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Inhibitory effects of 3'-deoxycytidine 5'-triphosphate and 3'-deoxyuridine 5'-triphosphate on DNA-dependent RNA polymerases I and II purified from Dictyostelium discoideum cells

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ABSTRACT

3'-Deoxycytidine 5'-triphosphate and 3'-deoxyuridine 5'-triphosphate were synthesized starting from cordycepin in good yield.
The inhibitory effects of these nucleotides were examined in comparison with that of cordycepin 5'-triphosphate (3'-dATP) using purified DNA-dependent RNA polymerases I and II from *Dictyostelium discoideum* cells. Both nucleotide analogues strongly and competitively inhibited the incorporations of CTP and UTP into RNA by the RNA polymerases. The Km and Ki values for CTP and 3'-dCTP were 6.3 µM and 3.0 µM,respectively, and those for UTP and 3'-dUTP were 6.3 µM and 2.0 µM, respectively. These two analogues will be useful in studies at the molecular level on the relationship of template and substrate in RNA synthesis with chromatin, isolated nuclei or permeable cells, because they do not have any effect on poly (rA) synthesis.

INTRODUCTION

Cordycepin (3'-deoxyadenosine) is a nucleoside antibiotic isolated from culture filtrates of *Cordyceps militalis* and *Aspergillus nidulans*.[2] This antibiotic is cytotoxic to both prokaryotes and eukaryotes. Cordycepin has been shown to affect RNA synthesis in both prokaryotes and eukaryotes by inhibiting the activities of several enzymes in the purine biosynthetic pathway,[3] DNA-dependent RNA synthesis[4] and polyadenylate synthesis.[5] Abelson and Penman reported some difference in the mode of inhibition of RNA synthesis by cordycepin (3'-dA) and 3'-deoxycytidine (3'-dC): 3'-dA inhibited both ribosomal RNA (rRNA) and messenger RNA (mRNA synthesis, while 3'-dC preferentially inhibited rRNA synthesis.[6] *In vitro* studies have shown that cordycepin 5'-triphosphate (3'-dATP) is the active form of the antibiotic cordycepin *in vivo* and that it inhibits DNA-dependent RNA polymerase from *M. lysodeicus*,[7] Ehrlich ascites cells and yeast.[8]

We previously showed that 1-β-D-arabinofuranosylthymine 5'-triphosphate (Ara TTP)[9-10] and 9-β-D-arabinofuranosylguanine 5'-triphosphate (Ara GTP)[11] have strong inhibitory effects on DNA polymerases from murine cells as well as

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Ara CTP and Ara ATP.

These results prompted us to synthesize 3'-deoxycytidine 5'-triphosphate (3'-dCTP) and 3'-deoxyuridine 5'-triphosphate (3'-dUTP), pyrimidine analogues of cordycepin 5'-triphosphate (3'-dATP), and to study the influences of these analogues on RNA synthesis *in vitro*.

This paper reports the chemical synthesis of 3'-dCTP and 3'-dUTP and the effects of these compounds on DNA-dependent RNA polymerses I and II isolated from the cellular slime mold *Dictyostelium discoideum*.

MATERIALS AND METHODS

CHEMICALS

^{3}H-UTP (16.6 Ci/mmole) was obtained from Radiochemical Centre, Amersham and ^{3}H-CTP (20.1 Ci/mmole) from New England Nuclear Co. ATP, CTP, UTP, GTP and α-amanitin were purchased from Boehringer Manheim. Calf thymus DNA (Type I), bovine serum albumin (crystallized) and phenylmethylsulfonyl fluoride were obtained from Sigma. Cordycepin was kindly provided from by Yamasa Shoyu Co. DEAE-Sephadex A-25 and DEAE-cellulose (DE-23)were from Pharmacia Fine Chemicals and Whatman, respectively. DNA-cellulose was prepared from native calf thymus DNA and cellulose (Biorad, Cellex 410) by the method of Litman.[12]

Preparation of cordycepin 5'-triphosphate (3'-dATP)

Cordycepin (1 g) was phosphorylated with tetrachloropyrophosphate in acetonitrile[13] and then purified by Dowex 1 (formate form) column chromatography. The yield of isolated 5'-monophosphate was 0.94 g as free acid (61%). UV, λmax in water, 259 nm and 0.01 N NaOH, 260 nm. Phosphate analysis: Calculated ε(P) = 13100; Found, 13800. Paper electrophoretic mobilities: in 300 mM triethylammonium bicarbonate, pH 8.5 (System A), 600 V for 40 min. $R_{2'\text{-dAMP}}$ = 1.01; in 30 mM potassium citrate, pH 4.1 (System B), $R_{2'\text{-dAMP}}$ = 1.04.
Paper chromatography, Rf = 0.46 (Solvent A) and Rf = 0.34 (Solvent B). Solvent A = isobutyric acid-0.5 N NH_4OH (5:3, v/v). Solvent B = n-propanol- cNH_4OH-H_2O (7:2:1). The resulting monophosphate (65 mg) was converted to its 5'-phosphoromorpholidate by reaction with freshly distilled morpholine in tertially butyl alcohol in the presence of dicyclohexylcarbodiimide.[14] The yield of morpholidate was 102 mg (78%) as the dicyclohexylcarboxyamidinium salt. The reaction of tri-n-butylammonium pyrophosphate followed by column chromatography on DEAE-cellulose afforded corresponding 5'-triphosphate in 50% yield.
UV, λmax in water, 259 nm. Paper chromatography, Rf = 0.53 (Solvent C) and 0.74 (Solvent D). Solvent C = ethanol-1 M sodium acetate (pH 7.5, 5:2, v/v).

Solvent D = ethanol-1 M sodium acetate (pH 7.5, 1:1, v/v). Paper electrophoresis: $R_{2'\text{-dATP}}$ = 1.03 in System A and 0.98 in System B. Phosphate analysis: Calculated ε(P) = 4700; Found ε(P) = 4400

Chemical synthesis of 3'-deoxycytidine 5'-monophosphate

3'-Deoxycytidine was synthesized from D-xylose by a 11 step process.[18,22] We synthesized this compound starting from cordycepin in only 4 steps by a much simpler and convenient method.[23] Phosphorylation of 3'-deoxycytidine was performed by similar method described above section.[13] Yield of phosphorylation reaction was 74%. UV, λ max in water, 280 nm. Phosphate analysis: Calculated ε (P) = 13100. Found: 13300. Paper chromatography, Rf = 0.67 (Solvent A), 0.34 (Solvent B).

Preparation of 3'-deoxyuridine 5'-monophosphate

3'-Deoxyuridine 5'- phosphate was prepared by the deamination of 3'-deoxycytidine 5'-monophosphate with nitrous acid followed by purification with DEAE-cellulose column chromatography and paper chromatography. Yield (67%). UV, λ max in water at 261 nm. Phosphate analysis: Calculated ε (P) = 10600; Found ε (P) = 10200. Paper chromatography: Rf = 0.75 (Solvent A) and 0.27 (Solvent B).

Preparation of 3'-deoxycytidine 5'-triphosphate (3'-dCTP) and 3'-deoxyuridine 5'-triphosphate (3'-dUTP)

Conversion of 5'-monophosphate to corresponding 5'-triphosphate was performed by the phosphoroimidazolidate method.[24]

3'-dCTP: UV, λ max in water at 270 nm and in 0.01 M HCl at 281 nm.
Phosphate analysis: Calculated ε (P) = 4400; Found ε (P) = 4600.
Paper electrophoretic mobilities: System A, $R_{2'\text{-dCTP}}$ = 0.95, System B, $R_{2'\text{-dCTP}}$ = 1.00.
Paper chromatography: Rf = 0.53 (Solvent C), Rf = 0.77 (Solvent D).

3'-dUTP: UV, λ max in water at 260 nm.
Phosphate analysis: Calculated ε (P) = 3500; Found ε (P) = 3600.
Paper electrophoretic mobilities: System A, R_{UTP} = 0.98; System B, R_{UTP} = 0.97
Paper chromatography: Rf = 0.21 (Solvent C), Rf = 0.42 (Solvent D).

Assay of RNA polymerases

The standard mixture for assay of RNA polymerases contained, in a final volume of 0.1 ml, 0.05 M Tris-HCl, pH 7.9, 1 mM dithiothreitol (DTT), 1 µCi of ^{3}H-UTP (16.6 Ci/mmole), 1.5 mM $MnCl_2$, 5 mM $MgCl_2$, 10 ug of native calf thymus DNA, 100 ug of bovine serum albumin (BSA) and 50 ul of enzyme fraction

as specified. The assay conditions used to determine the mode of inhibition by 3'-deoxynucleotide analogues were as described above, except that the concentrations of radioactive or non-radioactive substrates and $MgCl_2$ were as indicated in the legends to figures. The mixtures were incubated at 23°. Then 75 ul of the reaction mixture was transfered to Whatman DE-81 paper and the paper was immersed in 0.5 M Na_2HPO_4. Activity of RNA polymerase was expressed as the incorporation of radioactive nucleotide into RNA as described by Lindel et al.[15]

Culture of cells and preparation of RNA polymerases

D. discoideum strain NC-4 was grown in the presence of *E. coli* cells at 23°. The cells were harvested by centrifugation in the interphase-aggregation stage of development. Nuclei were isolated as described previously.[16]

RNA polymerases I and II were purified by the method of Takiya et al.[16] Unless otherwise noted, all procedure were carried out at 0° - 4°. Frozen nuclei (approx. 5 g) were thawed and homogenized in 20 ml of buffer A (0.05 M Tris-HCl, pH 7.9, 1 mM EDTA, 10% glycerol, 20 mM 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride). The homogenate was adjusted to 0.3 M ammonium sulfate concentration and the mixture was sonicated 3 times with a 15-second pulse. The sonicated homogenate was diluted with 2 volumes of buffer A and centrifuged for 30 min at 15000 x g. The resulting supernatant was diluted again with equal volume of buffer A, so that the concentration of ammonium sulfate was 0.05 M. The nuclear extract was then mixed with 4g (dry weight) of DEAE-cellulose previously equilibrated with buffer A containing 0.05 M ammonium sulfate. The mixture was gently stirred for 40 min and then centrifuged.
The precipitated cellulose was washed 3 times with 300 ml (in total) of the same buffer solution. Enzyme protein absorbed on DEAE-cellulose was eluted with buffer A containing 0.4 M ammonium sulfate and precipitated by adding 1.5 volume of saturated ammonium sulfate solution prepared with buffer B (0.05 M Tris-HCl, pH 7.9, 0.5 mM EDTA and 10% glycerol). The precipitate obtained by centrifugation was suspended in a small amount od buffer C (0.05 M Tris-HCl, pH 7.9, 0.5 mM EDTA, 25% glycerol and 10 mM 2-mercaptoethanol) and dialyzed against buffer C containing 0.05M ammonium sulfate.
The dialyzate was applied to a DEAE-Sephadex A-25 column (1.5 cm x 10 cm) previously equilibrated with buffer C containing 0.05 M ammonium sulfate and 2 mM DTT. The column was washed with three bed volumes of the same buffered solution and then RNA polymerases were eluted with a linear gradient of ammonium sulfate (0.05 M to 0.3 M) in buffer C with 2 mM DTT. The fractions of

RNA polymerases I and II were separately pooled and purified further by DNA-cellulose column chromatography. The RNA polymerase I and II were eluted from the column with 0.3 M KCl, and then dialyzed against buffer E (0.05 M Tris-HCl, pH 7.9, 0.1 mM EDTA, 25% glycerol and 10 mM 2-mercaptoethanol containing 0.05 M or 0.4 M KCl). The specific activities of the RNA polymerase I and II preparations thus obtained were 5400 and 4800 nmole UMP incorporation, respectively, per mg of protein in 40 min at 23°. The characteristics and subunit structures of these enzymes have been reported.[17]

RESULTS

Inhibitory effect of 3'-deoxyribonucleoside 5'-triphosphates on RNA polymerase I and II

The 3'-deoxyribonucleoside 5'-triphosphates, 3'-dCTP, 3'-dUTP and 3'-dATP

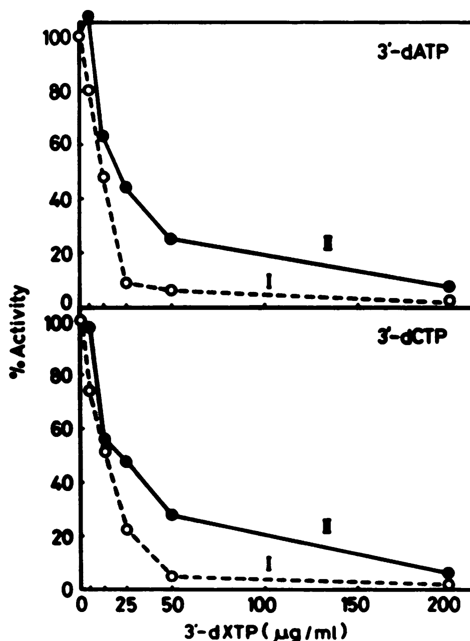

Fig. I. Inhibitions of RNA polymerases by 3'-dATP and 3'-dCTP
RNA polymerase I (o-------o) and II (●———●) were assayed as described in the Materials and Methods. The concentrations of 3'-dATP or 3'-dCTP added to the standard assay mixture are indicated in the figure. 100% incorporations of UMP by RNA polymerase I and II were 29.8 and 31.1 nmoles, respectively.

(cordycepin 5'-triphosphate) inhibited the activities of RNA polymerase I and II of the cellular slime mold *in vitro*. Fig I shows the dose-response curves of the inhibitory effects of 3'-dCTP and 3'-dATP on the RNA polymerase activity. The polymerase I activity was somewhat more sensitive than the polymerase II activity to low doses of the two analogues. In assay systems containing 0.5 mM each of the substrates, the concentrations of both analogues causing about 50% inhibition of enzyme activity (ED_{50}) were 12.5 µg/ml for polymerase I and 25 µg/ml for polymerase II. When the concentration of 3'-dCTP was increased to 50 µg/ml, the polymerase I activity was almost completely inhibited, whereas about 20% of the polymerase II activity remained.
A concentration of about 200 µg/ml of 3'-dCTP caused complete loss of polymerase II activity. Similar results were obtained with 3'-dUTP.

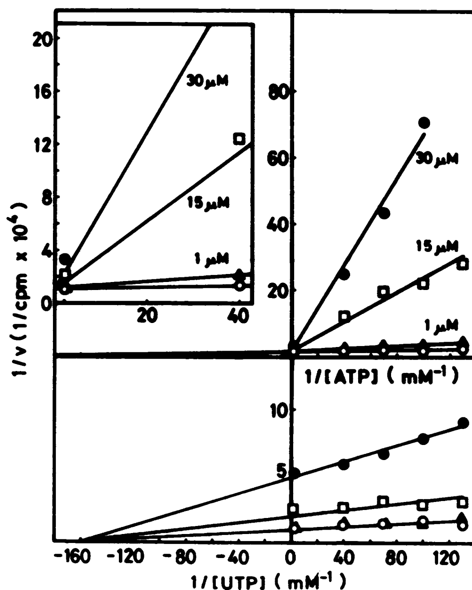

Fig II. Effects of 3'-dATP on the incorporations of ATP and UTP.
Reaction conditions were as described in the Materials and Methods. The assay mixture contained 0.5 mM each of GTP, UTP and ATP, 0.01 mM CTP plus 1 µCi of ^{3}H-CTP (20.1 Ci/mmole) and varying amounts of ATP and UTP. The reaction was carried out at 23° for 30 min. Concentration of 3'-dATP : 0 µM (o——o) 1 µM (▲——▲), 15 µM (□——□) and 30 µM (●——●).

Kinetic analysis of the inhibitory action of 3'-deoxyribonucleoside 5'-triphosphates

The mode of inhibition of RNA polymerase II by 3'-deoxyribonucleoside 5'-triphosphate (3'-NTP) was determined from Lineweaver-Burk plots.
The experimental data shown in Figs. II to IV. It is clear that 3'-dATP competitively inhibited the incorporation of AMP into RNA and non-competitively inhibited the incorporation of UMP.
As can be seen in Figs. III and V, 3'-dCTP and 3'-dUTP caused competitive inhibition with natural substrates bearing the corresponding bases and non-competitive inhibition with other substrates. The Michaelis constant (Km) of ATP and the inhibition constant (Ki) of 3'-dATP were estimated to be 5.6 µM and 0.8 µM, respectively, from Lineweaver-Burk plots and Dixon plots. The Km value of CTP and the Ki value of 3'-dCTP were 6.3 µM and 3.0 µM, respectively. In the same way, the Km and Ki values of UTP and 3'-dUTP were estimated to be 6.3 µM and 2.0 µM, respectively.

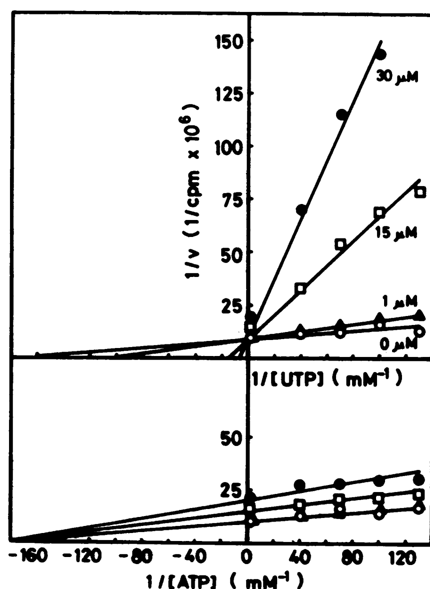

Fig. III. Effects of 3'-dCTP on the incorporations of CTP and ATP. Reaction conditions were as for Fig III except for the substrates. Assay mixtures contained 0.5 mM each of GTP, ATP and CTP, 0.01 mM UTP plus 1 µCi of ^{3}H-UTP (16.6 Ci/mmole) and various amounts of CTP or ATP. The concentrations of 3'-dCTP added are indicated in the figure.

DISCUSSION

The present results indicate that 3'-dCTP and 3'-dUTP as well as 3'-dATP strongly inhibit RNA polymerases of *D. discoideum*. Abelson and Penman showed in cultured mammalian cells that 3'-deoxycytidine inhibited rRNA synthesis without having any effect on HnRNA or mRNA synthesis while cordycepin (3'-dA) prevented both rRNA and mRNA synthesis without affecting the formation of HnRNA.[6] Since in our experiment 3'-dCTP inhibited the activities of RNA polymerase I and II, it seems likely that the inhibition of rRNA synthesis in cultured cells by 3'-dC reported by Abelson and Penman is caused by the inhibitory effect of 3'-dCTP, which is formed with cellular cytidine kinase system *in vivo*.[6] The fact that RNA polymerase II was inhibited by 3'-dCTP is contradictory with the *in vivo* finding of Abelson and Penman. A similar discrepancy has been reported on the effect of 3'-dATP. In experiments on *in vivo* labelling of RNA, 3'-dA preferentially inhibited the synthesis of rRNA or chromatin-associated poly (A), but scarcely affected the synthesis of HnRNA or free poly (A) chains.

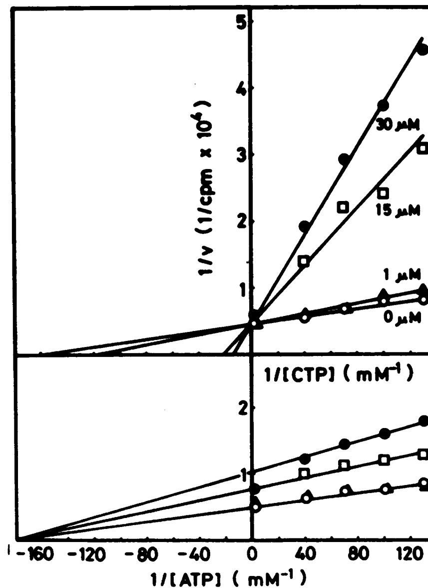

Fig.IV. Effects of 3'-dUTP on the incorporation of UTP and ATP. Reaction conditions were as for Fig. III, except for the substrates. The assay mixture contained 0.5 mM each of GTP, ATP and UTP, 0.01 mM CTP plus 1 µCi of ^{3}H-CTP (20.1 Ci/mmole) and various amounts of UTP or ATP. The concentrations of 3'-dUTP are indicated in the figure.

However, Horowitz et al. found that 3'-dATP inhibited the activities of RNA polymerase II and III on naked DNA-template and that of poly (A) polymerase, although it did not inhibit RNA polymerase I activity.[8] Rose et al. obtained consistent results in *in vitro* and *in vivo* experiments using chromatin.[19] This fact suggested that the structure of chromatin, or some unknown factor(s) influencing transcription, may be important for the modulation of RNA synthesis. This idea could explain the discrepancy in results on the effect of 3'-dCTP. Beach and Ross have shown that in cultured cells 3'-dA can prevent the appearence of newly synthesized globin mRNA regardless of its inhibitory effect on poly (A) synthesis.[21] This observation is consistent with our findings that 3'-dATP as well as 3'-dCTP and 3'-dUTP inhibit the activity of RNA polymerase I and II. In addition, it has been reported that calf thymus RNA polymerases I and II have the same sensitivity to 3'-dATP *in vitro*. These lines of evidence showing that 3'-dATP inhibits RNA polymerase activity support the observation that 3'-dA inhibits rRNA synthesis in intact cells, although the results were slightly different from those of Horowitz et al.[8] The results obtained in the present study suggest that the 3'-deoxyribofuranose moiety of nucleotides is important for the compounds to inhibit the activities of RNA polymerases. These new 3'-dCTP and 3'-dUTP should be useful in studies at a molecular level on the relationship of the template and substrate in RNA synthesis with chromatin, isolated nuclei or permeable cells, because these compounds do not affect poly (A) synthesis.

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