EFFECT OF CYTOSINE ARABINOSIDE 5'-TRIPHOSPHATE ON MAMMALIAN DNA POLYMERASE

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SUMMARY

Cytosine arabinoside 5'-triphosphate (araCTP) produced an inhibition of a partially purified DNA polymerase obtained from calf thymus. The purified DNA polymerase catalyzed the incorporation of ^{3}H-araCTP into an acid insoluble product (DNA) in the presence of dATP, dGTP, dTTP, $MgCl_2$, and denatured DNA. The addition of dCTP to the reaction mixture partially reversed the inhibition of DNA polymerase by araCTP and decreased the incorporation of ^{3}H-araCTP into DNA. About 90% of the radioactivity in the DNA product after treatment with micrococcal nuclease and spleen phosphodiesterase was released as ^{3}H-cytosine arabinoside, suggesting that most of the incorporation was at the 3'-hydroxyl terminal end and not within the polydeoxynucleotide chain.

INTRODUCTION

Cytosine arabinoside (1-β-D-arabinofuranosyl cytosine, araC) has been shown to be an inhibitor of growth of certain bacteria (1), mammalian cells (2), and some DNA viruses (3). In studies with mammalian cells araC produced a marked inhibition of DNA synthesis, but no significant inhibition of RNA synthesis (4). In these cells araC was phosphorylated mostly to araCTP (5), which has been demonstrated to be a potent inhibitor of a purified mammalian DNA polymerase; this inhibition produced by araCTP was competitive with dCTP (6). No incorporation of araCTP into DNA was observed with this enzyme (6), in contrast to some reports that araC was incorporated into DNA of mammalian cells to a very limited extent (7-9). In this communication data are presented that suggest that a partially purified mammalian DNA polymerase can catalyze the incorporation araCTP into DNA.

MATERIALS AND METHODS

Chemicals — Nonradioactive nucleotides were obtained from P-L Laboratories. Micrococcal nuclease, spleen phosphodiesterase, venom phosphodiesterase, and calf thymus DNA were obtained from Worthington Biochemical Corporation. Pancreatic DNAse I (DN-EP), pancreatic RNAse-A (type X-A), chicken intestine alkaline phosphatase type II, Crotalus adamanteus 5'-nucleotidase, and araC were obtained from Sigma. Radioactive nucleotides and ^{3}H-araC were obtained from Schwarz BioResearch, Inc. ^{3}H-araC was purified by descending chromatography on Whatman 3 MM in 86% n-butanol-concentrated NH_4OH (94.5:5.5).

Purification of DNA Polymerase — DNA polymerase was purified about 100-fold from calf thymus by the method of Yoneda and Bollum (10). The phosphocellulose and the hydroxylapatite fractions were obtained by this procedure.

DNA Polymerase Assay — DNA polymerase was assayed by the paper disc method (11) except that Millipore cellulose filters 0.22 μ pore size were used in place of Whatman No. 1 paper. The composition of the reaction mixture is given in each Table. Denatured DNA was obtained by heating a solution of DNA (500 μg/ml in 20 mM Tris-HCl, pH 8.0) in a boiling water bath for 5 minutes and placing immediately on ice. Under these conditions, a hyperchromic shift of approximately 35% at 260 mμ was attained. One unit of enzyme activity was defined as the amount of enzyme catalyzing the incorporation of 1 mμmole of radioactive nucleotide into an acid insoluble product per minute at 37°.

Synthesis of AraCTP — AraCTP was synthesized enzymatically from cytosine arabinoside 5'-monophosphate (araCMP) using partially purified dCMP kinase (12) and nucleoside diphosphokinase (13). AraCMP was a gift from Drs. M.Y. Chu and G.A. Fischer; it was synthesized chemically from araC (7). ^{3}H-araCTP was synthesized enzymatically from ^{3}H-araC using deoxycytidine kinase (14) and the enzymes mentioned above. The nucleotides were purified on Dowex 1 (carbonate) using a linear gradient of 0-0.8 M NH_4HCO_3 as the eluant (15).

Enzymatic Digestion of DNA — The Millipore filter containing the enzymatically synthesized DNA was washed, dried,

and placed in 20 mM Tris-HCl, pH 7.5 and 5 mM $CaCl_2$. Enzymatic digestion of the acid insoluble product was performed with micrococcal nuclease and spleen phosphodiesterase (16). The cytosine containing nucleotides and nucleosides were separated by thin layer chromatography on DEAE-cellulose using 0.01 N HCl as the solvent (17). The cytosine containing spots were scraped off the plates and placed directly in scintillation vials for radioactivity assay or they were eluted from the DEAE-cellulose with 0.4 M NH_4HCO_3, evaporated to dryness, treated with 5'-nucleotidase or alkaline phosphatase and identified by paper chromatography in 86% n-butanol-NH_4OH.

RESULTS

Data on the inhibition of DNA polymerase by araCTP are shown in Table I. AraCTP at a concentration of 10 μM produced about 38% inhibition of DNA polymerase. The addition of dCTP at a concentration of 500 μM reduced this inhibition to 26%. These observations confirm those of other investigators who

TABLE I

Inhibition of DNA Polymerase by AraCTP

The incubation mixture (0.1 ml) contained 4 μmoles of $KHPO_4$, pH 7.2, 0.6 μmoles of $MgCl_2$, 5 μg of denatured DNA, 0.2 μmole of dithiothreitol, 10 mμmoles each of dATP, dGTP, dCTP, and ^{3}H-dTTP (8 x 10^4 cpm), and 0.06 unit of DNA polymerase (hydroxylapatite fraction). The mixture was incubated at 37° for 10 minutes and assayed as described under "Methods".

Additions	mμmoles ^{3}H-dTTP incorporated into DNA
None	0.69
araCTP (5 μM)	0.52
araCTP (10 μM)	0.43
araCTP (25 μM)	0.26
araCTP (5 μM) plus dCTP (100 μM)	0.65
araCTP (10 μM) plus dCTP (500 μM)	0.51
araCTP (25 μM) plus dCTP (500 μM)	0.49

found that dCTP could reverse the inhibitory effects of araCTP on DNA polymerase (6, 18).

The properties of DNA polymerase were studied by following the incorporation of either ^{3}H-dCTP or ^{3}H-araCTP into an acid insoluble product (Table II). The DNA polymerase had an absolute dependence on $MgCl_2$ and DNA primer. RNA could not replace DNA as primer of the reaction. The addition of more denatured DNA to the complete reaction mixture produced a 30% increase in the incorporation of ^{3}H-dCTP and a 100% increase in the incorporation of ^{3}H-araCTP into DNA. The omission of one or more of the deoxynucleoside 5'-triphosphate substrates resulted in a considerable decrease in DNA polymerase activity. The detectable incorporation when only ^{3}H-dCTP or ^{3}H-araCTP

TABLE II

Properties of the Reaction

The incubation mixture (0.1 ml) contained 4 μmoles of $KHPO_4$, pH 7.2, 0.6 μmole of $MgCl_2$, 5 μg of denatured DNA, 0.2 μmole of dithiothreitol, 10 mμmoles each of dATP, dGTP, and dTTP, 3,000 μμmoles of ^{3}H-dCTP (1 x 10^5 cpm) or 500 μμmoles ^{3}H-araCTP (1 x 10^5 cpm) as indicated, and 0.03 unit of DNA polymerase (phosphocellulose fraction). The mixture was incubated at 37° for 10 minutes and assayed as described under "Methods".

Experimental Conditions	μμmoles incorporated into DNA	
	^{3}H-dCTP	^{3}H-araCTP
Complete	240	6
Omit $MgCl_2$	<10	<1
Omit DNA	<10	<1
Omit DNA, add RNA (10 μg)	<10	<1
Add DNA (5 μg)	310	12
Omit dATP	20	2
Omit dATP, dGTP	15	2
Omit dATP, dGTP, dTTP	12	2
Add pancreatic DNAse (25 μg)	<10	<1
Add pancreatic RNAse-A (10 μg)	230	7
Add dCTP (0.1 mM)	82	2

were used as substrate may have been due to the contamination of DNA polymerase with terminal deoxynucleotidyltransferase (19). The acid insoluble product was resistant to enzymatic digestion by RNAse-A, but not by pancreatic DNAse. The addition of nonradioactive dCTP decreased the incorporation of both ^{3}H-dCTP and ^{3}H-araCTP. Under all experimental conditions the incorporation of ^{3}H-dCTP into DNA was much greater than that of ^{3}H-araCTP.

The enzymatically synthesized DNA when either ^{3}H-dCTP or ^{3}H-araCTP was used as the radioactive substrate was treated with micrococcal nuclease and spleen phosphodiesterase. Analysis of the digest on DEAE-cellulose indicated that in the case of ^{3}H-dCTP 5% and 83% of the radioactivity was released as a nucleoside (^{3}H-deoxycytidine) and nucleotide (^{3}H-deoxycytidine 3'-monophosphate) respectively in comparison to ^{3}H-araCTP where 90% and 6% of the radioactivity was released as nucleoside (^{3}H-cytosine arabinoside) and nucleotide respectively. Enzymatic digestion of DNA with micrococcal nuclease and spleen phosphodiesterase produces mostly nucleoside

TABLE III

Enzymatic Digestion of DNA Product

The incubation mixture (0.22 ml) contained 8 μmoles of $KHPO_4$, pH 7.2; 1.2 μmoles of $MgCl_2$; 40 μg of denatured DNA; 0.4 μmoles of dithiothreitol; 20 mμmoles each of dATP, dGTP, and dTTP; 6 mμmoles of ^{3}H-dCTP (3×10^5 cpm) or 0.6 mμmoles of ^{3}H-araCTP (4×10^5 cpm) as indicated, and 0.2 units of DNA polymerase (phosphocellulose fraction). The mixture was incubated at 37° for 150 minutes, the acid insoluble product isolated and treated with micrococcal nuclease and spleen phosphodiesterase as described under "Methods".

Substrate	Nucleoside (cpm)	Nucleoside Monophosphate (cpm)	Total Radioactivity (cpm) *	3'-Hydroxyl Terminal (%)
^{3}H-dCTP	1,600	26,100	30,600	5
^{3}H-araCTP	22,600	1,400	25,000	90

* Amount of radioactivity in sample analyzed.

3'-monophosphates except for the nucleotide at the 3'-hydroxyl terminus which is released as a nucleoside (20). These results suggest that 90% of ^{3}H-araCTP was incorporated into the 3'-hydroxyl terminal position of the polydeoxynucleotide primer. Not all of the radioactivity placed on DEAE-cellulose was identified. Presumably some of the radioactivity was present in oligodeoxynucleotides as a result of incomplete enzymatic digestion of the DNA. In the case of ^{3}H-araCTP there was not enough radioactivity in the nucleoside monophosphate fraction to permit adequate identification of ^{3}H-cytosine arabinoside 3'-monophosphate and thus the actual amount of incorporation of ^{3}H-araCTP within the polydeoxynucleotide chain is unknown. There was no detectable monoester phosphatase activity associated with the micrococcal nuclease or the spleen phosphodiesterase when ^{3}H-araCMP was used as substrate. Enzymatic digestion of the DNA product with pancreatic DNAse and venom phosphodiesterase when ^{3}H-araCTP was used as the radioactive substrate released over 90% of the radioactivity as ^{3}H-araCMP.

DISCUSSION

The inhibition of DNA synthesis in mammalian cells by araC most probably involves specific interactions of this antimetabolite and its phosphorylated derivatives with the enzymes involved in DNA replication. One aspect of this inhibition involves the interaction of araCTP with the enzyme DNA polymerase; this produced an inhibition that appeared to be competitive with dCTP (6, 18). Another possible aspect of the inhibition of DNA synthesis involves the interaction of araCTP with the DNA template. Studies presented in this communication suggest that the incorporation of ^{3}H-araCTP into DNA takes place *in vitro* with a purified mammalian DNA polymerase. Enzymatic digestion of the acid insoluble product with micrococcal nuclease and spleen phosphodiesterase suggested that most of the incorporation of ^{3}H-araCTP was at the 3'-hydroxyl terminus and not within the polydeoxynucleotide chain. The inhibition of DNA synthesis could result from the addition of a single molecule of araCMP to the terminal position of the replicating polydeoxynucleotide template since this would probably prevent further addition of other nucleotides

and thus block the extension of the polydeoxynucleotide chain (6). Such a phenomenon, if it occurs *in vivo*, may be an important factor in the production of irreversible lethal effects in mammalian cells by short exposure to araC (7, 21).

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