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Comparison of Tyrosyl Transfer Ribonucleic Acid and Brome Mosaic Virus Tyrosyl Ribonucleic Acid as Amino Acid Donors in Protein Synthesis†

Jane M. Chen and T. C. Hall*

ABSTRACT: Several plant viruses possess RNA which can be aminoacylated in a tRNA-like manner at the 3′ terminus; for example, the RNA components of brome mosaic virus specifically bind tyrosine. The possibility that tyrosine, bound to the viral RNA, could be donated to nascent peptides was investigated using a cell-free system from wheat, a natural host for brome mosaic virus. This system was found to be efficient at mediating the transfer of tyrosine from tyrosyl-tRNA to peptides when programmed with a viral messenger RNA, a plant RNA fraction, and the synthetic heteronucleotide poly(A,C,U). However, only a very small amount of tyrosine was transferred by this system to peptidyl material when brome mosaic virus tyrosyl-RNA was supplied as the amino acid substrate. It appears unlikely, therefore, that the ability of brome mosaic virus to bind tyrosine is directly involved in peptide chain elongation.

The RNA from plant viruses belonging to three different groups has recently been found to be capable of accepting amino acids in a tRNA-like manner, the specific amino acid bound varying from one virus group to another. Thus, the RNA from TYMV[1] (Yot *et al.*, 1970), eggplant mosaic virus, and okra mosaic virus (Pinck *et al.*, 1972) binds valine. Histidine binds to TMV RNA (Öberg and Philipson, 1972) and the RNA from BMV and cowpea chlorotic mottle virus binds tyrosine (Hall *et al.*, 1972; T. C. Hall, unpublished observations).

No explanation of the biological function of this property is available, but the conditions for esterification, the specificity for a single amino acid, the ability for elongation factor binding to acylated *vs.* the absence of binding for deacylated RNA (Litvak *et al.*, 1973), and the resistance to RNase degradation of ribosome-bound RNA (Haenni *et al.*, 1973) all suggest that a tRNA-like function exists. Valine, bound to TYMV RNA, has been shown to be donated to nascent peptidyl material in a cell-free system from *Escherichia coli*, but much less efficiently than from valyl-tRNA, and with kinetics which suggested that partial cleavage of the viral RNA preceded the amino acid transfer (Haenni *et al.*, 1973).

In this article we describe experiments conducted to determine if BMV RNA could function as a tyrosine donor in protein synthesis and to compare its efficiency with that of tyrosyl-tRNA. Three physiological possibilities were examined. One was that the tyrosine-charged BMV RNA might function as a virus-specific tRNA and preferentially insert tyrosine into viral coat, or some other, protein. An alternative possibility was that tyrosine might be inserted into plant proteins in incorrect locations, the viral RNA functioning as a missense adaptor. Another possibility was that regulatory systems might be operating which precluded tyrosine transfer from charged viral RNA when the cell-free system was programmed with either viral or plant messenger, and for this reason a third set of experiments was carried out using copoly(A,C,U) as messenger.

Methods and Materials

Preparation of Synthetase Enzyme Fraction. Aminoacyl-tRNA synthetases were extracted from maturing (11–15 mm long) seeds of French Bean (*Phaseolus vulgaris* L. *cv.* Tendergreen) plants which had been cultured in an aerated liquid nutrient medium under a controlled environmental regime in the Madison Biotron. All extraction steps were at 0–4°. Freshly excised cotyledons (50 g) were ground for 90 sec, using a VirTis 45 homogenizer, into 100 ml of extraction buffer containing 0.1 M imidazole-Cl (pH 7.3), 5 mM dithiothreitol, 1 mM GSH, 10 mM $MgCl_2$, 0.3 M KCl, 10 g of insoluble polyvinylpyrrolidone, and 2 g of Dowex 50-X8 (Na^+ form). The grinding flask stood in crushed ice and N_2 was continually flushed through from a side arm. The crude extract was squeezed through acetate taffeta cloth and then centrifuged at 10,000 rpm (15 min) in a JA 20 rotor on a Beckman J 21 centrifuge. A lipid pellicle was removed from the surface; then the light green supernatant was applied (in batches of 15 ml) to a DEAE-Sephadex A-25 column (3 × 40 cm) which had been previously equilibrated with 0.1 M imidazole-Cl (pH 7.3) containing 10 mM $MgCl_2$, 0.3 M KCl, 5 mM dithiothreitol, and 1 mM GSH. For elution the buffer was brought to 1 M

† From the Department of Horticulture, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706. *Received July 5, 1973.* Supported by Grant GB-38652 from the National Science Foundation, Grant 1 R01 AI 11572-01 from the National Institutes of Health, and by an Institutional Grant from the American Cancer Society.

[1] Abbreviations used are: BMV, brome mosaic virus; TMV, tobacco mosaic virus; TYMV, turnip yellow mosaic virus.

KCl (Kirkegaard *et al.*, 1972); 3-ml fractions were collected and subsequently stored in 0.3-ml aliquots at −90°. Fractions 3–7 (after elution of the void volume) were used for aminoacylation of RNA.

Preparation of Nucleic Acids. BMV, RNA, tRNA, and tyrosylated RNAs were prepared as described previously (Hall *et al.*, 1972) except that the synthetase enzyme fraction described above was used for esterification of the RNAs. The DEAE-Sephadex A-25 column removed much of the tRNA bound to the synthetase, and contamination of the tyrosylated BMV RNA preparation by charged tRNA extracted from the enzyme preparation was found by sucrose density gradient centrifugation to represent 1% of the radioactivity of the charged viral RNA preparation.

An RNA fraction obtained from maturing bean seeds, designated B101 RNA, was found to be capable of stimulating amino acid incorporation when added to the wheat embryo system. This was a fraction which was insoluble in 3 M NaOAc (see method 3 in Tao and Hall, 1971b); after solubilization in water the RNA was dialyzed against 0.5 mM Tris-OAc (pH 8.0) and concentrated by ethanol precipitation.

The synthetic heteronucleotide poly(A,C,U) was purchased from Miles Laboratories, Inc.

Amino Acid Incorporation. Procedures for the isolation of wheat seed embryos and preparation of the S-23 ribosome–enzyme fraction were detailed by Shih and Kaesberg (1973). The protein content was measured by the method of Lowry *et al.* (1951). The detailed composition of the reaction mixtures is given in the figure legends, and in all cases the radioactive amino acid was L-[3,5-^{3}H]tyrosine (52 Ci/mmol). Since some deacylation occurred during storage of the charged RNAs, the actual amount bound was determined (Tao and Hall, 1971a) on the day of use, and this figure quoted as the radioactivity supplied. In no case was more than 15% deacylation found. In order that direct comparison could be made of tyrosine transfer within each set of experiments, similar levels of radioactivity and similar amounts of RNA were supplied in each reaction. All incubations were at 30°; samples were withdrawn at the times indicated and spotted onto Whatman 3MM filter paper disks (2.5-cm diameter) which had previously received 3 drops of 20% trichloroacetic acid. Amino acid was hydrolyzed from aminoacylated tRNA and viral RNA by heating to 80° in 5% trichloroacetic acid for 15 min. An ethanol–ether (1:1) and two ether washes followed to remove free amino acids and to dry the disks. Radioactivity was measured as described by Davies and Hall (1969), the counting efficiency for tritium on paper disks being 17%. The efficiency (E, per cent) with which tyrosine was transferred to nascent peptidyl material was calculated from $E = (V_t R_s / V_s R_t)100$, where V_t and R_t are the total volume and radioactivity of the incubation and V_s and R_s are the sample volume and radioactivity incorporated, respectively.

Results

Tyrosine Transfer to Viral Protein. The cell-free protein synthesizing system from wheat embryo (Marcus *et al.*, 1968; Klein *et al.*, 1972) is known to be capable of synthesizing BMV coat protein when BMV RNA is added as messenger (Shih and Kaesberg, 1973). When [^{3}H]tyrosine was supplied to this system, its incorporation into hot trichloroacetic acid insoluble material was readily detected (Figure 1a). Despite having been dialyzed, the system appeared to contain appreciable amounts of competing, unlabeled tyrosine since the addition of lower, but not limiting, amounts of isotopic

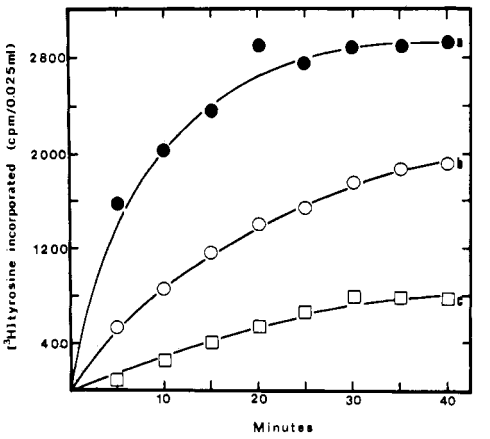

FIGURE 1: Incorporation of [^{3}H]tyrosine into viral protein. The reaction mixtures (0.28 ml) contained a buffer–cofactor solution of 1.43 mM ATP, 0.29 mM GTP, 2.86 mM phosphoenolpyruvate, 4.3 eu of pyruvate kinase (Calbiochem), 3 mM $Mg(OAc)_2$, 45 mM KCl, 2.14 mM dithiothreitol, 28.6 μM of each of 19 amino acids (tyrosine omitted), 3.86 mM Tris-OAc (pH 7.6), 4.3 mM Hepes–NaOH (pH 7.6), and an S-23 ribosome–enzyme preparation (1.4 mg of protein) from wheat embryo. In addition, each reaction contained 25 μg of BMV RNA, 30 μg of tRNA, and [^{3}H]tyrosine. Experiment a contained 960 pmol of tyrosine (1.89×10^7 cpm), experiment b 480 pmol of tyrosine (9.44×10^6 cpm), and experiment c 96 pmol of tyrosine (1.89×10^6 cpm); 1 fmol of tyrosine = 19.6 cpm.

tyrosine did not decrease the levels of radioactive tyrosine in the peptidyl product proportionately (Figure 1b,c).

The efficiency with which tyrosine was incorporated into viral protein was compared for free [^{3}H]tyrosine, [^{3}H]tyrosyl-tRNA, and BMV [^{3}H]tyrosyl-RNA (Figure 2). Virtually complete transfer of tyrosine from tRNA to the peptidyl product was observed (Figure 2a and Table I), there being no doubt that the charged tRNA functioned as a tyrosine donor in

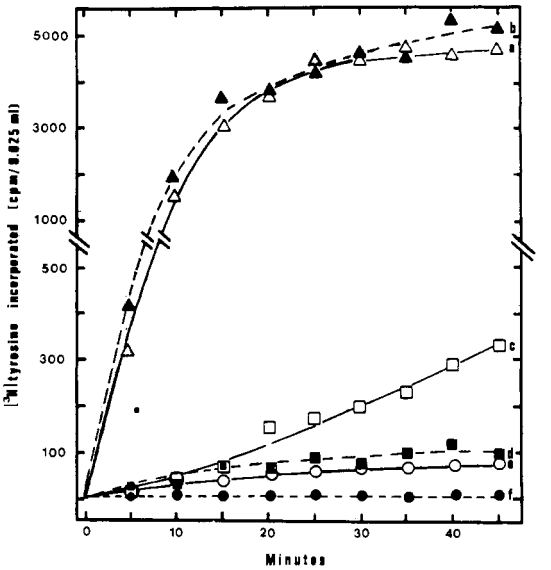

FIGURE 2: Transfer of free and bound [^{3}H]tyrosine into viral protein. All reaction mixtures (0.28 ml) contained the buffer–cofactor solution detailed in Figure 1. In addition, experiment a contained 28 μg of [^{3}H]tyrosyl-tRNA (46,700 cpm) and 25 μg of BMV RNA; experiment c contained 25 μg of BMV [^{3}H]tyrosyl-RNA (100,300 cpm) and 30 μg of tRNA; experiment e contained 3.84 pmol of [^{3}H]tyrosine (75,500 cpm), 25 μg of BMV RNA, and 30 μg of tRNA. Curves with filled symbols (b, d, and f) are the respective controls for experiments a, c, and e, and contained 400 nmol of unlabeled tyrosine in addition to the other materials; 1 fmol of tyrosine = 19.6 cpm.

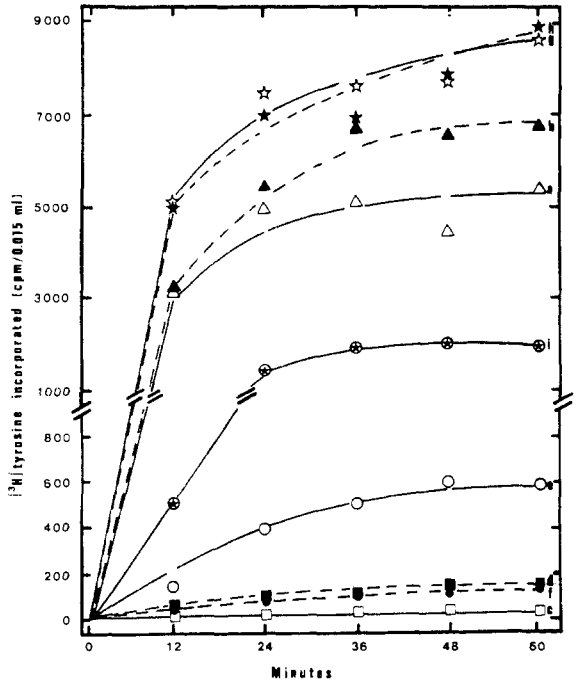

FIGURE 3: Incorporation of free and bound tyrosine in a plant RNA-directed system. All reaction mixtures (0.1 ml) contained 2.5 mM ATP, 0.25 mM GTP, 5 mM phosphoenolpyruvate, 4.5 mM $Mg(OAc)_2$, 90 mM KOAc, 0.5 mM dithiothreitol, 3.75 eu of pyruvate kinase, 2.5 nmol of each of 19 amino acids (tyrosine excluded), 5 mM Tris-OAc (pH 7.6), 20 mM Hepes–KOH (pH 7.6), and an S-23 ribosome–enzyme preparation (520 μg of protein). In addition, experiment a contained 15 μg of [^{3}H]tyrosyl-tRNA (64,300 cpm) and 23 μg of B101 RNA; experiment c contained 12 μg of BMV [^{3}H]tyrosyl-RNA (63,800 cpm), 23 μg of B101 RNA, and 15 μg of tRNA; experiment e contained 3.29 pmol of [^{3}H]tyrosine (63,400 cpm), 23 μg of B101 RNA, and 15 μg of tRNA; experiment g contained 15 μg of [^{3}H]tyrosyl-tRNA (64,300 cpm), 23 μg of B101 RNA, and 12 μg of BMV RNA; experiment i contained 3.29 pmol of [^{3}H]-tyrosine (63,400 cpm), 23 μg of B101 RNA, 12 μg of BMV RNA, and 15 μg of tRNA. Curves with filled symbols (b, d, f, and h) are the respective controls for experiments a, c, e, and g, and contained 100 nmol of unlabeled tyrosine in addition to the other materials: 1 fmol of tyrosine = 19.6 cpm.

TABLE I: Efficiency of Transfer of Free and Bound Tyrosine to Peptidyl Material in a Cell-Free System Directed by Three Different Messengers.[a]

		Efficiency of Tyrosine Transfer (%)[b]					
		Tyrosine		Tyrosyl-tRNA		BMV Tyrosyl-RNA	
Messenger	Data from	−	+	−	+	−	+
BMV RNA	Figure 2	0.9	0.1	107.6	100.0	1.9	1.0
B101 RNA	Figure 3	4.2	1.1	51.2	56.7	0.2	1.4
Poly(A,C,U)	Figure 4	0.3	0.1	16.8	25.9	0.5	0.1

[a] The efficiency of transfer was calculated from the equation given under Methods and Materials using the 25-min incubation time for BMV RNA experiments, the 24-min incubation time for B101 RNA, and the 26-min incubation time for poly(A,C,U). [b] For a radioactive substrate; the minus and plus signs indicate the absence and presence of unlabeled tyrosine in the reaction mixture.

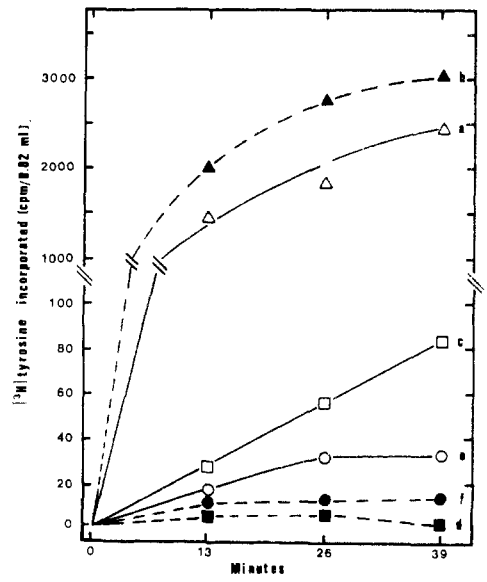

FIGURE 4: Incorporation of free and bound tyrosine in a copoly-(A,C,U)-directed system. All reaction mixtures (0.09 ml) contained 1.43 mM ATP, 0.29 mM GTP, 2.86 mM phosphoenolpyruvate, 4.3 eu of pyruvate kinase, 7.44 mM $Mg(OAc)_2$, 36.2 mM KCl, 101 μM each of Asn, Gln, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, and Thr, 1.67 mM dithiothreitol, 3.21 mM Tris-OAc (pH 7.6), 3.33 mM Hepes–NaOH (pH 7.6), and an S-23 enzyme–ribosome preparation (350 μg of protein). In addition, experiment a contained 10 μg of [^{3}H]tyrosyl-tRNA (48,500 cpm) and 105 μg of copoly(A,C,U); experiment c contained 7.1 μg of BMV [^{3}H]tyrosyl-RNA (53,200 cpm) and 105 μg of copoly(A,C,U); experiment e contained 2.56 pmol of [^{3}H]tyrosine (50,500 cpm) and 105 μg of copoly(A,C,U). Curves with filled symbols (b, d, and f) are the respective controls for experiments a, c, and e, and contained 100 nmol of unlabeled tyrosine in addition to the other materials; 1 fmol of tyrosine = 19.6 cpm.

peptide chain elongation. Free tyrosine competed poorly under these conditions, a 10^5-fold excess of nonradioactive tyrosine having little or no effect on the amount of radioactive tyrosine incorporated (Figure 2b). As expected, nonradioactive tyrosine was an effective competitor of free [^{3}H]tyrosine (Figure 2e,f). When viral RNA, charged with [^{3}H]tyrosine, was added to the incorporation system a low level of incorporation into peptidyl material was observed (Figure 2c), but the efficiency of transfer was very poor (Table I). The addition of excess unlabeled tyrosine together with the BMV [^{3}H]tyrosyl-RNA resulted in only a very small amount of incorporation (Figure 2d). This competition by free tyrosine suggested that much of the incorporation shown in Figure 2c actually resulted from hydrolysis of tyrosine from the viral RNA during the experiment. Even the small amount of incorporation seen in Figure 2d could be from tyrosyl-tRNA present as a 1% contamination in the charged viral RNA after the preparative aminoacylation (see Methods and Materials).

Donation of Tyrosine from tRNA and Viral RNA in the Synthesis of Plant Peptidyl Material. RNA prepared from maturing bean seed cotyledons (B101 RNA, see Methods and Materials) is capable of stimulating amino acid incorporation when added to the wheat embryo system (J. M. Chen and T. C. Hall, unpublished observations). Because no authentic plant mRNA was available we used B101 RNA as a messenger to test the ability of BMV tyrosyl-RNA to function as an amino acid donor in a plant RNA-stimulated incorporation system (Figure 3). The relative efficiencies of viral RNA and tRNA as amino acid donors (Table I) were similar to those detailed for the experiments using viral messenger: tyrosyl-

tRNA was competent in tyrosine donation to plant peptidyl material (Figure 3a); free tyrosine was incorporated to a small extent (Figure 3e); virtually no tyrosine was incorporated when BMV tyrosyl-RNA was added (Figure 3c).

The addition of BMV tyrosyl-RNA to this system is equivalent to adding two messengers (B101 RNA and BMV RNA), and there was a possibility that only one of them was being translated. For this reason additional experiments were made which contained B101 RNA plus an amount of viral RNA equivalent to that added as aminoacylated viral RNA in the experiment shown in Figure 3c. Incorporation of tyrosine from [^{3}H]tyrosyl-tRNA and from [^{3}H]tyrosine in the presence of both these messengers (Figure 3g–i) was higher than in the presence of B101 RNA alone, suggesting that both messages were in fact being translated.

Tyrosine Incorporation Using Poly(A,C,U) as Messenger. The ability of BMV tyrosyl-RNA to function as a tyrosine donor was also examined using poly(A,C,U) as the messenger (Figure 4). This was found to be a relatively inefficient message, possibly because of the frequency of ochre (UAA) termination sequences. However, tyrosyl-tRNA was again found to be a much better tyrosine donor than was the charged viral RNA (Table I).

Discussion

The efficiency with which tyrosine was transferred to nascent peptide from the several radioactive substrates used is summarized in Table I. Charged tRNA was, as expected, a very effective donor of tyrosine to peptidyl material, the actual efficiency being dependent on the activity of the incorporation system when coded by the different messengers. The BMV RNA directed wheat embryo system is known to be very efficient (Shih and Kaesberg, 1973), and the apparently complete donation of tyrosine by tRNA to peptidyl material is in agreement with this finding. In contrast to the high efficiency of tyrosine transfer from tRNA, the apparent incorporation from free [^{3}H]tyrosine was low, undoubtedly because of dilution with unlabeled tyrosine derived from the S-23 preparation (*cf.* Sela, 1969).

Very small amounts of tyrosine were transferred from BMV [^{3}H]tyrosyl-RNA to peptidyl material. Some 1% of the radioactivity present in the charged BMV RNA preparation supplied was known, from sucrose density gradient patterns, to be bound to tRNA present from the enzymic aminoacylation reaction. Since all experiments for each message were supplied with similar levels of radioactivity, it follows that 1% of the incorporation shown in the [^{3}H]tyrosyl-tRNA columns (Table I) should be subtracted from the incorporation observed when BMV [^{3}H]tyrosyl-RNA was added as substrate. Application of this correction to the experiments conducted in the presence of excess unlabeled tyrosine results in zero values for tyrosine transfer from aminoacylated BMV RNA for the system directed by viral message and near zero for the plant and synthetic heteronucleotide systems. Our results, therefore, appear to preclude the direct involvement of aminoacylated BMV RNA in peptide chain elongation.

Prochiantz and Haenni (1973) showed that tRNA maturation endonuclease from *Escherichia coli* can cleave TYMV RNA to yield a fragment of similar size to that of a tRNA. The possibility exists that the transfer of valine from TYMV RNA to peptidyl material (Haenni *et al.*, 1973) may be preceded by release of an RNA fragment containing the 3′ terminus. Since only trace amounts of tyrosine were transferred from BMV RNA to nascent peptidyl material, even in incubations lasting 1 hr, we conclude that our system does not contain an appropriate endonuclease or that BMV RNA fragments do not donate tyrosine.

The physiological function for aminoacylation of plant viral RNAs is elusive, and this has, perhaps, contributed to the lag in following up an earlier report of valine binding by TYMV RNA (Beljanski, 1965). In the case of RNA fragments from TMV, binding of serine and methionine has been reported, and for these amino acids a tRNA-like role in preferential initiation of viral message seems plausible (Sela, 1972), but this function does not appear to be applicable to other amino acids. Elongation factor EF1 from plant cells has been shown to react efficiently with TYMV valyl-RNA or TMV histidyl-RNA, but no interaction occurs with unacylated viral RNAs (Litvak *et al.*, 1973). EF1 is identical with bacterial factor EF Tu, which in turn has been shown to be identical with subunit III of Qβ replicase (Blumenthal *et al.*, 1972). Thus, interaction of viral RNA with this factor may indicate a tRNA-like function, or, alternatively, it may provide a mechanism for recognition of the viral RNA by the replicase.

The present data rule out direct participation of charged BMV RNA as a tyrosine donor in protein biosynthesis, although the possibility exists for an indirect function as a faulty adaptor molecule resulting in the release of nascent host peptide and mRNA, consequently freeing ribosomes for translation of a new (viral) message. We are currently investigating this possibility.

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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.