MOLECULAR PHARMACOLOGY, 8, 362-370

Kinetic and Template Studies with 1-β-D-Arabinofuranosylcytosine 5′-Triphosphate and Mammalian Deoxyribonucleic Acid Polymerase

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(Received December 7, 1971)

SUMMARY

Kinetic and template studies have been performed with 1-β-D-arabinofuranosylcytosine 5′-triphosphate (araCTP), dCTP, and calf thymus DNA polymerase. Using denatured DNA as the template, the estimated apparent K_m values for araCTP and dCTP were about the same; however, the estimated apparent V_{max} value for dCTP was about 8 times greater than the apparent V_{max} for araCTP. Both araCTP and dCTP appeared to compete for the same catalytic site of DNA polymerase. When poly(dA-dT) was used as the template, araCTP did not produce any detectable inhibition of the incorporation of [^{3}H]dTTP into acid-insoluble material. DNA polymerase catalyzed the incorporation of [^{3}H]araCTP into poly dC:poly dG, but not into poly(dA-dT). DNA containing [α-^{32}P]arabinofuranosylcytosine 5′-monophosphate ([α-^{32}P]araCMP) was synthesized enzymatically, using denatured DNA and DNA polymerase. Following the digestion of this [^{32}P]DNA with micrococcal nuclease and spleen phosphodiesterase to deoxynucleoside 3′-monophosphates, the radioactivity from [α-^{32}P]araCMP appeared in 3′-dAMP, 3′-dGMP, 3′-dTMP, and 3′-dCMP, suggesting that DNA polymerase could catalyze the formation of a phosphodiester bond between araCMP and each of the deoxynucleotides present in the DNA template.

The data from studies on the rate of release of [α-^{32}P]araCMP and [^{3}H]dCMP from DNA by snake venom phosphodiesterase and the incorporation of araCTP and dCTP into denatured, sonicated DNA are consistent with the proposal that the incorporation of araCTP into DNA in the reaction catalyzed by DNA polymerase produces termination of polynucleotide chain growth.

INTRODUCTION

1-β-D-Arabinofuranosylcytosine is a potent inhibitor of cellular proliferation and DNA synthesis in mammalian cells (1–4). This antimetabolite, which is an analogue of deoxycytidine, appears to act specifically to kill cells during the S phase of the cell cycle (5, 6). The rate of phosphorylation of araC[1] increases during the S phase (7) and is apparently due to an increase in the activity of deoxycytidine kinase (8), the enzyme that catalyzes the phosphorylation of this nucleoside analogue (9). Mammalian cells convert araC to its 5′-triphosphate derivative, araCTP (10, 11), which is presumably the active form of this antimetabolite in the cell. At the cellular level araCTP produces potent inhibition of DNA synthesis and is

This work was supported by a grant from the National Cancer Institute of Canada.

[1] The abbreviations used are: araC, 1-β-D-arabinofuranosylcytosine; araCMP and araCTP, the 5′-monophosphate and 5′-triphosphate of araC, respectively.

incorporated into DNA and RNA to a very limited extent (3, 12–14). At the enzymatic level araCTP acts as a potent competitive inhibitor with respect to dCTP in the DNA polymerase reaction (14, 15). Momparler (16) found that the mammalian DNA polymerase could catalyze the incorporation of araCTP into DNA and that the presence of araCMP at the 3′ end of the polydeoxynucleotide chain produced termination of chain growth. This observation that araCTP could act as a chain growth terminator was confirmed by Atkinson *et al.* (17), using DNA polymerase I from *Escherichia coli*. In studies with L-cells Graham and Whitmore (14) reported that only a small percentage of the [^{3}H]araCMP in the DNA was located in the terminal position and that most of the [^{3}H]araCMP was found in an internucleotide linkage. The differences between the enzymatic and cellular studies are difficult to resolve at the present moment because of the lack of information concerning the normal mechanisms of DNA replication in mammalian cells.

In this paper kinetic and template studies are reported with araCTP and mammalian DNA polymerase in order to elucidate further the mode of action of this antimetabolite. Data are also presented that are consistent with the previous finding that araCTP is a chain growth terminator.

EXPERIMENTAL PROCEDURE

Materials. Nonradioactive nucleotides were obtained from P-L Biochemicals. Radioactive nucleotides and [^{3}H]araC were obtained from New England Nuclear and Schwarz BioResearch, respectively. In some experiments nucleotides of different specific activities were made by adding different amounts of nonradioactive nucleotide to a constant amount of radioactive nucleotide. Micrococcal nuclease, spleen phosphodiesterase, and venom phosphodiesterase were obtained from Worthington Biochemical Corporation. Poly(dA-dT) and poly dC:poly dG were obtained from Miles Laboratories and dissolved at a concentration of 1.0 mg/ml in 10 mM NaCl containing 1 mM sodium citrate, pH 8. Native calf thymus DNA was a gift from Dr. J. H. Spencer and was prepared by the method of Kay *et al.* (18). Nonradioactive araC was obtained from Sigma Chemical Company. The [^{3}H]araC was purified by descending chromatography on Whatman No. 3MM paper in 86% 1-butanol–concentrated ammonium hydroxide (94.5:5.5) (12). DNA polymerase was purified about 100-fold by the method of Yoneda and Bollum (19). The phosphocellulose fraction obtained by this procedure was further purified by gel filtration chromatography to remove exonuclease and terminal deoxynucleotidyltransferase. The specific activity of the purified DNA polymerase was about 5.0 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 per minute at 37°.

DNA polymerase assay. DNA polymerase activity was assayed by the filter disc method (20), using Whatman GF/C glass fiber discs (2.4-cm diameter). The composition of the reaction mixture is given in each table and figure. Unless stated otherwise, the reaction was terminated by the addition of 3 ml of cold 5% trichloracetic acid and the mixture was placed on a glass fiber disc in a Millipore filter holder. The disc was washed with 5 ml of cold 5% trichloracetic acid and 10 ml of water, dried at 50°, and placed in a toluene-based scintillation mixture for radioactivity analysis.

Synthesis of araCTP. [^{3}H]AraCTP was synthesized enzymatically from [^{3}H]araC, using deoxycytidine kinase (9), dCMP kinase (21), and nucleoside diphosphokinase (22). The nucleotides were purified by column chromatography on DEAE-cellulose (bicarbonate), using a linear gradient of triethylammonium bicarbonate (0–0.5 M, pH 7.5–8.0). The salt was removed by evaporation under reduced pressure, and the [^{3}H]araCTP was suspended in 50% ethanol and stored at −20%. Nonradioactive araCTP was synthesized using this method with araC. [α-^{32}P]araCTP was prepared by the phosphorylation of araC with [γ-^{32}P]ATP, using deoxycytidine kinase. The [α-^{32}P]araCMP formed was isolated under the conditions described above and then phosphorylated with nonradioactive ATP, using dCMP kinase and nucleoside diphosphokinase to [α^{32}P]araCTP.

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 approximately 35% at 260 nm was attained. DNA containing [α-^{32}P]araCMP was synthesized enzymatically, using DNA polymerase and [α-^{32}P]araCTP as the radioactive substrate. The reaction mixture (0.5 ml) contained 20 μmoles of potassium phosphate (pH 7.2), 3.0 μmoles of $MgCl_2$, 200 μg of denatured DNA, 25 nmoles each of dATP, dGTP, and dTTP, 2.0 μmoles of 2-mercaptoethanol, 50 pmoles of [α-^{32}P]araCTP (2.3×10^5 cpm), and 1.2 units of DNA polymerase. The mixture was incubated at 37° for 30 min, and, following the addition of 600 μg of denatured DNA, 20 μmoles of EDTA (pH 8.0), and 1.0 ml of water, the mixture was heated at 100° for 1 min. The radioactive DNA was dialyzed against 1 liter of 10 mM NaCl containing 1.0 mM EDTA, pH 8.0, for 4 hr, and then overnight with 1 liter of fresh buffer. DNA containing both [α-^{32}P]araCMP and [^{3}H]dCMP was prepared as described above, with the following changes. At the end of the incubation with DNA polymerase, no denatured DNA or EDTA was added to the mixture. The dialyzed [^{32}P]DNA was concentrated to 0.1 ml by dialysis against Ficoll powder, and the concentrated DNA was used as the template in the reaction mixture described above, containing 100 nmoles of [^{3}H]dCTP (9×10^5 cpm) in place of [^{32}P]araCTP. The reaction mixture was incubated at 37° for 60 min, and 400 μg of denatured DNA, 10 μmoles of EDTA (pH 8.0), and 0.5 ml of water were added. The mixture was then dialyzed overnight against 1 liter of 10 mM NaCl containing 1.0 mM EDTA, pH 8.0, using two changes of this buffer.

Denatured, sonicated DNA was prepared by sonication of 40 ml of native calf thymus DNA (2.0 mg/ml in 100 mM NaCl and 10 mM sodium citrate, pH 7) for 30 min at 10° in an Artek Sonic Dismembrator at the maximum setting. This method produces fragments of DNA with an average molecular weight of about 400,000 (23). In order to obtain DNA molecules of uniform size, the sonicated DNA (14 ml) was placed on a column of Sepharose 6B ($19.4 \text{ cm}^2 \times 38$ cm) and eluted with potassium phosphate buffer, 50 mM, pH 7.2, containing 1.0 mM EDTA. Fractions containing 25 ml were collected at 30-min intervals, and the three fractions containing the highest absorbance at 280 nm were pooled. The pooled DNA (10.4 mg) was placed in a dialysis sac, concentrated with Ficoll powder to about 2 ml, and dialyzed against 500 ml of 10 mM NaCl and 1.0 mM sodium citrate, pH 7. This native, sonicated DNA was heated at 100° for 5 min and cooled immediately in ice water to form heat-denatured, sonicated DNA.

Enzymatic digestion of DNA. Snake venom phosphodiesterase (5.0 mg/ml), micrococcal nuclease (1.0 mg/ml, 7500 units/ml), and spleen phosphodiesterase (11 units/ml) were dissolved in Tris-acetate buffer, 100 mM, pH 8.0. DNA containing [5′-α-^{32}P]araCMP was digested to deoxynucleoside 3′-monophosphates with micrococcal nuclease and spleen phosphodiesterase as described by Josse *et al.* (24). After digestion the different deoxynucleotides were separated by chromatography on a column ($0.63 \text{ cm}^2 \times 52$ cm) of Dowex 50-X4, minus 400 mesh, using 0.2 M ammonium formate, pH 4.1, as the eluent (25). Fractions containing 2 ml were collected at 5-min intervals and assayed for radioactivity and absorbance at 257 nm.

RESULTS

Kinetic studies. The effect of araCTP on the rate of the DNA polymerase reaction in the presence of different concentrations of [^{3}H]dTTP is shown in Fig. 1. The data have been plotted according to the method of Lineweaver and Burk (26). The inhibition produced by araCTP appeared not to be competitive or noncompetitive with respect to [^{3}H]dTTP, but complex in nature. The apparent K_m for [^{3}H]dTTP obtained from this plot was 5.0 ± 0.5 μM.

The effect of araCTP on the reaction rate in the presence of different concentrations of [^{3}H]dCTP is shown in Fig. 2. The inhibition produced by araCTP appeared to be competitive with respect to [^{3}H]dCTP. The apparent K_m for [^{3}H]dCTP was 2.0 ± 0.5 μM, and the apparent K_i for araCTP was 1.5 ± 2.0 μM. Both the apparent K_m and K_i values

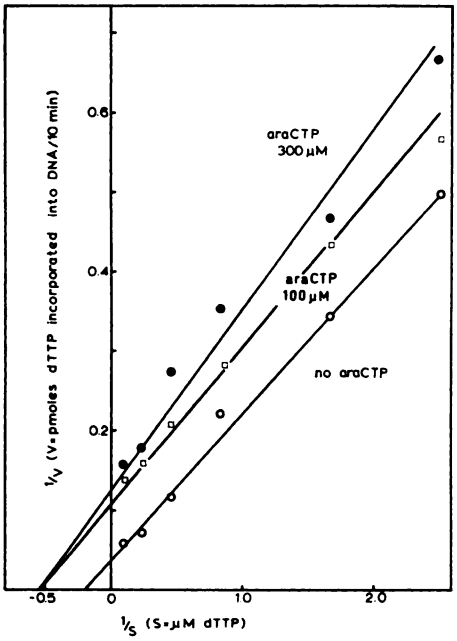

FIG. 1. *Effect of araCTP on reaction rate in the presence of different concentrations of dTTP*

The reaction mixture (0.1 ml) contained 4 μmoles of potassium phosphate (pH 7.2), 0.6 μmole of $MgCl_2$, 20 μg of denatured DNA, 5 nmoles each of dATP, dCTP, and dGTP, 0.05 unit of DNA polymerase, and the indicated concentrations of [^{3}H]dTTP (1.2×10^5 cpm) and araCTP. The mixture was incubated at 37° for 10 min and assayed as described under EXPERIMENTAL PROCEDURE.

of dCTP and araCTP, respectively, were similar to those reported by Furth and Cohen (15). The apparent V_{max} for the reaction using [^{3}H]dCTP as the radioactive substrate was about 340 pmoles/min/mg.

The effect of dCTP on the reaction rate in the presence of different concentrations of [^{3}H]araCTP is shown in Fig. 3. The inhibition produced by dCTP appeared to be competitive with respect to [^{3}H]araCTP. It should be noted that the amount of DNA polymerase used in the reaction mixture was about 6 times greater than the amount of enzyme used in Figs. 1 and 2. The apparent K_m for [^{3}H]araCTP was 2.0 ± 0.5 μM, and the apparent K_i for dCTP was 7.0 ± 2.0 μM. The apparent K_m value of [^{3}H]araCTP in Fig. 3 was similar to the apparent K_i value of araCTP in Fig. 2. In the case of dCTP the apparent K_i value of Fig. 3 was about 3 times greater than the apparent K_m value of Fig. 2. The apparent V_{max} for the reaction using [^{3}H]araCTP as the radioactive substrate was 40 pmoles/min/mg.

Template studies. The template requirements for araCTP inhibition of the DNA polymerase reaction using [^{3}H]dTTP as the radioactive substrate are shown in Table 1. At a concentration of 20 μM araCTP produced about 60% inhibition when denatured DNA was used as the template, but no inhibition when poly(dA-dT) was used as the template. Since there was no detectable incorporation of [^{3}H]dTTP into poly dC: poly dG, the effect of araCTP on the reaction using this template is unknown.

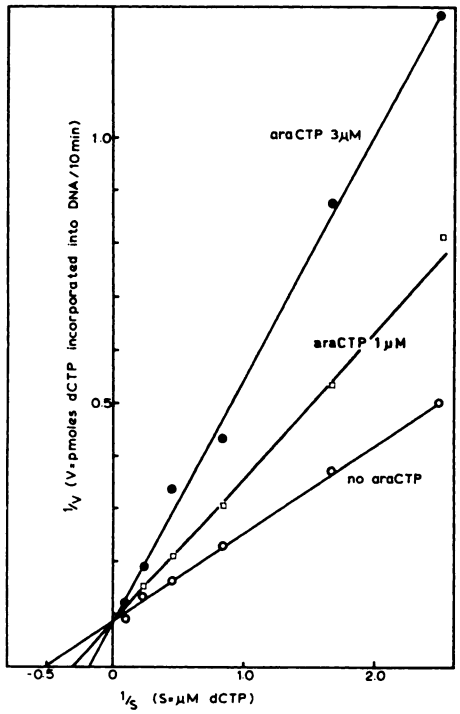

FIG. 2. *Effect of araCTP on reaction rate in the presence of different concentrations of dCTP*

The reaction mixture (0.1 ml) contained 4 μmoles of potassium phosphate (pH 7.2), 0.6 μmole of $MgCl_2$, 20 μg of denatured DNA, 5 nmoles each of dATP, dGTP, and dTTP, 0.05 unit of DNA polymerase, and the indicated concentrations of [^{3}H]dCTP (1.2×10^5 cpm) and araCTP. The mixture was incubated at 37° for 10 min and assayed as described under EXPERIMENTAL PROCEDURE.

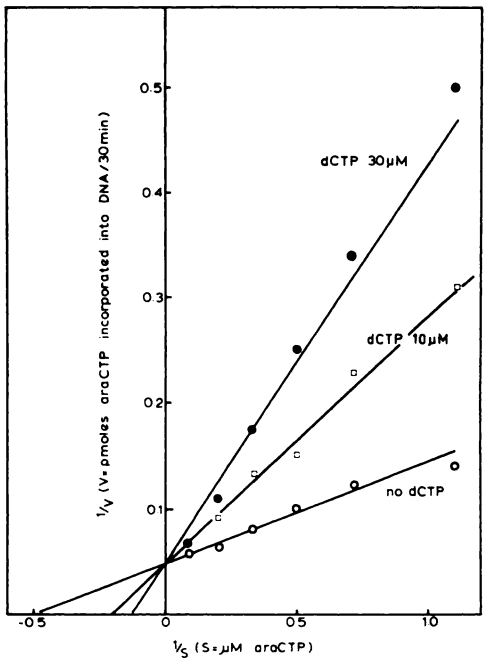

FIG. 3. *Effect of dCTP on reaction rate in the presence of different concentrations of araCTP*

The reaction mixture had the same composition as in Fig. 2, except that it contained 0.3 unit of DNA polymerase and the indicated concentrations of [^{3}H]araCTP (6.6×10^4 cpm) and dCTP. The mixture was incubated at 37° for 30 min and assayed as described under EXPERIMENTAL PROCEDURE.

The template requirements for [^{3}H] araCTP incorporation have been studied and compared with the template requirements for [^{3}H]dTTP and [^{3}H]dCTP incorporation (Table 2). When [^{3}H]dTTP and dATP were the substrates, [^{3}H]dTTP was incorporated into poly(dA-dT) and, to a lesser extent, into denatured DNA, but not into poly dC:poly dG. When [^{3}H]dCTP and dGTP were the substrates, [^{3}H]dCTP was incorporated into poly dC:poly dG and, to a lesser extent, into denatured DNA, but not into poly (dA-dT). Similar results were obtained when [^{3}H]araCTP and dGTP were used as substrates, except that more [^{3}H]araCTP was incorporated into denatured DNA than into poly dC:poly dG.

Analysis of DNA product. DNA labeled with [α-^{32}P]araCMP was synthesized enzymatically with DNA polymerase, using [α-^{32}P]araCTP as the radioactive substrate and denatured DNA as the template. The [^{32}P]DNA was digested with micrococcal nuclease and spleen phosphodiesterase to deoxynucleoside 3′-monophosphates, and the radioactivity in the various nucleotide fractions was assayed (Table 3). In this experiment the ^{32}P on the 5′hydroxyl group of araCMP ends up on the 3′-hydroxyl group of the nucleotide neighbor in the DNA template following enzymatic digestion (24). An equal amount of radioactivity was present in the 3′-dTMP and 3′-dAMP fractions, a smaller amount of radioactivity was present in the 3′-dGMP fraction, and a very low amount was present in the 3′-dCMP fraction.

The rate of conversion of [^{32}P]araCMP and [^{3}H]dCMP into the acid-soluble fraction from DNA by snake venom phosphodiesterase is shown in Fig. 4. In this figure the incorporation of [^{32}P]araCTP into denatured DNA was first catalyzed by DNA polymerase, followed by the incorporation of [^{3}H] dCTP into DNA. Snake venom is an exonuclease that attacks DNA at the 3′-hydroxyl end, releasing nucleoside 5′-monophosphates (27). It appeared that [^{32}P]araCMP was released more rapidly from DNA than [^{3}H]

TABLE 1

Template requirements for araCTP inhibition

The reaction mixture (0.1 ml) contained 4 μmoles of potassium phosphate (pH 7.2), 0.6 μmole of $MgCl_2$, 5 nmoles each of dATP, dGTP, dCTP, and [^{3}H]dTTP (1.0×10^5 cpm), 0.05 unit of DNA polymerase, 20 μg of either denatured DNA, poly(dA-dT), or poly dC:poly dG, as indicated, and the indicated concentration of araCTP. The mixture was incubated at 37° for 10 min and assayed as described under EXPERIMENTAL PROCEDURE.

Template	Addition	[^{3}H]dTTP incorporated
		pmoles
Denatured DNA	None	560
Denatured DNA	AraCTP (20 μM)	210
Poly(dA-dT)	None	1100
Poly(dA-dT)	AraCTP (20 μM)	1100
Poly dC:poly dG	None	<10
Poly dC:poly dG	AraCTP (20 μM)	<10

TABLE 2

Template requirements for araCTP incorporation

The reaction mixture (0.1 ml) contained 4 μmoles of potassium phosphate (pH 7.2), 0.6 μmole of $MgCl_2$, and 20 μg of either denatured DNA, poly(dA-dT), or poly dC:poly dG, as indicated. Where indicated, 5 nmoles each of [^{3}H]dTTP (1.0×10^5 cpm) and dATP and 0.05 unit of DNA polymerase were added to the mixture, which then was incubated at 37° for 10 min. Where indicated, either 4 nmoles of [^{3}H]dCTP (1.1×10^5 cpm) or 0.1 nmole of [^{3}H]araCTP (6.0×10^4 cpm) plus 5 nmoles of dGTP and 0.1 unit of DNA polymerase were added to the mixture, followed by incubation at 37° for 30 min. The amount of incorporation was assayed as described under EXPERIMENTAL PROCEDURE.

Substrates	Template activity		
	DNA	Poly(dA-dT)	Poly dC:poly dG
	pmoles incorporated		
[^{3}H]dTTP + dATP	20 ± 20	700 ± 20	<10
[^{3}H]dCTP + dGTP	50 ± 20	<10	410 ± 20
[^{3}H]AraCTP + dGTP	8 ± 1	<1	3 ± 1

TABLE 3

Enzymatic digestion of DNA labeled with [^{32}p]araCMP by micrococcal nuclease and spleen phosphodiesterase

DNA (300 μg) containing [^{32}P]araCMP (1.6×10^4 cpm) was digested with micrococcal nuclease and spleen phosphodiesterase as described by Josse *et al.* (24). After digestion the mixture was placed on a column of Dowex 50-X4, minus 400 mesh (0.63 cm^2 × 52 cm), and the deoxynucleoside 3′-monophosphates were eluted with 0.2 M ammonium formate, pH 4.1 (25). Fractions containing 2 ml were collected every 5 min and assayed for radioactivity.

Fraction	Total radioactivity
	cpm
Deoxythymidine 3′-monophosphate	5500
Deoxyguanosine 3′-monophosphate	3300
Deoxyadenosine 3′-monophosphate	5100
Deoxycytidine 3′-monophosphate	540

dCMP by the snake venom phosphodiesterase.

Incorporation studies. In Fig. 5 is shown a comparison of the incorporation of [^{3}H] araCTP and [^{3}H]dCTP into denatured, sonicated DNA with respect to time. The incorporation of [^{3}H]dCTP reached a plateau within 7 min (Fig. 5B), whereas the incorporation of [^{3}H]araCTP reached a plateau at about 30 min (Fig. 5A). The number of molecules of [^{3}H]dCTP incorporated into

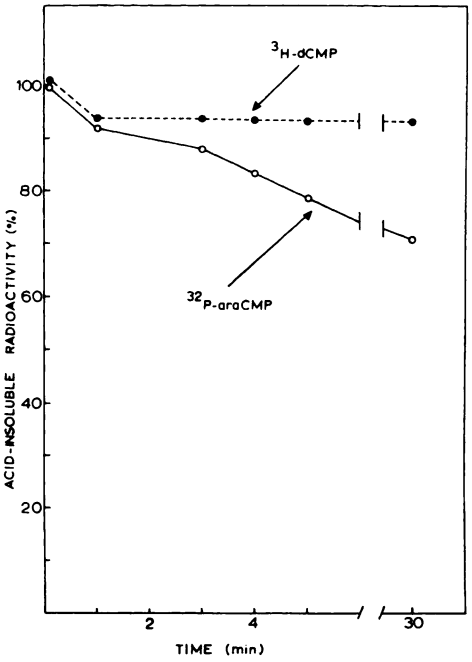

FIG. 4. *Enzymatic digestion of DNA labeled with [^{32}P]araCMP and [^{3}H]dCMP by snake venom phosphodiesterase*

The reaction mixture (0.5 ml) contained 25 μmoles of Tris-HCl (pH 8.0), 5 μmoles of $MgCl_2$, 220 μg of DNA labeled with [^{32}P]araCMP (4×10^3 cpm) and [^{3}H]dCMP (1.2×10^4 cpm), and 25 μg of snake venom phosphodiesterase. The mixture was incubated at 37°, and 0.05-ml aliquots were removed at the times indicated. After the addition of 20 μg of unlabeled DNA, the acid-insoluble radioactivity was assayed as described under EXPERIMENTAL PROCEDURE.

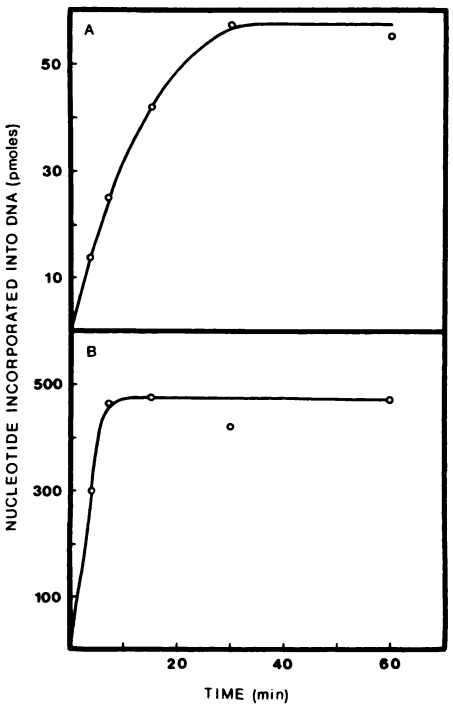

FIG. 5. *Incorporation of araCTP and dCTP into denatured, sonicated DNA*

The reaction mixture (0.1 ml) contained 4 μmoles of potassium phosphate (pH 7.2), 0.6 μmole of $MgCl_2$, 20 μg of denatured, sonicated DNA, 5 nmoles each of dATP, dGTP, and dTTP, and 2.0 units of DNA polymerase. To mixture A, 100 pmoles of [^{3}H]araCTP (3.2×10^4 cpm) were added and to mixture B, 1000 pmoles of [^{3}H]dCTP (3.8×10^4 cpm). The mixture was incubated at 37° for the times indicated and assayed as described under EXPERIMENTAL PROCEDURE.

denatured, sonicated DNA at its plateau level was about 10 times the number of molecules of [^{3}H]araCTP incorporated at its plateau level. Assuming that most of the denatured, sonicated DNA was single-stranded with "hairpin" loops (28), it was estimated that in the reaction mixture containing 20 μg of DNA there were about 100 pmoles of DNA. The number of molecules of [^{3}H]dCTP incorporated was greater, whereas the number of molecules of [^{3}H] araCTP incorporated was less, than the number of DNA molecules present in the reaction mixture.

DISCUSSION

Since araCTP was reported to be a potent inhibitor of mammalian DNA polymerase (14, 15), it was of interest to study in more detail the interactions of this antimetabolite with the polymerase enzyme. The reaction catalyzed by DNA polymerase is complex, requiring the presence of four different deoxynucleoside 5′-triphosphates, a cation, and a DNA template with available 3′-hydroxyl groups on one of the strands of the DNA duplex (28). In a previous report from this laboratory it was demonstrated that mammalian DNA polymerase could catalyze the incorporation of araCTP into DNA, and as a result of this incorporation termination of chain growth apparently occurred (16). Using DNA polymerase I from *E. coli*, Atkinson *et al.* (17) confirmed the observation that araCTP could act as a chain growth terminator. In this paper kinetic and template studies further support the proposal that DNA polymerase catalyzes the incorporation of araCTP into DNA and that this incorporation is not due to contamination of the enzyme preparation with terminal deoxynucleotidyltransferase (29). Also data are presented that are consistent with the previous observation that araCTP produces termination of chain growth in the DNA polymerase reaction.

The kinetic data obtained with DNA polymerase have been summarized in Table 4. The similarity in the apparent K_m values of dCTP and araCTP and the competitive inhibition observed between these two nucleotides (Figs. 2 and 3) strongly suggest that both dCTP and araCTP compete for the same catalytic site of DNA polymerase. However, the apparent V_{max} for dCTP was about 8 times that for araCTP. These data suggest that, even though dCTP and araCTP had about the same binding affinity for the catalytic site of DNA polymerase, this enzyme catalyzed the formation of a

TABLE 4

Summary of kinetic data

Substrate	Apparent K_m	Apparent V_{max}
	μM	*pmoles/min/mg*
dTTP	5.0 ± 0.5	930
dCTP	2.0 ± 0.5	340
AraCTP	2.0 ± 0.5	40

phosphodiester bond between araCTP and the 3′-terminal deoxynucleotide in the DNA template at a much slower rate than dCTP.

Data from the template studies suggested that the mammalian DNA polymerase was the enzyme involved in the incorporation of araCTP into DNA, since it appeared that a Watson-Crick base-pairing mechanism was implicated in these reactions (Tables 1 and 2). AraCTP did not inhibit the DNA polymerase reaction when poly(dA-dT) was used as the template, and this nucleotide analogue was incorporated into poly dC: poly dG, but not into poly(dA-dT).

Analysis of the products obtained from DNA labeled with [^{32}P]araCMP following enzymatic digestion with micrococcal nuclease and spleen phosphodiesterase indicated that the mammalian DNA polymerase could catalyze the formation of a phosphodiester bond between araCMP and each of the four deoxynucleotides present in the DNA template (Table 3). It is difficult to interpret the significance of the low amount of radioactivity present in the 3′-dCMP fraction as compared to the other 3′-deoxynucleotide fractions, since the relative amount of dCMP with respect to the other deoxynucleotides at the 3′-terminus of the replicating points of the denatured DNA template is unknown.

The data shown in Figs. 4 and 5 are consistent with the previous reports that araCTP acts as a chain growth terminator in the DNA polymerase reaction (16, 17). Since chain elongation in the reaction catalyzed by DNA polymerase takes place at the 3′-hydroxyl end of the primer strand of the DNA template (28), one would expect that those nucleotides incorporated last should be released first from the DNA template by enzymatic digestion with snake venom phosphodiesterase, an exonuclease that attacks the 3′-hydroxyl end of polynucleotides (27). In the experiment shown in Fig. 4, [^{32}P]araCMP was released more rapidly than [^{3}H]dCMP from DNA by snake venom phosphodiesterase, even though the [^{32}P]araCMP was incorporated into DNA before [^{3}H]dCMP. The DNA was not completely hydrolyzed by the snake venom phosphodiesterase, apparently because of inactivation of this enzyme during the incubation period. Although this experiment does not unequivocally prove that araCTP is a chain growth terminator, presumably the presence of [^{32}P]araCMP at the 3′-terminus of the DNA primer strand prevented the addition of [^{3}H]dCMP, which had to be incorporated into those polydeoxynucleotide chains that did not contain [^{32}P]araCMP.

The experiment on the incorporation of [^{3}H]araCTP and [^{3}H]dCTP into denatured, sonicated DNA indicated that the number of molecules of [^{3}H]araCTP incorporated was less than, whereas the number of molecules of [^{3}H]dCTP incorporated exceeded, the number of DNA molecules in the reaction mixture (Fig. 5). If araCTP produces termination of chain growth, one would expect only 1 molecule of araCTP to be incorporated for each molecule of single-stranded DNA in the "hairpin" conformation (28). The data indicate that only 1 molecule of [^{3}H]araCTP was incorporated for every 2 molecules of DNA; one possible explanation for this observation is that only about 50% of the DNA molecules were active templates for DNA polymerase.

Although chain termination appears to take place *in vitro* with araCTP and DNA polymerase, such an observation has not been clearly demonstrated *in vitro* with intact cells, because of the difficulty in isolation and characterization of the replicating region of the mammalian cell chromosome (30–32). Graham and Whitmore (14) have analyzed high molecular weight DNA labeled with [^{3}H]araCMP from L-cells and found only a small percentage of the [^{3}H]araCMP at the 3′-termini, whereas most of the [^{3}H]araCMP was located in the internucleotide linkages of DNA. The reasons for the differences between the enzymatic and cellular data concerning the chain growth termination activity of araCTP are not known, because of the lack of basic information on the normal mechanism of DNA replication in mammalian cells. It is possible that other enzymes are involved in DNA synthesis in the cell, which interact differently with araCTP than DNA polymerase, or that this latter enzyme may have different properties in the intracellular environment. It should be interesting to study the interaction of araCTP and the new low molecular weight DNA polymerase that has recently been isolated from mammalian cells (33, 34). Also, the interaction between araCMP-

terminated polydeoxynucleotides and DNA ligase (35) should be investigated.

At present there are not enough data available to determine the relationship between the incorporation of araC into cellular DNA and the cytotoxic effects produced by this antimetabolite. Recently Chu (36) has attempted to correlate the incorporation of araC into cellular RNA with cell death.

ACKNOWLEDGMENT

I would like to thank Miss Aurora Labitan for her excellent technical assistance in this work.

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