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Mechanism of tobacco mosaic virus assembly: Incorporation of 4S and 20S protein at pH 7.0 and 20°C

(protein–RNA interactions/initiation and elongation of reconstitution/metastable protein aggregates/analytical ultracentrifugation)

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ABSTRACT **The mechanism of assembly of tobacco mosaic virus (TMV) has been investigated at pH 7.0 and 20°C by analytical ultracentrifugation. Under these conditions the overall rates of interconversion of 4S and 20S TMV coat protein are sufficiently slow to make possible measurements of the concentrations of remaining 4S and 20S TMV coat protein after addition of homologous RNA to solutions containing, initially, various mass ratios of 20S protein to 4S protein. It has been possible to measure, by schlieren boundary analysis, the relative rates of incorporation of 4S and 20S TMV protein into the growing nucleoprotein rod over the range of initial 20S:4S protein mass ratios from 93:7 to 18:82. The results show that the amount of incorporation of 20S TMV protein depends on the initial 20S:4S mass ratio between ≈100% and 60% 20S protein but that reconstitution can proceed with ≈100% 20S TMV protein to form full virus-size rods. However, when the initial protein solutions have less than 60% 20S protein, ≈80% of the reconstituted nucleoprotein is preferentially formed from 4S coat protein. The remaining ≈20% appears to require preformed 20S coat protein. These results suggest that a larger region of RNA than previously estimated is involved in the rate-limiting nucleation step in assembly and may explain previously conflicting results concerning the elongation phase of assembly when starting with partially assembled rods.**

Since the early reconstitution of tobacco mosaic virus (TMV) *in vitro* by Fraenkel-Conrat and Williams (1), TMV has served as a paradigm for macromolecular self-assembly and is the first virus for which detailed structural aspects of assembly are being elucidated (2, 3). The isolated coat protein (TMVP) can exist in a variety of polymerization states and exists as an equilibrium mixture of 20S and 4S sedimenting boundaries at pH 7.0 and 20°C (4). Recent sedimentation experiments suggest that the so-called "20S" boundary varies from about 24S to 19S over a pH range of 6.5–7.2 (5). For convenience, we will continue to refer to this TMVP boundary as having a 20S sedimentation coefficient with the understanding that the boundary does not represent a species with a single molecular weight. Because TMVP exists in these various equilibrium states it is difficult to discern the relative roles of these various aggregation states in the elongation (rod growth) process, and controversy has developed regarding the role of preformed 20S aggregates in virus assembly. Although workers in various laboratories concur that the 20S aggregates serve as an obligate nucleus for initiation of assembly, there is disagreement concerning the direct participation of 20S TMVP in the subsequent elongation of virus rods. Details of experiments and conclusions from various laboratories have been presented in three reviews (6–8).

Previous studies at pH 7.0 and 20°C of the mechanism of rod elongation have all relied upon measurements of virus formation and have not involved measurements of TMVP reactants. Recent experiments at pH 6.5 and 6.5°C enabled us to measure directly the rates of incorporation into virus of the protein aggregates and to conclude that under these conditions 4S protein is incorporated 50–70 times more rapidly than 20S TMVP (9). Under the nearly physiological conditions used by other researchers [pH 7.0, 0.1 M ionic strength $K(H)PO_4$ at 20°C], the rate of virus assembly is too rapid to make direct kinetic measurements by centrifugation. However, it is possible to measure the concentrations of the free 20S and 4S protein aggregates before and after substantial reconstitution because the rate of formation and depolymerization of the 20S TMVP is much slower than the rate of virus assembly (8). This makes it possible to draw conclusions regarding the relative rates of assembly from the nonequilibrium protein composition produced as a result of incorporation of protein aggregates into virus.

MATERIALS AND METHODS

TMV (common strain), TMVP, and TMV RNA were prepared and purified as reported (9), with the same methods used for concentration determinations. Samples, rotors, and cells were carefully handled to maintain all samples at 20°C (9, 10) for analytical ultracentrifugation. The concentrations of sedimenting components were determined from schlieren patterns evaluated as described (10).

RESULTS

Reconstitution with Equilibrium Mass Ratios of 4S:20S TMVP. Representative schlieren sedimentation patterns of TMVP obtained before and after addition of TMV RNA to TMVP are shown in Fig. 1*A*. In this experiment the mass ratio of TMVP to RNA was 25:1, as compared with a ratio of 19:1 in the native virus. The corresponding sedimentation coefficients and concentrations of TMVP and of reconstituted TMV are given in Table 1, Exp. 52. The schlieren pattern seen in the reconstitution sample in Fig. 1*A* represents the nonequilibrium TMVP composition after 30–60 min of reconstitution. Photographs of transient TMVP concentrations like that in Fig. 1*A* can be obtained because the half-time for the overall 4S-to-20S reequilibration reaction is much greater than that for reconstitution or for this kind of sedimentation analysis (8, 12). The photographs at low speed showed the reconstituted virus boundary, which sediments at 173 S, the value expected for whole virus (13) (see Table 1, Exp. 52). The amount of reconstituted virus corresponds to a yield of about 51%, assuming that all the added RNA can completely participate in assembly. At higher speed at later time in the centrifugation experiment, the protein boundaries are fully resolved (Fig. 1*A*). The resulting nonequilibrium

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Abbreviations: TMV, tobacco mosaic virus; TMVP, tobacco mosaic virus protein; $K(H)PO_4$, potassium hydrogen orthophosphate at a given pH and ionic strength.

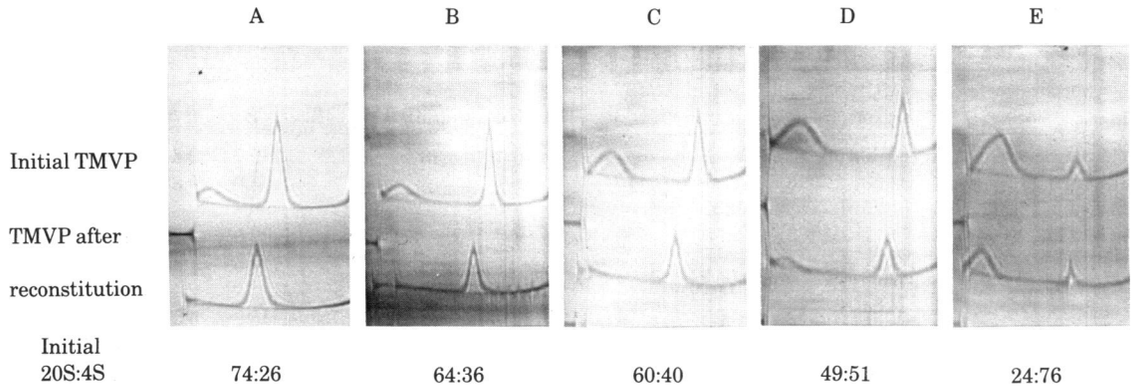

FIG. 1. Schlieren sedimentation patterns resulting from addition of RNA to TMVP at various initial ratios of 20S:4S protein. For each photograph, upper patterns are before and lower patterns after RNA addition. Centrifugation was begun ≈15 min after addition of RNA. The resulting protein compositions were determined at 52,000 rpm rotor speed after the reconstituted TMV had sedimented. (*A*) Exp. 52, TMVP at 5.09 mg/ml; (*B–E*) Exp. 66, TMVP at 4.6 mg/ml. Sedimentation coefficients and concentrations are given in Table 1. RNA concentration was 0.2 mg/ml in all solutions. All runs were at 20°C and pH 7.0 in 0.1 M ionic strength $K(H)PO_4$.

TMVP is composed of only the 4S and 20S boundaries. No discrete intermediate species were observed.

From the concentration changes summarized in Table 1 it can be seen that approximately 48% of the protein mass (29 disks per rod) in the reconstituted virus originates from protein in the 20S boundary.

Fig. 1*A* shows a strikingly large decrease in 4S concentration, from 1.26 to 0.21 mg/ml, after reconstitution. This corresponds to a change in the 20S:4S mass ratio from 76:24 initially to 93:7 shortly after assembly. At this final TMVP concentration, 3.14 mg/ml, the equilibrium 20S:4S ratio is 70:30 (5, 8). Sedimentation measurements at later times of the nonequilibrium TMVP after reconstitution revealed a slow TMVP reequilibration by depolymerization from 20 S to 4 S in agreement with Butler's observations (12). We have not studied this process in detail. However, no intermediate TMVP aggregates were observed during the reequilibration. Cooling of the reaction mixture to 0°C after reconstitution and then rewarming it to 20°C yielded full depolymerization of the unincorporated 20S protein, but not of the reconstituted 173S virus. The resulting TMVP was initially all 6S protein at 20°C. This transient species is usually observed initially after warming 4S TMVP but before the formation of the 20S boundary (4), demonstrating that the excess TMVP behaves in the expected fashion.

Reconstitution with Varying Mass Ratios of 20S:4S TMVP in Synthetic Mixtures. Durham (4) has shown that under the conditions used in the present experiments, the half-time for 20S protein formation at 20°C from 4S TMVP, previously at 0–5°C, is 3–5 hr. The half-time of the reverse reaction has been estimated by Butler (12), who found values of a few hours by dilution experiments. This slow interconversion has made it possible to prepare nonequilibrium mixtures of TMVP with varying 20S:4S ratios. The results of a series of experiments using a 20% excess of TMVP, assuming fully competent RNA, are shown in Fig. 1 *B–E* and Table 1, Exps. 66a–66d. This series of experiments was performed by using single protein and RNA stock solutions in order to reduce scatter of the data resulting from any variability in the quality of the RNA preparations. The extent of incorporation of 20S TMVP is seen to be essentially constant over the range of 1.1–2.8 mg/ml initial concentration of 20S TMVP, corresponding to a range of 20S:4S mass ratios from 24:76 to 64:36. At a higher total TMVP concentration (Table 1, Exp. 52) at which the 20S:4S ratio was 74:26, the extent of incorporation of 20S protein increased slightly. In contrast to this nearly constant extent of incorporation of 20S TMVP, the amount of 4S protein taken up during reconstitution increases with increasing initial concentration of 4S protein as does the concentration of reassembled virus. The yields of reconstituted virus range from 63% at a 20S:4S mass ratio of 64:36 to 79% at the corresponding ratio of 24:76.

The experiments with synthetic mixtures were extended to the higher TMVP:RNA mass ratio of 45:1, and the results are presented in Table 1 as Exps. 102b, 102c, 102e, 102f, and 104a–104j. The purpose of extending the experimental conditions was to ascertain whether or not the previous results depend on the TMVP:RNA mass ratio in the reconstitution mixture as might be expected if some TMVP component were being selectively removed, resulting in a 20S boundary consisting of aggregates that are not capable of reassembly with RNA. The overall results remain unchanged and are summarized for experiments 104a–104j in Fig. 2. The increase of 4S TMVP participation in assembly as a function of 4S protein concentration is quite marked in contrast to that of 20S TMVP, which is remarkably constant over the 20S:4S mass ratio range of 81:19 to 18:82.

Reconstitution with Varying Concentrations of TMVP at Equilibrium. In addition to the previous experiments using synthetic mixtures of 20S and 4S protein, Table 1 summarizes results of reconstitution experiments with RNA and equilibrium TMVP at varying concentrations. As before, nearly all the 4S protein was incorporated after addition of the RNA, resulting

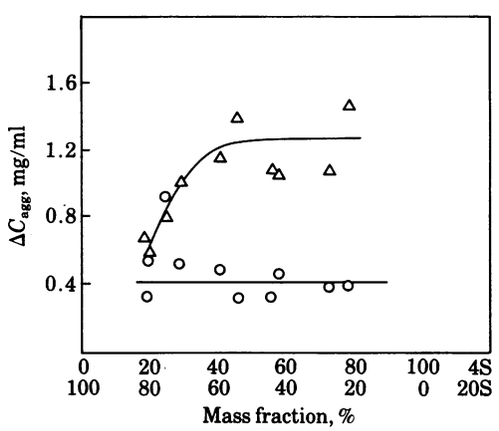

FIG. 2. Incorporation of 20S (○) or 4S (△) TMVP resulting from addition of RNA (0.1 mg/ml) to TMVP at 4.7 mg/ml with different 20S:4S mass ratios. All concentrations were determined from areas of schlieren sedimentation patterns similar to those shown in Fig. 1.

Table 1. Sedimentation analysis of TMV reconstitution

Exp.	Conc., mg/ml	Mass ratio (20S:4S)	TMVP $s_{20,w}$, S	TMVP C, mg/ml	TMVP + RNA $s_{20,w}$, S	TMVP + RNA C, mg/ml	$\Delta C_{20S}/C_{Recon}$
Reconstitution with equilibrium TMVP							
52	5.09	74:26	20.1	3.83	18.3	2.93	0.44
			5.2	1.26	3.8	0.21	
					173	2.05	
Reconstitution with synthetic mixtures of 4S and 20S TMVP*							
66a	4.42	64:36	19.8	2.83	18.4	2.14	0.29
			4.8	1.59	4	0	
					172	2.39	
66b	4.65	60:40	19.7	2.77	18.4	2.00	0.28
			4.3	1.88	4	0	
					168	2.79	
66c	4.93	49:51	19.9	2.43	18.7	1.78	0.23
			4.8	2.50	3.8	0.40	
					173	2.89	
66d†	4.58	24:76	20.3	1.11	21.6	0.45	0.22
			6.1	3.47	4.1	1.28	
					165	2.99	
102b	8.67	83:17	19.2	7.19	18.4	6.25	0.49
			4.6	1.48	4.3	0.61	
					167	1.91	
102c	8.68	65:35	19.7	5.65	19.4	5.00	0.38
			4.9	3.03	4.1	2.04	
					167	1.73	
102e	8.08	88:12	18.4	7.10	17.3	6.34	0.49
			4.0	0.98	4.3	0.26	
					164	1.56	
102f	8.66	71:29	20.0	6.2	19.8	5.55	0.38
			4.5	2.46	4.3	1.49	
					168	1.71	
104a	4.50	80:20	19.1	3.62	18.3	3.08	0.45
			4.0	0.88	4	0.29	
					174	1.19	
104b	4.31	81:19	19.0	3.50	18.4	3.17	0.31
			3.5	0.81	4	0.13	
					174	1.06	
104c	4.68	71:29	19.4	3.31	18.5	2.79	0.32
			3.8	1.37	3.1	0.35	
					175	1.62	
104d	4.69	75:25	19.6	3.53	18.4	2.60	0.51
			4.0	1.16	4	0.36	
					174	1.82	
104e	4.79	58:42	19.8	2.76	19.1	2.27	0.27
			4.6	2.03	4.5	0.82	
					176	1.79	
104f	4.97	54:46	20.1	2.69	19.2	2.38	0.17
			4.4	2.28	4.0	0.89	
					175	1.79	
104g	5.22	44:56	20.3	2.31	20.3	1.99	0.22
			5.1	2.91	4.7	1.82	
					175	1.48	
104h	4.67	42:58	20.4	1.94	20.0	1.48	0.29
			5.5	2.73	4.3	1.68	
					172	1.59	
104i	4.89	18:82	20.3	0.90	20.3	0.51	0.21
			5.6	3.99	4.9	2.62	
					173	1.85	
104j	4.73	27:73	20.4	1.26	20.4	0.88	0.25
			5.7	3.47	5.0	2.38	
					175	1.16	
Reconstitution with varying concentrations of TMVP at equilibrium							
86a	4.0	65:35	19.4	2.64	18.0	1.97	0.36
			4.3	1.36	4	0.26	
					165	1.86	
104k	4.38	80:20	19.2	3.5	18.3	1.65	0.64
			4.0	0.88	4	0	
					164	2.87	
54	4.86	69:31	19.3	3.38	18.3	2.49	0.36
			4.5	1.48	4	0	
					167	2.49	
70	6.76	81:19	18.9	5.52	18.2	2.69	0.66
			3.2	1.24	4	0	
					165	4.27	
86b	8.66	77:23	18.7	6.71	17.6	5.94	0.34
			4.9	1.95	4	0.56	
					162	2.27	
78	8.92	80:20	19.1	7.06	18.2	5.58	0.44
			4.5	1.86	4.7	0.15	
					165	3.35	

Sedimentation coefficients and concentrations resulting from addition of RNA to TMVP at 20°C, pH 7.0, 0.1 M ionic strength $K(H)PO_4$. RNA was at 0.1 mg/ml (Exps. 104a–104j), 0.2 mg/ml (Exps. 52, 54, 66, 78, 86, 102, and 104k), and 0.27 mg/ml (Exp. 70). The table is organized in terms of the sections presented in *Results*. The sedimentation coefficients and concentrations reported to three significant figures have estimated uncertainties of 3%. The concentrations of reconstituted virus obtained in these experiments were determined in each case by summing the concentration decreases of the 4S and 20S TMVP resulting from RNA addition and dividing by 0.95 in order to account for the contribution of the RNA mass. Centrifugation was begun ≈10 min after addition of RNA and dialysate, and schlieren patterns were photographed during the subsequent 1–2 hr of centrifugation. After this period of reaction the reconstitution is nearly complete, as judged by sedimentation, turbidity measurements, and electron microscopy (8, 11). Our electron microscopy measurements after 15 min indicate an average rod length greater than 2000 Å. Sedimentation measurements after 48 hr of reconstitution indicate a noticeable increase in the yield of material >160 S to greater than 85% based on the initial RNA concentration (unpublished results). Therefore, the sedimentation experiments reported here represent a time sampling late in the reconstitution reaction.

* Synthetic, nonequilibrium mixtures of 4S and 20S protein at 20°C and pH 7.0 in 0.1 M ionic strength $K(H)PO_4$ were prepared for ultracentrifuge analysis by mixing together different amounts of protein that had been equilibrated at 20°C for 12 hr with 4S protein warmed from 0°C to 20°C just prior to mixing. These mixtures, which resulted in solutions with different 20S:4S mass ratios at various concentrations, were divided into two equal portions, and a volume of RNA was added to one sample and an equal volume of protein dialysate to the other sample.

† This particular 20S:4S composition was obtained by warming a sample of TMVP at 4.6 mg/ml from 0°C to 20°C and, thus, instead of a 4S component this sample has a transient 6S boundary which has been observed by other workers (8).

in protein compositions with enhanced 20S:4S mass ratios. The data for all the experiments listed in Table 1 at different total protein concentrations are plotted in Fig. 3 as $(\Delta C_{20S}/C_T) \times 100$, the decrease in 20S concentration divided by the concentration of reassembled virus, against the initial 20S:4S TMVP mass ratios. There is a clear trend in the data indicating that, as the concentration of 4S protein is increased relative to that of the 20S TMVP, the percentage of the reassembled virus originating from 20S TMVP decreases to a plateau level. There does not appear to be any correlation of the fraction of 20S protein incorporated with the total initial 20S TMVP concentration.

Reconstitution with 20S Supernatant TMVP. The results shown in Fig. 1 agree with previous finding (8) of slow overall 4S $\rightleftharpoons$ 20S interconversion because it would otherwise not be possible to obtain nonequilibrium solutions by this method consist-

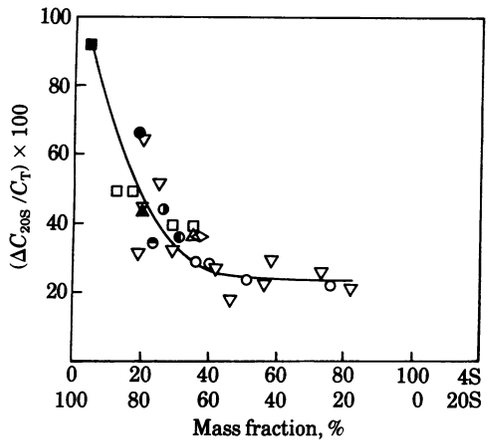

FIG. 3. Weight percentage of reconstituted virus originating from the 20S boundary as a function of the initial 20S:4S TMVP mass ratio. Total protein concentrations (experiment nos. in parentheses): ■, 1.61 mg/ml (72b); ◇, 2.69 mg/ml (72d); △, 4.0 mg/ml (86a); ○, 4.6 mg/ml (66); ▽, 4.7 mg/ml (104); ◐, 4.86 mg/ml (54); ◑, 5.09 mg/ml (52); ●, 6.76 mg/ml (70); ◒, 8.66 mg/ml (86b); □, 8.7 mg/ml (102); ▲, 8.92 mg/ml (78). RNA concentrations were (experiment nos. in parentheses): 0.088 mg/ml (72), 0.1 mg/ml (104), 0.2 mg/ml (52, 54, 66, 78, 86, and 102), and 0.27 mg/ml (70). Corresponding sedimentation values and concentrations of protein components are given in Table 1. For Exps. 72b and 72d, supernatant 20S protein (see text for details) was used as the source for 20S TMVP, and the corresponding sedimentation coefficients and TMVP concentrations are given in the text (Exp. 72b) and Fig. 4 (Exp. 72d).

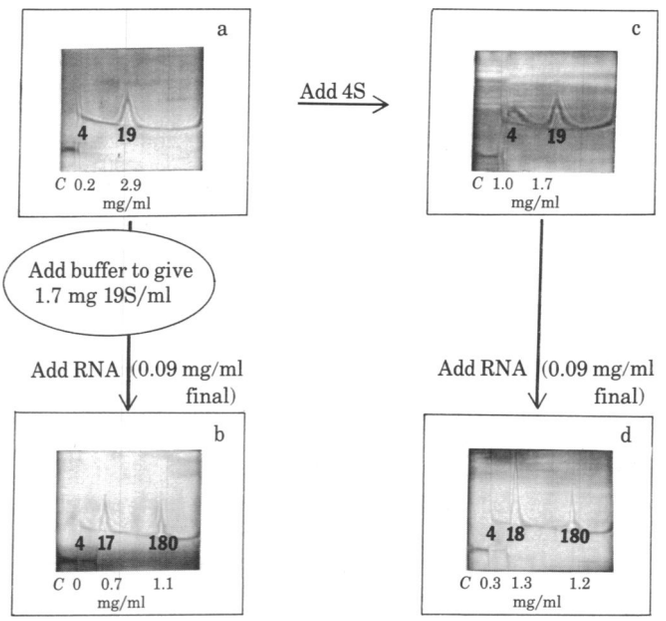

FIG. 4. Competition experiment at pH 7.0 and 20°C in 0.1 M ionic strength $K(H)PO_4$ involving reassembly of TMV RNA with 20S supernatant and 4S TMVP. Sedimentation coefficients are given in schlieren photographs for respective boundaries, and concentration of material in each boundary is given in mg/ml below each peak. (*a*) Supernatant 20S TMVP prepared about 45 min prior to addition of buffer and RNA; (*b*) supernatant 20S TMVP reassembled with TMV RNA; (*c*) synthetic mixture of 4S and 20S supernatant TMVP, prepared by adding 4S TMVP to the same 20S supernatant sample used in reconstitution experiment shown in *b*; (*d*) reconstitution of the sample shown in *c* with TMV RNA. Photographs were taken 20–30 min after each RNA or TMVP addition. All centrifugations were at 40,000 rpm in 12-mm double-sector cells.

ing of very little 4S protein (<8%). This conclusion is strengthened by the results of reconstitution experiments in which the reconstituted nucleoprotein was pelleted 10 min after addition of RNA. The supernatant, hereafter referred to as "20S supernatant," was then removed and added to two double-sector centrifuge cells. A volume of RNA was then added to one cell and an identical volume of reconstitution buffer to the other. Centrifugation of these samples was begun approximately 0.5 hr after the first addition of RNA. The 20S supernatant sample without RNA consisted solely of a 20.3S boundary at 1.61 mg/ml, which demonstrates that the time required for depolymerization of the 20S TMVP is substantially less than that for centrifugation because at this TMVP concentration there is 50% 4S and 50% 20S protein at equilibrium (5). The addition of RNA to the 20S supernatant (final TMVP:RNA mass ratio of 17:1) yielded a 157 ± 7S boundary at 0.71 mg/ml and a 18.9S boundary at 0.94 mg/ml, corresponding to a yield of about 46%, for the second RNA addition. Thus, ≈100% 20S TMVP is capable of forming reconstituted nucleoprotein rods of substantial length because $s_{20,w}$ values of about 160 S correspond to rod lengths of at least 2000 Å, as estimated by traditional computational methods (14).

Reconstitution with Synthetic Mixtures Prepared with 20S Supernatant. The results of the previous experiments demonstrate that both 4S and 20S aggregates can participate in the assembly of TMV after the initial nucleation of RNA by 20S aggregates. As a further test of the relative efficacy of these aggregates, reconstitution was carried out with a synthetic mixture of 20S supernatant and added 4S protein such that the resulting solution has the same concentration of 20S protein as the original 20S supernatant, as shown in Fig. 4.

Centrifugation of the synthetic mixture yielded the expected 20S:4S mass ratio with concentrations and sedimentation coefficients given in Fig. 4*c*. Addition of identical amounts of RNA to either 20S supernatant or to the synthetic 20S and 4S mixture resulted in samples before reconstitution with 20S:4S:RNA mass ratios of 19:0:1 or 19:11:1, respectively. Sedimentation analysis yielded the $s_{20,w}$ values and concentrations given in Fig. 4 *b* and *d*. Similar amounts of reconstituted material were formed in both samples with an $s_{20,w}$ value of about 180 S. Although this value differs from that of 157 S reported in the previous section, the difference between these values is probably not significant because the sedimentation experiments, which were not designed to characterize the reconstituted virus carefully, were conducted at high rotor speeds of either 40,000 or 52,000 rpm in order to resolve the TMVP boundaries rapidly. After reconstitution with 20S supernatant, the average sedimentation coefficient of the remaining protein is about 17 S, probably because of a concentration-dependent slow depolymerization. For the sample with 4S TMVP added, there is a reduction of 20S TMVP incorporation which can be accounted for by the competitive incorporation of 4S TMVP.

DISCUSSION

From a previous study of the rates of reconstitution at pH 6.5 and 6.5°C we concluded that 4S TMVP (assumed to be a trimer) is incorporated 50–70 times more rapidly than the 20S TMVP during elongation (9). The present results are clear in showing that, when TMV reconstitution is carried out with the usual equilibrium "disk preparation" (8) of about 25% 4S and 75% 20S TMVP, addition of RNA results in a preferential incorporation of 4S TMVP even in the presence of excess 20S TMVP. These conditions of pH, temperature, ionic strength, and TMVP:RNA mass ratios are identical to those used by others who have obtained conflicting results by using partially assembled nucleo-

protein rods to study the elongation reaction. In addition, these new experiments have enabled us to prepare nonequilibrium TMVP solutions with a greatly enhanced mass fraction (>94%) of the 20S aggregates, which we termed 20S supernatant.

Because the formation of the 20S boundary and the depolymerization to 4S protein are slow, the results with 20S supernatant TMVP show that the 20S TMVP can serve as the exclusive protein source for TMV assembly. This demonstrated ability of 20S TMVP to react with TMV RNA to form nucleoprotein rods 2000–3000 Å long is in accord with the stereochemical interpretation of the recent x-ray crystallographic studies of the 20S disk structure (2).

The results shown in Fig. 4, in which the concentration of the 20S TMVP was sufficient to coat all the RNA present, show clearly that the elongation phase of TMV reconstitution can proceed with either the 20S or the 4S protein components, with 4S TMVP adding more rapidly during the growth of the virus rod.

These conclusions are further supported by the results shown in Fig. 2, where it is seen that as the amount of 4S TMVP increases relative to that of 20S TMVP, the amount of reconstituted virus formed and the amount of 4S protein incorporated into virus increase, although the 20S TMVP was in excess over the RNA. Furthermore, separate experiments show that addition of excess RNA to 20S supernatant results in the complete incorporation of the 20S TMVP, demonstrating that all the aggregates that make up this boundary can reassemble with RNA (unpublished observations). Again it can be concluded that 4S protein is added more rapidly during elongation even in the presence of competent 20S TMVP.

In addition, reconstitution experiments at varying protein concentrations, including the synthetic mixtures of Exps. 66, 102, and 104, yield an interesting result. At large values of 4S:20S TMVP in Fig. 3, the percentage of the reassembled virus originating from 20S TMVP decreases to a constant value of about 22%. This amount of 20S TMVP would coat about 1400 nucleotides of RNA, assuming that all particles reconstituted are 3000 Å long. This length of RNA is similar to that reported for the short 3′ RNA tail plus the nucleation region of the so-called "inside-out" model for TMV assembly (15). This apparent similarity should be regarded with caution because the experiments reported here do not characterize the distribution of 20S binding sites, which may or may not be contiguous along the RNA strand. It has been demonstrated that the assembly reaction begins from an internal initiation site about 1000 nucleotides from the 3′ end (16) and proceeds in both directions. The growth in the 5′-to-3′ direction is much slower than in the 3′-to-5′ direction (11, 17). In particular, recent experiments by Butler and Lomonossoff (18) strongly suggest that the 4S TMVP adds to the short 3′ RNA tail preferentially, and thus an assembly mechanism involving competitive binding of 20S and 4S aggregates in the 3′-to-5′ direction appears to be likely. Our previous study at pH 6.5 (9) also demonstrated that the kinetics of the elongation of the virus rods is biphasic and, although we have not measured rate constants at pH 7.0 by the centrifuge method, the results of Fig. 3 and those of Butler and Lomonossoff (18) suggest that the kinetics may be biphasic due to the fact that the 20S and 4S aggregates are incorporated preferentially along different regions of the RNA chain.

In conclusion, the results presented here demonstrate that coat protein as either 4S or 20S TMVP can assemble with RNA to form essentially complete virus. We have confirmed that 20S protein is required for the nucleation phase of TMV reconstitution and find that for the elongation phase at pH 7.0 and 20°C there is preferential incorporation of 4S TMVP over about 80% of the virus length. However, the results presented here demonstrate that 20S TMVP can assemble with RNA to form essentially complete virus, but at a slower rate than does 4S TMVP.

These conclusions suggest a possible explanation for the conflicting results obtained previously by others concerning the relative rates of incorporation of 4S and 20S TMVP during the elongation phase of TMV assembly (7, 8). Those studies were done with partially assembled nucleoprotein rods in order to preclude kinetic effects due to the nucleation reaction. Although our finding that about 22% of the overall rod length requires 20S TMVP does not prove that all of this preassembled protein binds initially and contiguously along the RNA, such a possibility is likely. This kind of 20S protein binding might imply that the nucleation process of TMV assembly requires a larger number of two-layered disk structures per RNA strand than previously estimated (8) and, furthermore, would mean that measurements of the apparent elongation rates might be extremely sensitive to the length of the preformed partially assembled rods. Thus, partially assembled nucleoprotein rods with less than about 30 layers of subunits (i.e., ≈22% of the number in TMV) might still require 20S TMVP to overcome some part of the overall kinetic barrier involved in nucleation. Such partially assembled rods would reconstitute more rapidly with disk preparation TMVP (i.e., a mixture of 20S and 4S TMVP) than with 4S TMVP alone. Partially assembled nucleoprotein rods with more than about 30 layers of subunits would be expected to reconstitute somewhat faster with 4S TMVP than with disk preparation TMVP. In such a mechanism, 20S TMVP can be considered as an RNA-melting protein as well as a source of coat protein subunits.

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