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Sedimentation Equilibrium Measurements of the Intermediate-Size Tobacco Mosaic Virus Protein Polymers†

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ABSTRACT: Short-column sedimentation equilibrium methods have been applied for the first time to tobacco mosaic virus (TMV) protein (0.1 M ionic strength orthophosphate) at pH 6.5 and at pH 7.0 to estimate molecular weights. Previous sedimentation velocity experiments at pH 6.5, 20 °C have led to the conclusion that the major boundary with an $s^0_{20,w}$ value of 24.4 ± 0.1 S consists of a distribution of polymers which are mainly three-turn, 48-51-subunit helical rod aggregates. The directly measured z-average molecular weights together with sedimentation velocity data are entirely consistent with this assignment of a three-turn aggregate. Molecular weights have also been determined under two conditions where a large mass fraction of the protein sediments with an $s^0_{20,w}$ value of 20.3 ± 0.2 S. At pH 6.5, 6-8 °C, the aggregates in this boundary are metastable and correspond to 50-60% of the preparation. At pH 7.0, 20 °C at equilibrium, 65-75% of the protein sediments at 20.3 S. The 20.3S boundary is very similar under both conditions and is interpreted as being composed of a distribution of protein aggregates centered about 39 ± 2 subunits. This result is important in the interpretation of previous kinetic measurements of TMV self-assembly. The current view is that the 34-subunit structure of TMV protein, in the form of a cylindrical disk which is made up of two 17-subunit layers and has been characterized in single-crystal X-ray diffraction studies, plays a central role in the initial binding steps with RNA. The present results are not consistent with the view that there is a significant concentration of the TMV protein disk structure in solution under the usual conditions of TMV self-assembly.

The highly endothermic polymerization of tobacco mosaic virus protein (TMVP)[1] involves a single polypeptide species of 17 530 daltons and results in helical rods of varying length and similar shape to that of the native virus (TMV). At pH 7.0, 20 °C, 0.1 M ionic strength orthophosphate, the protein exists as a mixture of so-called 4S and 20S boundaries in an apparent weight ratio of approximately 30:70, respectively. The 20S boundary has been assigned as containing the obligatory nucleating species in TMV self-assembly (protein + RNA) (Butler & Klug, 1971). It has also been shown to act as the initiating material in the nucleation-controlled polymerization of TMVP to form helical viruslike rods (Schuster et al., 1979). This species has been referred to as a two-turn cylindrical disk [for a review, see Butler (1984) or Stubbs (1984)].

However, the only data which suggest a two-turn disk structure in low ionic strength solutions are from electron microscopy measurements (Crowther & Amos, 1971; Durham et al., 1971; Durham & Finch, 1972). The X-ray diffraction results of TMVP structure are derived from crystals in high ionic strength solutions where there is no doubt concerning the existence of a two-turn disk (17 subunits per turn) (Bloomer et al., 1978). Recent solution characterization of

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[1] Abbreviations: TMV, tobacco mosaic virus; TMVP, tobacco mosaic virus protein; A protein, TMVP in low states of aggregation, e.g., monomer through trimer, sedimenting at 3-5 S, depending upon the total concentration.

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20S TMVP shows a significant and continuous pH dependence of the sedimentation coefficient, varying from 20.3 S at pH 7.0, 20 °C to 24.4 S at pH 6.5, 20 °C (Schuster et al., 1980). This implies the existence of a progressive self-association reaction as a function of pH. Furthermore, previous $s_{20,w}$ measurements at pH 7.0, 20 °C and pH 6.5, 6–8 °C suggest that the 20S boundaries under these conditions are composed of similarly sized aggregates (Shire et al., 1979). The existence of a continuous size change involving a closed ring structure such as the cylindrical two-turn disk calls into question the assignment of the structure(s) present in solution under self-assembly conditions. Since there have been no quantitative measurements of molecular weight under any of these conditions, the present experiments are an attempt to characterize the size of the polymer(s) in the 20S boundary. Above pH 7.0, the apparent mass fraction of material in the 20S boundary decreases dramatically with a commensurate increase in the fraction of material in the 4S boundary. Below pH 6.5, aggregates with $s_{20,w}$ values larger than 24.4 S appear (presumably larger than three turns). At pH 6.5, 20 °C, the principal aggregate has an $s^0_{20,w}$ value of 24.4 S and presumably represents the initial stages of helical rod formation. Increased rod lengths are found at lower pH values. However, at 20 °C below pH values of about 6.7, TMVP self-association displays a strong heating rate dependence (Schuster et al., 1979), making it necessary to accurately control heating rates in order to obtain reproducible states of association.

Since intermediate-size association states of TMVP such as the 20S aggregates are required for efficient assembly of the coat protein as well as the virus, this study was undertaken to more fully characterize the protein under conditions of limited association.

Materials and Methods

TMVP was prepared according to the method of Stevens described by Scheele & Lauffer (1967) as modified by Shire et al. (1979b). Heating times were carefully controlled to minimize nonequilibrium polymers (Shire et al., 1979a). Specific sample preparations are described in the figure legends. All pH values were measured at 20 °C, and no corrections have been applied for temperature. Ionic strength is given in molar units.

Analytical Ultracentrifugation. Short-column (0.75-mm) sedimentation equilibrium experiments were performed (Correia, 1980) under three sets of conditions (pH 7.0, 20 °C; pH 6.5, 20 °C; pH 6.5, 6–8 °C) using pulsed laser interferometry (Paul & Yphantis, 1972; Laue et al., 1983, 1984; Yphantis et al., 1984) and a Kel-F centerpiece. Final photographs were taken after apparent equilibrium had been reached, typically in 4–8 h.[2] Interference patterns recorded on Kodak 35-mm technical pan film SO-115 were scanned automatically (Laue & Yphantis, 1979) to obtain the fringe displacement as a function of radial position. These data were fit by a linear least-squares orthonormalization routine (Correia, 1981), yielding accurate estimates of both the fringe gradient, $dc/d(r^2/2)$, and its curvature, $d^2c/d(r^2/2)^2$, at the midpoint of each channel. Since at sufficiently low angular velocity the concentration at the midpoint of a short-column cell approximates the loading concentration c_0 (Van Holde & Baldwin, 1958; Yphantis, 1960), one can estimate the apparent effective reduced average molecular weights: σ_w = gradient/c_0 and σ_z = curvature/gradient (Yphantis, 1964). These values are then used to infer the nonideality and/or the association behavior of the particular solute. (Under the conditions used here, TMVP is both nonideal and selfassociating.) The effective reduced molecular weight (σ_i) is defined as $M_i(1 - \bar{v}\rho)\omega^2/RT$; R is the gas constant, T is the absolute temperature, ω is the angular velocity, $\bar{v}$ is the partial specific volume of the solute, ρ is the density of the solution, and M_i (σ_i) is either M_w (σ_w) or M_z (σ_z) (Yphantis & Waugh, 1956). The apparent weight-average and z-average molecular weights (M_w and M_z, respectively) are obtained by assuming $\bar{v}$ to be independent of solute concentration. Details of this method appear elsewhere (Correia, 1980, 1981; J. J. Correia and D. A. Yphantis, unpublished results).

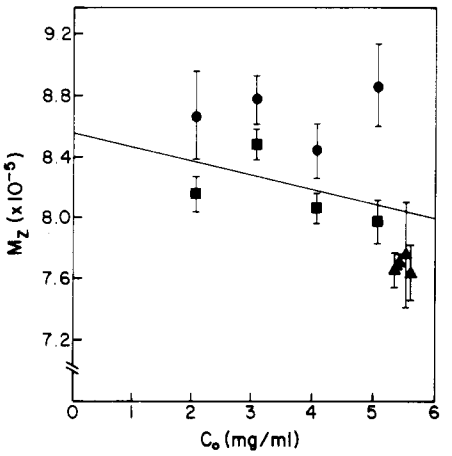

FIGURE 1: Apparent z-average molecular weights, M_z, presented as a function of loading concentration for TMVP at pH 6.5, 0.1 ionic strength potassium orthophosphate. The samples were warmed from 0 to 20 °C at the heating rates (dT/dt) and total protein concentrations indicated and then diluted to the loading concentrations at 20 °C: (●) 20 °C, 4400 rpm, dT/dt = 0.008 °C/min at 12.0 mg/mL; (■) 20 °C, 4400 rpm, dT/dt = 0.004 °C/min at 17.2 mg/mL; (▲) 20 °C, 4000 rpm, dT/dt = 0.006 °C/min at 13.0 mg/mL. [These are replicate measurements done on one solution (four channels) giving an apparent M_z = 770 300 ± 5300.] The line in Figure 1 is the expected behavior for a 49-subunit structure exhibiting nonideality equivalent to that for the excluded volume of an equivalent sphere (Tanford, 1961) and for a charge of 3– per monomer at pH 6.5 (Scheele & Lauffer, 1967; Roark & Yphantis, 1971).

Sedimentation velocity experiments were performed on the same solutions used for sedimentation equilibrium experiments using either interference fringe displacement or schlieren boundary areas to obtain the apparent relative amounts of material in the 4S and 20S boundaries. Protein concentrations (millimeters of fringe displacement) were determined by synthetic boundary experiments and converted to milligrams per milliliter by using an interpolated value of dn/dc = 0.188 × 10^{-3} (g/mL)$^{-1}$ at 514.5 nm, the wavelength of the argon ion laser [values used for interpolation were dn/dc = 0.1856 × 10^{-3} (g/mL)$^{-1}$ at 546 nm (Scheele & Lauffer, 1967) and dn/dc = 0.194 × 10^{-3} (g/mL)$^{-1}$ at 436 nm (Boedtker & Simmons, 1958)].

Results

The observed values of M_z as a function of the total protein concentration at pH 6.5 and 20 °C are presented in Figure 1. The TMVP solutions used were warmed from 0 to 20 °C

[2] Occasionally stock solutions at pH 7.0, 20 °C became turbid during the course of the experiments. Sedimentation velocity data subsequently indicated very rapidly sedimenting material in addition to 4S and 20S boundaries; the sedimentation equilibrium data indicated ever increasing molecular weights, in excess of (1–2) × 10^6. Consequently, any solutions that became turbid and any sedimentation equilibrium molecular weight data that did not become constant within 4–8 h were discarded. Autoclaving glassware and filtering cold buffer and protein solutions seemed to have no effect on this spurious aggregation (Durham & Finch, 1972). This turbidity is not observed when millimolar amounts of sodium azide are included to inhibit bacterial growth.

Table I: Apparent $s_{20,w}$ and Weight Fraction (f_{app}) of Sedimentation Velocity Boundaries in Solutions Used for Sedimentation Equilibrium Experiments

pH	T (°C)	expt	c_0	$s_{20,w}$ (S)	f_{app}	$s_{20,w}$ (S)	f_{app}
6.5	20	1[a]	5.0[d]	22.2	0.85	3–4[f]	0.08
			4.0	21.7	0.84	3–4	0.09
			3.0	22.1	0.85	3–4	0.08
			2.0	21.6	0.84	3–4	0.09
		2[b]	5.0	23.0	0.92	3.1	0.08
			4.0	23.2	0.86	3–4	0.14
			3.0	23.1	0.96	3–4	0.04
			2.0	23.0	0.84	3–4	0.16
		3[b]	5.3	22.9	0.95	2.5	0.05
	6–8[c]	1	5.0	19.3	0.65	3.8	0.35
			4.0	18.7	0.66	3.6	0.34
			3.0	18.8	0.64	3.4	0.36
			2.0	18.3	0.58	3.3	0.43
		2	5.0	19.1	0.66	3.7	0.34
			4.0	18.5	0.65	3.6	0.35
			3.0	18.9	0.66	3.6	0.34
			2.0	18.4	0.57	3.3	0.43
		3	5.3	*e*			
7.0	20	1	2–5	*e*			
		2	5.8	18.9	0.83	3.8	0.17
			4.0	19.6	0.77	3.5	0.23
			3.0	19.3	0.72	3.4	0.27
			2.0	19.1	0.63	3.5	0.37

[a] Sample warmed from 0 to 20 °C at 0.008 °C/min at 12 mg/mL and then diluted to loading concentrations at 20 °C. All concentrations c_0 are in units of milligrams per milliliter. [b] Sample warmed from 0 to 20 °C at 0.004 °C/min at 17.2 mg/mL and then diluted to loading concentrations at 20 °C. [c] Samples were cooled overnight (8–12 h) in a cold room along with the An J rotor to 6–8 °C after the pH 6.5, 20 °C run and then centrifuged at the appropriate speed. [d] These solutions contained approximately 7% of a 27S species that has been assigned the size of 3.5 turns of a 16.3-subunits/turn helix by mass conservation considerations (Shire et al., 1979). [e] Velocity sedimentation experiment not done. [f] The low concentration of material in the 4S boundary occasionally made accurate $s_{20,w}$ measurements difficult.

at different heating rates and concentrations as indicated in the legend of Figure 1. The larger apparent M_z values determined for the more rapidly warmed sample (circles) are consistent with prior sedimentation velocity experiments with TMVP warmed to 20 °C at different heating rates (Shire et al., 1979a). These results are consistent with the proposed overshoot polymerization model of Scheele & Schuster (1974). Sedimentation coefficients and apparent weight fractions (f_{app}) of these TMVP samples are given in Table I. The apparent weight fraction of material in the 24S boundary in the sample warmed at 0.004 °C/min (squares) varies from 84% to 96%, as determined by schlieren boundary areas. Assuming that all of the polymer sedimenting at 24S is a 49-mer and that all of the 4S protein is trimer results in a calculated average M_z of 853 000 ± 3300 (48.7 ± 0.2 subunits) for this range of weight fractions. The line in Figure 1 is the expected behavior for a 49-subunit structure exhibiting nonideality equivalent to that for the excluded volume of an equivalent sphere (Tanford, 1961) and for a charge of 3– per monomer at pH 6.5 (Scheele & Lauffer, 1967; Roark & Yphantis, 1971). Unweighted linear least-squares extrapolation of the sedimentation equilibrium data in Figure 1 (squares) to zero concentration results in an experimentally determined M_z value (M_z^0) of 851 000 ± 37 200 (48.6 ± 2.1 subunits), in excellent agreement with the M_z value inferred above. The third experiment performed under these conditions (triangles) is a replicate measurement of a solution at one loading concentration (5.3 mg/mL). The experimentally measured M_z^0, 770 300 ± 5300, indicates the reliability of these molecular weight measurements. These data are consistent with the data from the second experiment (squares). Combination of these two sets of data (eight points) results in a linearly extrapolated M_z^0 of 877 500 ± 23 900 (50.1 ± 1.4 subunits), also in agreement with the range of M_z values inferred above by assuming only trimers and 49-mers.

Cooling these TMVP samples to 6–8 °C results in the M_z variation with loading concentration presented in Figure 2.

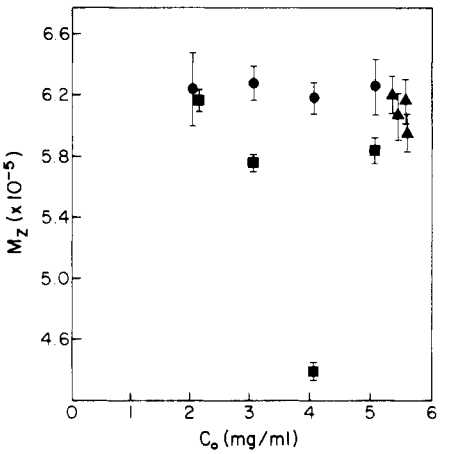

FIGURE 2: Apparent z-average molecular weights, M_z, presented as a function of loading concentration for TMVP at pH 6.5, 0.1 ionic strength potassium orthophosphate: (●) 8.4 °C, 5600 rpm; (■) 6.5 °C, 5600 rpm; (▲) 8 °C, 4400 rpm. [These are replicate measurements done on one solution (four channels) giving an apparent M_z = 610 500 ± 11 500.] Samples were cooled overnight (8–12 h) in a cold room along with the An J rotor to 6–8 °C after the pH 6.5, 20 °C run and then centrifuged at the appropriate speed.

Molecular weight measurements at pH 7.0, 20 °C (Figure 3, open symbols) and at pH 6.5, 6–8 °C suggest that the 20S boundaries under these two conditions are composed of aggregates of similar size. Sedimentation coefficients and apparent weight fractions for these samples are also presented in Table I. Note that the slightly lower average M_z values at pH 6.5, 6–8 °C than at pH 7.0, 20 °C are consistent with slightly smaller apparent weight fractions in the 20S boundary. Figure 3 also presents the weight-average molecular weight data plotted vs. loading concentration for the pH 7.0 and 20 °C solutions (see closed symbols).

Discussion

Interpretation of Results at pH 6.5 and 20 °C. The simplest

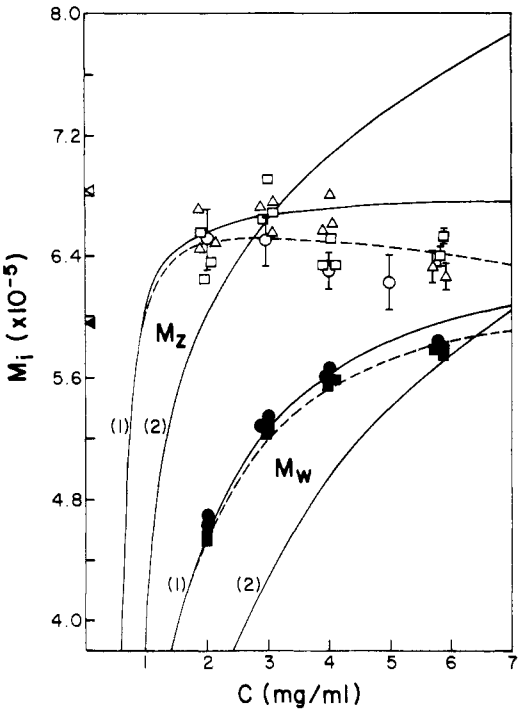

FIGURE 3: Apparent weight-average molecular weights, M_w (closed symbols), and apparent z-average molecular weights, M_z (open symbols), presented as a function of loading concentration (milligrams per milliliter) for TMVP at pH 7.0, 0.1 ionic strength potassium orthophosphate. Apparent M_w: (●) 20.7 °C, 3600 rpm; (■) 20.7 °C, 4400 rpm. Apparent M_z: (○) 20.35 °C, 5600 rpm; (△) 20.7 °C, 5600 rpm; (□) 20.7 °C, 4400 rpm. Dilutions were performed at 20 °C 24 h after the stock solution was warmed to 20 °C. The arrowheads correspond to the molecular weights of 34-subunit TMVP (closed arrowhead) or 39-subunit TMVP (open arrowhead). Simulations of sedimentation equilibrium experiments involving a trimer of TMVP reacting to give a 39-mer (model 1) or an equilibrium involving trimer to 34-mer to 68-mer (model 2) are also presented. The solid curves correspond to M_w or M_z values for model 1 or model 2. The dashed curves correspond to M_w or M_z values for model 1, using the same equilibrium constant, plus a correction for nonideality. The value of the second virial coefficient chosen ($B = 1.7 \times 10^{-5}$ cm^3 g^{-1} mol^{-1}) corresponds to a reasonable estimate of B due to nonideality, asymmetry, and charge for a 39-mer of TMVP at pH 7.0.

model we have found to adequately explain the pH 6.5, 20 °C data is that a single species of 49 ± 2 subunits exists in the 20S boundary. This assignment assumes all of the 4S material is trimer. We do not exclude some distribution of polymers centered about 49 subunits. Sedimentation velocity experiments at temperatures other than 20 °C, i.e., 18 and 24 °C, also show a common extrapolation to 24.4 S, suggesting unique structural stability of a three-turn helical rod aggregate at pH 6.5 (Adams et al., 1981).[3] The data point at 2 mg/mL in the second experiment at pH 6.5, 20 °C (squares in Figure 1) seems to deviate from the trend of the other data points in the second and third experiments. This deviation may reflect a concentration-dependent dissociation of the aggregates. (Alternatively, it may reflect fluctuations for unknown causes in the individual weight fractions of the 4S and 20S components for this specific solution sample.) Exclusion of this point from a linear fit of these data gives an extrapolated M_z at zero protein concentration of 943 200 ± 19 800 (53.8 ± 1.1 subunits). This gives a range of M_z values corresponding to 49–54 subunits (approximately three turns) for the 20S boundary at pH 6.5, 20 °C.

The results shown in Figure 1 and Table I are also consistent with the proposed overshoot polymerization mechanism for TMV protein assembly (Scheele & Schuster, 1974). Protein warmed at a heating rate (circles in Figure 1) of 0.008 °C/min resulted in the presence of ~7% by weight of a 27S boundary after dilution to the various loading concentrations. This boundary was absent in solutions warmed at 0.004 °C/min, and this is reflected in the lower M_z values in Figure 1 (squares). Apparently, under these conditions, the slower rate of nucleus formation can keep pace with the faster rate of rod propagation. Moreover, the large concentration dependence expected for nucleus formation would also decrease the amount of overshoot polymer formed. This is consistent with the sedimentation velocity and equilibrium results for the protein sample warmed at 0.004 °C/min at 17.2 mg/mL (see Table I). If the aggregates in the 27S boundary are assigned a molecular weight corresponding to an average of 3.5 turns, as previously assigned on a mass balance basis (Shire et al., 1979), then the molecular weight of the major species in the 24.4S boundary is also estimated to correspond to 49 subunits. (This calculation involves the weight fractions from Table I, an assignment of trimer to the 4S material, and the experimentally measured M_z values from Figure 1.)

Interpretation and Comparison of Results at pH 7.0, 20 °C and pH 6.5, 6–8 °C. The molecular weights at pH 7.0, 20 °C and pH 6.5, 6–8 °C are not consistent with the 20S boundary being composed solely of a 34-subunit structure, as would be required if the 20S polymer were uniquely the two-turn cylindrical disk aggregate. Rather, these molecular weight data, combined with the compositional data from Table I, are consistent with an equilibrium between A protein and a (39 ± 2)-subunit structure.[4] The number of subunits in the polymer (39) under these conditions is estimated by using the definition of z-average molecular weight, the f_{app} values from Table I, and the assumption that A protein is a trimer. Compositional assignments of this type have previously been used to analyze TMVP aggregates under these same conditions (Shire et al., 1979, 1981). The pH 6.5, 6–8 °C solutions are known to be metastable and, within several weeks, will decay completely to A protein (Shire et al., 1979). Thus, we are taking a "snapshot" of the molecular weight profile under these low-temperature conditions. We suspect that the very low molecular weight data point in Figure 2 (square at 4 mg/mL) may reflect a more rapid polymer dissociation than usual or perhaps even convection in the equilibrium experiment. The nonequilibrium nature of these solutions obviates more extensive analysis of the low-temperature, pH 6.5 data.

The pH 7.0 solutions appear to be nonideal and self-associating.[5] Exchange measurements with radioactively labeled TMVP suggest a rapid reaction between A protein and the

[3] This is, however, highly pH dependent. A single preliminary experiment (four replicates of a solution at c_0 = 5.3 mg/mL) performed at pH 6.6, 20 °C (conditions that gave 93.4% of a 22.5S species) gave a measured M_z of 740 400 ± 11 700, or approximately two subunits less than at pH 6.5 and 5.3 mg/mL (see the triangles in Figure 1).

[4] Katzel (1981) has inferred the existence of heptamer as well as trimer at pH 7.0, 5.5–15 °C in the 4 S boundary ("A protein"). A complete explanation of the assembly of TMV and TMVP must include the existence of all the molecular species. However, under these conditions (about 63–83% higher aggregate), the existence of heptamer is inconsequential to interpretation of the molecular weight observations.

[5] Apparently this is also true of the pH 6.5, 20 °C solutions, where the amount of polymer is >84%, resulting in only a slight increase of M_z with concentration due to polymerization. Since this increase in M_z is small, the effects of nonideality are predominant, yielding the observed decrease of the apparent M_z with concentration. Apparently this is not so in the more rapidly warmed solution (dT/dt at 0.008 °C/min) where small variations in the concentration of overshoot material can greatly affect the M_z values measured.

20S boundary polymers (Richards & Williams, 1972). Yet it is known that at pH 7.0, 20 °C the formation of the 20S material from 4S material initially at 0 °C is a slow process with a half-time of 5 h. When the more sensitive interference optical system is used, the sedimentation velocity patterns at pH 7.0 and 20 °C have a raised base line between the boundaries [data not shown; cf. Laue (1981)]. The presence of a concentration gradient between the 4S and 20S boundaries suggests that there may be an equilibrium between different polymer forms of the coat protein. A "micelle" system with appreciable intermediates would exhibit such a mass transport behavior, the term micelle implying cooperative effects that give rise to a bimodal molecular weight distribution (Tai & Kegeles, 1984). Kinetic effects cannot be ruled out. Similar results could be seen if there were a distribution of molecules not at equilibrium.

Alternative Models for Interpretation of the pH 7.0, 20 °C Data. The most likely alternative interpretation of these molecular weight data would involve higher aggregates of disk, i.e., 68-mer, 102-mer, etc. A mixture of nonreacting two-turn disk (34 subunits) and three-turn aggregate (49–51 subunits) or of two-turn disk and four-turn disk (68 subunits) would be apparent in the sedimentation velocity experiments. The larger polymers should resolve readily from the predominant 34-subunit species (Durham, 1972).

Reacting boundaries involving "dimers", i.e., 34-mer → 68-mer, do not resolve as separate sedimentation peaks unless they are kinetically controlled reactions (Bethune & Kegels, 1961; Oberhauser et al., 1965). Rather, a skewing of the trailing or leading edge of the boundary occurs, depending upon the extent of association. At low extents of association, the leading edge of the boundary should be skewed, indicating the presence of 68-mer. This is not apparent [cf. Schuster et al. (1979)] although concentration dependence sharpens the leading edge of a reacting boundary. A skewed trailing edge would be obscured by the raised base line between the 4S and 20S boundaries. The original electron microscopy experiments that identified the double or two-turn disk were performed with solutions at pH 7.0, 20 °C, 0.1 M ionic strength, conditions that gave a 4S boundary and a symmetric 19S boundary (Durham et al., 1971; Durham & Finch, 1972). However, dimers and trimers of disk or helical structures appeared frequently in the micrographs [cf. Figure 6 of Durham et al. (1971); cf. plate 1 of Durham & Finch (1972)].

A number of workers have reported molecular weight measurements of TMVP at temperatures and pH values that differ from the experimental conditions reported here (Durham, 1972; Durham & Klug, 1972; Westover & Stevens, 1977; Stevens & Loga, 1977). Direct comparisons with our results are thus difficult. In most instances where a large polymer species is required to fit the molecular weight data, a 34-subunit disk structure has been invoked (Durham & Klug, 1972; Stevens & Loga, 1977), although an indefinite polymerization of trimers has also been proposed to occur (Stevens & Loga, 1977). To date, no published molecular weight data have required the inclusion of structures larger than 34-mers. It is known that in solutions of high ionic strength and at high pH, disks stack to form irregular rods (Durham & Finch, 1972). Durham & Klug (1972) claimed a monomer–trimer–34-mer would not fit their M_w molecular weight data (Durham, 1972) at temperatures less than 15 °C. Rather, their model involves a nucleated condensation up to 34 subunits and a terminal ring closure. The logical extension of that model would involve higher aggregates of disk.

The curves presented in Figure 3 show simulations of a sedimentation equilibrium experiment with a solution of TMVP trimers in equilibrium with 39-mers, model 1, or trimers in equilibrium with 34-mers that are in turn in equilibriuim with 68-mers, model 2. These are to be compared with the experimental data at pH 7.0 and 20 °C. The curves presented are derived from least-squares fits of the data and are the best estimates generated by trial and error within the constraint that between 17% and 37% of the material is A protein, i.e., 4 S.[6] It is apparent that the dimerization of disk models will not fit both the M_w and the M_z data whereas the single N-mer model will. Thus, the data favor a single aggregate species in equilibrium with a trimer model, although a distribution of polymers cannot be excluded and in fact is required by the rapid exchange results of Richards & Williams (1972). Nonideality is not included here. A reasonable estimate of the second virial coefficient will slightly improve the agreement between model 1 and the data. This is demonstrated by the dashed lines which correspond to model 1 plus a realistic correction for nonideality due to excluded volume, asymmetry, and the charge of a 39-mer of TMVP at pH 7.0. (It must be stressed that these fits do not specify a unique model but rather demonstrate the reasonableness of model 1.)

Sedimentation velocity experiments under various conditions, pH 6.5–7.4, 18 °C, suggest that the composition of the 20S boundary can vary and that the time to attain chemical equilibrium is strongly dependent upon the sample history (Adams et al., 1981). In particular, TMV protein samples warmed to 20 °C at high concentration (20.6 mg/mL) and subsequently diluted (prewarmed) have different compositions as estimated by sedimentation velocity compared with samples which are first diluted and then warmed to 20 °C (prediluted). Furthermore, the measured sedimentation coefficients as well as the weight fractions of the so-called 4S and 20S boundaries differ. The compositions and sedimentation coefficients for the prewarmed and prediluted samples eventually become equivalent over time, reflecting a redistribution of protein aggregate size within the 20S boundary (S. Shire and T. M. Schuster, unpublished results). However, the initial rates of reconstitution of prewarmed and prediluted TMV protein with RNA are quite different (S. Shire and T. M. Schuster, unpublished results) and this undoubtedly reflects differences in the concentration of nucleation species. It can be inferred that this observation is not due simply to a disk aggregation model where the relative amounts of 34-mer to 68-mer may vary. The presence of more than 7% nonreacting 68-mer is *not* consistent with the obtained molecular weight data (see Figure 3, model 2). Therefore, a more likely interpretation of the results is that the 20S boundary consists of a distribution of aggregates and that the size of the majority of the polymers in the 20S boundary varies with temperature and pH, shifting to 39 ± 2 subunits at pH 7.0 and 20 °C. This is also consistent with previous reconstitution experiments at pH 7.0 (Shire et al., 1981). Preliminary UV difference spectroscopy data show little difference between 20S and 24S TMVP, suggesting that 20S TMVP at pH 7.0 is similar in structure to 24S TMVP at pH 6.5 and thus, by inference, is also helical (Potschka, 1981). Recent circular dichroism measurements of TMV

[6] Nonlinear least-squares procedures exist for fitting multiple channel, multiple speed sedimentation equilibrium data to a unique model (Johnson et al., 1981). Short-column experiments span only a limited concentration range in each channel and thus do not provide enough information to determine unique fits with complex models (such as those involving association and nonideality). Nonetheless, it should be noted that all attempts to fit the pH 7.0, 20 °C data with model 2 gave results very similar to those indicated in Figure 3 (see model 2).

protein [see following paper (Raghavendra et al., 1985)] under the same virus assembly conditions used here and under disk crystallizing conditions have revealed significant spectral (structural) differences, indicating that the disk structure seen in crystals is not the same aggregate present in the 20S boundary in solution. Since aggregates larger than 34 subunits cannot be in the form of the closed cylindrical disk that has been characterized in TMVP crystals (Bloomer et al., 1978), it is difficult to imagine any structure other than a "two-plus" turn helical array of subunits that can be consistent with both present molecular weight measurements and previous electron micrographs.

Summary of the TMVP Results. At pH 6.5, 20 °C, the molecular weight data are consistent with an equilibrium between a trimer and a (49 ± 2)-subunit short helical rod structure. At pH 7.0, 20 °C and at pH 6.5, 6–8 °C, the molecular weight data are consistent with an equilibrium between a trimer and a (39 ± 2)-subunit helical rod structure. Under all three conditions, a distribution of sizes of helical aggregates cannot be excluded, and, in fact, such a distribution is required to explain the sedimentation velocity profiles and the radioactively labeled TMVP exchange results (Richards & Williams, 1972). Simulations of the sedimentation equilibrium experiments show that a model involving a 34-subunit dimerization reaction is unable to fit the molecular weight data at pH 7.0, 20 °C. Thus, the pH 7.0 and the low-temperature pH 6.5 data are not consistent with the assignment of the 20S boundary as being composed of a 34-subunit disk structure. It must be emphasized that a kinetic pathway of TMV assembly involving a 34-subunit disk as an intermediate species is not necessarily excluded by these data. Low concentrations of disk may exist and, in the presence of TMV RNA, may serve as a nucleating species for assembly. Further work is needed to substantiate or to disprove the existence in solution of this postulated kinetic intermediate.

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