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Effect of Cordycepin Triphosphate on In Vitro RNA Synthesis by Plant Viral Replicases

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In vitro RNA synthesis by tobacco mosaic virus and cowpea chlorotic mottle virus replicase were inhibited by cordycepin triphosphate. Inhibition could be overcome with higher concentrations of ATP in assay mixtures but not with UTP. Products synthesized in vitro by tobacco mosaic virus RNA replicase in the presence of inhibitor revealed replicative form but not replicative intermediate RNAs. These results suggest that cordycepin triphosphate competes specifically with ATP and results in premature termination of viral RNA synthesis in vitro.

Cordycepin (3'-deoxyadenosine), the first nucleoside antibiotic, inhibits synthesis of specific cellular (2) and viral (7, 8, 13) RNAs, while synthesis of other RNAs is unaffected (2, 4, 6, 8, 10). This antibiotic's inhibitory effects are probably due to its conversion in vitro to the nucleoside triphosphate that inhibits RNA polymerases (5). The inhibition is caused by premature termination of RNA synthesis, but incorporation of 3'-deoxyadenosine monophosphate (3'-dAMP) in place of AMP has not been established. However, AMP, not 3'-dAMP, has been found to be the terminal nucleoside in RNA chains prematurely terminated by cordycepin in vivo (2). Using picornavirus polymerase complexes, Panicalli and Nair (9) demonstrated that the presence of cordycepin triphosphate (3'-dATP) inhibited RNA synthesis in vitro and that the inhibitor promptly and specifically competes with ATP, preventing in vitro incorporation of AMP residues into virus-specified single-stranded RNA.

Although numerous inhibitors have been reported to block replication of plant viruses (12), the molecular basis of inhibition is uncertain. In synchronously infected tobacco mosaic virus (TMV)-infected tobacco leaves or cowpea chlorotic mottle virus (CCMV)-infected cowpea leaves (3), viral replication becomes sensitive to cordycepin beginning at 12 h after infection and continuing to 48 h (unpublished data). The time course of cordycepin inhibition paralleled that of virus-specified RNA and replicase synthesis. To further investigate the site and mechanism of action of cordycepin, we studied the effect of 3'-dATP on inhibition of in vitro RNA synthesis by two different plant virus replicases with different properties, those from TMV and CCMV. Our results suggest that 3'-dATP specifically competes with ATP and prevents in vitro incorporation of AMP into replicative form and intermediate RNAs.

3'-dATP and other biochemicals were obtained from Sigma Chemical Co.; [^{3}H]ATP (49 Ci/mmol) and [^{3}H]UTP (32 Ci/mmol) were obtained from ICN Pharmaceuticals. Primary leaves of cowpea *Vigna unguiculata* (L.) walp. California Blackeye were inoculated with crude sap from plants infected with CCMV and harvested 4 days later for extraction. CCMV-infected cowpea leaves were homogenized in 0.05 M Tris-hydrochloride (pH 7.4 at 4°C), 0.01 M KCl, 0.001 M EDTA, and 2.5 mM dithiothreitol (5 ml/g of tissue) in a chilled mortar. The homogenate was filtered through two layers of Miracloth, and the filtrate was centrifuged at 1,000 × *g* for 10 min. The supernatant fraction was adjusted to 20% glycerol and centrifuged at 31,000 × *g* for 30 min. The pellet was suspended in 0.05 M Tris-hydrochloride (pH 8.0 at 4°C), 0.5 M KCl, 0.01 M $MgCl_2$, 0.001 M EDTA, 5% glycerol, and 2.5 mM dithiothreitol (0.1 ml/g of tissue) with the aid of a tissue homogenizer and sedimented at 31,000 × *g* for 30 min. The pellet was suspended in fresh buffer and centrifuged again. The final 31,000 × *g* pellet was suspended in 10 mM Tris-hydrochloride (pH 8.0 at 35°C), 10 mM KCl, 25 mM $(NH_4)_2SO_4$, 5% glycerol, and 2.5 mM dithiothreitol (0.1 ml/g of tissue) and used as the source of bound CCMV replicase (15). A similar procedure was used for the preparation of TMV replicase, except different buffers were used (14). The standard RNA assay mixture contained 100 mM Tris-hydrochloride (pH 8.0 at 35°C), 10 mM $MgCl_2$, 7.5 mM dithiothreitol, 25 mM $(NH_4)_2SO_4$, 10 μg of actinomycin D per ml, 0.1 μmol each of CTP and GTP per ml, 1 to 2 nmol of [^{3}H]UTP per ml, and varying concentrations of ATP (0.1 to 1.0 μmol/ml). The reaction was initiated by adding

the enzyme and was terminated by transferring two 40-μl samples onto a 2.3-cm disk of Whatman 3MM filter paper, which was then placed into cold 5% trichloroacetic acid containing 1% sodium pyrophosphate and 0.02% uracil. The disks were further processed, and radioactivity was determined as previously described (16). Each data point represents the average of duplicate samples minus the average of the zero time values. RNA was isolated from scaled-up reaction mixtures and analyzed on polyacrylamide gels (15).

The 31,000 × *g* membrane fractions were prepared from plants infected with TMV or CCMV and from uninfected plants. [^{3}H]AMP polymerization was determined either in a modified RNA polymerase mixture where ATP was the sole nucleotide present, to measure polyadenylic acid polymerase activity, or in standard assay mixtures containing all four nucleoside triphosphates, to measure total viral RNA polymerase activity. [^{3}H]AMP polymerization in complete assay mixtures in the presence or absence of 3'-dATP is shown in Table 1. The CCMV and TMV replicase activities were inhibited in the presence of 0.75 mM 3'-dATP. Similar enzyme preparations from uninfected plants showed low levels of [^{3}H]AMP incorporation, which was sensitive to the presence of 3'-dATP. When ATP was the sole nucleotide, incorporation of [^{3}H]-AMP was similar to that detected in healthy plants when all four nucleotides were present. The polyadenylic acid polymerase activity was inhibited by 0.75 mM 3'-dATP. [^{3}H]AMP polymerization was nucleotide specific since only background radioactivity was detected when UTP was the sole nucleoside triphosphate.

TABLE 1. *Effect of 3'-dATP on ^{3}H-labeled nucleoside monophosphate incorporation*

Reaction mixture[a]	Enzyme source	[^{3}H]NMP[b] incorporation (cpm)/40-μl reaction mixture	
		−3'-dATP	+3'-dATP
Complete [^{3}H]ATP	Healthy tobacco	307	236
	TMV	1,835	1,231
	Healthy cowpea	291	200
	CCMV	2,197	1,643
[^{3}H]ATP only	Healthy tobacco	414	277
	TMV	324	192
	Healthy cowpea	206	158
	CCMV	380	238
[^{3}H]UTP only	Healthy tobacco	60	67
	TMV	87	59
	Healthy cowpea	54	47
	CCMV	49	81

[a] Reaction mixtures were incubated under standard conditions as described in the text.
[b] [^{3}H]NMP, ^{3}H-labeled nucleoside monophosphate.

The above results suggest that [^{3}H]AMP polymerization activity present in membranes from healthy plants could interfere with quantitation of viral replicase activity and its inhibition by 3'-dATP. Therefore, we chose to use [^{3}H]UTP in place of [^{3}H]ATP in assay mixtures. The presence of 0.75 mM or 1.5 mM 3'-dATP in assay mixtures strongly inhibited [^{3}H]UMP incorporation by CCMV and TMV replicases (Fig. 1). No [^{3}H]UMP incorporation could be detected in comparable fractions from healthy plants.

Inhibition of [^{3}H]UMP polymerization by 3'-dATP could be reversed with increasing concentrations of ATP in the initial assay mixture (Fig. 2). The Lineweaver-Burk plot of [^{3}H]UMP polymerization in the presence of excess ATP (Fig. 2, insert) suggests competitive inhibition. The inhibition was not overcome when increasing concentrations of UTP were added to the initial assay mixtures. Addition of excess ATP 15 min after the inhibitor did not reverse inhibition (not shown). Similar results were obtained for CCMV replicase (not shown).

The products of in vitro RNA synthesis by TMV replicase are replicative form (Fig. 3, gel slice no. 7 to 9) and replicative intermediate (Fig. 3, gel slice no. 2 to 5) RNAs with no apparent synthesis of free single-stranded TMV RNA (1,

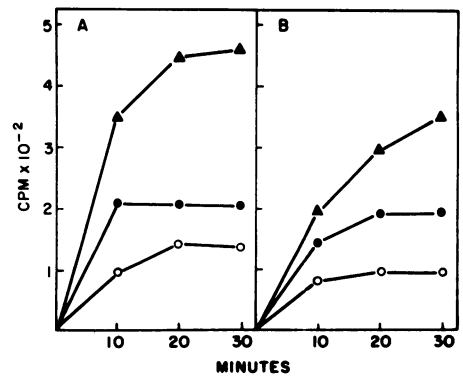

FIG. 1. *3'-dATP inhibition of [^{3}H]UMP polymerization by plant viral replicases. Complete assay mixtures containing viral replicases were incubated in the presence or absence of 3'-dATP. Samples were removed at intervals, and total trichloroacetic acid acid-insoluble radioactivity was determined as described in the text. [^{3}H]UMP polymerization by TMV replicase (A) or CCMV replicase (B) in the absence (▲) or presence of 0.8 mM (●) or 1.6 mM (○) 3'-dATP.*

16). RNA products synthesized in vitro in the presence of 3'-dATP were altered (Fig. 3). Replicative intermediate RNA was detected in low amounts in RNA isolated from reaction mixtures containing the inhibitor. However, additional double-stranded RNAs smaller than the replicative form (Fig. 3, gel slice no. 11 to 15) were detected in products isolated from reaction mixtures containing 3'-dATP. The origin of these virus-specific RNAs is unclear.

3'-dATP inhibited TMV and CCMV replicases in vitro. The inhibition could be overcome by increasing ATP but not UTP concentrations in assay mixtures. It appears that the incorporation of AMP into growing viral RNA chains is specifically blocked by 3'-dATP. Other evidence supports this hypothesis: in vitro RNA synthesis in the presence of 3'-dATP by poliovirus and rhinovirus polymerase complexes was specifically inhibited (9), and 3'-dATP inhibits transcription of polyadenylic acid from a polyuridylic acid template by procaryotic DNA-dependent RNA polymerase (11). 3'-dATP reduced the in vitro synthesis of TMV replicative intermediate RNA to a greater extent than the replicative form (Fig. 3). These results are consistent with the proposal that cordycepin's mode of action is by termination of RNA chain elongation. From these results and others (5, 9), it is not possible to determine whether or not 3'-dAMP is incorporated into RNA. The failure to reverse inhibition by addition of excess ATP 15 min after addition of the inhibitor is consistent with irreversible binding of the inhibitor to viral replicase or incorporation of 3'-dAMP into RNA. In vivo data suggest that 3'-dAMP is not incorporated into RNA chains (2).

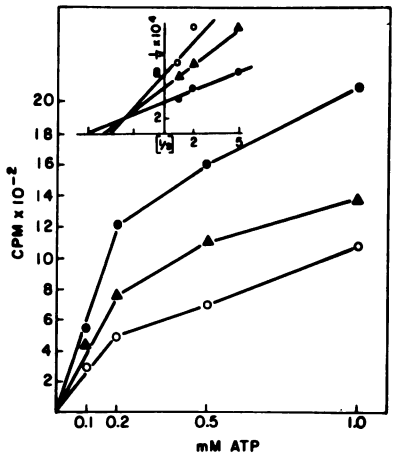

FIG. 2. *Effect of various ATP concentrations on 3'-dATP inhibition of in vitro [³H]UMP polymerization by TMV replicase. Assay mixtures containing TMV replicase and increasing concentrations of ATP were incubated in the presence or absence of 3'-dATP at 35°C for 15 min. Samples were removed, and acid-insoluble radioactivity was determined as described in the text. No 3'-dATP (●); 0.25 mM 3'-dATP (▲); 0.5 mM 3'-dATP (○). The insert shows a double-reciprocal plot of the data.*

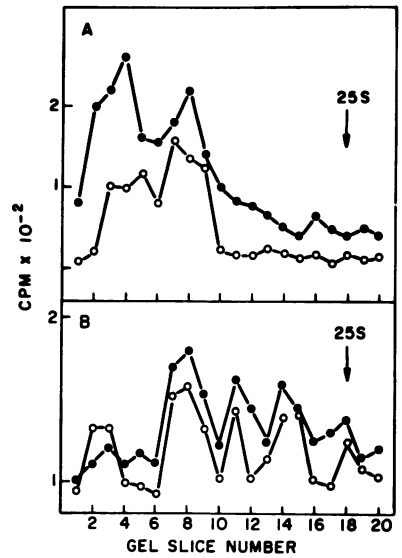

FIG. 3. *Polyacrylamide gel electrophoresis of the in vitro product of TMV replicase. RNA was extracted from scaled-up reaction mixtures with (B) or without (A) 3'-dATP. A portion of RNA was treated with RNase (○) prior to analysis on 3% acrylamide gels. Migration is from left to right. TMV replicative intermediate and TMV replicative form RNAs are located in gel slices no. 2 to 5 and 7 to 9, respectively.*

The plant viral replicase sensitivity to 3'-dATP is similar to the inhibitor's action upon DNA-dependent RNA polymerase II (5), poliovirus polymerase, and rhinovirus polymerase (9). This sensitivity to 3'-dATP may indicate similarities in site(s) in the polymerase ATP-utilizing enzymes where ATP and 3'-dATP act.

When ATP was the sole nucleotide precursor present, the incorporation obtained with viral replicases was comparable to that obtained with control cell preparations. Both activities were inhibited by 3'-dATP. There appears to be no virus-specific polyadenylic acid polymerase activity.

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