Time-Course of Tobacco Mosaic Virus-Induced RNA Synthesis in Synchronously Infected Tobacco Leaves

W. O. Dawson and D. E. Schlegel

Department of Plant Pathology and Cell Interaction Group, University of California, Riverside 92502, and Department of Plant Pathology, University of California, Berkeley 94720.

This work was supported in part by National Science Foundation Grant GB 43403.

Accepted for publication 22 September 1975.

ABSTRACT

DAWSON, W. O., and D. E. SCHLEGEL. 1976. Time-course of tobacco mosaic virus-induced RNA synthesis in synchronously infected tobacco leaves. Phytopathology 66: 437-442

The time-course of tobacco mosaic virus (TMV)-induced RNA synthesis was examined in synchronously infected tobacco leaves which were infected at a nonpermissive temperature (3 C) using the differential temperature inoculation procedure. Single-stranded (SS) TMV-RNA, LiCl-soluble double-stranded (LS-DS) RNA, and the double-stranded RNA remaining after RNase treatment of the LiCl- precipitable RNA (LP-DS RNA) each was first detected during the labeling period 6-8 hours after the shift of the infected leaves to the permissive temperature. This coincided with the first detection of infectivity. Afterwards, the rate of synthesis of each species of RNA increased exponentially and in parallel, with the ratios of each species of RNA remaining constant during the early phase of the replication cycle. The maximum rate of synthesis of LS-DS RNA and LP-DS RNA occurred at 18-20 hours, and that of SS TMV-RNA at 24-26 hours. After the maximum rate, the synthesis of SS TMV-RNA declined, but the rate of synthesis of LS-DS RNA continued to increase slightly and the rate of synthesis of LP-DS RNA remained constant.

In a synchronous tobacco mosaic virus (TMV) infection in tobacco cells, the replication cycle consists of three phases; an initial eclipse period where there is no increase in infectivity, a period of exponential increase, followed by a rapid linear increase in virus (5, 14). Tobacco mosaic virus increases exponentially beginning about 8 hours after infection until about 24 hours when a rapid and linear phase of accumulation begins which produces more than 95% of the progeny virus.

The mechanism of synthesis of RNA of plant viruses is thought to proceed through a double-stranded intermediate in an asymmetrical manner similar to that established for small RNA viruses in animal and bacterial systems (2). An entirely double-stranded replicative form RNA (RF) and a multistranded replication intermediate RNA (RI) have been described from phenol-extracted RNA from TMV-infected tissues (6, 12). Even though kinetics of TMV-RNA synthesis has been extensively examined (7, 8, 11, 15), the manner in which RF and RI function in the replication of TMV-RNA is not fully understood. Due to the lack of suitable systems of synchronous virus replication, information concerning the kinetics of TMV-RNA synthesis in relation to a single replication cycle was not available. Nilsson-Tillgren (11) and Kielland-Brandt and Nilsson-Tillgren (8) examined TMV-RNA synthesis in systemically infected leaves in which the infection is near synchrony. However, the precise time of infection was not known, so that the kinetics of TMV-RNA synthesis could not be correlated to time of infection. As this paper was being prepared, the paper of Aoki and Takebe (1) appeared, showing the time-course of TMV-RNA synthesis in synchronously infected tobacco protoplasts.

The system of synchronous virus synthesis described previously (4, 5) permits the examination of the time-course of viral RNA synthesis in leaf tissues. In this paper we examine the time of initiation of TMV-induced RNA synthesis and the kinetics of synthesis of each species of viral RNA during the eclipse, exponential, and linear phases of TMV replication in cells of intact leaves and compare them to that reported from TMV in tobacco protoplasts (1).

MATERIALS AND METHODS

Young tobacco *(Nicotiana tabacum* L. 'Xanthi') leaves were systemically inoculated at 3 C (DTI-3C leaves) with TMV, strain U1, using the differential temperature procedure described previously (5). Virus replication was initiated by moving the upper leaves to a plant growth chamber at 25 C with a 14-hour photoperiod of 20,000 lx. Disks were removed from samples to be radioactively labeled for infectivity assays as described previously (5).

Labeling procedure.—Temperature-synchronized DTI-3C leaves (5) were detached at intervals after the temperature shift to 25 C, midveins were removed, and the leaves were diced into approximately 5 mm strips. The strips were vacuum infiltrated with 40 μCi/ml ^{3}H-uridine (8 Ci/mM) and 65 μg/ml actinomycin D and maintained in that solution on ice for 10 minutes. The strips were then removed to petri dishes and incubated at 25 C and 20,000 lx for 2 hours, after which they were stored at −20 C until extracted.

RNA extraction procedure.—Frozen tissue (5 g) was blended on ice in a VirTis homogenizer in 12 ml of cold 0.01 M Tris-HCl buffer, pH 7.6, 0.05 M NaCl, 1% sodium dodecyl sulfate (SDS) and 12 ml water saturated phenol, containing 10% M-cresol and 0.1% 8-hydroxyquinoline. The aqueous phase was recovered after centrifugation at 10,000 g for 10 minutes, made 0.5 M NaCl, and stirred 15

minutes at room temperature after addition of 10 ml of the above phenol solution. After centrifugation, the aqueous phase was precipitated with the addition of two volumes of ethanol and allowed to stand at −20 C overnight. The precipitate was dissolved in 2 ml STE buffer (0.05 M Tris, 0.1 M NaCl, 0.001 M EDTA, pH 6.85) and dialyzed against that buffer overnight. The solution was then made 2 N with respect to LiCl, stored overnight at 5 C, and the precipitate was collected by centrifugation at 10,000 *g* for 10 minutes, and dissolved in 1.0 ml E buffer (0.04 M Tris-acetate, 0.02 M sodium acetate, 0.002 M EDTA, pH 7.8) containing 0.2% SDS and 20% sucrose. The LiCl supernatant and 0.8 ml of the LiCl precipitate were each precipitated with two volumes

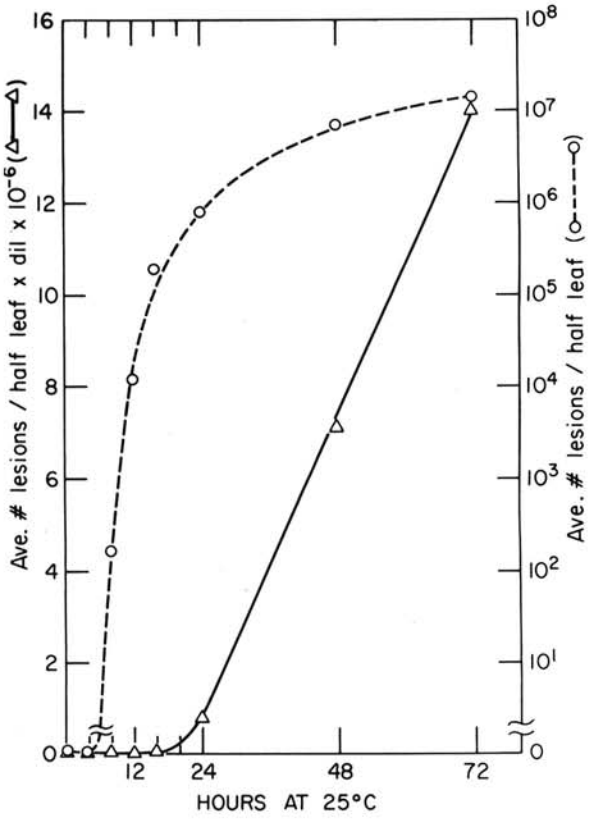

Fig. 1. Time-course increase of infectivity in DTI-3C leaves beginning when the temperature of the leaves was shifted from 3 C to 25 C. Data is plotted on linear (solid line) and logarithmic (dashed line) scales.

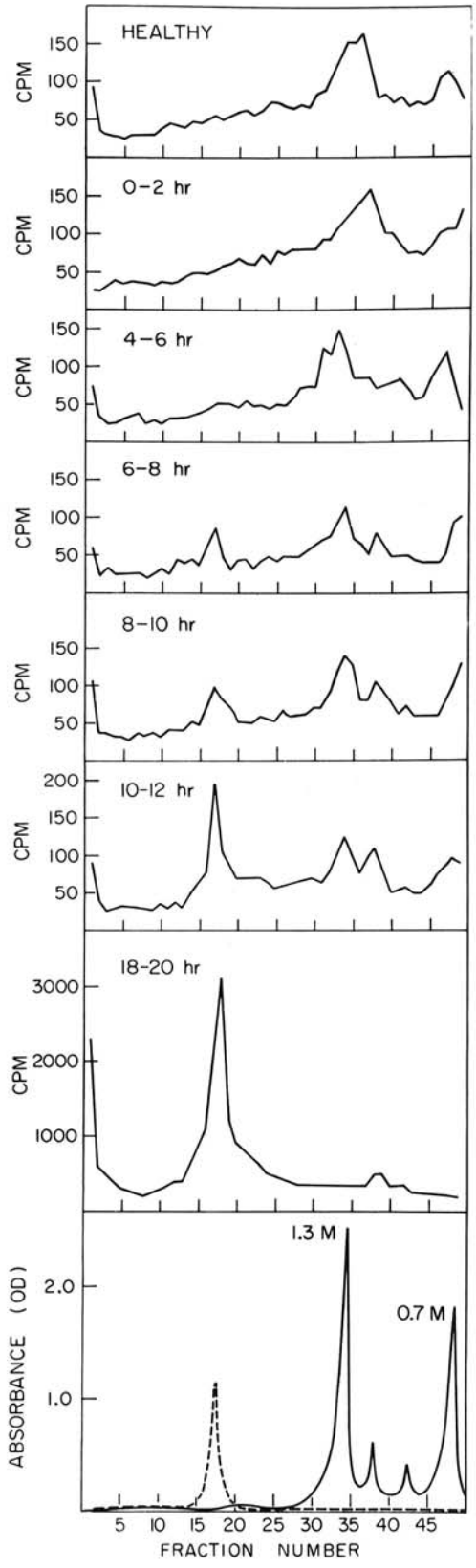

Fig. 2. Incorporation of [^{3}H] uridine into LiCl-precipitated RNA labeled and extracted as described in Materials and Methods. The RNA (50 μl) was electrophoresed on 2.5% polyacrylamide gels at 7 mA/gel for 5 hours. The gels were then scanned at 260 nm and sliced into 2-mm fractions of which radioactivity was determined. The tops of the gels are on the left. Bottom panel: dashed line shows electrophoretic profile of TMV-RNA extracted from purified virus particles. Solid line shows the electrophoretic profile of ribosomal RNA similar to that which occurred from the 260-nm scan of each of the above gels.

of ethanol. Each precipitate was dissolved in 1.0 ml 0.01 M $MgCl_2$ and incubated 1 hour at 37 C with 50 μg/ml DNase (RNase-free) after which each was precipitated with two volumes of ethanol. The LiCl supernatant RNA and the LiCl precipitate RNA each was dissolved in 0.5 ml of 0.2 M NaCl, 0.01 M Tris, 0.01 M $MgCl_2$, pH 7.6, and incubated with RNase A (5 μg/ml) at 37 C for 30 minutes. The samples were then precipitated with two volumes of ethanol and dissolved in 0.2 ml E buffer plus 20% sucrose and 0.2% SDS.

Gel electrophoresis.—Polyacrylamide gel electrophoresis was performed on 2.5% acrylamide gels essentially by the procedure of Loening (9). Fifty μliters of the LiCl precipitate RNA, 150 μliters of the LiCl-precipitate RNase-treated RNA, and 200 μliters of the LiCl-supernatant RNA were layered onto gels and run at 7 mA per tube for 5 hours at room temperature with electrophoresis buffer containing 0.2% SDS. After electrophoresis, the gels were scanned at 260 nm with a Beckman Acta II spectrophotometer equipped with a gel scanner.

Determination of radioactivity.—Gels were sliced into 2-mm-thick slices that were placed in glass scintillation vials containing 0.75 ml of 30% H_2O_2 and were incubated overnight at 70 C. Ten ml of a PPO-dimethyl-POPOP scintillation fluid containing 33% Triton X-100 and 67% toluene was added to each vial. After setting at least 24 hours in the dark, the vials were counted in a Beckman LS-100C liquid scintillation counter.

RESULTS

Detection of early viral RNA synthesis.—The time of initiation of TMV-induced RNA synthesis was examined in synchronously infected tobacco leaves. Due to slight variation between experiments, an infectivity curve for each experiment was determined to accurately correlate new RNA synthesis to infectivity increases. Figure 1 shows the infectivity curve of a typical experiment. An eclipse period before detectable infectivity occurred until 8 hours after the shift of the leaves to the permissive temperature, after which infectivity increased exponentially the next 16 hours, and then the virus increased rapidly and linearly. Virus-induced RNA synthesis was most closely examined during the early periods of synthesis (0-24 hours) when the replication process appeared to be developing.

Single-stranded (SS) TMV-RNA has a molecular weight of 2.07×10^6 daltons (13). To detect small amounts of radioactivity in the area of SS TMV-RNA on polyacrylamide gels when tissue was labeled for periods shorter than 4 hours, it was necessary to inhibit host ribosomal RNA synthesis with actinomycin D, because a ribosomal RNA precursor which is $2.3 - 2.5 \times 10^6$ daltons (10) concealed the SS TMV-RNA peak.

A prominent peak of radioactivity which co-electrophoresed with virion RNA and was susceptible to RNase was observed on gels from RNA of tissue which was labeled 18-20 hours after the temperature shift to 25 C (Fig. 2). This was a time near the end of the exponential phase and the beginning of the rapid linear phase of infectivity increase. At earlier labeling periods, this peak of ^{3}H-uridine incorporation was progressively less prominent. The first detectable peak which corresponded

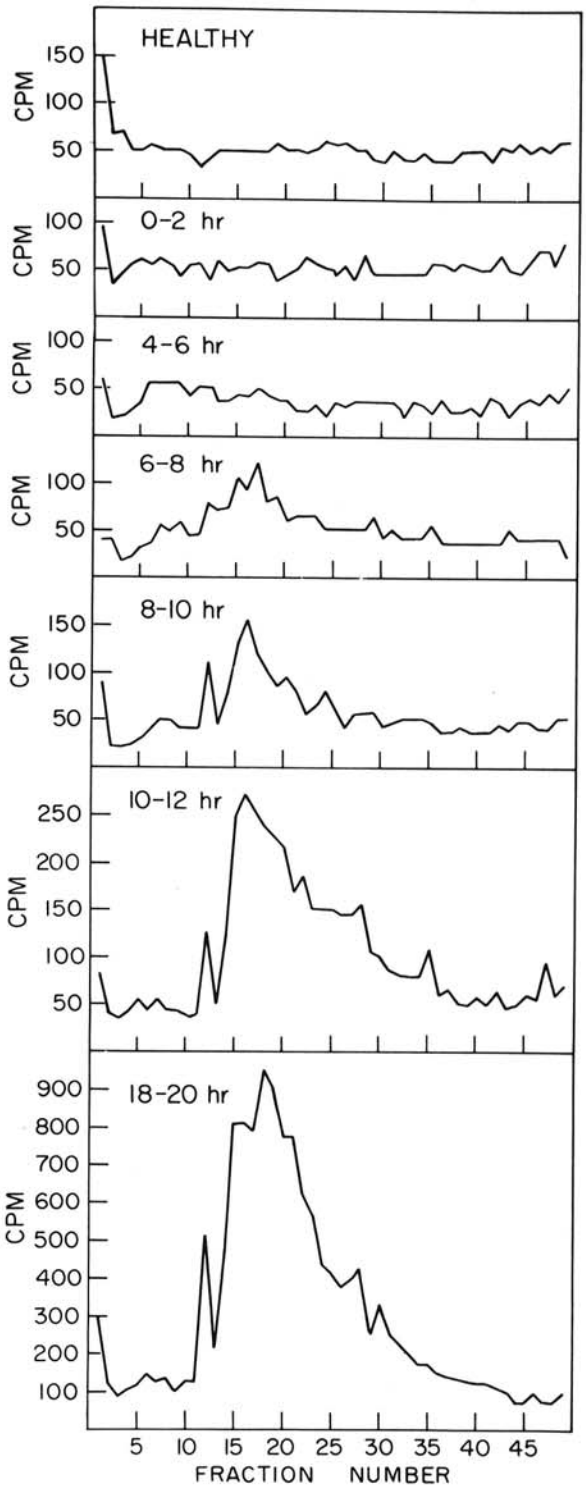

Fig. 3. Incorporation of [^{3}H] uridine into LiCl-soluble RNA that was labeled and extracted as described in Materials and Methods. The RNA (200 μliters) was electrophoresed, scanned at 260 nm, and radioactivity determined as in Fig. 2.

to SS TMV-RNA was found at 6-8 hours, when a small but reproducible peak was observed. This corresponded to the first detectable infectivity which was found at 8 hours. No radioactive peak was ever found at 4-6 hours or earlier.

The time of initiation of TMV-induced double-stranded RNA synthesis was also monitored. The double-stranded RNA was divided into two fractions: (i) LiCl-soluble, double-stranded (LS-DS) RNA that was resistant to RNase; and (ii) the double-stranded RNA recoverable after RNase digestion of the LiCl precipitate (LP-DS). The LS-DS RNA should be comparable to RF described by Nilsson-Tillgren (12) and Jackson et al. (6); however, our preparations of LS-DS RNA were not homogeneous (Fig. 3) because the RNA was extracted with a VirTis homogenizer instead of powdering tissue frozen with liquid nitrogen in a mortar and pestle which gives homogeneous RF. However, the VirTis extraction gave higher yields of RNA which we considered necessary to detect the initial synthesis of TMV-induced RNA. There was a sharp peak at fraction 12 (Fig. 3) which had a MW (approximately 4×10^6 daltons, estimated using double-stranded RNA markers from bacteriophage $\phi 6$, kindly supplied by J. S. Semancik) expected for RF and a population of smaller, more heterogeneous double-stranded RNA which moved more rapidly during electrophoresis. However, the LS-DS RNA banded as a homogeneous peak at 1.60 g/cc in Cs_2SO_4 gradients and each of the peaks after electrophoresis on acrylamide gels specifically hybridized to TMV-RNA.

The LP-DS RNA should be representative of the RI described by Nilsson-Tillgren (12) and Jackson et al. (6). The LP-DS RNA was RNase treated, which according to Jackson et al. (6), converts the RI to a RF-like form. However, the LP-DS was also heterogeneous with few molecules being as large as RF, similar to that described by Aoki and Takebe (1). The LP-DS RNA also banded homogeneously at 1.60 g/cc in a Cs_2SO_4 gradient and each peak (Fig. 4) hybridized specifically to TMV-RNA.

The time-course of synthesis of LS-DS RNA is shown in Fig. 3. A distinct and reproducible population of peaks was first observed from RNA labeled at 6-8 hours, and became larger at each successive labeling period. This corresponded to the first detection of SS TMV-RNA and infectivity. No peak was ever observed at the 4-6 hour labeling period.

A distinct and reproducible LP-DS was first detected in the 6- to 8-hour labeling period (Fig. 4). At later labeling periods, that species of virus-specific RNA became progressively larger.

Rates of synthesis of TMV-induced RNA's.—The synthesis rate of SS TMV-RNA increased exponentially, beginning at the 6- to 8-hour label after the temperature shift to 25 C, until the maximum rate was attained at 24-26 hours (Fig. 5). Afterwards, the rate of synthesis began to decline. The maximum synthesis rate of SS TMV-RNA occurred at the time the linear infectivity increase was beginning (Fig. 1), and although the infectivity increased rapidly from 24 to 72 hours, the rate of synthesis decreased.

The rate of synthesis of LS-DS RNA increased exponentially until the 18-20 hours labeling period, after which the rate continued to slowly increase (Fig. 5). In other experiments, the rate of synthesis of LS-DS RNA

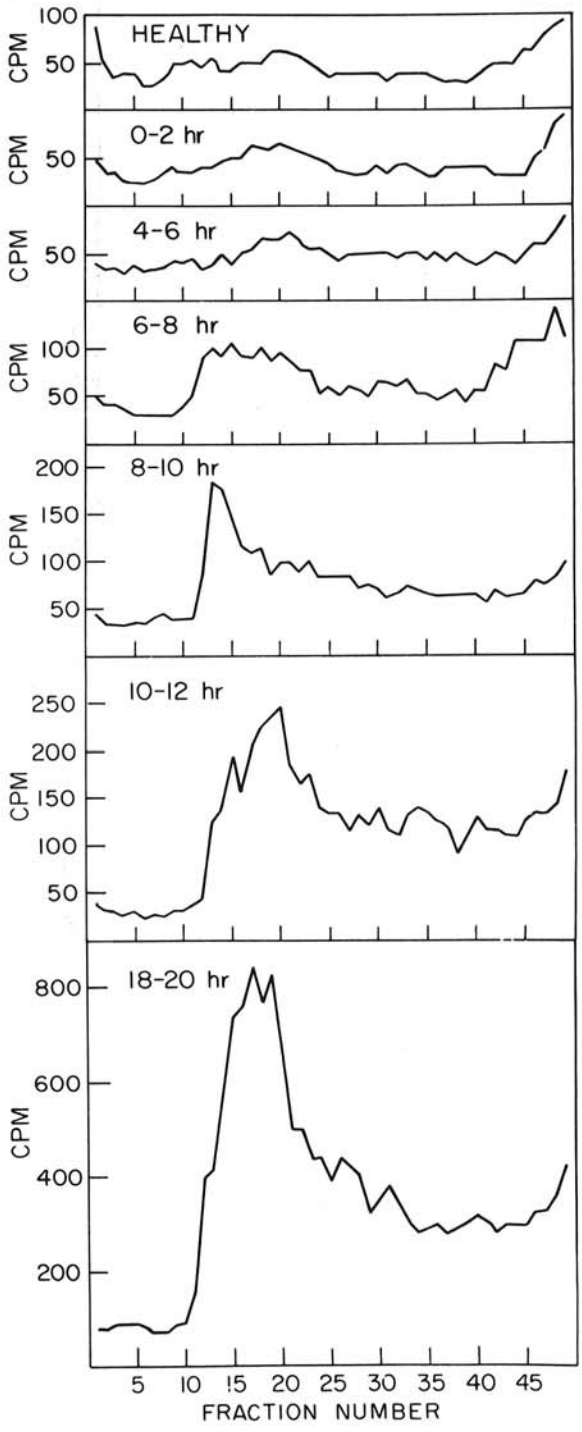

Fig. 4. Incorporation of [^{3}H] uridine into LiCl-precipitated RNA that was RNase-treated (5 μg/ml RNase A, 37 C, 30 minutes). Tissue was labeled and RNA was extracted as described in Materials and Methods. The RNA (150 μliters) was electrophoresed, scanned at 260 nm, and radioactivity determined as in Fig. 2.

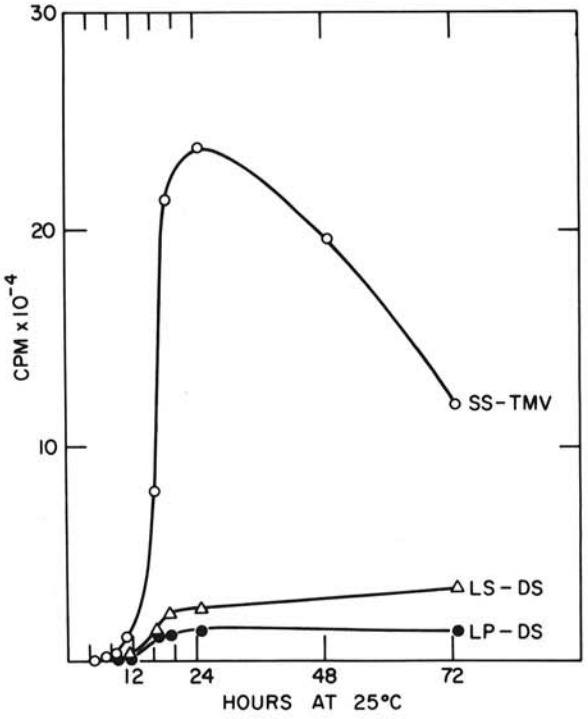

Fig. 5. Rates of incorporation of [^{3}H] uridine into SS TMV-RNA, LS-DS RNA, and LP-DS RNA, labeled 2 hours at 25 C. The areas under peaks of radioactivity corresponding to each type of RNA were determined by planimetry and normalized to CPM/g fresh tissue.

usually remained constant after a maximum at about 20 hours. Although the rate of synthesis of SS TMV-RNA decreased after 24 hours, the rate of synthesis of LS-DS did not decrease.

The ratio of the rate of synthesis of LS-DS RNA to that of SS TMV-RNA remained almost constant during the first 24 hours with approximately 10% of the total virus-induced radioactivity incorporated in LS-DS RNA. The range of several different experiments was 5-15%. At later times when the rate of synthesis of SS TMV-RNA declined, the percentage of incorporation into LS-DS RNA became larger.

The rate of synthesis of LP-DS RNA was slightly lower than that of LS-DS RNA. It increased exponentially until 18-20 hours, after which the rate remained nearly constant. The percentage of total counts incorporated into virus-specific RNA's which was found in LP-DS RNA ranged from 2-10% and remained nearly constant within any one experiment.

Integrated rates.—When the synthesis rates were integrated, curves resulted which represent the amount of product made with respect to time. This does not measure the amounts of RNA species which accumulate. If a species of RNA turns over, as the double-stranded forms do (Dawson and Schlegel, *unpublished*), less of that RNA would accumulate than was synthesized. When plotted on a logarithmic scale, it is easy to visualize the initial increases of the different species of TMV-induced RNA's. Exponential increase of SS TMV-RNA began at 6-8 hours and continued until 18-20 hours (Fig. 6), very similar to the infectivity increase (Fig. 1). Both double-stranded forms began at the same time as single strands and followed similarly shaped curves. Disregarding turnover and looking only at the amount of each species produced, each species was produced logarithmically between 6 and 20 hours, after which each was produced linearly. There was no accumulation of either type of double strand before the synthesis of single strands.

DISCUSSION

The system of synchronous virus synthesis provided by infecting leaves at a nonpermissive temperature and allowing synthesis to begin when the leaves were moved to a permissive temperature (5) allowed the examination of TMV-induced RNA synthesis in relation to the virus replication cycle in cells of intact leaves. The synthesis of SS TMV-RNA was first detected at 6-8 hours after the shift to the permissive temperature. Synthesis continued to increase exponentially until 18-20 hours when the accumulation of SS TMV-RNA became approximately linear. The vast majority of viral RNA accumulated during the linear phase. A similar pattern of SS TMV-RNA synthesis was reported in tobacco protoplasts (1), except that the protoplasts were incubated at a higher

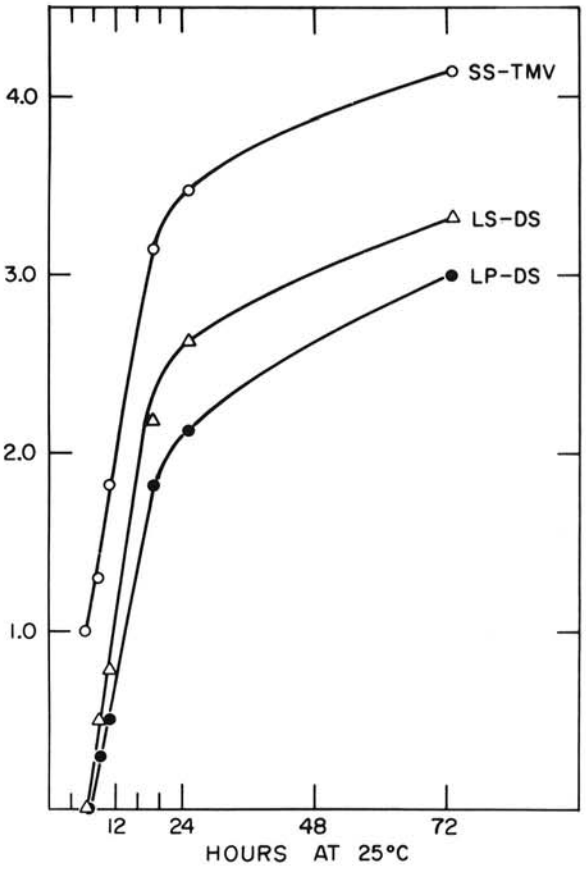

Fig. 6. Integrated rates of synthesis of SS TMV-RNA, LS-DS RNA, and LP-DS RNA. Rate curves were integrated by planimetry.

temperature (28 C) and the pattern occurred earlier. Although on a different time scale, the RNA synthesis of small RNA viruses of bacteria and animals follow similar patterns in one-step replication cycles (3).

If the double-stranded structures found in TMV-infected tissues are intermediates of TMV-RNA synthesis, their kinetics of synthesis should be compatible with the increase of progeny RNA. In synchronously infected leaves, LS-DS RNA and LP-DS RNA were first detected at 6-8 hours, the same time that SS TMV-RNA and infectivity were first detected. The synthesis of TMV-induced double-stranded RNA paralleled SS TMV-RNA synthesis in a manner similar to that of polio virus in a single-step infection cycle (3). Synthesis of both single-stranded and double-stranded RNA's increased exponentially during the early phase of infection and then began to accumulate much more rapidly during the linear phase. During the early phases of synthesis, before synthesis of SS TMV-RNA began decreasing, the ratio of synthesis of LS-DS RNA and LP-DS RNA to SS TMV-RNA remained nearly constant. This is in contrast to the report of TMV synthesis in protoplasts in which 40 percent of the earliest detectable viral RNA was double stranded (1).

During the early stage of infection in protoplasts, Aoki and Takebe (1) found assembly of TMV particles to occur 4-5 hours later than SS TMV-RNA synthesis, resulting in the accumulation of substantial amounts of free viral RNA. We did not observe this in our experiments. We found the accumulation curve of SS TMV-RNA to be almost identical to the encapsulated TMV infectivity curve. This is supported by RNA infectivity assays which demonstrate only minute amounts of free-RNA infectivity in synchronously infected leaves (5). Also, the observation that when cycloheximide treatment began at 48 hours, the amount of infectious virus doubled between 48 and 72 hours in the presence of cycloheximide without the accumulation of measurable amounts of free RNA suggests that a substantial amount of replicase and coat protein were already synthesized at 48 hours (4). These data suggest that there is little delay between SS TMV-RNA synthesis and encapsulation in synchronously infected tobacco leaves and that the rate of encapsulation is dependent upon the rate of SS TMV-RNA synthesis.

Data in this paper and that of Aoki and Takebe (1) demonstrate that the kinetics of viral RNA synthesis in synchronously infected plant cells, except for being on a different time scale, are essentially the same as that of RNA synthesis of small RNA viruses in synchronously infected animal and bacterial cells.

LITERATURE CITED

1. AOKI, S., and I. TAKEBE. 1975. Replication of tobacco mosaic virus RNA in tobacco mesophyll protoplasts inoculated in vitro. Virology 65:343-354.
2. BISHOP, J. M., and L. LEVENTOW. 1971. Replicative forms of viral RNA. Structure and function. Progr. Med. Virol. 13:1-82.
3. DARNELL, J. E., M. GIRARD, D. BALTIMORE, D. F. SUMMERS, and J. V. MAIZEL. 1967. The synthesis and translation of poliovirus RNA. Pages 375-401 *in* J. S. Colter and W. Parachych, eds. The Molecular Biology of Viruses. Academic Press, New York. 730 p.
4. DAWSON, W. O., and D. E. SCHLEGEL. 1976. The sequence of inhibition of tobacco mosaic virus synthesis by actinomycin D, 2-thiouracil, and cycloheximide in a synchronous infection. Phytopathology 66:177-181.
5. DAWSON, W. O., D. E. SCHLEGEL, and M. C. Y. LUNG. 1975. Synthesis of tobacco mosaic virus in intact tobacco leaves systemically inoculated by differential temperature treatment. Virology 65:565-573.
6. JACKSON, A. O., D. M. MITCHELL, and A. SIEGEL. 1971. Replication of tobacco mosaic virus. I. Isolation and characterization of double-stranded forms of ribonucleic acid. Virology 45:182-191.
7. JACKSON, A. O., M. ZAITLIN, A. SIEGEL, and R. I. B. FRANCKI. 1972. Replication of tobacco mosaic virus. III. Viral RNA metabolism in separated leaf cells. Virology 48:655-665.
8. KIELLAND-BRANDT, M. C., and T. NILSSON-TILLGREN. 1973. Studies on the biosynthesis of TMV. V. Determination of TMV RNA and its complementary RNA at different times after infection. Mol. Gen. Genet. 121: 229-238.
9. LOENING, U. E. 1967. The fractionation of high-molecular-weight ribonucleic acid by polyacrylamide-gel electrophoresis. Biochem. J. 102:251-257.
10. LOENING, U. E., W. K. JONES, and M. L. BIRNSTIEL. 1969. Properties of the ribosomal RNA precursors in Xenopus laevis; comparison to the precursors in mammals and plants. J. Mol. Biol. 45:353-366.
11. NILSSON-TILLGREN, T. 1969. Studies on the biosynthesis of TMV. II. On the RNA synthesis of infected cells. Mol. Gen. Genet. 105:191-202.
12. NILSSON-TILLGREN, T. 1970. Studies on the biosynthesis of TMV. III. Isolation and characterization of the replicative form and the replicative intermediate RNA. Mol. Gen. Genet. 109:246-256.
13. REIJNDERS, L., A. M. J. AALBERS, A. VAN KAMMEN, and R. W. J. THURING. 1974. Molecular weights of plant viral RNAs determined by gel electrophoresis under denaturing conditions. Virology 60:515-521.
14. TAKEBE, I., Y. OTSUKI, and S. AOKI. 1971. Infection of isolated tobacco mesophyll protoplasts by tobacco mosaic virus. Colloq. Int. Cent. Nat. Rech. Sci. 193:503-511.
15. WYATT, S. 1973. Appearance and accumulation of double-stranded viral RNA in tobacco leaves inoculated with tobacco mosaic virus. Ph.D. Dissertation, University of Kentucky, Lexington. 77 p.