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Tobacco Rattle Virus RNA-Protein Interactions

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Tobacco rattle virus RNA–protein interactions

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[Plate 24]

For the purpose of attempting to generalize the rules concerning morphogenesis of helical viruses, the *in vitro* reconstitution of the CAM strain of TRV was studied.

The conditions for reconstitution and the importance of the aggregation state of the protein for initiation and elongation are compared with those of TMV.

The initiation step consisting of the binding of RNA with the 36S disk of protein was easily accomplished. The polarity and the specificity of encapsidation of TRV RNA by homologous and heterologous viral protein is discussed.

1. Introduction

Tobacco rattle virus (TRV) is a multicomponent RNA plant virus which consists of two types of helical particles. Long particles of the virus are infectious but they produce only free long RNA. Short particles alone are not infectious but they are known to have the information for the viral coat protein. The long RNA may contain the information for a RNA replicase system (Harrison & Nixon 1959; Lister & Bracker 1969; Sanger 1968). The first virus to be reconstituted *in vitro* from its protein and RNA was tobacco mosaic virus (TMV) (Fraenkel-Conrat & Williams 1955). Two other helically constructed viruses, have also been reconstituted, barley stripe mosaic virus (Atabekov, Nonikov, Vishnichenko & Kabtanova 1970) and the C-isolate of TRV (Semancik & Reynolds 1969; Morris & Semancik 1973). We have previously reported conditions for the complete reconstitution of the CAM isolate of TRV (Abou Haidar, Pfeiffer, Fritsch & Hirth 1973).

In an effort to define the morphogenic stages in the *in vitro* reconstitution of TRV, we then made the following study. First, the different states of aggregation of TRV-protein were examined. Secondly, a comparison was made between TRV and other helical viruses, particularly TMV, with respect to the 'initiation complex' between the RNA and the protein and the elongation process. Finally we investigated the problem of the polarity of reconstitution and the specificity of the recognition process again making comparisons between the two helical viruses, TMV and TRV.

2. Materials and results

(*a*) *States of aggregation of TRV protein*

The analysis of coat protein under different conditions of pH, temperature and ionic strength (Fritsch, Witz, Abou Haider & Hirth 1973) resulted in the formation of only 2 types of aggregates, the dissociated protein (2–4S) and disks (36S). The 2S represents the monomeric form and the 36S probably corresponds to a two-layer disk as seen in the electron microscope.

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The phase diagram of TRV-protein is given in figure 1. At pH values below 4 and ionic strength of 0.2 M the protein is denaturated and precipitated. At pH 5.5 to pH 9.0 and ionic strength between 0.01 and 0.8, the predominant component is the 36S disk with the 4S component representing less than 10%. Between pH 4.7 and 5.2 at very low ionic strength, the 36S was always predominant. At pH 5.0 and ionic strength of 0.1, both components were present in approximately equal amounts. Between pH 4.7 and pH 5.0, an equilibrium between the 4S and 36S disks is observed. In this region of the phase diagram, we have also observed a component sedimenting at 22–25S. It was only present in minute amounts and never coexisted with 36S disks. Its significance and structure are not known. Offord (1966) and Semancik & Reynolds (1969) noticed an aggregation of TRV protein at 30 °C. We have obtained at pH 8.0 and 30 °C and ionic strength of 0.1 some undefined aggregates sedimenting at 47S and 73S. The nature of these aggregates is not known. We also irreproducibly obtained the stacked disks but a helical protein structure was never found. The TRV-protein disk seems to be very stable because it can be obtained in a wide range of pH (5.0–10.0) and from 0.01 to 0.8 ionic strength at 1–30 °C. The stability of the disk is probably due to strong bonding between the structural subunits. This lack of polymorphism of TRV protein in comparison with TMV protein is probably due to the stability of disks and the weakness of interactions between the surface region of disks. This property of TRV–protein plays a crucial role in the assembly of TRV (see below).

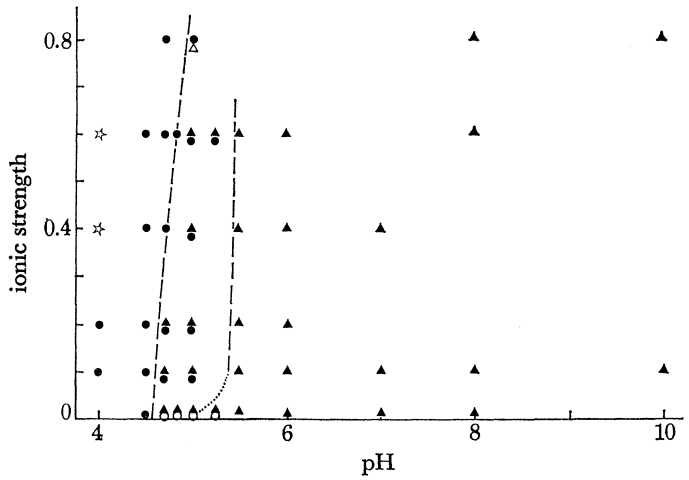

FIGURE 1. Diagram showing the range of existence of the different types of TRV protein aggregates as a function of pH and ionic strength at 2°. Symbols: ●, ○, 4S protein; ▲, △ 36S aggregate. The relative amounts of 4S and 36S components are indicated according to the following convention: ▲, More than 90% of 36S; ●, more than 90% of 4S; (▲/●), approx. equal amounts of 4S and 36S (from 30 to 70% of 4S or 36S); (▲/○), 10–30% of 4S; (△/●), 10–30% of 36S. The symbol ☆ indicates that the protein is denatured and precipitates.

(*b*) *Reconstitution of TRV*

We first attempted to repeat the reconstitution experiments described by Semancik & Reynolds (1969) for the C isolate. The failure of these attempts for our CAM isolate led us to reinvestigate the conditions of reconstitution.

In fact at pH 8.0, we obtained disks and short particles sometimes accompanied by particles longer than 50 nm, but standard long particles were never obtained. The discrepancy between our results and those of Semancik & Reynolds (1969) could simply arise from the use of the

CAM strain which is serogically different from the C isolate and has an amino-acid composition significantly different from the other strains of TRV (Miki & Okada 1970). We tried to reconstitute TRV in other conditions of pH and ionic strength. In the pH range of 5.0–8.5 and ionic strength varying between 0.01 and 0.6, our results were very similar in all conditions, i.e. formation of the 'initiation complex' and some elongation.

The 36S aggregate was shown to form and dissociate in the pH range 4.6–5.0. This implies that some side chain groups of the amino acids in the TRV protein subunits are titrating in this pH range and led us to try to reconstitute the TRV short and long particles under these conditions.

Total TRV–RNA was mixed with TRV protein in the ratio 1/10 at pH 4.7, 0.5 ionic strength phosphate buffer and incubated for 20 h at 1 °C. Analytical sedimentation of the reconstituted material after RNase treatment indicates the formation of different states of reconstitution, particularly two peaks with values of 145S and 245S corresponding to short and long TRV-particles. By electron microscopy and isopycnic sedimentation, we confirmed the presence of complete short and long TRV-particles. After RNase A treatment, the reconstituted particles were infectious. However the yield of reconstitution was always low (8–15 %). We note that the temperature has an important effect on the reconstitution since if we increased the temperature from 1 to 10 °C the reconstitution did not occur.

(*c*) *Partial and polar TRV-reconstitution*

The conditions described above at pH 8.0 give rise to partial reconstitution. We investigated the formation of the initiated and short particles by adding the RNA to protein disks in the ratio 1/2 in borate buffer 0.02 M, pH 8.0. After 6 h incubation at 1–2 °C, the protein disks which were not stabilized by the protein–RNA interactions were dissociated by dialysis against citric acid-Na_2HPO_4 buffer pH 4.5. In conditions where there is no formation of protein disks, the initiation did not occur, e.g. at pH 4.5 at different ionic strengths. These results confirmed those of Butler & Klug (1971) concerning the role of TMV protein disks in the initiation of the assembly of TMV.

We investigated the polarity of the reconstitution of TRV by using two techniques: the fixation of [^{14}C]dimedone (Glitz & Sigman 1970) on TRV-short and long separated RNAs and the isolation of the sequence –C–C–C–OH specific for the 3′-OH end (Minson & Darby 1973). It was first confirmed that the oxidation by the $NaIO_4$ and the fixation of [^{14}C]dimedone on TRV short and long separated RNAs did not give rise to degradation of the RNAs as was described by Thouvenel, Guilley, Stussi & Hirth (1971). [^{14}C]Dimedone labelled RNA was partially encapsidated as described above to generate only initiation complexes with little elongation (figure 2*b*, plate 24). The A_{260}/A_{280} ratio was 1.9 indicating the presence of considerable non encapsidated RNA. After RNase T1 treatment the radioactivity of the supernatant (i.e. the non encapsidated RNA) and of the pellet (the RNA encapsidated by the protein) was estimated. In the case of short TRV–RNA (table 1), 81 % of the radioactivity was in the supernatant and 19 % in the pellet. With long TRV–RNA 66 % of the radioactivity was in the supernatant and 34 % in the pellet. With TMV as a control, 94 % of the radioactivity was in the supernatant and only 6 % in the pellet.

An examination of the partial encapsidation of [^{14}C]dimedone-labelled RNA was made by electron microscopy using platinium shadowing in order to see the RNA strands protruding from the nucleoprotein rods or disks (figure 2*a*). The encapsidation of the TRV–RNA by

TRV–protein was seen always to begin from one end of the RNA molecules. We have never observed either encapsidation from both ends of the RNA or two tailed particles.

Recently, Zimmern (1975) showed that TMV–RNA terminates with the structure $m^7G^{5'}ppp5'Gp$ in which the m^7G has free 2′,3′ hydroxyls which can be oxidized by periodate treatment. However, Thouvenel *et al.* (1971) had found that the reaction of [^{14}C]dimedone with TMV–RNA was always equimolar and that 94% of the dimedone was found in the supernatant. It is possible that the reaction of [^{14}C]dimedone at the 5′-OH end did not occur because of steric hindrance or for some other unknown reason. Work is in progress to investigate the possibility of reacting dimedone at the 5′-OH end.

TABLE 1. DISTRIBUTION OF RADIOACTIVITY OF RNA LABELLED WITH [^{14}C]DIMEDONE FOLLOWING RECONSTITUTION AND RNASE T_1 TREATMENT

	percentage of total radioactivity	
RNA	supernatant	pellet
TRV (short)	81	19
TRV (long)	66	34
TMV	94	6

With TRV-short and long RNA, no attempt has been made to find a blocked 5′ terminal end group. However, our results do show that the 3′-OH ends of TRV–RNAs are not recognized and encapsidated by TRV-protein. Thus, it was demonstrated by Minson & Darby (1973) that the TRV-short and long RNAs have the sequence –C–C–COH at their 3′-OH end. This oligonucleotide, which terminates in a 3′hydroxyl group and has no uridylate or guanylate residues, is the only product in T_1 RNase digest that has a residual positive charge, and therefore migrates towards the cathode during electrophoresis at pH 3.5. All other products, which have negative or neutral charges, migrate towards the anode or remain at the origin. Separated short and long TRV–RNAs labelled with ^{32}P were partially encapsidated by TRV protein as described above. Oligonucleotides from T^1 RNase digests of the encapsidated and non encapsidated RNAs were fractionated by electrophoresis at pH 3.5. The 3′-OH terminal oligo-

DESCRIPTION OF PLATE 24

FIGURE 2. Electron micrographs of TRV–RNA partially encapsidated by TRV coat protein. Reconstitution performed in 0.02 M borate buffer pH 8 at 1–2 °C for 6 h followed by dialysis against citric acid-Na_2HPO_4 buffer pH 4.5. (*a*) selected particles from a platinium shadowed preparation showing RNA tails protruding from nucleoprotein rods or disks. (*b*) rods negatively stained by 1% uranyl acetate. The material was purified by sucrose gradient centrifugation.

FIGURE 4. Electron micrographs of particles negatively stained by uranyl acetate (1%). (*a*) particles formed from TRV-long RNA and TMV-protein. (*b*) particles formed from TRV-short RNA and TMV-protein. Both samples were obtained from reconstitution under our standard conditions for *in vitro* reconstitution of TMV: Na pyrophosphate buffer pH 7.25, ionic strength 0.5 for 12 h at 24 °C. The material was purified by two cycles of high speed centrifugation and RNase T_1 treatment.

FIGURE 6. Electron micrographs of heterologous reconstituted material from TRV-protein and TMV–RNA. Reconstitution was performed in 0.02 M buffer pH 8.0 at 1–2 °C for 12 h followed by dialysis against pH 4.5 citric acid-Na_2HPO_4 buffer. The RNA/protein ratio was 1/2. (*a*) selected particles and disks from platinum shadowed specimens from TRV-protein disks and short rods. (*b*) preparations negatively stained with 1% uranyl acetate.

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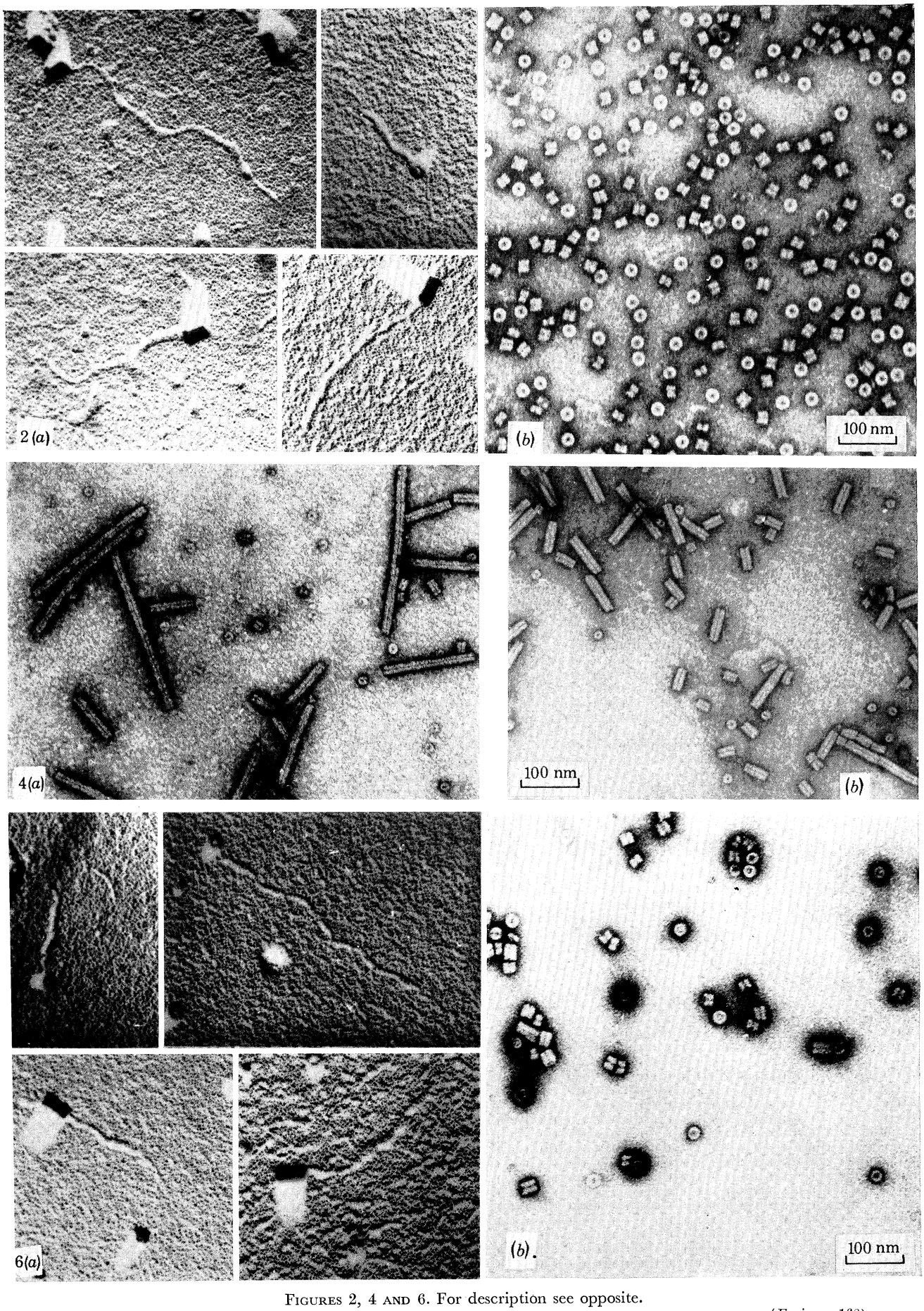

FIGURES 2, 4 AND 6. For description see opposite.

(*Facing p.* 168)

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nucleotide was present only in the non encapsidated parts of the TRV short (figure 3*c*) or long (figure 3*e*) RNAs.

These data show clearly that the 3′-OH ends of short or long TRV–RNA were not encapsidated by TRV protein. In addition, these results and those obtained with [^{14}C]dimedone and electron microscopy suggest that the reconstitution of TRV-short and long particles is a polar process, probably from the 5′-OH terminal to the 3′OH end.

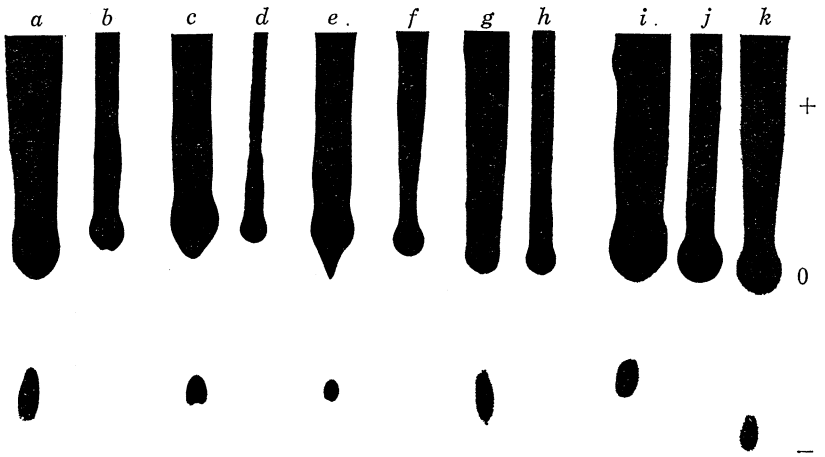

FIGURE 3. Electrophoresis of a total T_1 RNase digest of TRV ^{32}P short or long RNA and TMV–RNA on Whatman 3 MM paper in 0.5% pyridine/acetic acid buffer at pH 3.5 (*a*) to (*i*) or pH 2.5 (*j*) and (*k*) for 1 h at 50 V/cm. (*a*) TRV-total RNA as control; (*b*) TRV-protein encapsidated parts of TRV-short RNA and (*d*) of TRV-long RNA; (*c*) the parts of TRV-short RNA and (*e*) TRV-long RNA not encapsidated by TRV-protein; (*f*) parts of TRV-short RNA and (*h*) TRV-long RNA encapsidated by TMV-protein. (*g*) parts of short TRV–RNA or long TRV–RNA (*i*) not encapsidated by TMVprotein. (*j*) parts of TMV–RNA encapsidated by TRV protein. (*k*) parts of TMV–RNA not encapsidated by TRV-protein. All samples of TRV or TMV–RNAs were digested with RNase T_1 (1 unit/20 μg RNA) for 30 min at 37 °C before electrophoresis.

(*d*) *Encapsidation of short and long TRV–RNAs by TMV-protein: evidence for the polarity of heterologous reconstitution*

Separated TRV-short and long RNAs were encapsidated by TMV-protein under our standard conditions for TMV reconstitution pH 7.25, ionic strength 0.5 Na-pyrophosphate at 24 °C for 24 h. The spectrophotometric study shows that the A_{260}/A_{280} ratio of reconstituted material was 1.30–1.35 indicating that the reconstitution was partial in some cases. After T_1 RNase treatment, the buoyant densities of the reconstituted material and standard TMV were compared by equilibrium sedimentation on CsCl gradient. The reconstituted material sedimented to the same position in the gradient as standard TMV. This result shows that the density and thus the RNA content of reconstituted material were identical to those of native TMV. We defined the yield of reconstitution by the ratio of the amount of reconstituted nucleoprotein to the theoretical amount of nucleoprotein which could be obtained on the basis of the quantities of RNA used. By this criteria, the yield of heterologous reconstitution was 30% with short TRV–RNA and 50–60% with long TRV–RNA. Electron microscopic examination of the reconstitution products confirmed the existence of TMV-like particles (figure 4, plate 24). The length distribution of heterologous reconstituted nucleoprotein particles is shown in the histograms in figure 5.

To study the polarity of the heterologous reconstitution, we used conditions for only partial reconstitution (RNA/protein ratio 1/10, 12 h at 24 °C). The partially reconstituted material was purified by chromatography on hydroxyapatite columns according to Guilley *et al.* (1972) The oligonucleotide –C–C–COH specific for the 3′-OH ends of short and long TRV–RNAs was only found in the non-encapsidated parts either of short or long TRV–RNA (figure 3*g* and *i*). Therefore, the TMV protein did not recognize the 3′-OH end of TRV–RNA and it is probable that the heterologous reconstitution between TRV-short and long TRV–RNA and TMV protein is a polar process. The encapsidation seems to grow from the 5′ terminal towards the 3′-OH end.

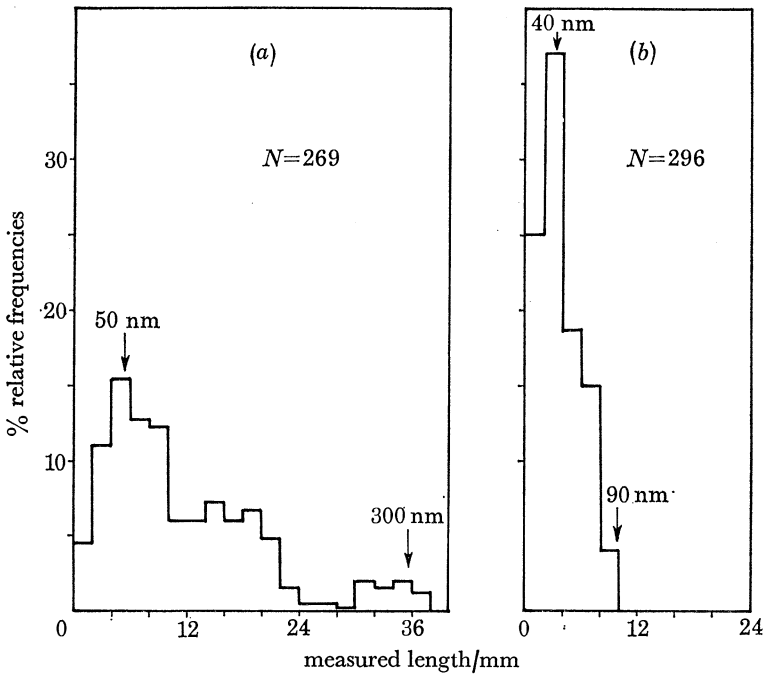

FIGURE 5. Histograms of length distribution of reconstituted particles originating from TRV-long RNA (*a*) and from TRV-short RNA (*b*) with TMV-protein. Reconstitution was performed under our standard conditions for TMV reconstitution. The class interval is 2 mm (= 17 nm) at ×120000 magnification. The length of the particles is given with a precision of ±8.5 nm.

(*e*) *Recognition and partial encapsidation of TMV–RNA by TRV-protein*

TRV-protein in 0.02 M borate buffer pH 8.0, consisting mostly of disks, was mixed with TMV–RNA in the proportion 1/4. The mixture was incubated for 12 h at 1–2 °C, dialysed against pH 4.5 citric acid phosphate buffer to dissociate the protein disks which were not stabilized by RNA-protein interactions and ultracentrifuged to eliminate dissociated protein. The presence of partially encapsidated RNA molecules was indicated by an A_{260}/A_{280} ratio of 1.9 and by electron microscopy using platinum shadowing (figure 6*a*, plate 24).

Negatively stained preparations of reconstituted material show the formation of short rods and disks – TRV-like particles (figure 6*b*).

Analysis of the T_1-oligonucleotides, originating from TRV-protein encapsidated regions (figure 3*j*) and the non-encapsidated regions of TMV–RNA (figure 3*k*), shows that the sequence –C–C–AOH (which is specific for TMV–RNA 3′-OH end Guilley *et al.* (1975) was not recognized and encapsidated by TRV-protein. In this case, the encapsidation of TMV–RNA by TRV-protein seems to be a polar process.

3. Discussion

TRV-protein follows the general rule of helical viruses namely, TRV-protein subunits can be polymerized into protein disks. This observation shows that the bonding between protein subunits is a specific property of the protein subunit not only with TMV (Durham, Finch & Klug 1971) but also with TRV. The TRV-disk seems to be a stable state of aggregation and the pairing between the subunits of the layers of the disk has an importance in the formation of helically constructed viruses. The important feature of TRV-protein in comparison with TMV protein, is the lack of polymorphism and the presence of disks over a wide range of pH, ionic strength and temperature. The 36S disk might undergo a conformational change in the presence of TRV–RNA, similar to that proposed for the TMV protein disk (Butler & Klug 1971), and specifically recognize the initiation sequence of the RNA molecules of short and long TRV particles. The TRV-protein disk did not recognize the 3′-OH end either of TRV-short or long RNA or of TMV–RNA. On the other hand, TMV-protein did not recognize the 3′-OH end of both short or long TRV–RNA but recognizes and encapsidates poly A (Fraenkel-Conrat & Singer 1964) and 'SERF' (Guilley *et al.* 1975) which is rich in adenosine and poor in cytosine.

The mechanism of initiation involves the specificity of the recognition sequence. It is possible that TMV and TRV possess certain features in common with respect to their initiation sites.

These results and those obtained by electron microscopic observation, that the RNA molecules were always protruding from one end, suggest that the 'initiation sites' of TRV and TMV are probably located at the 5′ termini of the RNAs and that the elongation process proceeds towards the 3′-OH end.

The failure of attempts to reconstitute the CAM strain of TRV at pH 8.0 was not due to the initiation process, which seems to proceed correctly, but to the elongation process which does not take place because the TRV-protein does not undergo the configurational change to become active.

The fact that the elongation process is more efficient for the CAM strain at pH 4.7 than at pH 8.0 might be explained by the destabilization of the protein disk giving rise to some configurational changes allowing the elongation process. This seems to be true because in these conditions a 20S configuration was observed. We do not know if the elongation occurred from the 4S or from another discrete unstable form of the protein. It is to be noted from the reported experiments that carboxyl-carboxylate bonds do not apparently play any role in protein–protein association at low pH, rather some repulsive forces contributing to the destabilization of the 36S aggregate arise. On the other hand low temperature greatly diminishes the hydrophobic forces. Both pH and temperature act in the same way: that is they lower the interaction forces between protein subunits and allow the protein aggregate to undergo the conformational changes necessary especially for the elongation process.

None of the conditions of reconstitution of TRV yet described (Semancik & Reynolds 1969; Morris & Semancik 1972; Abou Haidar *et al.* 1973) are comparable to physiological conditions.

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2 (*a*)

(*b*) 100 nm

4(*a*)

100 nm (*b*)

6(*a*)

(*b*) 100 nm

FIGURES 2, 4 AND 6. For description see opposite.