}}{RT_m} + \text{constant} \qquad (6)$$

Thus, the slope of a plot of nA_0 vs. $1/T_m$ should be equal to $\Delta H_{vH}/R$. It should be emphasized that this variation of T_m with protein concentration is expected for a very wide range of processes, including simple two-state processes.

Table III: van't Hoff Enthalpies Estimated from the Curves in Figure 5

protein concn (mg mL^{-1})	55	20	7.4	2.7
ΔH_{vH} (kcal mol^{-1})	100	91	74	57
T_m at pH 6.75 (°C)	13.0	14.8	16.8	19.5

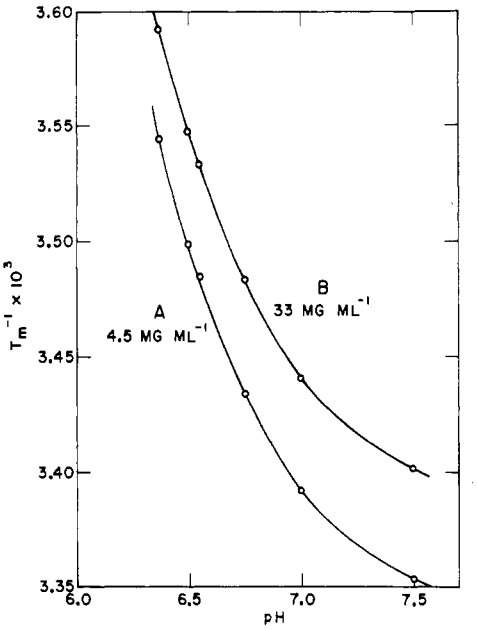

FIGURE 9: Variation of T_m, the temperature of maximal excess heat capacity, with pH at protein concentrations of 4.5 and 33 mg mL^{-1}. The values plotted in this figure were read from the curves in Figure 5.

Since the curves in Figure 5 are parallel, van't Hoff enthalpies obtained in this way are independent of pH. The values obtained by visual estimation of the slopes of the curves are listed in Table III and are smaller than the van't Hoff enthalpies obtained by fitting DSC curves to the isodesmic model. Presumably, if a fully satisfactory model for the polymerization were available, the discrepancies between van't Hoff enthalpies estimated by different procedures would disappear. The increase in ΔH_{vH} with increasing protein concentration is expected on the basis of the concentration dependence of the average degree of polymerization of the A protein (Vogel et al., 1979).

Effect of pH. It is obvious from the curves in Figure 3 that pH has a profound effect on the variation of the extent of polymerization with temperature. The variation of T_m at constant A_0 with pH, as read from the curves in Figure 5, is shown in Figure 9. It is generally agreed that polymerization at low pH leads to helical polymers while polymerization at high pH yields double disks. The smooth variation of T_m with pH indicates that there is a continuous transition between these two extremes as the pH is varied.

Returning again to the isodesmic model for the polymerization, we expressed the effect of pH by replacing eq 1 and 2 by eq 7 and 8:

$$A_1 + A_1 \rightleftarrows A_2 + iH^+ \quad (7)$$

$$A_2 + A_1 \rightleftarrows A_3 + iH^+ \quad (8)$$

The treatment in the preceding section holds if K_p is replaced by $K_p/a_H{}^i$, where a_H is the hydrogen ion activity. Then, it follows that

$$\frac{K_m A_0}{a_H{}^i} = \text{constant} \quad (9)$$

and that at constant A_0

$$\frac{1}{T_m} = \frac{2.303iR}{\Delta H_{vH}} \text{pH} + \text{constant} \quad (10)$$

This result holds for a wide range of processes other than isodesmic polymerization. Values of i obtained from estimating the slopes of the curves in Figures 5 and 9 are given in Table IV. These values are subject to uncertainties of perhaps as much as 25% because of the errors inherent in graphical estimations of slopes, and pertain not to the monomer unit but to the actual polymerizing unit.

Scheele & Lauffer (1967) reported pH titrations of TMV-P which indicated the binding of about one hydrogen ion per monomer unit on polymerization at pH 6.5 and none at pH 7.0. Vogel & Jaenicke (1974) found that one hydrogen ion is taken up by the protein in the formation of double disks from the A protein. Butler et al. (1972) published titration curves from which it may be concluded that between 0 and 35 °C about 1.3 H^+ are bound at pH 6.35 and about 0.3 H^+ is bound at pH 7.5. Similar figures are obtained from the titration curves given by Shalaby & Lauffer (1977). The values of i given in Table IV show a decrease in magnitude between pH 6.37 and 7.50 similar to that observed in these titration experiments. The facts that our values for i are larger than the titration values, and increase in magnitude with increasing protein concentration, can be qualitatively understood in terms of the size of the actual polymerizing unit as discussed above.

Enthalpy of Polymerization. The enthalpies of polymerization, obtained by integration of the DSC curves, are listed in Table II. As noted above, protons are bound by the protein during polymerization. The resulting removal of protons from the buffer makes a significant contribution to the observed enthalpies. The heat of the second ionization of phosphoric acid, determination at 25 °C under approximately the same conditions as used in the present work, is +1.13 ± 0.02 kcal mol^{-1} (Watt & Sturtevant, 1969; Paabo et al., 1965). If we assume that the changes in protonation indicated by titration curves, rather than those deduced earlier from the DSC data, should be used in estimating the buffer corrections, and that the enthalpy obtained by integrating a DSC curve is to be assigned to T_m, we find buffer corrections ranging from −2300 cal (mol of monomer)$^{-1}$ at low concentration to −2900 cal (mol of monomer)$^{-1}$ at high concentration at pH 6.37, and from −300 to −340 cal (mol of monomer)$^{-1}$ at pH 7.5.

Even after the buffer correction is applied, the calorimetric heats still include the enthalpies of protonation of groups in the protein. It seems likely that these groups are carboxyl groups (Caspar, 1963) having abnormally high pK values; if this is so, the enthalpies of protonation would presumably be rather small. In any case, the information necessary for attempting to estimate the contributions of protein protonation to the overall enthalpies is not available.

The polymerization enthalpies corrected for buffer heats are listed in Table II and are plotted in Figure 10 as a function of protein concentration. Included in the figure are error bars

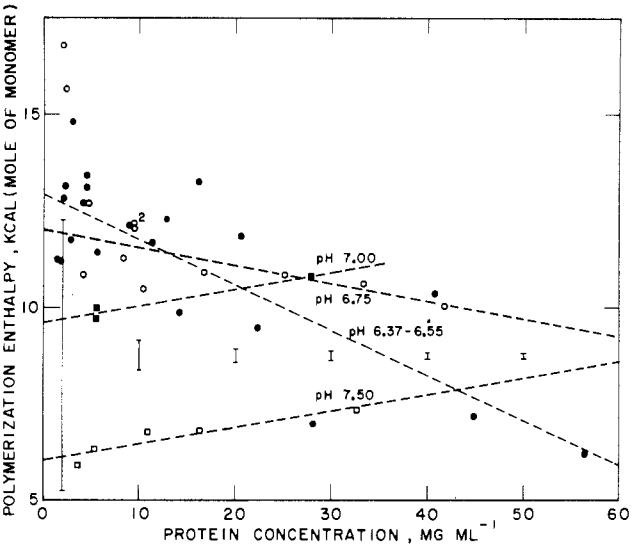

FIGURE 10: Enthalpy of polymerization, after correction for buffer heats, plotted against protein concentration. (●) pH 6.37, 6.50, and 6.55; (○) pH 6.75; (■) pH 7.00; (□) pH 7.50. The dashed lines were obtained by linear least-squares analysis, with the coefficients listed in Table V. The error bars correspond to a calorimetric uncertainty of 28 cal K^{-1} for the cell and its contents at a scan rate of 0.5 K min^{-1}.

at several concentrations which show the purely calorimetric uncertainties based on a conservative estimate of 28 μcal K^{-1} (for cell plus contents) at a scan rate of 0.5 K min^{-1} for the useful sensitivity of the Privalov calorimeter. It is evident that while calorimetric uncertainty may be the limiting factor in the accuracy of the heat data at very low protein concentration other factors are much more important at high concentration. Among these may be incomplete control of the state of polymerization at both the start and completion of the DSC experiment (Shalaby & Lauffer, 1977; Vogel et al., 1977; Schuster et al., 1979; Shire et al., 1979). Sedimentation velocity controls were employed to estimate the fractions of material in each preparation which were already polymerized at low temperature, or did not become polymerized at high temperature. Uncertainties in these estimates may introduce significant errors. A reasonable estimate of the accuracy of the enthalpy values given in Table II is afforded by the standard deviations listed in Table V.

The enthalpy values obtained at pH 6.37, 6.50, and 6.55 have been grouped together in Figure 10 since they seem to follow similar trends. At low pH, the enthalpy decreases sharply as the concentration is increased while at high pH it increases slightly. The lines in the figure were determined by unweighted least squaring eq 11. Values for the coefficients

$$\Delta H_{cal(corr)} = \Delta H_0 - b \text{ (mg mL}^{-1}\text{)} \quad (11)$$

and the standard deviations of the points from the lines are given in Table V.

There is a significant decrease in the apparent heat capacity of the protein due to polymerization at pH 6.37–6.75, very little change at pH 7.00, and an increase at pH 7.50. The observed changes are listed in Table II. Since the temperature of polymerization varies with protein concentration, we should expect some variation in enthalpies to result from the nonvanishing changes in apparent heat capacity. Differentiation of eq 6 and utilization of the definition of the heat capacity change, ΔC, as the temperature derivative of the enthalpy give

$$\frac{d\Delta H_0}{dA_0} = -\frac{RT_m{}^2\Delta C}{A_0\Delta H_{vH}} \quad (12)$$

Table IV: Estimation of Change in Protonation of TMV-P on Polymerization from the Slopes of the Curves in Figures 5 and 9

pH	protein concn (mg mL^{-1})	ΔH_{vH} (kcal mol^{-1})	no. of protons liberated, i
7.50	4.5	65	−0.7
7.50	33	97	−1.1
6.37	4.5	64	−5.6
6.37	33	96	−7.7

Table V: Coefficients in Equation 11

pH	ΔH_0 (kcal mol^{-1})	b (kcal mL mol^{-1} mg^{-1})	standard deviation
6.37–6.55	12.93 ± 0.28[b]	−0.1176	±1.24
6.75[a]	12.01 ± 0.21	−0.0463	±0.63
7.50	6.04 ± 0.12	+0.0426	±0.21

[a] The values at 2.09 and 2.35 mg mL^{-1} were omitted from the least squaring. [b] Standard error.

For pH 6.75, C = −350 cal K^{-1} mol^{-1}; taking as typical values T_m = 290 K, ΔH_{vH} = 100 kcal mol^{-1}, and A_0 = 20 mg mL^{-1} gives $d\Delta H/(dA_0)$ = +0.029 kcal mol^{-1} (mg mL^{-1})$^{-1}$. This is of opposite sign to the observed dependence at this pH, indicating that the decreases due to other causes are even larger than those shown in the figure.

There is evidence that the degree of polymerization of the protein at low temperature, the so-called A protein, varies with protein concentration from an average of 3 to perhaps as high as 12 (Vogel et al., 1979). Although the sedimentation studies of Ansevin & Lauffer (1959) showed that the A protein dissociates into monomeric units at extremely low protein concentrations, we may assume, for example, on the basis of osmotic pressure measurements by Banerjee & Lauffer (1966) and Paglini & Lauffer (1968), as well as our own sedimentation experiments, that the lowest aggregate encountered in our work has an average molecular weight corresponding to the trimer and that the extrapolated enthalpies, ΔH_0, at "zero" protein concentration refer to polymerization of "trimers".

The variation with protein concentration of the low-temperature state of aggregation can have a pronounced effect on the apparent enthalpy of polymerization. Consider, for example, a much oversimplified model in which the number of monomer–monomer contacts, all assumed to be characterized by the same increase in enthalpy, ΔQ, in an n-mer is $n-1$. At low concentration, the polymer growth process would be

$$(3\text{-mer})_{n-1} + 3\text{-mer} \rightleftarrows (3\text{-mer})_n \qquad (13)$$

with the formation of one new contact per 3-mer unit. For this process, $\Delta H_{app} = \Delta Q/3$ = 12000 cal mol^{-1}, the limiting value at low concentration (pH 6.75). If at A_0 = 50 mg mL^{-1} the A protein is largely heptameric, the growth process would be

$$(7\text{-mer})_{n-1} + 7\text{-mer} \rightleftarrows (7\text{-mer})_n \qquad (14)$$

and the observed enthalpy would be $\Delta H_{app} = \Delta Q/7$ = 5000 cal mol^{-1}. According to this view of the variation of enthalpy with concentration, there appears to be very little change in the low-temperature state of aggregation as the concentration is changed at pH 7.50. This conclusion is not in agreement with the results of Vogel et al. (1979).

The extrapolated enthalpies, ΔH_0, are strongly dependent on pH. Although experimental uncertainties and the limited number of values for ΔH_0 make the following interpretation somewhat arbitrary, it is interesting that ΔH_0 varies with pH in the manner expected if the form of the polymerization, either to helical rods or to double disks as end products, is

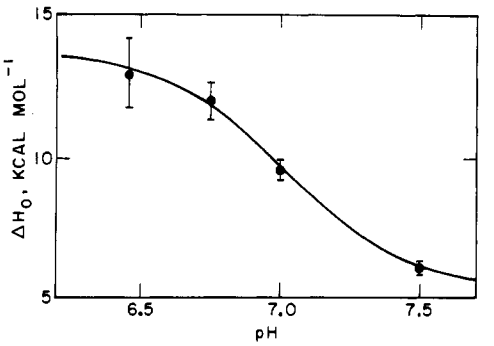

FIGURE 11: Intercepts of the least-squared lines of Figure 10 plotted against the pH. The curve was calculated on the sasumption that the value of the intercept is controlled by the ionization of two groups on the protein having pK values symmetric about the value 7.02, with the limiting values of the intercept equal to 13.7 and 5.3 kcal mol^{-1} at low and high pH, respectively.

controlled by the ionization of a pair of groups having pK values symmetric about the value 7.02, with the limiting value of ΔH_0 being 13.7 kcal mol^{-1} at low pH and 5.3 kcal mol^{-1} at high pH (Figure 11). There are more monomer–monomer contacts in a helical polymer than in a double disk, but probably not 13.7/5.3 = 2.6 times as many. The large difference between the low-pH and high-pH values of ΔH_0 must therefore reflect major chemical differences in the types of interactions occurring in the two forms of polymerized protein. This conclusion is in qualitative agreement with the known differences between the low-pH helices, presumably of the same structure as determined in the intact virus (Stubbs et al., 1977), and the high-pH double disks (Champness et al., 1976; Bloomer et al., 1978).

Srinivasan & Lauffer (1970) measured by isothermal calorimetry at 23 °C the heat absorbed as the pH of a 2.5 mg mL^{-1} solution of TMV-P was lowered from pH 7.5 to various values down to 3.0. They observed a sharp increase in the heat absorbed in a narrow range at pH 6.3, amounting to 0.725 cal g^{-1} or 12.7 kcal mol^{-1}. This agrees remarkably well with our estimate of 12.9 kcal mol^{-1} for the enthalpy of polymerization at low concentration and low pH.

Another calorimetric estimate of the heat of polymerization was made by Stauffer et al. (1970). These authors measured the heat of dilution at 23 °C and pH 7.5 of a 50 mg mL^{-1} solution of TMV-P to various final concentrations in the range 2–4 mg mL^{-1}, finding values on extrapolation to zero concentration of 0.49–0.57 cal g^{-1}. These values correspond to 8.6–10.0 kcal mol^{-1}. Extrapolation of our data at pH 7.50 to 50 mg mL^{-1} gives 8.15 kcal mol^{-1}, in reasonable agreement.

Apparent Heat Capacities. The heat capacities of solutions of unpolymerized TMV-P are less than those of the buffer by 0.342 ± 0.010, 0.326 ± 0.010, and 0.318 ± 0.008 cal K^{-1} (g of protein)$^{-1}$ in the calorimeter cell at 0, 5, and 10 °C, respectively. From these figures, the apparent specific heat of the protein, c_p, can be evaluated by means of the relation (Privalov & Khechinashvili, 1974)

$$\frac{c_p}{v_p} = \frac{c_b}{v_b} - \frac{\Delta}{v_p} \qquad (15)$$

where v_p is the apparent specific volume of the protein, c_b/v_b is the volume specific heat of the buffer solution, and Δ is the quantity for which values are given above. If we use the value v_p = 0.734 given by Jaenicke & Lauffer (1969a) for 20 °C and assume a temperature coefficient for v_p of 0.005 mL g^{-1} K^{-1}, and if for c_b/v_b we use the values for water, 1.0072, 1.0036, and 1.0010 cal K^{-1} cm^{-3} at 0, 5, and 10 °C, we obtain c_p = 0.39 ± 0.01, 0.40 ± 0.01, and 0.41 ± 0.01 cal K^{-1} g^{-1}

at 0, 5, and 10 °C, respectively. These values are unusually high for a protein, values of 0.31 ± 0.02 being reported by Privalov & Khechinashvili (1974), and may be due either to a large degree of exposure of hydrophobic groups to the solvent (Edsall, 1935; Kauzmann, 1959) or to the existence of an unusually high number of soft internal vibrational modes (Sturtevant, 1977), or a combination of both causes. In contrast, the temperature coefficient of the apparent specific heat, $dc_{app}/(dT)$, is smaller than usual for globular proteins. As mentioned in the discussion of the base-line problem, $dc_{app}/(dT)$ ranges from 0.006 to 0.0018 cal K^{-2} g^{-1}, whereas Privalov & Khechinashvili (1974) report 0.0022 ± 0.0005 cal K^{-2} g^{-1} for five proteins. The low values for $dc_{app}/(dT)$ constitute an indication that no significant aggregation takes place in the temperature range before the start of the main DSC peak.

The decrease in the apparent specific heat accompanying polymerization at low pH, amounting to about 0.020 cal K^{-1} g^{-1}, is not an unusual value for a protein reaction (Sturtevant, 1977). Jaenicke & Lauffer (1969b), employing the spring balance method, estimated that there is a loss of 0.033 g of H_2O per g of TMV-P on polymerization at pH 6.5–6.8. According to the specific heat data of Suurkuusk (1974) at 25 °C, there is, on the average, a decrease in specific heat of 0.31 cal K^{-1} g^{-1} per g of water of hydration removed from a protein. The loss measured by Jaenicke and Lauffer should thus lead to a decrease in the apparent specific heat of the protein of 0.010 cal K^{-1} g^{-1}. The rest of the decrease, coupled with the positive entropy of polymerization, is consistent with the view that hydrophobic interactions are important in causing the polymerization.

The increase in apparent specific heat observed at pH 7.50, about 0.009 cal K^{-1} g^{-1}, is difficult to understand. It is in the direction expected if the exposure of charged groups to the solvent is decreased by polymerization. In any case, it indicates, as does the small enthalpy change at this pH, that the polymerization at high pH is very different in chemical character from that at low pH.

Acknowledgments

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