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# KINETIC STUDIES WITH 5-AZACYTIDINE-5′-TRIPHOSPHATE AND DNA-DEPENDENT RNA POLYMERASE*

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**Abstract**—In order to further understand the biochemical mode of action of 5-azacytidine, a potent antileukemic agent, kinetic studies were performed with 5-azacytidine-5′-triphosphate (5-aza-CTP) and purified DNA-dependent RNA polymerase from *Escherichia coli* and calf thymus. RNA polymerase could catalyze the incorporation of the fradulent nucleotide, 5-aza-CTP, into RNA. The apparent $K_m$ value for 5-aza-CTP was estimated to be 350 and 390 for the *E. coli* and calf thymus enzymes respectively. The $K_m$ value for 5-aza-CTP was about 18-fold greater than the $K_m$ value for CTP (20 $\mu$M). The apparent $V_{max}$ value for CTP was about 2-fold greater than the $V_{max}$ value for 5-aza-CTP. 5-Aza-CTP was a weak competitive inhibitor with respect to CTP; the apparent $K_i$ value for 5-aza-CTP was estimated to be 680 and 810 $\mu$M for the *E. coli* and calf thymus enzymes respectively. On the other hand, CTP was a potent competitive inhibitor with respect to 5-aza-CTP; the apparent $K_i$ value of CTP was estimated to be 16 $\mu$M. 5-Aza-CTP did not appear to inhibit the incorporation of UTP into RNA in the reaction catalyzed by RNA polymerase. These data suggest that the inhibition of RNA synthesis in cells by 5-aza-cytidine is not produced by the inhibition of RNA polymerase by 5-aza-CTP.

5-Aza-C,‡ the triazine analog of cytidine, is a potent cytotoxic agent to mammalian cells [1, 2]. Cells in the S phase of the cell cycle are most sensitive to the cytotoxic action of 5-aza-C [2, 3]. The biochemical mode of action of 5-aza-C is complex; this nucleoside analog inhibits protein, RNA and DNA synthesis [4, 5] and pyrimidine biosynthesis [6]. 5-Aza-C also produces degradation of polyribosomes [7, 8] blocks induction of certain liver enzymes by steroids [9], and modifies the biological activity of tRNA [1, 11]. The active form of 5-aza-C in the cell is most likely a nucleotide, since 5-aza-C-resistant cells have been shown to be deficient in uridine-cytidine kinase [12], the enzyme that catalyzes the phosphorylation of this nucleoside analog to 5-aza-CMP [13]. The predominant nucleotide form of 5-aza-C in the cell is 5-aza-CTP [4]. It is not clear whether the biological activity produced by 5-aza-C in cells is due to the incorporation of this analog into RNA [4] or to the inhibition of specific enzymes by the 5-aza-C nucleotides. In this report, in order to further understand the biochemical mode of action of 5-aza-C, we have studied the kinetic interaction of 5-aza-CTP with purified DNA-dependent RNA polymerase from *Escherichia coli* and calf thymus.

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‡ Abbreviations: 5-aza-C, 5-azacytidine; 5-aza-CMP, 5-azacytidine 5′-monophosphate; 5-aza-CTP, 5-azacytidine 5′-triphosphate; and PIPES, piperazine-$N$,$N'$-bis-(2-ethanesulfonic acid).

## MATERIALS AND METHODS

### *Materials*

Nonradioactive nucleotides were obtained from P-L Laboratories, Milwaukee, Wis. 5-Aza-C (NSC 102816), supplied through the Chemical and Drug Procurement Section, Chemotherapy, National Cancer Institute, Bethesda, Md., was filtered through a DEAE-cellulose disc to remove impurities resulting from chemical breakdown immediately prior to its use for 5-aza-C phosphorylation. The tritium-labeled pyrimidine nucleotides were obtained from Schwarz/Mann, Orangeburg, N.Y. [$\gamma$-$^{32}$P]ATP was purchased from ICN Pharmaceuticals, Inc., Irvine, Calif. Dowex AG1-X8 (capacity, 3.2 m-equiv/g) was supplied by BioRad Laboratories, Richmond, Calif. PIPES buffer was obtained from CalBiochem, San Diego, Calif. *E. coli* K12 RNA polymerase with a specificity of 1000 units/mg of protein was obtained from Miles Laboratories, Inc., Kankakee, 111. Calf thymus RNA polymerase B was purified about 100-fold by the method of Kedinger *et al.* [14]. The specific activity of the purified RNA polymerase was about 2.4 units/mg. One unit of enzyme activity was defined as the amount of enzyme catalyzing the incorporation of 1.0 nmole of radioactive nucleotide into an acid-insoluble product/10 min at 37°.

### *Methods*

*RNA polymerase assay.* The assay mixture contained in 0.1 ml, 5 $\mu$moles of PIPES–HCl, pH 6.8; 0.5 $\mu$mole $\beta$-mercaptoethanol; 0.25 $\mu$mole $MnCl_2$; 50 nmoles each of ATP, GTP and UTP or CTP; 5 nmoles [$^3$H]CTP ($6.9 \times 10^5$ cpm), or [$^3$H]UTP ($2.8 \times 10^5$ cpm), 20 $\mu$g of heat-denatured calf thymus

DNA and 0.5 to 2.5 units of *E. coli* RNA polymerase or calf thymus RNA polymerase [14]. The mixture was incubated for 10 min at 37° and the reaction was stopped by adding 3 ml of cold 5% TCA. After 10 min the precipitate was collected on a Whatman GF/C glass filter (2.4 cm diameter) and washed three times with 5 ml of cold 5% TCA and once with 5 ml ethanol. When [$\alpha$-$^{32}$P]5-aza-CTP was used as one of the substrates in RNA polymerase assay, the precipitate which was collected on a Whatman GF/C glass filter was washed three times with 5 ml of cold 5% TCA containing 10 mM sodium pyrophosphate and once with 5 ml ethanol. The disc was dried and counted in a scintillation fluid (10 ml) containing 3.9 g 2,5-diphenyloxazole (PPO) and 80 mg *p*-bis(O-methylstyryl)benzene (bis-MSB) in 1 liter toluene.

*Synthesis of 5-aza-CTP.* 5-Aza-CTP was synthesized enzymatically from 5-aza-C [15], using uridine-cytidine kinase [13], CMP kinase [16], and nucleoside diphosphokinase [17]. The nucleotides were purified by column chromatography on Dowex AG1-X8 (formate), using a linear gradient of ammonium formate (0 to 1.2 M, pH 4.1). The nucleotides were concentrated by lyophilization and desalted by column chromatography on P-2 polyacryl-amide gel. The lyophilized powder of 5-aza-CTP was dissolved in $H_2O$ (pH 5.5) and stored at −60°. [$\alpha$-$^{32}$P]5-aza-CTP was prepared by the phosphorylation of 5-aza-C with [$\gamma$-$^{32}$P]ATP using uridine-cytidine kinase. The [$\alpha$-$^{32}$P]5-aza-CMP formed was isolated under the conditions described above and then phosphorylated with nonradioactive ATP, using CMP kinase and nucleoside diphosphokinase, to [$\alpha$-$^{32}$P]5-aza-CTP.

*Preparation of DNA.* Denatured DNA was obtained by heating a solution of native calf thymus DNA (1.0 mg/ml in 10 mM NaCl and 1.0 mM EDTA, pH 8.0) at 100° for 15 min and placing it immediately on ice. Under these conditions, a hyperchromic shift of 35 per cent at 260 nm was attained.

## RESULTS

The effect of 5-aza-CTP on DNA-dependent RNA polymerase from *E. coli* and calf thymus when [$^3$H]CTP or [$^3$H]UTP was used as the radioactive substrate is shown in Table 1. 5-Aza-CTP appeared to inhibit only the incorporation of [$^3$H]CTP, but not [$^3$H]UTP, into RNA. At a concentration of 100 $\mu$M, 5-aza-CTP inhibited the incorporation of [$^3$H]CTP into RNA by 38.3 per cent for *E. coli* RNA polymerase. For calf thymus RNA polymerase, 100 $\mu$M 5-aza-CTP inhibited the incorporation of [$^3$H]CTP into RNA by 34.0 per cent. For both enzymes, no significant inhibition of incorporation of [$^3$H]UTP into RNA could be detected with 5-aza-CTP at a concentration of 100 $\mu$M.

The effect of 5-aza-CTP on the rate of *E. coli* RNA polymerase reaction in the presence of different concentrations of [$^3$H]CTP is shown in Fig. 1. The data have been plotted according to the method of Lineweaver and Burk [18]. The inhibition produced by 5-aza-CTP was competitive with respect to [$^3$H]CTP. The apparent $K_m$ for [$^3$H]CTP was 20 $\mu$M. The apparent $K_i$ for 5-aza-CTP was estimated to be 680

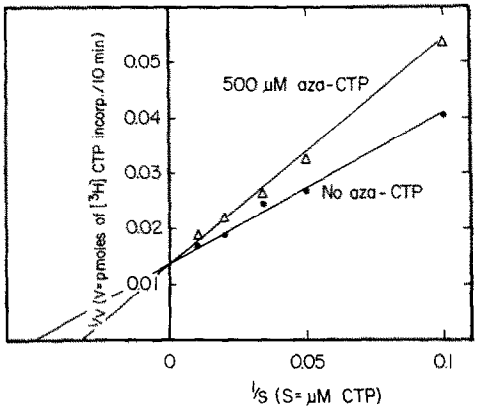


Fig. 1. Effect of various concentrations of CTP on the inhibition produced by 5-aza-CTP with *E. coli* RNA polymerase. The reaction mixture (0.1 ml) contained 10 $\mu$moles PIPES–HCl, pH 6.8; 0.5 $\mu$mole $\beta$-mercaptoethanol; 0.5 $\mu$mole $MnCl_2$; 20 $\mu$g of denatured calf thymus DNA; 50 nmoles each of ATP, GTP and UTP; 0.4 unit of *E. coli* RNA polymerase; and the indicated concentrations of [$^3$H]CTP ($5.2 \times 10^5$ cpm) and 5-aza-CTP. The mixture was incubated at 37° for 10 min.

Table 1. Effect of 5-aza-CTP on DNA-dependent RNA polymerase reaction*

| Enzyme | 5-Aza-CTP concn ($\mu$M) | Radioactive substrate [$^3$H]CTP Incorporation (pmoles) | [$^3$H]CTP Inhibition (%) | [$^3$H]UTP Incorporation (pmoles) | [$^3$H]UTP Inhibition (%) |
|---|---|---|---|---|---|
| *E. coli* RNA polymerase | 0 | 105.1 | 0 | 79.8 | 0 |
| | 20 | 99.2 | 16.8 | 82.5 | 0 |
| | 50 | 77.7 | 26.2 | 81.4 | 0 |
| | 100 | 65.0 | 38.3 | 82.5 | 0 |
| Calf thymus RNA polymerase | 0 | 35.6 | 0 | 29.0 | 0 |
| | 20 | 30.9 | 13.1 | 29.1 | 0 |
| | 50 | 28.2 | 21.8 | 29.2 | 0 |
| | 100 | 23.5 | 34.0 | 29.1 | 0 |

* The incubation mixture (0.1 ml) contained 5 $\mu$moles PIPES–HCl (pH 6.6), 0.25 $\mu$mole $MnCl_2$, 50 nmoles each of ATP, GTP and UTP (or CTP), 20 $\mu$g of heat-denatured calf thymus DNA, 0.3 unit of *E. coli* RNA polymerase or 0.5 unit of calf thymus RNA polymerase or 0.5 unit of calf thymus RNA polymerase, 5 nmoles [$^3$H]CTP ($6.9 \times 10^5$ cpm) or 5 nmoles [$^3$H]UTP ($5.2 \times 10^5$ cpm) and 5-aza-CTP as indicated. The mixture was incubated at 37° for 10 min, and the RNA polymerase assay was performed as described in Methods.

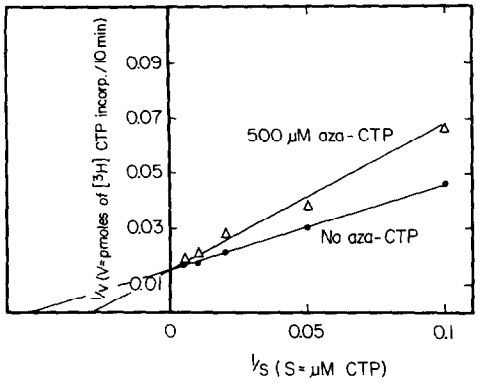


Fig. 2. Effect of various concentrations of CTP on the inhibition produced by 5-aza-CTP with calf thymus RNA polymerase. The reaction mixture (0.1 ml) contained 10 μmoles PIPES–HCl, pH 6.8; 0.5 μmole $MnCl_2$; 20 μg of denatured calf thymus DNA; 50 nmoles each of ATP, GTP and UTP; 0.5 μmole β-mercaptoethanol; 0.5 unit of calf thymus RNA polymerase; and the indicated concentrations of [$^3$H]CTP ($6.9 \times 10^5$ cpm) and 5-aza-CTP. The mixture was incubated at 37° for 10 min.

μM. The $V_{max}$ for the reaction using [$^3$H]CTP as the substrate was about 167 pmoles/10 min.

The effect of 5-aza-CTP on the rate of calf thymus RNA polymerase reaction in the presence of different concentrations of [$^3$H]CTP is shown in Fig. 2. The inhibition produced by 5-aza-CTP was competitive with respect to [$^3$H]CTP. The apparent $K_m$ value for [$^3$H]CTP was 20 μM and the apparent $K_i$ value for 5-aza-CTP was estimated to be 810 μM. The $V_{max}$ for the reaction using [$^3$H]CTP as the radioactive substrate was 148 pmoles/10 min.

The effect of CTP on the rate of *E. coli* RNA polymerase reaction in the presence of different concentrations of [α-$^{32}$P]5-aza-CTP is shown in Fig. 3. The inhibition produced by CTP was competitive with respect to [α-$^{32}$P]5-aza-CTP. It should be noted that the amount of *E. coli* RNA polymerase used in the reaction mixture was about 5.5 times greater than the amount of enzyme used in Fig. 1. The apparent $K_m$ for [α-$^{32}$P]5-aza-CTP was 350 μM and the apparent $K_i$ for CTP was estimated to be 16 μM. The apparent $V_{max}$ for the reaction using [α-$^{32}$P]5-aza-CTP as the radioactive substrate was 69 pmoles/10 min.

The effect of CTP on the rate of calf thymus RNA polymerase reaction in the presence of different concentrations of [α-$^{32}$P]5-aza-CTP is shown in Fig. 4. The inhibition produced by CTP was competitive with respect to [α-$^{32}$P]5-aza-CTP. The amount of calf thymus RNA polymerase used in the reaction mixture was about 5.0 times greater than the amount of enzyme used in Fig. 2. The apparent $K_m$ for [α-$^{32}$P]5-aza-CTP was 370–420 μM and the apparent $K_i$ for CTP was estimated to be 16 μM. The apparent $V_{max}$ for the reaction using [α-$^{32}$P]5-aza-CTP as the radioactive substrate was 67 pmoles/10 min.

## DISCUSSION

5-Aza-C, a potent cytotoxic agent [1, 2], appears to have a complex biochemical mechanism of action since this antimetabolite inhibits protein, RNA and DNA synthesis [4, 5], blocks pyrimidine biosynthesis [6], and produces a degradation of polyribosomes [7, 8]. It is not known whether the biological effects produced by 5-aza-C are due to its incorporation into nucleic acids or to its inhibition of specific enzymes. Since 5-aza-C must first be phosphorylated

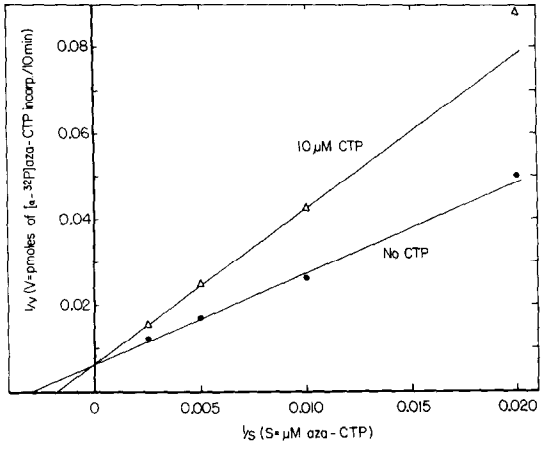


Fig. 3. Effect of various concentrations of 5-aza-CTP on the inhibition produced by CTP with *E. coli* RNA polymerase. The reaction mixture (0.1 ml) contained 10 μmoles PIPES–HCl, pH 6.8; 0.5 μmole $MnCl_2$; 20 μg of dentured calf thymus DNA; 50 nmoles each of ATP, GTP and UTP; 0.5 μmole β-mercaptoethanol; 2.2 units of *E. coli* RNA polymerase; and the indicated concentrations of [α-$^{32}$P]5-aza-CTP ($2.8 \times 10^5$ cpm) and CTP. The mixture was incubated at 37° for 10 min.

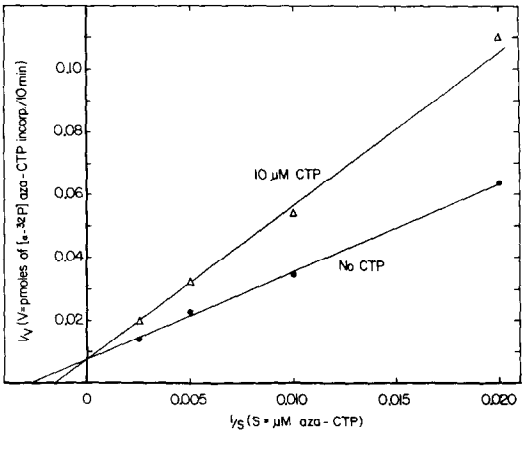


Fig. 4. Effect of various concentrations of 5-aza-CTP on the inhibition produced by CTP with calf thymus RNA polymerase. The reaction mixture (0.1 ml) contained 10 μmoles PIPES–HCl, pH 6.8; 0.5 μmole $MnCl_2$; 20 μg of denatured calf thymus DNA; 50 nmoles each of ATP, GTP and UTP; 0.5 μmole β-mercaptoethanol; 2.5 units of calf thymus RNA polymerase; and the indicated concentrations of [α-$^{32}$P]5-aza-CTP and CTP. The mixture was incubated at 37° for 10 min.

to exert its biological activity [12] and since 5-aza-CTP is the predominate nucleotide form of this drug in the cell [4], we have investigated the effect of 5-aza-CTP on purified DNA-dependent RNA polymerase from both *E. coli* and calf thymus.

The kinetic studies of the RNA polymerase reaction (Figs. 1–4) showed a very high $K_m$ value for 5-aza-CTP (350 and 390 $\mu$M for *E. coli* and calf thymus RNA polymerase respectively) as compared to the $K_m$ for CTP (20 $\mu$M). This high $K_m$ value for 5-aza-CTP may be due to the presence of the triazine ring in 5-aza-C, which apparently reduces the binding affinity of this nucleotide analog for the catalytic site of both *E. coli* and calf thymus RNA polymerases. A similar observation was reported in kinetic studies with the 5-aza-C and uridine-cytidine kinase reaction [13]; the $K_m$ value for 5-aza-C phosphorylation was much greater than the $K_m$ value for cytidine. The apparent $V_{max}$ value for 5-aza-CTP, which was about half the $V_{max}$ value for CTP in both *E. coli* and calf thymus RNA polymerase reactions, further indicates that the enzyme has more difficulty in catalyzing the incorporation of 5-aza-CTP into RNA than CTP.

The selective inhibition of incorporation of [$^3$H]CTP, but not [$^3$H]UTP, into RNA by 5-aza-CTP (see Table 1) confirms not only the correct structure of the nucleotide analog synthesized enzymatically from 5-aza-C [15], but also suggests that 5-aza-CTP competes with CTP for the same catalytic site on RNA polymerase. As shown in Figs. 1–4, 5-aza-CTP was a weak competitive inhibitor with respect to the natural substrate, CTP, in the RNA polymerase reaction. The $K_i$ value for 5-aza-CTP was 680 and 810 $\mu$M for *E. coli* and calf thymus RNA polymerase respectively. On the other hand, the low $K_i$ value for CTP (17 $\mu$M) suggests that CTP is a potent competitive inhibitor with respect to 5-aza-CTP incorporation into RNA (see Figs. 1 and 2).

The results reported in this paper on the effect of 5-aza-CTP on RNA polymerase are consistent with the published data on the effect of 5-aza-C on RNA metabolism in cells since this nucleoside analog was shown to be a weak inhibitor of cellular RNA synthesis [2, 4, 5]. Since tRNA isolated from 5-aza-C-treated cells has reduced amino acceptor activity [10] and reduced capacity to stimulate protein synthesis in a cell-free system [11], perhaps the incorporation of 5-aza-C nucleotides into nucleic acids has a far more important biological effect than the inhibition of RNA polymerase by this nucleotide analog.

There are three major classes of DNA-dependent RNA polymerase in eukaryotic cells [19]. We have performed our kinetic studies with 5-aza-CTP using RNA polymerase of class B [14]. It is possible that this nucleotide analog may interact differently with the other classes of RNA polymerase. Also, the enzyme kinetics with respect to 5-aza-CTP may be complex depending on whether the RNA polymerase used in the study is involved in the process of initiation of RNA synthesis and/or polynucleotide chain elongation.

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