The Sequence of Inhibition of Tobacco Mosaic Virus Synthesis by Actinomycin D, 2-Thiouracil, and Cycloheximide in a Synchronous Infection

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ABSTRACT

The time-courses of inhibition of tobacco mosaic virus (TMV) synthesis in synchronous infections by actinomycin D (AMD), 2-thiouracil (2TU), and cycloheximide (CX) were determined. Young tobacco leaves were systemically infected at 3 C (DTI-3C leaves) or 12 C (DTI-12C leaves) using the differential temperature inoculation procedure. Three degrees and 12 C inhibited different steps of TMV replication. After transfer of the DTI leaves to 25 C, TMV replication became resistant to different inhibitors sequentially. In DTI-3C leaves, the AMD-sensitive step occurred prior to the transfer to 25 C. When 2TU treatment began during the first 3 hours at 25 C, TMV synthesis was reduced about 95%, but when treatment began at later times the amount of inhibition rapidly declined to no inhibition by 12 hours. Cycloheximide inhibited TMV replication (>95%) when treatment began at 12 hours or before, after which the inhibition declined until little inhibition occurred by 48 hours at 25 C. In DTI-12C leaves, neither AMD nor 2TU inhibited TMV replication when treatment began at any time after the transfer from 12 C to 25 C. Immediately after the transfer to 25 C, CX inhibited TMV synthesis maximally, but the amount of inhibition began immediately declining when treatments began at later times. This establishes the sequence of inhibition of TMV replication as:

Infection → AMD → 3 C → 2TU → 12 C → CX → TMV-RNA → Virus.

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Determination of the time-courses of action of different inhibitors of animal virus replication in relation to the single-step infection cycle has been useful in elucidating the mechanisms of the inhibitors and in understanding the molecular biology of virus replication (3, 11). Although numerous inhibitors have been reported which block replication of plant viruses (10), their time-courses of inhibition have not been determined in relation to the replication cycle, because until recently, systems which provide synchronous multiplication of viruses in plant systems were not available.

In a system where virus multiplies synchronously, each step of virus replication should occur simultaneously in all infected cells. If different inhibitors block different steps of replication, their time-courses of inhibition might be different. This would define at what stage of replication each inhibitor is effective and would facilitate the understanding of their mechanisms of inhibition.

Previously, we reported a procedure that synchronized the tobacco mosaic virus (TMV) infection in intact leaves (5, 7). Young leaves maintained at a non-permissive temperature for virus replication (5 C or 12 C) were infected by virus which moved systemically from lower leaves of the same plants maintained at 27 C. After sufficient inoculum had moved into the upper leaves at the low temperature, they were moved to a permissive temperature that allowed virus synthesis in all infected cells to begin simultaneously. A shorter eclipse period in the leaves infected at 12 C (DTI-12C leaves) than in leaves infected at 5 C (DTI-5C leaves) suggested that 12 C blocked an early step of TMV replication that was different and occurred later than the step blocked by 5 C. In this paper we examined the time-courses of inhibition of different inhibitors of TMV synthesis in DTI leaves after they were shifted from 3 C or 12 C to a permissive temperature (25 C). We chose to examine actinomycin D (AMD), cycloheximide (CX), and 2-thiouracil (2TU). AMD, which inhibits DNA-directed RNA synthesis, has been shown to inhibit synthesis of the cowpea strain of TMV when treatment began soon after infection (8). CX, which blocks protein synthesis on plant cytoplasmic ribosomes, blocks TMV synthesis when added at concentrations which block protein synthesis (2). 2TU has long been recognized as one of the most potent inhibitors of TMV multiplication (4).

The data reported in this paper demonstrate that 3 C and 12 C block different early steps of TMV replication and that the time-courses of the inhibitors occur sequentially and during almost mutually exclusive periods in DTI leaves. Both phenomena support the contention that TMV replicates synchronously in DTI leaves.

MATERIALS AND METHODS.—Tobacco plants *(Nicotiana tabacum* L. 'Xanthi') were infected with U1 strain of TMV by the differential temperature inoculation procedure described previously (7), after which they were incubated in a plant growth chamber at 25 C with a 14-hour photoperiod of 20,000 lx. Seven-mm diameter disks were removed from the DTI leaves at intervals, beginning at the time of transfer to 25 C. Ten disks per treatment were vacuum infiltrated with the inhibitor solution at 0 C, and then allowed to dry on paper towels for 10-15 minutes. The disks were then floated on the inhibitor solutions in 3.5-cm petri dishes in the 25 C chamber. Control disks were similarly treated with distilled water. At 72 hours after the transfer to 25 C, all disks were removed from solution and frozen at −20 C. Infectivity was assayed by the half-leaf method on *Phaseolus*

vulgaris 'Pinto' as described previously (7).

Asynchronously infected plants were obtained by mechanically inoculating leaves with 0.5 mg TMV per ml in 0.01 M potassium phosphate buffer, pH 7.0, plus 1% Celite, followed by a distilled water rinse. Disks were removed and treated as described above, except that the disks were incubated at 25 C for 96 hours.

RESULTS.—*Time-courses of inhibition of TMV synthesis in an asynchronous infection by AMD, CX, and 2TU.*—Fully expanded leaves of greenhouse grown tobacco plants were mechanically inoculated with TMV and incubated at 25 C. At intervals after inoculation, disks were removed from the inoculated leaves, vacuum infiltrated and floated on AMD (50 μg/ml), CX (10 μg/ml), or 2TU (1.0 mM) at 25 C until 96 hours after inoculation. The disks were then assayed for infectivity and compared to distilled water treated controls. When treatment began immediately after inoculation, AMD, CX, and 2TU strongly inhibited TMV synthesis (Table 1). When treatments began at later times, the inhibition was less. By 48 hours after inoculation, AMD no longer inhibited TMV synthesis, although CX and 2TU inhibited synthesis 38% and 43%, respectively.

The effect of these inhibitors upon asynchronous infections in young tobacco leaves of plants that had been pretreated with differential temperatures was examined. Healthy plants were maintained in the differential temperature chamber (see reference 7) with the lower leaves at 27 C and the upper leaves at 3 C or 12 C. After 10 days, the plants were removed from the chamber and the upper leaves that had been maintained at the low temperature were mechanically inoculated with TMV and incubated at 25 C. Disks were removed at intervals after inoculation and treated as above. The pretreatment with differential temperatures had little effect upon the inhibition of TMV synthesis in these leaves by AMD, CX, or 2TU (Table 1).

Time-courses of inhibition of TMV synthesis in a synchronous infection by AMD, CX, and 2TU.—The time-courses of inhibition of TMV synthesis in DTI-3C and DTI-12C leaves by AMD (50 μg/ml), CX (10 μg/ml) and 2TU (1.0 mM) was determined beginning at the time they were transferred to 25 C. Disks were removed from the leaves at intervals after the transfer to 25 C and vacuum infiltrated and floated on the inhibitor solutions until 72 hours after the transfer to 25 C. Infectivity in inhibitor-treated disks was compared to that in distilled water treated disks.

1) DTI-3C leaves.—In these experiments the young leaves were infected at 3 C instead of at 5 C as described in the previous paper (7). DTI-3C leaves exhibited the same infectivity curves as DTI-5C leaves and appeared to be identical by all other measurements. For these experiments 3 C was chosen because it represented a greater difference from 12 C, and because a convenient cold room which maintained this constant temperature was available.

In DTI-3C leaves, AMD did not inhibit TMV synthesis (Fig. 1) regardless of when treatment began, immediately before (0 hours) or at intervals after the transfer to 25 C, although AMD inhibited TMV synthesis in mechanically inoculated leaves (Table 1).

2TU inhibited TMV synthesis about 95% when treatment began within the first 3 hours at the permissive temperature (Fig. 1). As treatments began at progressively later times, between 3 and 12 hours, the amount of inhibition rapidly declined until no inhibition occurred when treatment began at 12 hours or later. The first detectable infectivity was found at 8 hours. At 6 hours before any infectivity could be detected, enough products of the 2TU-sensitive function were produced to synthesize 60% as much virus as in control disks. At 12 hours after the transfer to 25 C when a minimal amount of virus had accumulated, synthesis was totally resistant to

TABLE 1. Time-course of inhibition of tobacco mosaic virus synthesis in mechanically inoculated leaves

Treatment	Time after inoculation (Hours)	Inhibition (%) by 50 μg/ml AMD	10 μg/ml CX	1 mM 2TU
GH→MI→25 C[a]	0	92	96	99
	24	18	84	75
	48	0	38	43
	72	0	3	8
3 C→MI→25 C[b]	0	72	98	92
	24	12	74	76
	52	0	42	48
12 C→MI→25 C[c]	0	77	94	96
	24	6	90	72
	52	0	76	35

[a]Plants were removed from the greenhouse (GH), mechanically inoculated (MI), and incubated at 25 C.

[b]Healthy plants were pretreated with differential temperature treatment with the young leaves at 3 C (7) after which the upper leaves were mechanically inoculated (MI) and incubated at 25 C.

[c]Healthy plants were pretreated with differential temperature treatment with the young leaves at 12 C (7) after which the upper leaves were mechanically inoculated (MI) and incubated at 25 C.

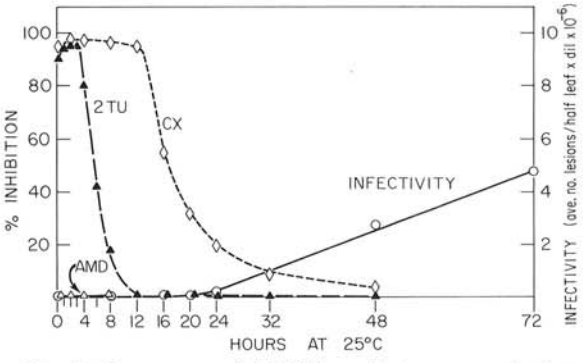

Fig. 1. Time-course of inhibition of tobacco mosaic virus (TMV) synthesis by actinomycin D (AMD) (50 μg/ml), 2-thiouracil (2TU) (1.0 mM), or cycloheximide (CX) (10 μg/ml) in young tobacco leaves systemically inoculated at 3 C, (DTI-3C), then transferred to 25 C. Leaf disks (7 mm in diameter) were removed from DTI-3C leaves at intervals after the shift to 25 C and continuously treated (vacuum infiltration, 10 disks per treatment) with one of the inhibitors until 72 hours at 25 C, when the infectivity of TMV from the leaf disks exposed to each treatment was compared to that from similar disks treated with distilled water.

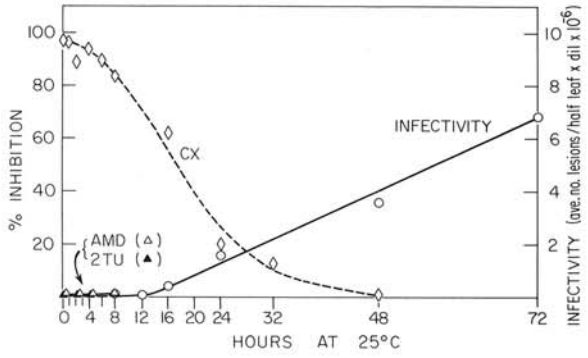

Fig. 2. Time-course of inhibition of tobacco mosaic virus (TMV) synthesis by actinomycin D (AMD) (50 μg/ml), 2-thiouracil (2TU) (1.0 mM), or cycloheximide (CX) (10 μg/ml) in young tobacco leaves systemically inoculated at 12 C, (DTI-12C), then transferred to 25 C. Leaf disks (7 mm in diameter) were removed from DTI-12C leaves at intervals after the shift to 25 C and continuously treated (vacuum infiltration, 10 disks per treatment) with one of the inhibitors until 72 hours at 25 C, when the infectivity of TMV from the leaf disks exposed to each treatment was compared to that from similar disks treated with distilled water.

2TU. More than 99% of the normal amount of virus was synthesized in the presence of 2TU.

Cycloheximide inhibited TMV synthesis in DTI-3C leaves about 95% when treatment began within the first 12 hours at 25 C (Fig. 1). When treatments began progressively later than 12 hours, synthesis became more resistant to CX, until by 48 hours there was little inhibition. When treatment began at 20 hours, CX inhibited synthesis by only 30%, although the linear accumulation of virus had not begun. This suggests that sufficient amounts of replicase and coat protein had accumulated by 20 hours to produce in the presence of cycloheximide about 70% as much virus as nontreated disks. Similarly, there was little inhibition by CX when treatment began at 48 hours, although approximately half of the virus that accumulated by 72 hours was synthesized between 48 and 72 hours. The time-course of CX inhibition of TMV infectivity of extracted RNA from infected tissue was similar to that of encapsidated virus (infectivity of expressed sap) from CX-treated tissue indicating that CX did not cause an accumulation of free virus RNA.

2) DTI-12C leaves.—Actinomycin-D also did not inhibit TMV synthesis in DTI-12C leaves when treatment began immediately before the transfer to 25 C or at various times after the transfer (Fig. 2). Although 2TU initially inhibited synthesis in DTI-3C leaves, it did not inhibit TMV synthesis in DTI-12C leaves. The total amount of virus synthesized in DTI-12C leaves after the transfer to 25 C occurred in the presence of 2TU. The 2TU-sensitive step occurred at 12 C.

Cycloheximide did inhibit TMV synthesis in DTI-12C leaves by about 90% when treatment began at the time of transfer of the leaves to 25 C. Unlike in DTI-3C leaves, the amount of inhibition in DTI-12C leaves began decreasing immediately after the transfer to 25 C. By 48 hours, CX caused little inhibition although approximately 50% of the virus was synthesized between 48 and 72 hours.

In DTI-12C leaves, the AMD- and 2TU-sensitive steps occurred at 12 C. Replication of TMV became resistant to CX beginning immediately after the temperature shift to 25 C.

DISCUSSION.—The procedure used to determine the time-courses of inhibition of different virus inhibitors by adding them to different leaf disks at intervals after infection and maintaining the disks on the inhibitors continuously for the duration of the experiment measures only inhibition and non-inhibition. It measures the development of resistance of the infection to the inhibitor, but not the time-course of sensitivity to the inhibitor. If an inhibitor blocks more than one step of virus replication, the time-course of inhibition represents only the last step blocked. It is conceivable that CX blocks both the 2TU- and CX-sensitive steps.

The time that an inhibitor-sensitive step begins to become resistant to the inhibitor is the time at which that step begins to produce a functional product (the time that step begins). Although CX totally inhibited TMV synthesis in DTI-3C leaves when treatment began at 12 hours or earlier, the (last) CX-sensitive step only commenced as it began to become resistant to CX. When the inhibitor-sensitive step becomes totally resistant to the inhibitor, then sufficient product of that step has been produced to allow normal levels of virus synthesis to occur. Thus the inhibitor-sensitive step occurs between the beginning of resistance and total resistance to the inhibitor.

Synthesis of TMV was inhibited at specific steps of replication by different nonpermissive low temperatures. When leaves infected at 3 C were transferred to 25 C, TMV synthesis was totally resistant and thus beyond the AMD-sensitive step which occurred in mechanically inoculated leaves soon after infection. However, the infection was totally inhibited by 2TU and CX when treatment began immediately after the transfer to the permissive temperature. Thus, 3 C blocked TMV replication between the AMD and 2TU-sensitive steps. Twelve degrees blocked TMV synthesis at a later step. Both the AMD- and 2TU-sensitive steps occurred at 12 C. Upon shifting the DTI-12C leaves to 25 C, synthesis was inhibited only by CX. Thus, 12 C blocked TMV synthesis between the 2TU-sensitive and CX-sensitive steps.

After transfer of the DTI leaves to the permissive temperature, the steps of replication occurred sequentially and during almost mutually exclusive periods. In DTI-3C leaves, the AMD-sensitive step occurred at 3 C, before the leaves were transferred to 25 C. A 2TU-sensitive step occurred between 3 and 12 hours after the transfer to 25 C. Since the 2TU-sensitive step occurred soon after the shift to the permissive temperature, 3 C and 2TU may block the same step. A CX-sensitive step occurred between 12 and 48 hours after the temperature shift. The 2TU-sensitive step was over before the CX-sensitive step began. In DTI-12C leaves, the AMD- and 2TU-sensitive steps occurred prior to the transfer to 25 C and the CX-sensitive function began immediately after the transfer to 25 C. Combining the data of both experiments, a sequence emerges. An AMD-sensitive step immediately follows infection. This is followed by a 3 C-sensitive step which is the same or is followed by the 2TU-sensitive function. Then 12 C blocks

a function which is the same or is followed by the (last) CX-sensitive step. Ending the sequence in TMV-RNA synthesis and virion production.

Although there has been much work on the effect of 2TU upon TMV multiplication, the mechanism of inhibition is not known, and many of the most frequently propounded hypotheses of its mechanism are not consistent with data reported here. Sinha (10) lists 2TU as a compound which affects the late stages of TMV replication. However, 2TU clearly inhibits TMV during the early stages of replication. The infection is resistant to 2TU at 12 hours, a time before the synthesis of an appreciable amount of virus or infectious RNA. Ralph et al. (9) reported that 2TU inhibited the synthesis of double-stranded TMV-RNA and proposed that the site of action of 2TU inhibition was the blockage of synthesis of double-stranded TMV-RNA. However, syntheses of single-stranded TMV-RNA and both LiCl-soluble and LiCl-precipitable double-stranded RNA's are first detected at 6-8 hours, then logarithmically increased during the next 14 hours, after which occurred a rapid and linear synthesis rate which produced the majority of each RNA species. At 6 hours, before the synthesis of detectable amounts of TMV-induced RNA species, 2TU inhibited TMV synthesis less than 50%. When 2TU treatment began at 12 hours, before 95% of both single-stranded and double-stranded TMV RNA's was produced, it did not inhibit TMV synthesis. The vast majority of both single-stranded and double-stranded TMV RNA species was synthesized in the presence of 2TU. Thus, 2TU inhibits replication at some step earlier than the synthesis of TMV-RNA.

Data in this paper confirm the earlier suggestion that AMD blocks an early event of TMV replication (8). The AMD-sensitive step was the first detectable event of virus replication. It occurred at least 6-8 hours earlier than the detection of infectious TMV-RNA (7) or the detection of virus-induced RNA synthesis (6).

The time-course of cycloheximide inhibition paralleled that of TMV-induced RNA synthesis in a manner consistent with the hypothesis that CX inhibits the synthesis of TMV-RNA replicase that produces progeny RNA. As TMV-induced RNA synthesis began, the infection began to become resistant to CX.

When CX was added at later times, to DTI-3C leaves between 16 and 48 hours or to DTI-12C leaves between 8 and 48 hours, substantial amounts of TMV were synthesized in the presence of CX. This suggests that TMV-RNA replicase and coat protein may be synthesized and accumulate prior to TMV-RNA synthesis. Baltimore et al. (1) demonstrated that poliovirus RNA synthesis and particle assembly continue after protein synthesis is blocked by CX. The observation that TMV-RNA synthesis closely paralleled encapsidated infectivity accumulation supports this suggestion for TMV. Synthesis of TMV-RNA in tobacco callus continued uninhibited by CX for at least 6 hours although protein synthesis was inhibited within one hour, implying that enough replicase was present for RNA synthesis to continue in the presence of CX (2). However, in the callus system, assembly was inhibited as rapidly as protein synthesis suggesting that coat protein did not accumulate.

In an earlier paper describing the differential temperature inoculation procedure (7), the similarity of the growth curves of TMV in DTI leaves to those of viruses in other systems exhibiting synchronous multiplication suggested that TMV synthesis is synchronous in DTI leaves. Data in this paper confirm that supposition:

(i) Three degrees and 12 C blocked TMV synthesis in DTI leaves at specific steps. When DTI leaves were moved to a permissive temperature, synthesis began at the same step of replication in all infected cells.

(ii) The time-courses of inhibition by different inhibitors upon TMV synthesis in DTI leaves occurred sequentially and during discrete non-overlapping periods.

(iii) All cells that normally become infected with TMV were apparently infected in the DTI leaves prior to the shift to the permissive temperature. 2TU blocks a specific step of TMV replication. It should prevent synthesis in cells that are not infected or are not beyond the 2TU-sensitive step at the time treatment begins, thus preventing the spread of virus multiplication from cell to cell. In asynchronous infections, 2TU-resistance occurs gradually, paralleling the spread of progeny virus to uninfected cells (Table 1 and unpublished data). In DTI-3C leaves, 2TU blocked synthesis in all cells, but after 12 hours at 25 C, there appeared to be no secondary spread of virus to uninfected cells in which the 2TU-sensitive step had not occurred. In DTI-12C leaves, synthesis in all cells at the permissive temperature was totally resistant to 2TU, but the same amount of virus accumulated as in nontreated tissue.

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