Inhibition of Deoxyribonucleic Acid Polymerases from Murine Cells and Oncornavirus by 5-Alkylated Derivatives of 1-β-D-Arabinofuranosyluracil 5′-Triphosphate: Substituent Effects on Inhibitory Action†

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ABSTRACT: The effects of 1-β-D-arabinofuranosyluracil 5′-triphosphate (ara-UTP) and a series of its 5-alkylated derivatives on the activity of deoxyribonucleic acid (DNA) polymerases from mouse cells and oncornavirus were examined in comparison with those of 1-β-D-arabinofuranosylthymine 5′-triphosphate (ara-TTP) [Matsukage, A., Ono, K., Ohashi, A., Takahashi, T., Nakayama, C., & Saneyoshi, M. (1978) *Cancer Res. 38*, 3076–3079]. The newly synthesized and tested compounds in the present studies were ara-UTP, 1-β-D-arabinofuranosyl-5-ethyluracil 5′-triphosphate (ara-EUTP), 1-β-D-arabinofuranosyl-5-propyluracil 5′-triphosphate (ara-PUTP), and 1-β-D-arabinofuranosyl-5-butyluracil 5′-triphosphate (ara-BUTP). These compounds did not replace dTTP as a substrate for the DNA polymerases but inhibited the activities of DNA polymerases α, β, and γ. Inhibition by these compounds was due to competition or partial competition with dTTP for the substrate-binding site of the enzyme. The inhibition constant (K_i) varied depending on the combination of inhibitor, template·primer, and enzyme species. The polymerases, however, showed the following characteristics different from each other in response to these inhibitors. (1) DNA polymerase α was more sensitive to ara-UTP and ara-TTP than to ara-EUTP, ara-PUTP, and ara-BUTP. (2) ara-EUTP was less inhibitory than the other inhibitors to DNA polymerase β. K_i values of DNA polymerase β for the inhibitors were in the range of 1.4–33 μM and in the order ara-UTP < ara-TTP < ara-EUTP > ara-PUTP ≥ ara-BUTP. (3) In contrast to DNA polymerases α and β, DNA polymerase γ was more sensitive to the inhibitors with longer 5-alkyl side chains. K_i's of these compounds extended over the range of 0.03–18 μM and were ordered ara-UTP > ara-TTP > ara-EUTP > ara-PUTP ≥ ara-BUTP. (4) Oncornaviral DNA polymerase was inhibited only by ara-UTP and ara-TTP. Relationships between the chemical structures of the analogues and their inhibitory effects are discussed according to inductive, steric, and hydrophobic characteristics of the substituents on the 5 position of the uracil nucleus.

D-Arabinosyl nucleosides such as ara-C[1] and ara-A have long been used clinically as potent carcinostatic and antiviral agents. The effectiveness of these compounds was primarily due to inhibition of DNA synthesis. ara-C and ara-A were thought to be converted in vivo to their corresponding triphosphates to inhibit DNA synthesis [see review by Cozzarelli (1977)]. In this triphosphate form, ara-C and ara-A could compete with the respective natural substrate, dCTP or dATP, for the same substrate-binding site on the DNA polymerase (Dicioccio & Srivastava, 1977; Furth & Cohen, 1967, 1968; Müller et al., 1975, 1977; Wist, 1979; Yoshida et al., 1977).

On the contrary, ara-T did not inhibit DNA synthesis of intact mammalian cells (Aswell et al., 1977; Gentry & Aswell, 1975), and ara-G was ineffective in preventing the growth of ascites tumors (Brink & LePage, 1964). Our previous reports (Matsukage et al., 1978a,b; Ono et al., 1977) showed, however, that ara-TTP, a 5-methyl derivative of ara-UTP and an analogue of dTTP, strongly inhibited the activities of DNA polymerases α, β, and γ and oncornaviral DNA polymerase. Therefore, the inhibitory effect of ara-T on herpes virus DNA replication in cultured cells (Aswell et al., 1977; Gentry & Aswell, 1975) can be explained by assuming that the kinase system induced by herpes virus infection converts ara-T to its active form, ara-TTP. This ara-TTP inhibited by competing with dTTP for incorporation into DNA. Similarly, it was shown that ara-GTP was a strong competitive inhibitor to dGTP for all the DNA polymerases tested (Ono et al., 1979b). It was thus concluded that all four kinds of D-arabinosyl nucleoside triphosphates (ara-ATP, ara-CTP, ara-GTP, ara-TTP) could inhibit DNA synthesis.

A series of such derivatives were designed and synthesized in order to obtain further systematic information on the recognition by various DNA polymerases of 5-alkylated ara-UTP derivatives. These alkyl groups have the same inductive effect as and stronger steric and hydrophobic effects on the uracil nucleus than those of the methyl group in ara-TTP (Hansch et al., 1973). These derivatives were examined for their inhibitory effects on various DNA polymerases in comparison with the mother compound ara-UTP.

In this paper, we report that all of the newly synthesized compounds inhibited DNA polymerases α, β, and γ and that only ara-UTP and ara-TTP were inhibitory to RLV DNA polymerase. Furthermore, each of the DNA polymerases was

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[1] Abbreviations used: ara-C, 1-β-D-arabinofuranosylcytosine; ara-A, 9-β-D-arabinofuranosyladenine; ara-T, 1-β-D-arabinofuranosylthymine; ara-G, 9-β-D-arabinofuranosylguanine; ara-TTP, 1-β-D-arabinofuranosylthymine 5′-triphosphate; ara-GTP, 9-β-D-arabinofuranosylguanine 5′-triphosphate; ara-UTP, 1-β-D-arabinofuranosyluracil 5′-triphosphate; ara-EUPT, 1-β-D-arabinofuranosyl-5-ethyluracil 5′-triphosphate; ara-PUTP, 1-β-D-arabinofuranosyl-5-propyluracil 5′-triphosphate; ara-BUTP, 1-β-D-arabinofuranosyl-5-butyluracil 5′-triphosphate; RLV, Rauscher murine leukemia virus; dNTP, deoxynucleoside triphosphate; DNA polymerase, deoxynucleoside triphosphate:DNA deoxynucleotidyltransferase (EC 2.7.7.7); DEAE, diethylaminoethyl; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

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Table I: UV Spectral and Chemical Properties of 5-Alkyl ara-UTP's

analogues	$\lambda_{max}^{H_2O}$ (nm)	phosphate analyses, ϵ(p) calcd	phosphate analyses, ϵ(p) found	R_f's on paper chromatography[a] (R_{UTP}) solvent A	solvent B	solvent C	paper electrophoretic mobilities[b] (R_{UTP})
ara-UTP	262	3300	3000	1.2	1.1		1.0
ara-TTP	268	3200	3100	1.3	1.3		1.0
ara-EUTP	267	3300	2900	1.4	2.5		1.0
ara-PUTP	270	3300	3200	1.8		2.0	0.9
ara-BUTP	271	3300	3200	1.8		2.0	0.9

[a] Paper chromatography was performed by the ascending technique using Toyo No. 51A filter paper. Solvent A is ethanol–0.5 M ammonium acetate, pH 7.5 (1:1 v/v); solvent B is isobutyric acid–0.5 M ammonia (66:34 v/v); solvent C is isobutyric acid–0.5 M ammonia (5:3 v/v). [b] Paper electrophoresis was carried out with Toyo No. 51A filter paper in 30 mM sodium acetate, pH 4.0, or 50 mM sodium citrate, pH 3.0, at 600 V for 40 min.

shown to have a different response to these compounds. These results are discussed relative to the substituent effects on the inhibition of enzyme activity. The information obtained in the present study is useful not only for designing further inhibitors of the DNA polymerases but also for elucidation of the active sites of the enzymes.

Materials and Methods

Preparation of 5-Alkyl ara-UTP's. 1-β-D-Arabinofuranosyl-5-alkyluracils (Nakayama et al., 1979) were phosphorylated by phosphorus oxychloride according to the method of Yoshikawa et al. (1969). The resulting 5′-monophosphate (triethylammonium salt, ~0.01 mmol) was dissolved in 1 mL of anhydrous dimethylformamide, *N,N′*-carbonyldiimidazole (5 equiv) was added to this solution, and the solution was stirred for 2 h at room temperature (Maeda et al., 1977). Then, 4 equiv of methanol was added to decompose the excess reagent, and the solution was stirred further for 30 min. To the reaction mixture was added tri-*n*-butylammonium pyrophosphate (10 equiv) in anhydrous dimethylformamide, and the mixture was stirred for 2 h at room temperature. The reaction mixture was diluted with water to 100 mL and was applied to a column of DEAE-cellulose (bicarbonate form, 1.5 × 18 cm). The column was washed with water, and elution was performed with a linear gradient of triethylammonium bicarbonate, pH 7.9 (0–0.5 M; total volume, 500 mL). Fractions containing 5′-triphosphate were collected and evaporated to dryness. The resulting 5-alkyl ara-UTP's were further purified by paper chromatography or paper electrophoresis. Yields of 5-alkyl ara-UTP's from the corresponding nucleosides were 51–70%.

UV Spectral and Chemical Properties of 5-Alkyl ara-UTP's. UV spectral and chemical properties of 5-alkyl ara-UTP's including $\lambda_{max}^{H_2O}$, phosphate analyses, R_f values on paper chromatography, and paper electrophoretic mobilities are summarized in Table I.

Other Chemicals. [^{3}H]dTTP was purchased from the Radiochemical Centre, Amersham, England. Unlabeled dNTP's and $(dT)_{12-18}$ were obtained from Boehringer Mannheim GmbH, Mannheim, West Germany. $(rA)_n$ was the product of Miles Laboratories, Elkhart, IN, and $(dA)_n$ was from P-L Biochemicals, Inc., Milwaukee, WI. Calf thymus DNA from Sigma Chemical Co., St. Louis, MO., was activated according to the method described earlier (Schlabach et al., 1971). DEAE-cellulose paper (DE81) was from Whatman Ltd., Springfield Mill, Maidstone, Kent, England.

Preparation of DNA Polymerases. DNA polymerases α and γ were purified from mouse myeloma MOPC 104E as previously described (Matsukage et al., 1975, 1976), and the preparations obtained from the DNA–cellulose column chromatography were used throughout the present studies. Nearly homogeneous DNA polymerase β was obtained from rat ascites hepatoma AH130 cells as previously described (Ono et al., 1979a). RLV was obtained from the culture medium of an established virus-producing cell line, R-17, and RNA-dependent DNA polymerase was purified according to the method described earlier (Nakajima et al., 1974).

Assays for DNA Polymerase Activity. DNA polymerase α was assayed with activated calf thymus DNA or $(dA)_n \cdot (dT)_{12-18}$ as the template·primer. DNA polymerases β and γ were assayed with activated calf thymus DNA, $(rA)_n \cdot (dT)_{12-18}$, or $(dA)_n \cdot (dT)_{12-18}$. RLV DNA polymerase was assayed with activated calf thymus DNA or $(rA)_n \cdot (dT)_{12-18}$. All assay conditions (summarized in Table II) were optimized with respect to the ratios and concentrations of all the template·primers used as well as to pH and divalent and monovalent cation concentrations. All incubations (50 μL) were carried out at 37 °C for 10–30 min, and the reaction was stopped by adding 15 μL of 0.2 M EDTA and immersing the mixture in ice. Then, 50 μL of the mixture was transferred to DE81 fiber paper and processed for counting radioactivity as previously described (Lindell et al., 1967). The concentrations of [^{3}H]dTTP and the inhibitor were varied on experiments on the K_m (Michaelis constant), K_i (inhibition constant), and mode of inhibition. In all of the kinetic experiments, the incorporation of [^{3}H]dTMP was proportional to the incubation time.

Results

Effects of ara-UTP and 5-Alkylated ara-UTP's on Mouse Cellular DNA Polymerases α, β, and γ and DNA Polymerase from Oncornavirus. ara-UTP and 5-alkylated ara-UTP's were tested as substrates for DNA synthesis. As shown in Figure 1A, DNA polymerase α incorporated 17 pmol of [^{3}H]dGMP into DNA in 30 min in the complete system, which contained all four dNTP's and activated calf thymus DNA. When 5 μM ara-UTP was added to the complete system, ~50% inhibition was observed throughout the reaction period. Furthermore, when 5 μM ara-UTP was added and dTTP was omitted, only 1.7 pmol of [^{3}H]dGMP was incorporated in 30 min. This value was much less than that of the complete system and less than that observed when neither dTTP nor ara-UTP was added (4 pmol). These results indicate that ara-UTP was not effective in place of dTTP as a substrate for DNA polymerase α. ara-BUTP, also, could not substitute for dTTP as a substrate for DNA polymerase α (Figure 1B). Similar results showing that ara-EUTP and ara-PUTP could not substitute for dTTP were obtained with DNA polymerases β and γ (Figure 1C,D). In the case of RLV DNA polymerase, no inhibition by these 5-alkylated ara-UTP's was observed with activated calf thymus DNA as the template·primer (data not shown).

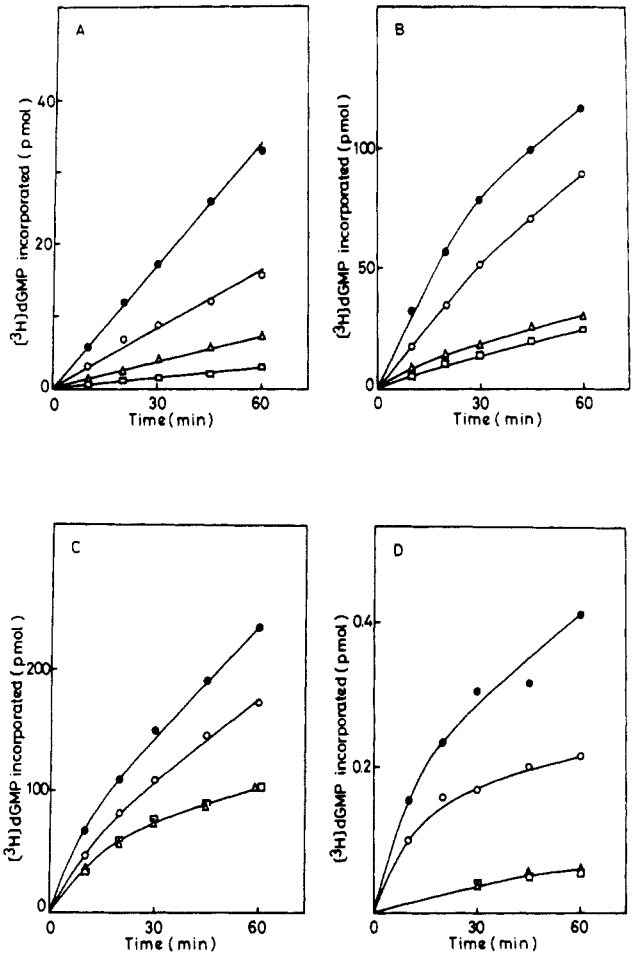

FIGURE 1: Effects of ara-UTP and 5-alkylated ara-UTP's as substrate or inhibitor of various DNA polymerases. DNA polymerase activity was measured by determining incorporation of [^{3}H]dGMP with activated calf thymus DNA as template·primer under the assay conditions described under Materials and Methods. Reactions (200 μL) were started by adding enzyme, and 30-μL aliquots taken at the times indicated were directly applied to DE81 filter paper and processed for counting as described in the text. Complete system containing all four dNTP's without arabinosyl compound (●); complete system plus arabinosyl compund (○); complete system minus dTTP (△); complete system minus dTTP plus arabinosyl compound (□). DNA polymerases and arabinosyl compounds tested are as follows: (A) DNA polymerase α and 5 μM ara-UTP; (B) DNA polymerase α and 10 μM ara-BUTP; (C) DNA polymerase β and 50 μM ara-EUTP; (D) DNA polymerase γ and 5 μM ara-PUTP. The specific activity of [^{3}H]dGTP was 200 cpm/pmol in (A), (B), and (C) and 2640 cpm/pmol in (D).

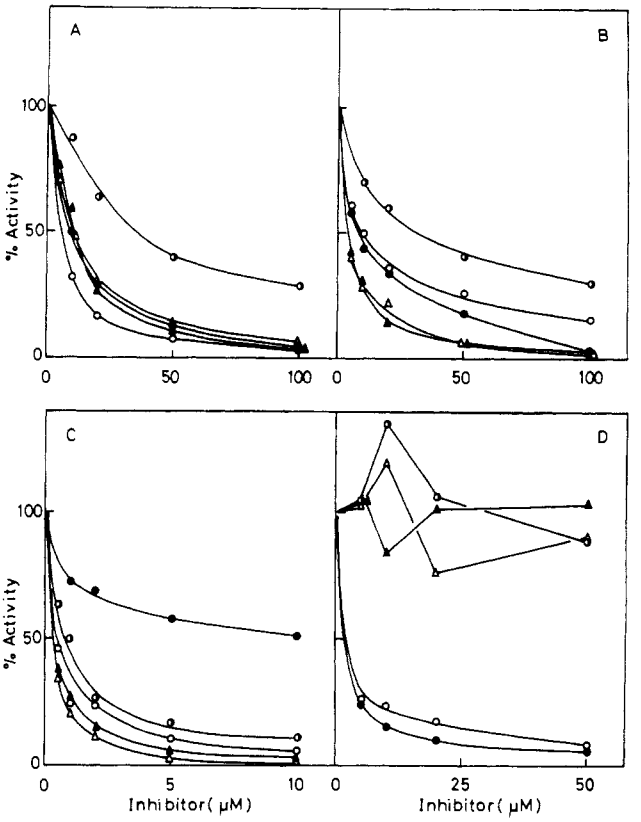

FIGURE 2: Effects of ara-UTP and 5-alkylated ara-UTP's on the activities of various DNA polymerases. The enzymes used were DNA polymerases α (A), β (B), and γ (C) and RLV DNA polymerase (D). DNA polymerase activities were measured under the conditions described under Materials and Methods by determining incorporation of [^{3}H]dTMP with either activated calf thymus DNA (A) or $(rA)_n$·$(dT)_{12-18}$ (B, C, and D). Incubation times were 30, 20, 10, and 20 min in (A–D), respectively. Concentrations of inhibitors are indicated in the figure. ara-UTP (●), ara-TTP (○), ara-EUTP (◑), ara-PUTP (△), and ara-BUTP (▲). The specific activities of [^{3}H]dTTP were 1000, 600, 6000, and 180 cpm/pmol in (A–D), respectively. 100% values (pmol) were 23.3 in (A), 180.2 in (B), 16.7 in (C), and 31.3 in (D).

ara-UTP and 5-alkylated ara-UTP's strongly inhibited the polymerization of normal substrate. As shown in Figure 2 and Table III, these compounds inhibited all the activities tested: DNA polymerases α, β, and γ with activated calf thymus DNA or with $(dA)_n$·$(dT)_{12-18}$ as the template·primer; DNA polymerases β and γ and viral DNA polyerases with $(rA)_n$·$(dT)_{12-18}$. Exceptionally, these compounds did not inhibit viral DNA polymerase with activated calf thymus DNA as the template·primer (data not shown). Viral DNA polymerase was inhibited only by ara-UTP and ara-TTP with $(rA)_n$·$(dT)_{12-18}$ and slightly inhibited by ara-UTP with activated calf thymus DNA (Table III). Results showed that a relatively low concentration of ara-UTP or its 5-alkylated derivatives was enough to inhibit the incorporation of dTTP, especially when synthetic polynucleotides were used as template·primers.

Analysis of Mode of Inhibition and Determination of Kinetic Constants. The activity of DNA polymerase which had been preincubated with an inhibitor was restored by the addition of excess dTTP (data not shown). Since this indicates that the inhibitor was bound to the enzyme reversibly, the reaction was analyzed kinetically. Typical examples of DNA polymerases α, β, and γ with ara-UTP, ara-EUTP, ara-PUTP, and ara-BUTP are shown in Figure 3. Inhibition of DNA polymerase α by ara-UTP was examined with activated calf thymus DNA and [^{3}H]dTTP. The double-reciprocal plot of Figure 3A shows the K_m for dTTP was 9.2 μM and ara-UTP inhibited by competition with dTTP. The K_i of ara-UTP for DNA polymerase α was 0.5 μM from a Dixon plot (Table III). The competition between dTTP and ara-UTP indicates that both nucleotides bind to the same active site of DNA polymerase α. A competitive type of inhibition was also observed with a combination of DNA polymerase β and ara-EUTP (Figure 3B) as well as with that of DNA polymerase γ and ara-PUTP or ara-BUTP (Figure 3C,D). However, the kinetics displayed in Figure 3A,D were not simply competitive but rather showed complexity as indicated by the concave curves. The results imply that, under the assay conditions with ara-UTP or ara-BUTP, DNA polymerases α (Figure 3A) and γ (Figure 3D) reveal a cooperative property among multiple dTTP-binding sites of these enzymes. In addition, ara-UTP, ara-TTP, and ara-EUTP caused a mixed type of inhibition of DNA polymerase α with $(dA)_n$·$(dT)_{12-18}$. An example with ara-TTP is shown in Figure 4. K_i values for these compounds were also lower than the K_m for dTTP (7.8 μM) Table III).

Table II: Assay Conditions for Various DNA Polymerases[a]

DNA polymerase	template·primer	reaction components and their concn: template·primer concn[b] (μg/mL)	Tris buffer pH	Tris buffer concn (mM)	[³H]-dTTP concn (μM)	unlabeled dNTP concn (μM)	divalent cation and concn (mM)	monovalent cation (K^+) concn (mM)	DTT[c] concn (mM)
α	activated calf thymus DNA	80	8.5	50	10	other three dNTP's, 10 each	Mg^{2+}, 4		1
	$(dA)_n\cdot(dT)_{12-18}$	20 (5:1)[d]	7.5	50	10		Mn^{2+}, 0.5	50	1
β	activated calf thymus DNA	200	9.0	100	10	other three dNTP's, 10 each	Mg^{2+}, 10	30	5
	$(rA)_n\cdot(dT)_{12-18}$	10 (1:2)[e]	9.0	100	10		Mn^{2+}, 0.2	100	5
	$(dA)_n\cdot(dT)_{12-18}$	6 (2:1)	9.0	100	10		Mn^{2+}, 0.2	100	5
γ	activated calf thymus DNA	100	7.5	100	1	other three dNTP's, 10 each	Mg^{2+}, 5	150	5
	$(rA)_n\cdot(dT)_{12-18}$	10 (10:1)	7.5	100	1		Mn^{2+}, 0.1	70	5
	$(dA)_n\cdot(dT)_{12-18}$	10 (10:1)	8.5	100	1		Mg^{2+}, 2	5	5
Rauscher leukemia viral	activated calf thymus DNA	100	8.0	100	10	other three dNTP's, 10 each	Mg^{2+}, 10	30	5
	$(rA)_n\cdot(dT)_{12-18}$	10 (10:1)	8.5	100	10		Mn^{2+}, 0.2	130	5

[a] All reaction mixtures except those for viral enzyme contained 15% (v/v) glycerol and 400 μg of bovine serum albumin per milliliter. [b] Concentration with respect to the template in the case of synthetic homopolymers. [c] DTT, dithiothreitol. [d] Numbers in parentheses are base ratios of template to primer. [e] Details of this condition appear elsewhere (Ono et al., 1979a).

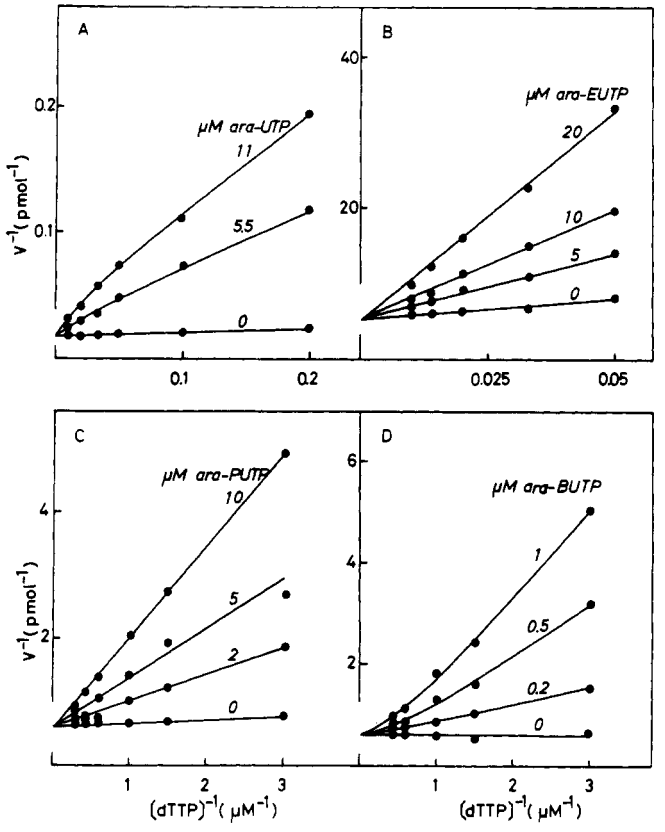

FIGURE 3: Analysis of inhibition of various DNA polymerases by ara-UTP and its 5-alkylated derivatives. The enzymes used were DNA polymerases α (A), β (B), and γ (C and D). All reactions were carried out for 10 min with either activated calf thymus DNA (A and C), $(dA)_n\cdot(dT)_{12-18}$ (B), or $(rA)_n\cdot(dT)_{12-18}$ (D) under the assay conditions described under Materials and Methods, except that various concentrations of [³H]dTTP [360 cpm/pmol in (A), 80 cpm/pmol in (B), and 6000 cpm/pmol in (C) and (D)] were used as substrate in the presence of various concentrations of ara-UTP (A), ara-EUTP (B), ara-PUTP (C), and ara-BUTP (D) as indicated in the figure. The figure represents double-reciprocal plots.

Summary of Effects of ara-UTP and 5-Alkylated ara-UTP's on Mouse Cellular and Viral DNA Polymerases. The K_m's, K_i's, and modes of inhibition of all the compounds tested are summarized in Table III. All the DNA polymerase activities, measured by following incorporation of [³H]dTMP, were inhibited, in most cases competitively (competitive type inhibition) or in some cases partially competitively (mixed type inhibition), by these compounds. Furthermore, except for DNA polymerase β and γ and viral enzyme with activated calf thymus DNA, the K_i's for these inhibitors were lower than the K_m's for dTTP. These results indicate that, in most cases, 5-alkylated derivatives of ara-UTP have a stronger affinity than does dTTP for the substrate-binding site of DNA polymerases.

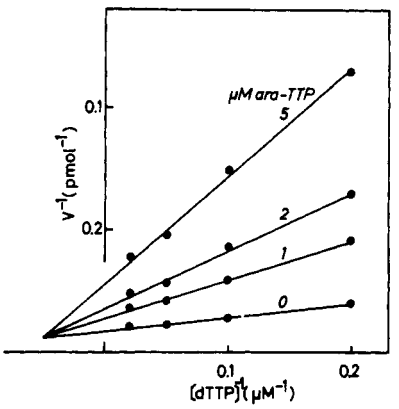

FIGURE 4: Analysis of inhibition of DNA polymerase α by ara-TTP. Reactions were carried out for 10 min with $(dA)_n\cdot(dT)_{12-18}$ under the conditions described in the text, except that various concentrations of [³H]dTTP (360 cpm/pmol) were used in the presence of 0, 1, 2, and 5 μM ara-TTP. The figure represents a double-reciprocal plot.

From the results in Table III, three characteristics with respect to the combinations of DNA polymerase and template·primer can be drawn: (1) in the cases of DNA polymerase α with activated calf thymus DNA and DNA polymerase β with $(rA)_n\cdot(dT)_{12-18}$, ara-EUTP is less effective than ara-UTP and ara-TTP, but the inhibitors with longer 5-alkyl side chains (ara-PUTP and ara-BUTP) are more effective than ara-EUTP; (2) inhibitors with longer 5-alkyl side chains are effective than those with shorter side chains on DNA polymerase γ with activated DNA and $(rA)_n\cdot(dT)_{12-18}$; (3) only ara-UTP and ara-TTP are inhibitory to RLV DNA polymerase with $(rA)_n\cdot(dT)_{12-18}$. These results clearly indicate that the DNA polymerases have properties different from each other in re-

Table III: Characterization of Inhibition of Various DNA Polymerases by ara-UTP and 5-Alkylated ara-UTP's

DNA polymerase	template·primer	K_m for [^{3}H]dTTP (μM)	inhibition by ara-UTP K_i (μM)	mode[a]	ara-TTP K_i (μM)	mode	ara-EUTP K_i (μM)	mode	ara-PUTP K_i (μM)	mode	ara-BUTP K_i (μM)	mode
α	activated calf thymus DNA	9.2	0.5	C	0.3	C	12	C	5	C	4	C
	$(dA)_n \cdot (dT)_{12-18}$	7.8	0.5	M	0.55	M	1.2	M	2.5	C	3.0	C
β	activated calf thymus DNA	8.3	5.5	C	17	C	33	C	13	C	16	C
	$(rA)_n \cdot (dT)_{12-18}$	45	4.5	C	5	C	16	C	3.5	C	1.5	C
	$(dA)_n \cdot (dT)_{12-18}$	5.0	1.4	C	1.7	C	1.9	C	1.5	C	1.5	C
γ	activated calf thymus DNA	0.29	18	C	4	C	3	C	0.5	C	0.4	C
	$(rA)_n \cdot (dT)_{12-18}$	0.18	0.33	C	0.06	C	0.05	C	0.03	C	0.03	C
	$(dA)_n \cdot (dT)_{12-18}$	2.0	1.0	C	0.32	C	0.27	C	0.34	C	0.25	C
Rauscher leukemia viral	activated calf thymus DNA	18.9	110	M		NI		NI		NI		NI
	$(rA)_n \cdot (dT)_{12-18}$	24	1.5	C	2.0	C		NI		NI		NI

[a] C, competitive; M, mixed; NI, no inhibition.

Table IV: Various Constants of Aromatic Substituents[a]

5 substituent	F[b]	π[b]	MR[b]
H	0	0	0.103
CH_3	−0.04	0.56	0.565
CH_2CH_3	−0.05	1.02	1.030
$CH_2CH_2CH_3$	−0.06	1.55	1.496
$CH_2CH_2CH_2CH_3$	−0.06		1.959

[a] Taken from Hansch et al. (1973). [b] F, polar constant representing inductive effects; π, lipophilic constant denoting hydrophobic effects; MR, molar refractivity scaled by 0.1, representing steric effects.

sponse to the 5-substituted ara-UTP's.

Discussion

ara-UTP and all of its 5-alkylated derivatives examined in this study were shown to be competitive inhibitors of dTTP for DNA polymerases α, β, and γ. The extent of inhibition varied, however, depending on the combination of inhibitor, template·primer, and enzyme species. As shown in Table III, DNA polymerase α, with activated DNA as the template·primer, was strongly inhibited by ara-UTP and ara-TTP. The addition of one carbon unit to the methyl group caused a 40-fold larger K_i value than that of ara-TTP. This large K_i value decreased again to $^1/_2$–$^1/_3$ of that of ara-EUTP for *n*-propyl and *n*-butyl substituents. This finding suggests the apparent importance of the hydrophobic effect of *n*-propyl and *n*-butyl substitution on the K_i value. In the case of ethyl substitution, steric interference might have occurred in the interaction between the analogue and the active site of DNA polymerase α and consequently given a larger K_i value than that of ara-TTP. When $(dA)_n \cdot (dT)_{12-18}$ was used as a template·primer, however, only a weak steric but no hydrophobic effect of the substituent was observed. Hansch and co-workers have previously well reviewed and summarized the substituent constants necessary for correlating structure with reactivity (Hansch et al., 1973). The data in Table IV are inductive (F), hydrophobic (π), and steric (MR) parameters of the 5-alkylated derivatives of ara-UTP studied.

In the case of DNA polymerase β, an interesting difference was observed between two template-primers, activated DNA and $(rA)_n \cdot (dT)_{12-18}$. When activated DNA was used, the resulting K_i value showed the contribution of steric effects of the substituents, because all the substituents with methyl to butyl groups gave similar larger K_i's than that of ara-UTP. The similar results that 5-alkyl substitutents have larger K_i's than that of ara-UTP were also obtained when $(dA)_n \cdot (dT)_{12-18}$ was used as template·primer. With these template·primers, the steric effect of the alkyl substituent may cause the change in affinity of the analogue to the active site of DNA polymerase β. On the other hand, when $(rA)_n \cdot (dT)_{12-18}$ was used as a template·primer, K_i values of *n*-propyl and *n*-butyl derivatives were shown to be much smaller than that of the ethyl derivative, indicating that a hydrophobic interaction is involved in the combination of $(rA)_n \cdot (dT)_{12-18}$ and *n*-propyl or *n*-butyl derivative. The difference in K_i values with activated DNA from those with $(rA)_n \cdot (dT)_{12-18}$ may depend on the template used, because both activated DNA and $(dA)_n \cdot (dT)_{12-18}$ are polydeoxyribonucleotide templates, whereas $(rA)_n \cdot (dT)_{12-18}$ is a polyribonucleotide template.

In contrast to DNA polymerases α and β, polymerase γ showed a unique response to these 5-alkyl substituents. When activated DNA was used as a template·primer, the inhibitory effect was increased by increasing carbon units of the alkyl group on the 5-position of ara-UTP. The K_i value gradually decreased from 18 (ara-UTP) to 4 μM for the methyl substituent and further to almost 0.4 μM for *n*-propyl and *n*-butyl substituents. These results suggest that hydrophobic interactions and steric effects of the substituents affect the affinities of these compounds to DNA polymerase γ. Thus, the active site of polymerase γ seems to be permissive to these longer alkyl groups, since no steric hindrance indicated by smaller K_i values was observed by extension of the 5-alkyl side chain. Similar results that the analogues with longer 5-alkyl substituents have a stronger affinity to polymerase γ were obtained with $(rA)_n \cdot (dT)_{12-18}$ as the template·primer. Exceptionally, no significant difference was observed between the K_i of ara-TTP and those of the other three 5-alkylated ara-UTP's when examined with $(dA)_n \cdot (dT)_{12-18}$ as the template·primer.

In the case of oncornaviral DNA polymerase, ara-UTP and ara-TTP gave K_i values smaller than the K_m of dTTP ($^1/_{16}$ and $^1/_{12}$, respectively) when assayed with $(rA)_n \cdot (dT)_{12-18}$ as the template·primer, while the ethyl to *n*-butyl derivatives were completely inactive. Thus, oncornaviral DNA polymerase seems to be most strict with regard to substrate analogues and is nonpermissive to derivatives with side chains longer than the methyl group.

Each of the DNA polymerases was shown to have specific characteristics in response to a series of 5-alkylated derivatives of ara-UTP. This response varied depending on the template·primer, suggesting that functional expresssion of the active

site(s) of DNA polymerase is (are) directed by the template, probably through conformational change of the enzyme molecule. Thus, these analogues may be useful for identification of the DNA polymerases. Among the compounds, ara-PUTP and ara-BUTP, having smaller K_i values, are expected to be strong and specific inhibitors for DNA polymerase γ. Furthermore, these analogues may be useful for understanding structural features of the active sites of various DNA polymerases. For example, the fact that DNA polymerase γ is highly reactive with various 5-alkyl substituents of ara-UTP probably through hydrophobic interactions suggests that presence of the corresponding hydrophobic counterpart in the active site of the enzyme.

The results presented in this paper were obtained from in vitro experiments with purified DNA polymerases and arabinosylnucleoside triphosphates, and, therefore, the data do not give direct information for in vivo utilization of ara-U derivatives. However, if the cellular kinase system can phosphorylate ara-U and its 5-alkylated derivatives, the resulting triphosphates are expected to inhibit DNA synthesis in vivo. By analogy with the case of ara-T (Aswell et al., 1977; Gentry & Aswell, 1975), herpes virus infected cells appear to induce thymidine kinase active on ara-U and its derivatives. Alternatively, 5-alkylated ara-U analogues may be formed from the corresponding ara-C derivatives by the action of deaminases as observed with the conversion of ara-C to ara-U or ara-UMP in vivo experiments with mice (Chou et al., 1975). These are the further problems to be studied. Anyway, the information obtained in the present studies should be quite useful for designing new nucleotide analogues that may be species-specific inhibitors of the DNA polymerases.

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