Vol. 83, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 14, 1978 Pages 1099-1104

EFFECTS OF 3'-DEOXYADENOSINE TRIPHOSPHATE ON IN VITRO RNA SYNTHESIS OF PLANT RNA-DEPENDENT RNA POLYMERASES

James L. White

Department of Plant Pathology, University of California, Riverside, California 92521

Received June 9,1978

SUMMARY. 3'-deoxyadenosine triphosphate inhibited *in vitro* [^{3}H]UMP incorporation by RNA-dependent RNA polymerases from tobacco and cowpea plants. The inhibition of [^{3}H]UMP incorporation could be reversed by simultaneous addition of higher ATP concentrations but not with increasing concentrations of UTP or when excess ATP was added 10 min after the inhibitor. These results suggest 3'-deoxyadenosine triphosphate competes specifically with ATP in reaction mixtures and results in premature termination of RNA synthesis *in vitro* by RNA-dependent RNA polymerase.

INTRODUCTION. Most cellular RNA is synthesized by DNA-dependent RNA polymerases. However, RNA-dependent RNA polymerases have been extracted from chinese cabbage (1), tobacco leaves (2), cowpea leaves (3), and tobacco callus (4). The enzymatic activity is found predominantly in 100,000 xg supernatant and produces small (4-6S), predominantly dsRNA (2,3). The enzyme exhibits greater activity in the presence of Mg^{++} than Mn^{++}, possess an endogenous RNA template, and is completely dependent upon the presence of all four nucleotides for activity. The template-free enzyme, MW approximately 160,000 daltons, prefers RNA over DNA as a template (5).

The antibiotic 3'-deoxyadenosine (cordycepin) inhibits synthesis of certain cellular (6) and viral RNA (7,8) while other RNA synthesis is resistant to the antibiotic (6,8,9). The inhibitory effects of this antibiotic are generally ascribed to its conversion *in vitro* to the nucleoside triphosphate which acts as a potent inhibitor of DNA-directed RNA polymerases (10,11). This inhibition is caused by premature termination of RNA chain elongation. Whether chain termination results from incorporation of 3'-dAMP

Abbreviations: 3'-dATP = 3'-deoxyadenosine triphosphate;
3'Ado = 3'-deoxyadenosine.

0006-291X/78/0833-1099$01.00/0

in place of AMP is not certain since AMP rather than 3'-AMP has been found to be the end residue in RNA chains prematurely terminated by 3'Ado (6). Since 3'Ado inhibits in vivo synthesis of some RNAs while others are resistant, the effect of 3'-dATP on the in vitro RNA synthesis by RNA-dependent RNA polymerase was investigated. These results suggest that 3'-dATP promptly and specifically competes with ATP and prevents AMP incorporation in RNA.

MATERIALS AND METHODS. 3'-dATP and other biochemicals were obtained from Sigma Chemical Co., [^{3}H]UTP was obtained from New England Nuclear. Primary leaves of cowpea [Vigna unguiculata (L.) walp. var. California blackeye] and leaves from 10-15 cm high tobacco (Nicotiana tabacum var. Xanthi) were used as enzyme source. For the preparation soluble RNA polymerase from 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 DTT (5 ml per g of tissue) in a chilled mortar. The homogenate was filtered through 2 layers of Miracloth and the filtrate centrifuged at 1,000 g for 10 min. The supernatant fraction was adjusted resulting supernatant fluid was adjusted to pH 4.7 with 1 N HCl, allowed to sit at 4 C for 15 min, then centrifuged at 20,000 g for 20 min. The pellet was resuspended 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 DTT (0.1 ml per g tissue). This solution was used as the source of the soluble RNA polymerase. The enzyme from tobacco was isolated by the above procedures except different buffers were employed (4). The standard RNA polymerase assay mixture contained 100 mM Tris-hydrochloride (pH 8.0 at 35 C), 10 mM $MgCl_2$, 7.5 mM DTT, 25 mM $(NH_4)_2SO_4$, 10 μg/ml actinomycin D, 0.5 μmoles/ml each CTP and GTP, 1 nmole [3H]UTP, and various concentrations of ATP (0.1-1.0 μmoles/ml). The standard reaction mixture was 0.1 ml. The reaction was initiated by adding the enzyme and was terminated by transferring two 50 μl samples onto a 2.3 cm disc of Whatman 3 MM filter paper which was then placed into cold 5% trichloroacetic acid contianing 1% sodium pyrophosphate and 0.02% uracil. The discs were further processed and radioactivity determined as previously described (3). RNA was isolated from scaled-up reaction mixtures and analyzed on polyacrylamide gels (3).

RESULTS. Inhibition of [^{3}H] UMP incorporation. Soluble RNA polymerases from tobacco (Fig. 1A) and cowpea (Fig. 1B) were inhibited by 3'-dATP. Approximately 20% of the control [^{3}H]UMP incorporation occurred in the presence of 1.25 mM 3'-dATP. This amount of residual activity is proportional to the relative concentration of ATP and 3'-dATP used, 0.5 mM and 1.25 mM, respectively.

Reversal of 3'-dATP inhibition with ATP. The effect of the concentration of ATP or UTP in the reaction mixture relative to inhibition by 3'-dATP of [^{3}H]UMP incorporation by soluble RNA polymerase was investigated. The inhibition kinetics indicated that the effect was reversed with simultaneous

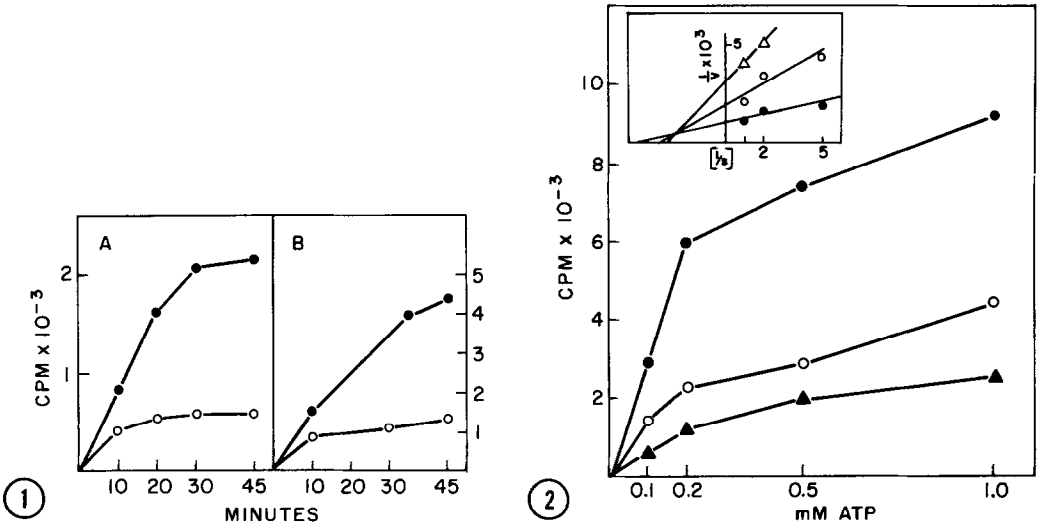
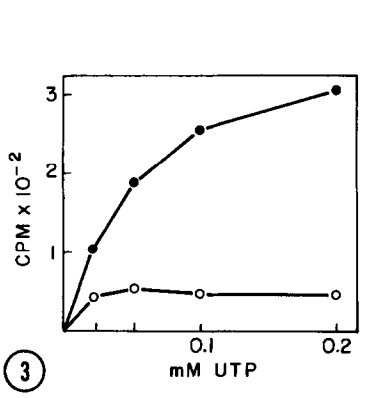


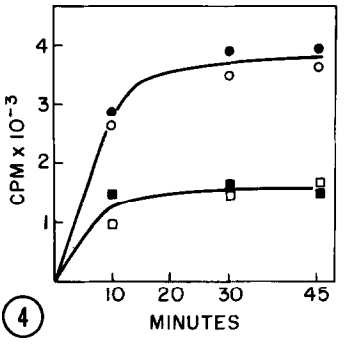
Figure 1. 3'-dATP inhibition of [^{3}H]UMP inocorporation by soluble RNA polymerases. Complete reaction mixtures containing RNA polymerase were incubated with or without 3'-dATP. Acid-insoluble radioactivity of samples removed at intervals was determined. [^{3}H]UMP incorporation by soluble RNA polymerases from tobacco (A) or cowpea (B) in the absence (●) or presence(o) of 0.25 mM 3'-dATP.

Figure 2. Effect of increasing concentrations of 3'-dATP inhibition of in vitro [^{3}H]UMP incorporation. Reaction mixtures containing tobacco polymerase, a constant amount of [^{3}H]UMP and increasing amounts of ATP were incubated in the presence or absence of 3'-dATP for 15 min at 35 C. Acid-insoluble radioactivity of samples removed was determined. Each point represents the average of duplicate samples minus the average of zero-time values. No 3'-dATP (●); 0.25 mM 3'-dATP (o); 0.5 mM 3'-dATP ( ). The insert shows a double-reciprocal plot of the data.

addition of increasing amounts of ATP with constant amounts of inhibitor (Fig. 2). The Lineweaver-Burk plot of [^{3}H]UMP incorporation obtained in the presence of excess ATP indicated competitive inhibition. The inhibition was not reversible when increasing UTP concentrations were added along with the inhibitor (Fig. 3) nor when additional ATP was added 10 min after the addition of the inhibitor (Fig. 4). In reaction mixtures containing 3'-dATP, near maximal inhibition occurred within 10 min (Fig. 4). Similar results were obtained with soluble RNA polymerase from tobacco.

RNA products synthesized in vitro. The product synthesized in vitro by soluble RNA polymerase from tobacco is 4-6S RNAs (Fig. 5) (2,3). The product





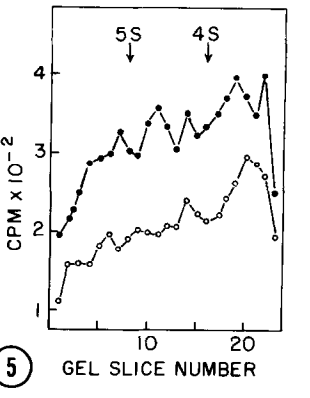


Figure 3. Effect of increasing concentration of UTP on 3'-dATP inhibition of *in vitro* [^{3}H]UMP incorporation in reaction mixtures containing cowpea RNA polymerase and increasing amounts of [^{3}H]UTP of constant specific activity 100 μCi/nmole were incubated in the presence (o) or absence (●) of 0.25 mM 3'-dATP for 30 min at 35 C. Acid-insoluble radioactivity of samples removed from reaction mixtures was determined. Each point represents the average of duplicate samples minus the average of zero-time values.

Figure 4. Effect of addition of excess ATP 10 min after 3'-dATP inhibition of *in vitro* [^{3}H]UMP incorporation. Complete reaction mixtures containing cowpea RNA polymerase and [^{3}H]UTP were incubated at 35 C for 10 min with (■) or without (●) 0.125 mM 3'-dATP. Excess ATP was then added to the 3'-dATP-inhibited reaction mixture ( ) and the one of the control reaction mixtures (o) and incubation continued. Samples were further processed as described in Fig. 2 legend.

Figure 5. Polyacrylamide gel electrophoresis of the *in vitro* product of soluble RNA polymerase from tobacco. RNA was extracted from scaled-up reaction mixtures with (●) or without (o) 3'-dATP and analyzed on 10% acrylamide gels. Migration is from left to right; 4S=transfer RNA.

is approximately 80% resistant to RNase treatment (not shown). The *in vitro* product isolated from reaction mixtures containing 3'-dATP revealed reduced synthesis of the heterogenous product. There was no difference in the size distribution or in the RNase-sensitivity of the product synthesized in the presence of 3'-dATP when compared to products synthesized in the absence of the inhibitor.

DISCUSSION. 3'-dATP inhibited the RNA-dependent RNA polymerases from plant tissues. This inhibition is due to direct competition between inhibitor and ATP. In the *in vitro* assay, inhibition could be reversed by increasing ATP but not UTP concentration in the reaction mixture. Three mechanisms have

been proposed to explain 3'-dATP inhibition of RNA polymerases: (1) 3'-dAMP is incorporated into RNA chains causing termination; (2) 3'-dATP binds irreversibly to the enzyme; (3) 3'-dATP inhibits initiation of RNA synthesis. These results cannot distinguish whether or not 3'-dATP is incorporated into RNA. Observations that inhibition was not reversed by the addition of excess ATP 10 min after the inhibitor is consistent with either 3'-dATP incorporation into RNA or irreversible binding of the inhibitor. However, the effect of 3'Ado in vivo indicated RNA synthesis in the presence of 3'Ado is terminated with AMP instead of 3'-dAMP suggesting that 3'-dAMP is not incorporated into RNA (6). Since soluble RNA polymerases do not reinitiate RNA synthesis in vitro, 3'-dATP role as an inhibitor of initiation remains to be resolved.

Eukaryotic ATP-utilizing enzymes involved in nucleic acid synthesis and processing show differing sensitivities to 3'-dATP. DNA-dependent RNA polymerase II, which synthesizes heterogenous nuclear RNA, is specifically inhibited by 3'-dATP. The poly (A) synthesizing enzymes, ATP-polynucleotidylexotransferase, were not inhibited by low concentrations of 3'-dATP (11). Soluble RNA-dependent RNA polymerase shows sensitivity to 3'-dATP similar to that reported for RNA polymerase II. Further investigations are required into the structure of soluble RNA polymerase and function of dsRNA in healthy cells before the role of this enzyme can be resolved.

ACKNOWLEDGMENTS. I gratefully acknowledge Dr. William O. Dawson for his support and assistance in preparation of this manuscript, and George Grantham for his technical assistance. This research was supported in part by National Science Foundation Grant PCM 76-15867.

REFERENCES.

1. Astier-Manifacier, S., Cornuet, P. (1971). Biochim. Biophys. Acta, 232, 484-493.
2. Duda, C. T., Zaitlin, M., Siegel, A. (1973). Biochim. Biophys. Acta, 319, 67-71.
3. White, J. L., Dawson, W. O. (1978). Virology, in press.
4. White, J. L., Murakishi, H. H. (1977). J. Virol., 21, 484-492.
5. Duda, C. T. (1976). Ann. Rev. Plant Physiol., 27, 119-132.
6. Brawerman, G. (1976). Prog. Nucleic Acid Res. Mol. Biol., 17, 117-148.
7. Nair, C. N., Panicali, D. L. (1976). J. Virol., 20, 170-176.

8. Nevins, J. R., Joklik, W. H. (1975). Virology, 63, 1-14.
9. Ehrenfield, E. (1974). J. Virol., 13, 1055-1060.
10. Harris, B., Dure, L. S. (1974). Biochemistry, 13, 5463-5467.
11. Maale, G., Stein, G., Mans, R. (1975). Nature (London) 255, 80-82.