Shih, D. S., and Kaesberg, P. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 1799.
Tao, K. L., and Hall, T. C. (1971a), *Biochem. J. 121*, 495.
Tao, K. L., and Hall, T. C. (1971b), *Biochem. J. 125*, 975.
Yot, P., Pinck, M., Haenni, A-L., Duranton, H. M., and Chapeville, F. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 1345.

Assembly of Tobacco Mosaic Virus Rods *in Vitro*. Elongation of Partially Assembled Rods†

Kenneth E. Richards and Robley C. Williams*

ABSTRACT: The elongation phase of the *in vitro* assembly of tobacco mosaic virus was examined by the use of partially assembled rods. The rates of rod elongation in reaction mixtures buffered by sodium phosphate and by sodium pyrophosphate were measured by turbidimetric methods and by electron microscopy. The mixtures contained partially assembled rods and added protein in two different states of polymerization: disc protein (consisting of a 70:30 mixture of 20-S discs and the 4-S A-protein complex) and A-protein alone. It was found that elongation rates are about the same regardless of which type of protein is added and remain essentially unchanged when its concentration is varied fivefold. It is concluded that, at least in phosphate buffer, one or more of the components of the A-protein complex is fully capable of yielding the observed rates of rod elongation, regardless of whether or not discs are also present. It is suggested that the component of the A-protein complex that is directly incorporated in the rod elongation process is most likely to be the monomer.

It was first shown by Fraenkel-Conrat and Williams (1955) that protein and RNA isolated from tobacco mosaic virus (TMV)[1] will spontaneously reassemble, under appropriate reaction conditions, to form rods that are physically and biologically indistinguishable from the native virus. The detailed mechanism of the assembly has been reported upon by Butler and Klug (1971) who concluded that the initiation of rod assembly required the presence of protein "discs,"[2] and that subsequent elongation of the rods proceeds by addition of protein in the disc form to their growing ends. Okada and Ohno (1972) and Ohno *et al.* (1972a), however, reported that full length TMV rods, as gauged by infectivity assay, could be formed from partially reconstituted rods under conditions where the formation of discs could not occur, and concluded that elongation of TMV rods proceeds by addition of protein units distinctly smaller than discs. We have previously reported (Richards and Williams, 1972) some results of experiments which support the conclusion of Okada and Ohno: that elongation of previously initiated rods proceeds primarily, if not exclusively, by addition of small protein units, in the range of size of the "A-protein."[3]

Our earlier experiments utilized quantitative electron microscopy (Backus and Williams, 1950) for assessing TMV rod initiation and elongation; *i.e.*, for counting and measuring the lengths of recognizable rods formed during an assembly reaction. The conclusions of Butler and Klug (1971), however, were primarily based on observations of an increase in turbidity which takes place during the first few minutes of assembly. We have now reinvestigated the kinetics of elongation of TMV rods by means of turbidimetry as well as electron microscopy. Elongation rates have been examined in reaction mixtures buffered by sodium phosphate, and by sodium pyrophosphate, the former because the behavior of TMV protein in its presence is well characterized (Durham, 1972; Durham and Finch, 1972; Durham and Klug, 1972), the latter in order to make more direct comparisons with the experimental results of Butler and Klug (1971). We have used partially assembled rods (PAR) as the substrate material for further assembly in order to study rod elongation in the absence of the perturbing effects of rod initiation that would take place if free RNA were present in the reaction mixture. Our results confirm and expand our earlier finding (Richards and Williams, 1972) that TMV rod elongation takes place with equal rapidity in the presence or absence of discs, and our conclusion that a small protein unit, of the size of A-protein, is the one primarily involved in the *in vitro* elongation of TMV rods.

Materials and Methods

Preparation of RNA and Protein. TMV, purified by differential centrifugation (Knight, 1963), was a gift from Dr. C. A. Knight. Its RNA was phenol extracted in the presence of bentonite and EDTA (Fraenkel-Conrat *et al.*, 1961) and was fractionated by means of sucrose gradient centrifugation in order to eliminate most of the molecules of less than full length. To do this, the RNA, at 2–3 mg/ml in 1 mM EDTA (pH 7), was first heated to 60° for 90 sec, followed by rapid

† From the Virus Laboratory and Department of Molecular Biology, University of California, Berkeley, California 94720. *Received June 6, 1973.* This work was supported by U. S. Public Health Service Research Grant CA 02245 from the National Cancer Institute and Postdoctoral Fellowship AI 50591 from the National Institute of Allergy and Infectious Diseases.

[1] Abbreviations used are: TMV, tobacco mosaic virus; PAR, partially assembled rods of TMV; μ, ionic strength.

[2] "Discs" are protein aggregates having a 20-S sedimentation rate and composed of 34 monomeric units of TMV protein arranged in a 2-turn disc. When mixed with TMV RNA under appropriate reaction conditions a disc will bind as a unit to RNA to form an "initiated" rod.

[3] "A-protein" is a multicomponent equilibrium mixture in which the monomer, trimer, and diminishing amounts of higher aggregates are present. It exhibits a 4-S sedimentation boundary when prepared as described in Materials and Methods.

chilling, and then layered upon a 5–20% sucrose gradient (0.15 M sodium acetate–1 mM EDTA (pH 6.5) buffer) for centrifugation in the cold at 75,000*g* for 17 hr. The portion of the RNA band near its peak was collected and concentrated by ethanol precipitation. The efficacy of the fractionation was tested by extended reassembly with a preparation of TMV protein containing discs and A-protein ("disc protein"); at the end of 30 min some 50–60% of the reconstituted rods were full length (300 nm).

TMV protein was prepared as previously described (Richards and Williams, 1972) and stored in the cold as "protein helix" at pH 5. The protein used for elongation studies in phosphate buffer was prepared with a slight modification: the 0.1 M ethanolamine (pH 11) buffer used to depolymerize the virus, and the sodium acetate buffer in which the protein was stored, were both supplemented with 1 mM EDTA.

Before being used in the assembly reaction mixtures samples of the stored protein were diluted in water to 5 mg/ml and were dialyzed in the cold against a large volume of 41 mM (0.1 ionic strength) sodium phosphate buffer at pH 7. The solution was then divided into two parts. One portion was maintained at 4°, while the other was incubated at 20° for 8–16 hr. The former material, called "A-protein," showed a single 4-S boundary in the ultracentrifuge. The latter displayed a sedimentation pattern in which 60–70% of the material sedimented at 20 S and the remainder at about 4 S. It is referred to as "disc protein."[4]

Bentonite suspensions were prepared by the method of Fraenkel-Conrat *et al.* (1961) and were stored in the presence of 1 mM EDTA.

Partially assembled rods (PAR) were prepared thus: 0.3 ml of fractionated RNA (2.5–3.0 mg/ml) was added to 2.7 ml of disc protein (5 mg/ml, 0.1 μ sodium phosphate buffer (pH 7)) and incubated for 3 min at 20°. The reaction was terminated by addition of 5 ml of ice-cold 0.1 M sodium phosphate (pH 8.6) containing 20 μg of bentonite, and rapidly chilled to 0°. The PAR were sedimented in the cold at 81,000*g* for 2 hr, followed by suspension of the pellet in 0.3 ml of H_2O. Appropriately diluted aliquots of the PAR preparation were spray deposited (Backus and Williams, 1950) for electron microscopy. Protein concentrations in two samples of PAR were determined by Lowry assay.

Elongation of the PAR brought about by addition of protein was studied in 0.1 M sodium pyrophosphate buffer (pH 7.3, 26°) and in 0.1 μ sodium phosphate buffer (pH 7, 20°). In the former case, disc protein, or A-protein, initially in sodium phosphate buffer, was mixed with 0.125 M sodium pyrophosphate (pH 7.3) to yield a final buffer concentration of 0.10 M and the desired protein concentration, usually 1 mg/ml. PAR, at a concentration of 3–6 $\times$ 10^{14} rods/ml (see below) and in an amount of 10–30 μl/ml of reaction mixture, were added to this solution. If elongation was to be followed by turbidimetry the components were mixed in a cuvette (1-cm light path) and the OD changes at 310 nm were followed in a thermostatically controlled Cary 14 spectrophotometer during the initial 5–10 min of reaction.

For turbidimetric studies of the reaction in sodium phosphate buffer, the protein (disc protein or A-protein) in 0.1 μ phosphate buffer (pH 7) was mixed with the PAR in the same amounts as before. Prior to mixing, however, the A-protein solution was allowed to warm to 20° for 1–2 min; the disc protein was already at that temperature.

As a control experiment on the quality of the protein preparations, as gauged by the ability of disc protein to form rods when mixed with RNA, and the relative inability of A-protein so to do, turbidity measurements were made on reaction mixtures containing protein to which RNA (rather than PAR) was added.

Electron Microscopy. The number of PAR per unit volume of reaction mixture, and the length distribution of the rods, were determined following spray deposition of samples upon filmed, electron microscope grids. Samples were withdrawn from the reaction mixtures, at predetermined times, and quickly diluted 200-fold in dilution buffer (0.1 M sodium bicarbonate (pH 7.8, 23°)) containing polystyrene latex spheres, 100 nm in diameter, at a known number concentration. Spray deposition was performed on collodion-filmed grids, followed by rotary shadowing with Ur, and electron microscopic examination. The number of rods/ml and their lengths were determined from micrographs of spray-drop patterns, and the concentration of protein in the PAR samples was calculated from the measurements. Enough drop patterns from each sample were photographed to allow at least 200 rods and 400 polystyrene latex spheres to be measured. The number concentration of the latex spheres (known to have a density of 1.055 g cm^{-3}) was calculated from measurements of the mass of a sample volume after thorough drying, and determination of average particle diameter in the electron microscope.

Results

TMV Protein and RNA. The rod initiation characteristics of the disc protein and the A-protein, and the potential of the RNA for directing rod assembly, were checked turbidimetrically after adding disc protein, and A-protein, to RNA-containing reaction mixtures (Materials and Methods). Figure 2A shows the results, obtained in pyrophosphate buffer, when only A-protein was added. Little, if any, increase in turbidity over the period of observation is seen. The reaction mixture to which disc protein was added showed a rate of change of turbidity, $d\tau/dt$, of about 0.026 min^{-1} ($d\tau/dt = 2.303(dOD/dt)$). These results are essentially in accord with those reported by Butler and Klug (1971) under comparable reaction conditions. The results for similar mixtures, when phosphate buffer was used, are shown in Figure 5A. Addition of A-protein resulted in a much smaller initial rate of turbidity increase than did the addition of disc protein. Since aggregation of A-protein to discs takes place slowly in this buffer (Durham, 1972; K. E. Richards, personal observation), the failure of the A-protein preparation to cause an appreciable turbidity rise is presumably due to its initial freedom from discs.

Partially Assembled Rods (PAR). Preparations of PAR had an OD_{260}/OD_{280} of 1.4–1.5, indicating that the rods were associated with about twice as much RNA (OD_{260}/OD_{280} = 1.95) as is native TMV (OD_{260}/OD_{280} = 1.2). Electron microscopy showed the PAR preparation consisted of rods generally less than 300 nm long (the length of native TMV particles) and frequently associated with a strand of what is presumed to be RNA extending from one end (Figure 1). The rods were of varying lengths; usually, however, the great majority of them were shorter than 200 nm. Their number concentration, determined by electron microscopy, was adjusted to fall within 3–6 $\times$ 10^{14} rods/ml.

[4] "Disc protein" is an equilibrium mixture of 20-S "discs" and A-protein. The relative proportions of the two depend upon total protein concentration; in the concentration range used here about 70% by weight are discs.

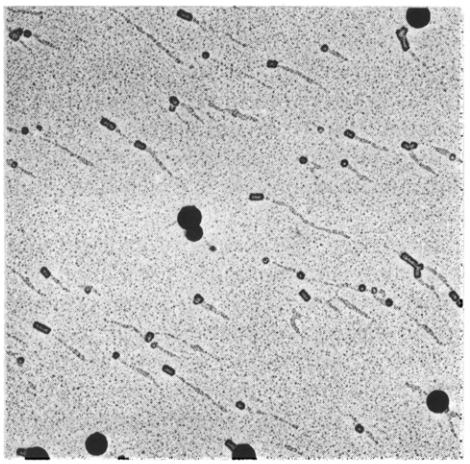

FIGURE 1: An electron micrograph of a preparation of partially assembled rods (PAR) to which 100-nm polystyrene latex spheres have been added. The specimen has been rotary shadowed with uranium (×33,000).

The protein concentrations in the two samples of PAR that were tested by Lowry assay were found to be within 10–15% of that calculated from the electron microscopic determinations of the lengths and number concentrations of the PAR.

Elongation of PAR in Pyrophosphate Buffer. In Figure 2B is shown the turbidity change which took place when disc protein, or A-protein at the same concentration, was mixed with PAR. It is seen that when PAR, rather than free RNA, is used as the nucleic acid source the addition of either disc protein or A-protein results in a comparably rapid turbidity rise. It should be noted (Figure 2B) that a preparation of PAR alone, used as a control, increases very slightly in turbidity as times goes on. This occurrence, interpreted as a slight increase in the average length of the PAR, probably arises from attachment of small amounts of contaminant protein to the RNA pendant from the rods.

Figure 3A shows the length distribution of the PAR used in the above experiment, while Figure 3B shows the distribution after the reaction, with either disc or A-protein, has proceeded essentially to equilibrium (13 min). A knowledge of the number concentration of the PAR and their initial length distribution can be combined with the observed $d\tau/dt$ to permit calculation to be made of the initial, average rate of rod elongation (see Appendix for calculations). This value was found to be 50–60 nm/min. It should be noted, however, that this is a figure averaged over all the PAR in the reaction mixture, some of which are actually incapable of growth because they contain no pendant RNA. When correction is made (see Appendix) for the effect of these inactive rods the rate of elongation is estimated to be 70–80 nm/min.

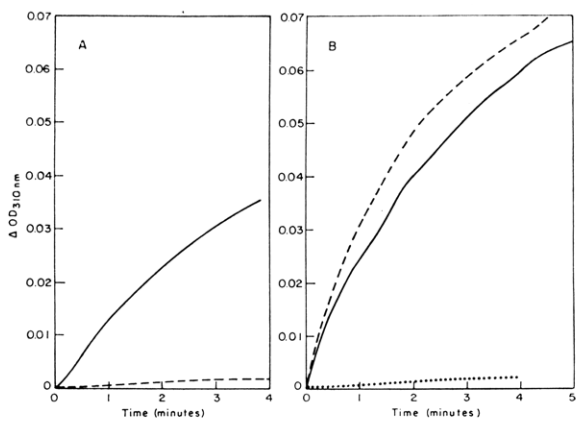

FIGURE 2: Turbidimetric measurements of the assembly of TMV RNA and of PAR, with disc protein (—) and with A-protein (- - - -) in pyrophosphate buffer. (A) Assembly with RNA. Reaction conditions: 0.1 M sodium pyrophosphate (pH 7.3), 26°; protein concentration 0.8 mg/ml; RNA concentration 0.046 mg/ml. (B) Assembly with PAR. Reaction conditions were the same as in Figure 2A except that the nucleic acid component was the pendant RNA associated with PAR at a concentration of 1.3×10^{13} particles/ml. Partially assembled rods alone (no added protein) at a concentration of 1.3×10^{13} particles/ml (. . . . . .).

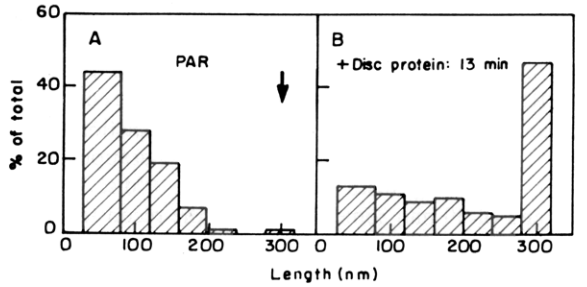

FIGURE 3: The length distribution of the PAR used in the experiment shown in Figure 2B before any further elongation (A), and after elongation for 13 min with disc protein (B). The length distribution after 13 min of elongation with A-protein (not shown here) was similar to that obtained with disc protein.

The initial rate of elongation of PAR was also assessed by electron microscopy. Figure 4 shows the length distribution of the PAR in the starting material (A), and after reaction for 1, 2, and 13 min when either A-protein (B-D) or disc protein (E-G) had been added. As was found in the turbidimetric studies, it appears from the histograms in Figure 4 that the use of A-protein as the protein source resulted in a rate of rod elongation at least as great as that obtained from use of disc protein. The rate of elongation, uncorrected for the effect of inactive PAR, was found to be about 55 nm/min, a value quite similar to that found from turbidimetry.

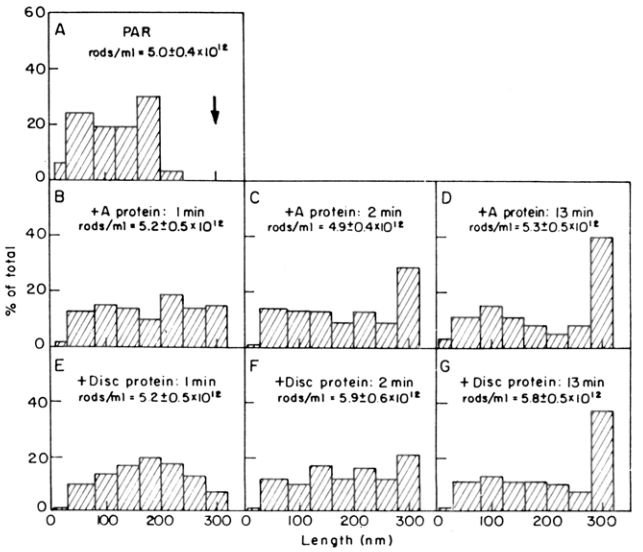

FIGURE 4: The length distribution of PAR before start of the reaction (A) and after elongation for 1, 2, or 13 min with either A-protein (B–D) or disc protein (E–G). Reaction conditions: 0.1 M sodium pyrophosphate (pH 7.3), 26°; protein concentration 1 mg/ml. The arrow [Figure 4A] marks the length expected for full-length particles.

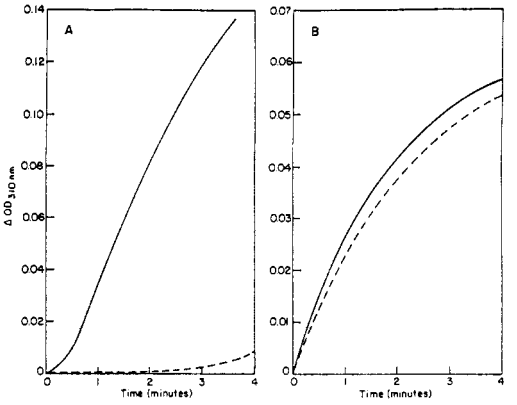

FIGURE 5: Turbidimetric measurements of the assembly of TMV RNA and of PAR, with disc protein (—) and with A-protein (- - - - -) in phosphate buffer. (A) Assembly with RNA. Reaction conditions: 0.1 μ sodium phosphate (pH 7), 20°; protein concentration 4 mg/ml; RNA concentration 0.072 mg/ml. (B) Assembly with PAR. Reaction conditions were the same as in (A) except that PAR at a concentration of 9.8×10^{12} particles/ml was used as the nucleic acid source. The length distribution of the starting PAR preparation (not shown here) was similar to that shown in Figure 4A.

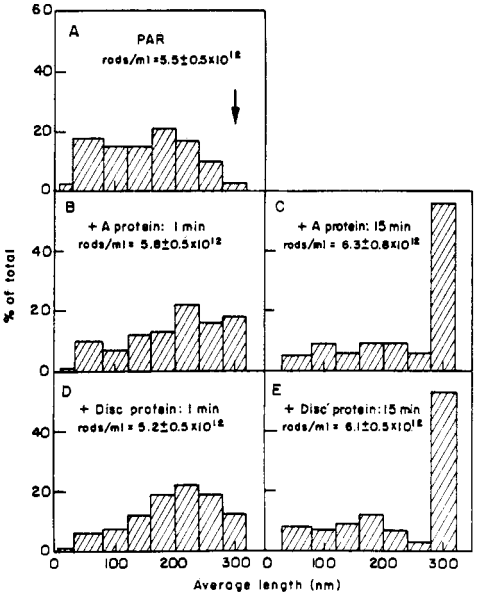

FIGURE 6: The length distribution of PAR before the start of the reaction (A) and after elongation for 1 min or 15 min with A-protein (B and C) or with disc protein (D and E). Reaction conditions: 0.1 μ sodium phosphate (pH 7), 20°; protein concentration 4 mg/ml.

The number concentration of PAR in the starting material of each sample used for elongation studies was compared with that after prolonged incubation with disc protein, to see if any "new" rods were initiated in this interval. The number concentration was found to increase by 20% or less, indicating that the PAR preparations were slightly contaminated with RNA molecules having free 5′-ends (see Discussion).

Elongation of PAR in Phosphate Buffer. Figure 5B shows the turbidimetric results when either A-protein or disc protein was added to the PAR-containing reaction mixture. The two rates of turbidity change are seen to be closely similar. The initial rate of rod elongation, uncorrected for inactive rods, was calculated to be about 60 nm/min; the estimate of corrected rate was 70–80 nm/min.

The results of number and length measurements of PAR in the electron microscope, before and after elongation, are shown in Figure 6. Again, the rates of growth were found to be quite similar, following addition of either A-protein or disc protein. The uncorrected rate of elongation was calculated to be 40 nm/min, two-thirds the rate found by turbidimetry.

Effect of Protein Concentration on PAR Elongation. Figure 7 shows the changes in turbidity which occurred when PAR and either A-protein (Figure 7A) or disc protein (Figure 7B) in phosphate buffer at the indicated concentrations were mixed with PAR. In both cases, over the concentration range used, the initial $d\tau/dt$ was relatively insensitive to amount of protein added. The reaction rate in pyrophosphate was found to be similarly insensitive to A-protein or disc protein concentration, an increase in concentration from 0.3 to 1.6 mg/ml resulting in only a 30% increase in $d\tau/dt$.

Effect of Bentonite on PAR Elongation. In order to protect the PAR from possible nucleolytic degradation during purification an addition of 20 μg of EDTA-extracted bentonite was made before the rods were purified by centrifugation. Since the bentonite cosedimented with the PAR, any further elongation of them in the reaction mixtures took place in the presence of small amounts of bentonite. Bentonite at this concentration had no discernible effect on the sedimentation pattern of either disc or A-protein, as seen in the analytical ultracentrifuge. Assembly experiments performed in either pyrophosphate or phosphate reaction buffers containing free RNA and either disc or A-protein showed that bentonite did not alter the rates of rod assembly. Finally, some recent experiments not reported here have shown that PAR prepared both

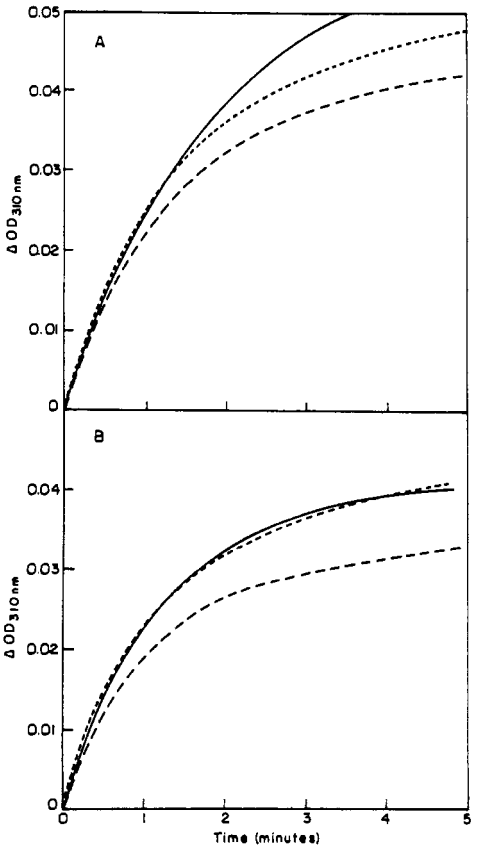

FIGURE 7: Elongation of PAR in phosphate buffer with A-protein (A) or with disc protein (B), showing the effect of protein concentration. Reaction conditions were the same as in Figure 5. Protein concentration: 7 mg/ml (—); 4 mg/ml (. . . . .); 1.5 mg/ml (- - - - -); PAR concentration, 8.1×10^{12} particles/ml.

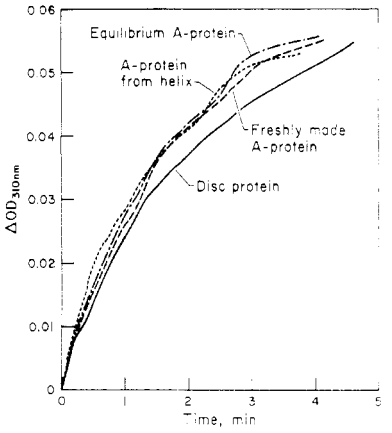

FIGURE 8: Elongation of PAR in pyrophosphate buffer: effect of mode of preparation of the A-protein. Reaction conditions were the same as in Figure 2: protein concentration, 1 mg/ml; PAR concentration, 1.5×10^{13} particles/ml. Disc protein and equilibrium A-protein were made as described in Materials and Methods. Freshly made A-protein (Richards and Williams, 1972) was prepared by chilling disc protein in 0.1 μ phosphate buffer (pH 7) at 0° for 15 min. A-protein was also generated directly from TMV protein helix (Butler and Klug, 1971) by introducing helix protein (at pH 5) directly into the pyrophosphate reconstitution buffer.

with, and without, bentonite behaved essentially the same in tests of rates of elongation. We conclude, therefore, that the presence of bentonite in the PAR preparations used in the experiments detailed above had no appreciable effect on the results.

Effect of Mode of A-protein Preparation on PAR Elongation. In order to respond to questions that have been raised (see Discussion) about our earlier (Richards and Williams, 1972) use of A-protein that had been prepared by brief chilling of disc protein, we have performed experiments with A-protein prepared by three different methods. The A-protein used in all experiments reported above ("equilibrium" A-protein) was kept exclusively in this form for at least 20 hr before use in reaction mixtures. Another, preparatively distinctive A-protein ("freshly made" A-protein) like that used previously (Richards and Williams, 1972), was prepared by cooling disc protein in 0.1 μ phosphate buffer (pH 7) at 0° for 15 min. Still another ("helix" protein) was prepared by depolymerizing a protein preparation which had been stored as TMV protein helix (Butler and Klug, 1971). All three types of A-protein were tested for capacity to elongate PAR. As Figure 8 shows, all three were indistinguishable in their ability to effect elongation.

Discussion

We have shown that in a reaction mixture buffered by either phosphate or pyrophosphate the elongation of partially assembled rods (PAR) proceeds at closely the same rate whether the species of protein added is A-protein or disc protein. It has previously been shown (Durham and Klug, 1971) that the material in the A-protein complex, in phosphate buffer, does not aggregate to discs in the time span of the elongation observations (<5 min). It therefore follows that when a phosphate-buffered reaction mixture contains PAR and A-protein only, the sole protein participant in elongation is one or more components of the A-protein complex. This conclusion is less certain from the experiments performed with pyrophosphate buffer, inasmuch as it has been demonstrated (Lonchampt *et al.*, 1972) that when A-protein is placed in this buffer some of it quickly aggregates to larger structures, as yet poorly characterized. It seems to us unlikely, however, that such aggregation rapidly produces "discs," *i.e.*, 20-S structures that are capable of initiating TMV assembly, since the addition of A-protein to RNA in pyrophosphate results in negligible turbidity increase (Figure 2A). Whatever the mechanism of TMV rod elongation in pyrophosphate buffer, therefore, it seems not to be one that requires discs (as defined) as the units of assembly.

The above conclusion, that discs are not required for rod elongation, is in accord with that proposed by Okada and Ohno and their colleagues (Okada and Ohno, 1972; Ohno *et al.*, 1972a,b). They showed that when partially assembled rods were used as the RNA source they could be elongated to full-length, infective TMV rods in the presence of A-protein under conditions where this material did not aggregate to discs. While their results and conclusions are like ours, they relate very little to the kinetics of rod elongation, since only the final formation of infective rods was studied.

Our finding that discs are not necessary for the growth of once-initiated TMV rods is contrary to the conclusions drawn by Butler and Klug (1971) that only the protein in the disc form is incorporated into growing rods, and the conclusion of Butler (1972) that the disc is the "kinetically favored" species in rod elongation. These authors examined the assembly of TMV rods by using TMV protein and free RNA as starting materials, mixing them in pyrophosphate buffer, and following the initial stages of the assembly reaction by turbidimetry. The initial rise in turbidity, however, is brought about by the simultaneous effects of both rod initiation and rod elongation. We feel, therefore, that the interpretations made by Butler and Klug remain uncertain until it is demonstrated that rod initiation is extremely rapid under the conditions of their experiments and is essentially complete before the turbidimetric measurements begin.

Protein Species Involved in TMV Rod Elongation. When A-protein is the only protein species available for rod elongation, as in the experiments shown in Figure 5B (- - - -) and Figure 6B,C, its role as the only participant in rod growth is clear. But when the protein additive is disc protein the possibility that discs participate directly in rod growth cannot be excluded. Their involvement is not necessary, however, at least in the early stages of the elongation process, since there is initially ample A-protein present in the disc protein to provide for rod growth at the observed rate. It is known that in an equilibrium mixture such as disc protein (Durham, 1972) the concentration of the smaller aggregates (A-protein) remains relatively constant as total concentration is decreased. Consequently, as the A-protein is incorporated into the growing rods in a reaction mixture, thereby decreasing the total protein concentration, it might be expected that a supply of A-protein sufficient for rod growth would be provided by depolymerization of discs. In support of this suggestion is the report by Butler and Klug (1971) that as rod elongation proceeds in a TMV assembly mixture the concentration of discs (20-S material) diminishes while that of A-protein (4-S material) stays substantially constant.

It is conceivable, of course, that in a reaction mixture containing disc protein both kinds of aggregates, the discs and the A-protein material, could participate in rod elongation. Unfortunately, there seems to be no way to establish whether or not such a dual mechanism of assembly operates. Such evidence as there is seems to favor the idea that only the A-protein material participates, since it is capable of doing so when no discs are present.

A significant aspect of the experiments reported here is that

the rate of rod elongation, in phosphate buffer, is relatively insensitive to protein concentration even when the only species present is A-protein (Figure 7). This observation may indicate that, in the concentration range employed, the reaction rate is not collision-frequency controlled. Alternatively, it may be accounted for by the effect of the rapid polymerization–depolymerization interactions known to take place within the A-protein complex (Durham and Klug, 1972). Calculation of the relative concentrations of the components of A-protein in phosphate buffer at pH 7, based on equations developed by Durham and Klug (1972), shows that when total A-protein concentration is allowed to vary from 1 to 5 mg/ml the monomer concentration changes less than 10% from its mean value of about 0.5 mg/ml. The concentration of the other A-protein species (trimer. . . *n*-mer) changes much more nearly in proportion to changes in total A-protein concentration. If the monomer were the main species active in rod elongation and if, indeed, its concentration remains almost constant while that of the whole A-protein complex is varied, the observed independence of elongation rate on A-protein concentration would simply be a consequence of this constancy. On these grounds we suggest the monomer as a likely candidate for the protein species that is active in TMV rod elongation. Such a role for the monomer has the additional attraction of avoiding the topological problem, earlier recognized (Butler and Klug, 1971), of fitting two-ringed assemblies into the one-start TMV helix.

Effect of Free RNA in the PAR Preparation. It is desirable that a preparation of PAR used to study TMV reassembly be made up of particles with maximal growth potential and contain a minimal amount of RNA with free 5′ ends. The former condition requires that the initial rod elongation process (Materials and Methods) be stopped at a time when the great majority of the rods still have a pendant strand of RNA of appreciable length. The latter condition requires, however, that the initiation process be extended long enough so that all the 5′ ends of the starting, free RNA are bound to protein. Too long an incubation time results in decreased reliability of the calculated rate of elongation, since the effect of "dead" rods, incapable of further growth, will result in apparent growth rates that are fictitiously low and a correction (see Appendix) for the effect of these rods is uncertain at best.

The effect of too short an incubation time will be to leave free RNA in the PAR preparative assembly mixture where it will cosediment with the PAR during isolation. If substantial amounts of it are present in those assembly mixtures that contain disc protein (and PAR) the growth rates will be either too large or too small depending on the method of observing and calculating them. If turbidimetry is employed they will be too large, since the measured rate of turbidity change will include the effect of the creation of new elongation centers by the binding of discs to the free 5′ ends of the RNA. If rod elongation is measured by electron microscopy the apparent growth rates will be too small. Those rods that have formed and grown during only the first minute of the reaction will be included in the length measurements, but since they will be somewhat shorter than the average PAR, their inclusion will depress the calculated growth rate.

We have assessed the amount of contaminant RNA having free 5′ ends by comparing the numbers of rods in samples of starting PAR with the numbers in samples which have experienced extended incubation with disc protein. The difference is taken to be the number of rods that could be initiated during incubation. We have found that no more than 15% of the total rods counted are newly formed ones. This amount of contamination by RNA is too small to have significant effect on the comparisons of growth rates of PAR.

"A-protein."* In a recent publication Butler and Klug (1972) questioned our earlier work on TMV assembly (Richards and Williams, 1972), stating that the A-protein we then used, here called "freshly made" A-protein, behaved in an assembly mixture in a unique manner quite unlike "equilibrium" A-protein. They proposed that freshly made A-protein as prepared by them, which they termed A*-protein, may possess a "memory" of its previous existence in the disc form and, under appropriate conditions, be capable of rapid polymerization back to the disc-like state. They speculated that our freshly made A-protein (which they took to be A*-protein) had similarly undergone rapid conversion to disc-like form, and that the rapid elongation rates observed by us were due to incorporation of material that was solely discs. Butler and Klug (1972) recognized, however, that the "discs" they presumed were rapidly produced by polymerization of our "freshly made" A-protein had to be somewhat anomalous in character since they had been shown to be incapable of rod initiation.

As is shown in Results, the prediction (Butler and Klug, 1972) that equilibrium A-protein would not accomplish rapid rod elongation has not been borne out. Indeed, experiments done with three preparatively distinctive forms of A-protein show that all are quite comparably effective in elongation of PAR (Figure 8), thus the "memory" hypothesis of Butler and Klug (1972) is not applicable to these experiments. It has since become clear (P. J. G. Butler, personal communication) that the origin of the apparent contradiction lay in a misunderstanding about the manner of preparation of "freshly made" A-protein. Our material was prepared by chilling disc protein to 0° in 0.1 μ phosphate buffer before introduction into the pyrophosphate-containing reaction mixture at 26°. The A*-protein of Butler and Klug (1972), however, was generated by introducing disc protein (in its usual phosphate buffer) into 0.1 M pyrophosphate buffer and *then* chilling. In view of the unique behavior of TMV protein in pyrophosphate of high ionic strength (Lonchampt *et al.*, 1972), it is not surprising that material so prepared would have properties strikingly different from those of any of the A-proteins we have prepared. Indeed, it is possible that chilling at 0° for 15 min in 0.1 M pyrophosphate buffer is not sufficient to depolymerize the discs, and that much of the material remains in that form. If that were so, such discs would act as rod-initiation centers and reaction events taking place in a mixture of A*-protein and free RNA would show the observed, relatively rapid rise of turbidity (Butler and Klug, 1972).

Acknowledgment

We wish to thank P. J. G. Butler and A. Klug for making several of their manuscripts available to us prior to publication.

Appendix

The rate of elongation of partially assembled TMV rods can be calculated from the classical equation relating turbidity, τ, with weight average molecular weight, $\overline{M}_w$

$$\tau = Hc\overline{M}_wQ \quad (1)$$

where H is an optical constant, c is the concentration of solute in the reaction mixture, and Q is the particle dissipation factor

TABLE I: Rate of Elongation of PAR Calculated from Turbidimetry.

Buffer	Form of Protein	Initial $d\tau/dt$ (min^{-1})	$\Sigma C_i(2Q_i + S_iD_i/\lambda')$ (g/ml ($\times 10^{-5}$))	Apparent Rate of Elongation[a] (daltons/min ($\times 10^6$))	nm/min
0.1 M sodium pyrophosphate (pH 7.3)	A-protein				
	Expt 1	0.099	30	6.9	54
	Expt 2	0.130	37	7.3	58
	Disc protein				
	Expt 1	0.092	30	6.4	50
	Expt 2	0.102	37	5.8	46
0.1 μ sodium phosphate (pH 7)	A-protein				
	Expt 3	0.073	23	6.6	52
	Expt 4	0.068	18	7.9	62
	Disc protein				
	Expt 3	0.083	23	7.5	59
	Expt 4	0.062	18	7.3	58

[a] Uncorrected for the effect of inactive rods.

(Doty and Steiner, 1950). The factor Q, whose role is analogous to that of $P(\theta)$ in the classical light-scattering equation, corrects for internal interference of the scattering centers which occurs with significant effect if the centers are larger than $^1/_{10}$ the wavelength of the incident light. Q-corrected values of turbidity measurements on polydisperse particles, such as are found in the reaction mixture, can be obtained if the solute material is categorized into monodisperse elements, the appropriate Q factor ascertained for each category and applied to correct the turbidity contribution of that category, and the turbidity of the entire solution calculated by summing up the corrected turbidity contributions of all the categories.

Let the molecular weights, lengths, and concentrations of the partially assembled rods (at $t = 0$) be written as: M_i,D_i, and C_i, molecular weight, length, and concentration of the partially assembled rods of the ith category; M_e, molecular weight of the protein unit involved in rod elongation; Q_i, the particle dissipation factor for rods of length D_i. Equation 1 may be written as

$$\tau = H\Sigma C_i M_i Q_i + HC_p M_p \tag{2}$$

where C_p and M_p are the concentration and molecular weight of the unassembled protein in the solution (for which $Q = 1$).

After differentiating eq 2 and substituting $dc_p/dt = -\Sigma dc_i/dt$

$$d\tau/dt = H\Sigma\left(C_iM_i\frac{dQ_i}{dt} + C_iQ_i\frac{dM_i}{dt} + (Q_iM_i - M_p)\frac{dc_i}{dt}\right) \tag{3}$$

For rods the lengths of those considered here, Q_iM_i is 6×10^6 daltons or more, and M_p, which is certainly no larger than 6×10^5 daltons, is, therefore, small compared to Q_iM_i in the last term of eq 3. Hence, as a first approximation

$$d\tau/dt = H\Sigma\left(C_iM_i\frac{dQ_i}{dt} + C_iQ_i\frac{dM_i}{dt} + Q_iM_i\frac{dc_i}{dt}\right) \tag{4}$$

Let N_i be the number of rods per unit volume in the ith length category and dn/dt the average, initial rate of rod elongation, expressed in terms of protein subunits added per unit time.

Then

$$dM_i/dt = M_e(dn/dt) \tag{5}$$

$$dC_i/dt = N_iM_e(dn/dt) = (C_i/M_i)M_e(dn/dt) \tag{6}$$

Doty and Steiner (1950) have graphically evaluated Q as a function of D/λ', where D is the rod length and λ' is the wavelength of light in the solution. For any value of D/λ' the time rate of change of Q is related to the time rate of change of change of D/λ' by

$$\frac{dQ}{dt} = S\left(\frac{dD/\lambda'}{dt}\right) \tag{7}$$

where S is the slope of the curve relating Q and D/λ' evaluated at a particular value of D/λ' (Doty and Steiner, 1950; their Figure 3). Equation 8 may be rewritten for each i category, and expressed in terms of molecular weight, rather than length

$$\frac{dQ_i}{dt} = \frac{S_i}{\lambda' k}\frac{dM_i}{dt} = \frac{S_i}{\lambda' k}M_e\frac{dn}{dt} \tag{8}$$

where k is the mass per unit length of TMV ($= 1.33 \times 10^5$ daltons/nm).

Substituting eq 5, 6, and 8 into eq 4, and rearranging terms

$$\frac{d\tau}{dt} = HM_e\frac{dn}{dt}\left[\Sigma C_i\left(2Q_i + S_i\frac{D_i}{\lambda'}\right)\right] \tag{9}$$

Therefore, $M_e(dn/dt)$, the rate of elongation of the rods in daltons/minute, may be expressed as

$$M_e\frac{dn}{dt} = \frac{d\tau/dt}{H\Sigma C_i[2Q_i + S_i(D_i/\lambda')]} \tag{10}$$

and evaluated. H is taken to be 4.80×10^{-5} cm^2/g^2 (Butler, 1972), while λ' is taken to be 228 nm (observations performed at 310-nm wavelength in air; refractive index of solution = 1.36). The value of $d\tau/dt$ is the initial rate of turbidity change, C_i and D_i are obtained from the electron microscopic determinations of rod numbers and lengths, while S_i and Q_i are taken from Doty and Steiner (1950).

Calculations from eq 10 of the rate of elongation of partially assembled rods give the *average* for all the rods in the PAR preparation (Table I). Only a fraction of the rods, however, will be capable of further growth, those rods having a pendant RNA "tail." The presence of tailless rods results in a calculated average growth rate that is less than the true one. We estimate from measurements of the length distribution of the PAR at $t = 0$, and after further growth has reached completion (*e.g.*, see Figures 3A and 3B), that the active rods comprise ~70% of the total number of PAR. The average rate of growth for those rods that are active is estimated to be about 80 nm/min.

References

Backus, R. C., and Williams, R. C. (1950), *J. Appl. Phys. 21*, 11.

Butler, P. J. G. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36*, 461.

Butler, P. J. G. (1972), *J. Mol. Biol. 72*, 25.

Butler, P. J. G., and Klug, A. (1971), *Nature (London), New Biol. 229*, 47.

Butler, P. J. G., and Klug, A. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 2950.

Doty, P., and Steiner, R. F. (1950), *J. Chem. Phys. 18*, 1211.

Durham, A. C. H. (1972), *J. Mol. Biol. 67*, 289.

Durham, A. C. H., and Finch, J. T. (1972), *J. Mol. Biol. 67*, 307.

Durham, A. C. H., and Klug, A. (1971), *Nature (London), New Biol. 229*, 42.

Durham, A. C. H., and Klug, A. (1972), *J. Mol. Biol. 67*, 315.

Fraenkel-Conrat, H., Singer, B., and Tsugita, A. (1961), *Virology 14*, 54.

Fraenkel-Conrat, H., and Williams, R. C. (1955), *Proc. Nat. Acad. Sci. U. S. 41*, 690.

Knight, C. A. (1963), *Protoplasmatologia 4*, 1.

Lonchampt, M., Lebeurier, G., and Hirth, L. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 22*, 297.

Ohno, T., Inoue, H., and Okada, Y. (1972a), *Proc. Nat. Acad. Sci. U. S. 69*, 3680.

Ohno, T., Yamaura, R., Kuriyama, K., Inoue, H., and Okada, Y. (1972b), *Virology 50*, 76.

Okada, Y., and Ohno, T. (1972), *Mol. Gen. Genet. 114*, 205.

Richards, K. E., and Williams, R. C. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1121.

Phosphorylation of the Sarcoplasmic Reticulum Membrane by Orthophosphate. Inhibition by Calcium Ions†

Hatisaburo Masuda‡ and Leopoldo de Meis*

ABSTRACT: A protein of the sarcoplasmic reticulum membrane of skeletal muscle is phosphorylated by orthophosphate at pH 6.0 in the presence of Mg^{2+}. The orthophosphate concentration required for half-maximal phosphorylation is 1.06 mM. This reaction is strongly inhibited by Ca^{2+} and to a lesser extent by Na^+ and K^+. ATP and ADP compete with orthophosphate for the membrane phosphorylating sites, ATP being a more effective inhibitor than ADP. It is suggested that nucleoside triphosphates and orthophosphate interact with the same site on the membrane and that the binding of Ca^{2+} determines which of them will phosphorylate the membrane.

Fragmented SRV[1] isolated from skeletal muscle retain a highly efficient ATP-dependent Ca^{2+} transport system (Hasselbach and Makinose, 1961; Hasselbach, 1964). Makinose (1969) has shown that in the process of ATP hydrolysis, the γ-phosphate of ATP is covalently bound to a membrane protein (E). The phosphoprotein (E~P) represents an intermediate in the sequence of reactions leading to Ca^{2+} transport and phosphate liberation. Makinose (1969) has therefore proposed the following reaction sequence.

$$ATP + E \overset{Ca^{2+}}{\rightleftharpoons} E\sim P + ADP \quad (1)$$

$$E\sim P \longrightarrow E + P_i \quad (2)$$

Recently it has been shown that under specific conditions, the Ca^{2+} pump of the SRV can be reversed, *i.e.*, that reaction 2 shown above is reversible. When SRV previously loaded with calcium oxalate or calcium phosphate are incubated in a medium containing ADP, Mg^{2+}, and [^{32}P]P_i, it is observed that [^{32}P]P_i interacts with the membrane forming E~P (Makinose, 1972), Ca^{2+} is released at a very high rate (Barlogie *et al.*, 1971), and [γ-^{32}P$_i$]ATP is formed (Makinose, 1971; Makinose and Hasselbach, 1971). In these experiments it was

† From the Instituto de Biofísica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, GB, Brazil. *Received June 11, 1973.* This investigation was supported in part by the Conselho Nacional de Pesquisas, Brazil (T.C. 16,293 and T.C. 16,398), by the Conselho de Ensino para Graduados da U.F.R.J., and by the Banco Nacional de Desenvolvimento Econômico (FUNTEC 143).

‡ Recipient of a fellowship from the Coordenação do Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

[1] Abbreviations used are: SRV, sarcoplasmic reticulum vesicles; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.