Inhibitory Effects of Various 5-Halogenated Derivatives of 1-β-D-Arabinofuranosyluracil 5′-Triphosphate on DNA Polymerases from Murine Cells and Oncornavirus: Substituent Effects on Inhibitory Action†

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ABSTRACT: The effects of four 5-halogenated derivatives of 1-β-D-arabinofuranosyluracil 5′-triphosphate (aUTP) on the activity of DNA polymerases from mouse cells and oncornavirus were examined in comparison with that of the mother compound, aUTP, and also with those of the sister compounds, 5-alkylated derivatives of aUTP [Ono, K., Ohashi, A., Ogasawara, M., Matsukage, A., Takahashi, T., Nakayama, C., & Saneyoshi, M. (1981) *Biochemistry 20*, 5088]. The newly synthesized and tested compounds in this study were 1-β-D-arabinofuranosyl-5-fluorouracil 5′-triphosphate (aFUTP), 1-β-D-arabinofuranosyl-5-chlorouracil 5′-triphosphate (aClUTP), 1-β-D-arabinofuranosyl-5-bromouracil 5′-triphosphate (aBrUTP), and 1-β-D-arabinofuranosyl-5-iodouracil 5′-triphosphate (aIUTP). The results were summarized as follows: (1) These compounds could not be used as substrate in place of dTTP but inhibited the activities of DNA polymerases α, β, and γ and viral DNA polymerase. (2) Inhibition by these compounds was due to competition with dTTP for the substrate-binding site of the enzyme. (3) The inhibition constants (K_i's) of DNA polymerase α for aFUTP, aBrUTP, and aIUTP were in a similar range (0.3–0.6 μM), whereas K_i values for aClUTP (1.33–1.45 μM) were higher than those for the other inhibitors. (4) K_i's of DNA polymerase β for these compounds varied depending on the template·primer. With activated DNA, K_i's were in the range of 13–60 μM, whereas those with $(dA)_n \cdot (dT)_{12-18}$ (0.7–2.0 μM) were much lower than those with activated DNA and those with $(rA)_n \cdot (dT)_{12-18}$ (1.5–10.5 μM). (5) Viral DNA polymerase was inhibited by these compounds only with $(rA)_n \cdot (dT)_{12-18}$ as the template·primer, and the K_i values extended over the range 5–120 μM. (6) In general, DNA polymerases α and β and viral DNA polymerase showed similar characteristics in response to these inhibitors; aClUTP was less inhibitory to these polymerases than the others, and the K_i values were usually ordered as follows: aFUTP < aClUTP > aBrUTP ≥ aIUTP. (7) On the contrary, the inhibitory effects of these compounds on DNA polymerase γ increased by introducing a halogen of higher atomic weight on the 5 position of the base. K_i's of DNA polymerase γ for these inhibitors showed the lowest values with $(rA)_n \cdot (dT)_{12-18}$ as the template·primer (0.015–0.25 μM), which were much lower than those with $(dA)_n \cdot (dT)_{12-18}$ (0.15–6.0 μM) and those with activated DNA (1–27 μM). Relationships between the chemical structures of the analogues and their inhibitory effects are discussed according to inductive, hydrophobic, and steric characteristics of the substituents on the 5 position of the uracil nucleus. It was concluded that both steric and hydrophobic effects of the halogen substituents were important for the inhibition of all DNA polymerases and that the inductive effect of halogen atoms was clearly observed for DNA polymerase β with activated DNA and for RLV DNA polymerase with $(rA)_n \cdot (dT)_{12-18}$.

Inhibitors of DNA replication are useful not only for the chemotherapy or prophylaxis of neoplasia but also for an understanding of the mechanism of DNA synthesis. Among various kinds of DNA polymerase inhibitors, a nucleotide analogue such as aCTP[1] or ddTTP has been used as a powerful tool for the characterization and identification of each of the DNA polymerases (Waqar et al., 1978; Edenberg et al., 1978). By the use of these nucleotide inhibitors and aphidicolin, a tetracyclic diterpenoid (Ohashi et al., 1978), enzymatic properties and biological roles of the DNA polymerases α, β, and γ have been clarified.

Our current interest has been focused on finding new nucleotide inhibitors and modifying their structure to obtain more specific inhibitors for each of the eukaryotic DNA polymerases. During the work on this line, we found that aTTP had a strong inhibitory effect on DNA polymerases from murine cells and oncornavirus (Matsukage et al., 1978a,b). In addition, we recently demonstrated that several 5-alkylated derivatives of aUTP such as aEUTP, aPUTP, and aBUTP exerted differential inhibitory action on various DNA polymerases. For example, DNA polymerase γ was more sensitive to the inhibitors with longer 5-alkyl side chains, while aEUTP was less inhibitory than other inhibitors to DNA polymerases α and β and to oncornaviral DNA polymerase (Ono et al., 1981). These 5-alkyl substituents have similar electron-donating effects on the uracil moiety of the nucleotide analogues (Hansch et al., 1973). From the results, we concluded that

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[1] Abbreviations: aCTP, 1-β-D-arabinofuranosylcytosine 5′-triphosphate; ddTTP, 2′,3′-dideoxythymidine 5′-triphosphate; aTTP, 1-β-D-arabinofuranosylthymine 5′-triphosphate; aEUTP, 1-β-D-arabinofuranosyl-5-ethyluracil 5′-triphosphate; aPUTP, 1-β-D-arabinofuranosyl-5-propyluracil 5′-triphosphate; aBUTP, 1-β-D-arabinofuranosyl-5-butyluracil 5′-triphosphate; aU, 1-β-D-arabinofuranosyluracil; aClU, 1-β-D-arabinofuranosyl-5-chlorouracil; aBrU, 1-β-D-arabinofuranosyl-5-bromouracil; aIU, 1-β-D-arabinofuranosyl-5-iodouracil; aUTP, 1-β-D-arabinofuranosyluracil 5′-triphosphate; aFUTP, 1-β-D-arabinofuranosyl-5-fluorouracil 5′-triphosphate; aClUTP, 1-β-D-arabinofuranosyl-5-chlorouracil 5′-triphosphate; aBrUTP, 1-β-D-arabinofuranosyl-5-bromouracil 5′-triphosphate; aIUTP, 1-β-D-arabinofuranosyl-5-iodouracil 5′-triphosphate; RLV, Rauscher murine leukemia virus; DNA polymerase, deoxynucleoside triphosphate:DNA deoxynucleotidyltransferase (EC 2.7.7.7); EDTA, ethylenediaminetetraacetic acid.

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Table I: Physical Constants and Elemental Analysis of 5-Halogenated aU's

5-halogenated aU	mp (°C)	elemental composition	calcd (found) for C	H	N	X[b]
aClU	235–237	$C_9H_{11}N_2O_6Cl$	38.78 (38.69)	3.99 (3.94)	10.05 (10.14)	12.72 (12.56)
aBrU	195–198[a]	$C_9H_{11}N_2O_6Br$	33.45 (33.27)	3.44 (3.40)	8.67 (8.53)	24.73 (24.47)
aIU	224–227[a]	$C_9H_{11}N_2O_6I$	29.20 (29.23)	3.00 (3.17)	7.57 (7.43)	34.2 (34.23)

[a] Decomposition occurs at these temperatures. [b] X represents halogen atoms.

Table II: UV Spectral and Chemical Properties of 5-Halogenated aUTP's

analogue	$\lambda_{max}^{H_2O}$ (nm)	phosphate analysis ε (P) calcd	found	R_f on paper chromatography[a] (R_{UTP}) solvent A	solvent B	paper electrophoretic mobility[b] (R_{UTP})
aFUTP	270	2700	2900	1.0	1.2	1.0
aClUTP	278	3300	3000	1.1	1.1	0.9
aBrUTP	281	3100	2800	1.0	1.1	1.0
aIUTP	288	2500	2400	1.2	1.2	0.9

[a] Paper chromatography was performed by ascending technique using Toyo No. 51A filter paper. Solvent A is isobutyric acid–0.5 M ammonium hydroxide (5:3 v/v); solvent B is ethanol–0.5 M ammonium acetate, pH 7.5 (1:1 v/v). [b] Paper electrophoresis was carried out with Toyo No. 51A filter paper in 50 mM sodium acetate, pH 4.0, or 50 mM sodium citrate, pH 3.5, at 600 V for 40 min.

both steric and hydrophobic characteristics of the 5-alkyl groups greatly affected the inhibitory powers of these compounds and consequently gave differential inhibitory effects, depending on the different DNA polymerase species (Ono et al., 1981).

In contrast to the 5-alkylated compounds, halogen substituents on the 5 position of aUTP have an electron-withdrawing effect on the uracil moiety, but they still have considerable hydrophobic and steric effects like 5-alkylated aUTP's (Hansch et al., 1973). It should be pointed out that 5-halogenated aU's have been reported to have antiviral activity against some DNA viruses such as herpes simplex virus, vaccinia virus (Renis et al., 1968; Renis, 1970), and cytomegalovirus (Sidwell et al., 1972). These observations prompted us to examine whether and how 5-halogenated aUTP's inhibit the activity of DNA polymerases.

The present study describes the characteristic properties of these 5-halogenated aUTP's in the inhibitory action on the DNA polymerases. The results are discussed in comparison with those of our previous study on inhibitory effects of 5-alkyl aUTP's on various DNA polymerases (Ono et al., 1981). The findings provide new information for designing further inhibitors of the DNA polymerases.

Materials and Methods

Preparation of 5-Halogenated aUTP's. 1-β-D-Arabinofuranosyl-5-fluorouracil was synthesized as reported previously (Saneyoshi et al., 1978). Other 5-halogenated aU's were prepared by direct halogenation methods (Prusoff, 1959; Visser et al., 1960; Wang, 1962). The physical constants and the results of elemental analysis of these compounds were listed in Table I. Phosphorylation of these 5-halogenated arabinosylnucleosides was performed under the similar conditions as reported previously (Ono et al., 1981).

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

Other Chemicals. [^{3}H]dTTP was purchased from the Radiochemical Centre, Amersham, England. Unlabeled deoxynucleoside triphosphates 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 (Matsukage et al., 1976) and γ (Matsukage et al., 1975) were purified from mouse myeloma MOPC 104E as previously described, and preparations obtained from DNA–cellulose column chromatography were used throughout the present studies. Nearly homogeneous DNA polymerase β was purified 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 (Ishimoto et al., 1971), and RNA-dependent DNA polymerase was purified according to the method described earlier (Nakajima et al., 1974).

Assay for DNA Polymerase Activity. DNA polymerase α was assayed with activated calf thymus DNA or $(dA)_n$·$(dT)_{12-18}$ as the template·primer. DNA polymerases β and γ were assayed with activated calf thymus DNA, $(rA)_n$·$(dT)_{12-18}$, or $(dA)_n$·$(dT)_{12-18}$. RLV DNA polymerase was assayed with activated calf thymus DNA or $(rA)_n$·$(dT)_{12-18}$. Details of the assay conditions which 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 were described previously (Ono et al., 1981). 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 filter paper and processed for counting radioactivity as previously described (Lindell et al., 1967). The concentrations of [^{3}H]dTTP and the inhibitor were varied in experiments on the K_m, K_i, and mode of inhibition. In all of the kinetic experiments, the incorporation of [^{3}H]dTMP was proportional to the incubation time.

Results

Inhibition of Mouse Cellular DNA Polymerases α, β, and γ and DNA Polymerase from Oncornavirus by 5-Halogenated aUTP's. 5-Halogenated aUTP's were tested for use as sub-

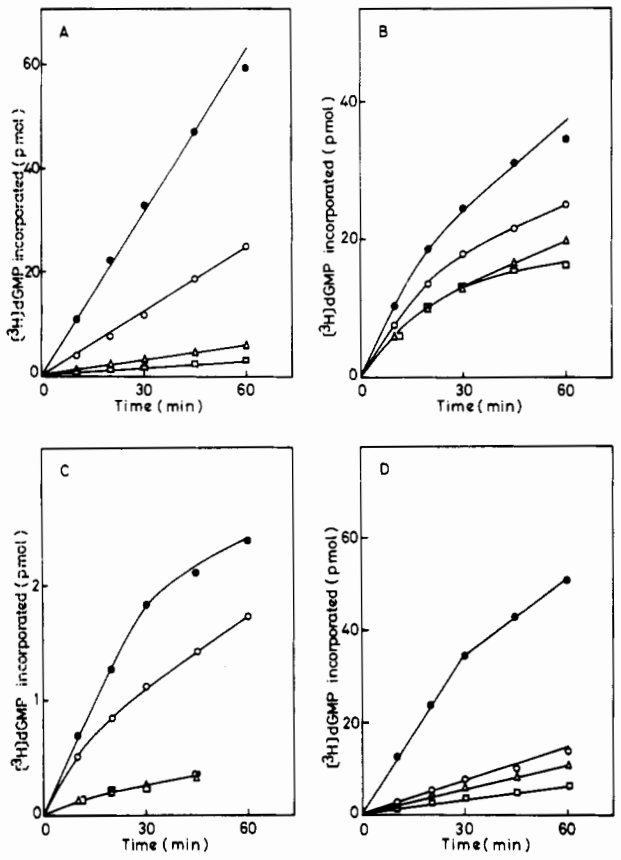

FIGURE 1: Effects of 5-halogenated aUTP's as substrate or inhibitor of various DNA polymerases. DNA polymerase activity was measured by determining incorporation of [^{3}H]dGMP with activated calf thymus DNA as template·primer under the assay conditions described under Materials and Methods. Reactions (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 compound (○); complete system minus dTTP (△); complete system minus dTTP plus arabinosyl compound (□). DNA polymerases and arabinosyl compounds tested are as follows: in (A), DNA polymerase α and 10 μM aFUTP; in (B), DNA polymerase β and 30 μM aClUTP; in (C), DNA polymerase γ and 10 μM aBrUTP; in (D), DNA polymerase α and 10 μM aIUTP. Specific activity of [^{3}H]dGTP was 200 cpm/pmol in (A), (B), and (D) and 6000 cpm/pmol in (C).

strates for DNA synthesis. As shown in Figure 1A, the incorporation of [^{3}H]dGMP by DNA polymerase α in the complete system was inhibited by 64% in the presence of 10 μM aFUTP. When 10 μM aFUTP was added to the reaction without dTTP, only 1.6 pmol of [^{3}H]dGMP was incorporated in 30 min. This value was much less than that of the complete system (32.3 pmol) or that without dTTP and aFUTP (3.1 pmol). These results indicate that aFUTP could not be used in place of dTTP as a substrate for DNA polymerase α. Also, it is seen that aIUTP cannot serve as a substrate for DNA polymerase α (Figure 1D). Similarly, aClUTP could not substitute for dTTP in the reaction by DNA polymerase β, nor could aBrUTP be used as a substrate in the reaction by DNA polymerase γ (Figure 1B,C).

As shown in Figure 2A–D and Table III, all four 5-halogenated aUTP's inhibited all the activities tested: DNA polymerases α, β, and γ with either activated calf thymus DNA or $(dA)_n\cdot(dT)_{12-18}$; also DNA polymerases β and γ and viral DNA polymerase with $(rA)_n\cdot(dT)_{12-18}$. Exceptionally, these compounds slightly enhanced the activity of viral DNA polymerase in the assay system containing activated calf thymus DNA as the template·primer (data not shown). Results showed that concentrations of these compounds relatively

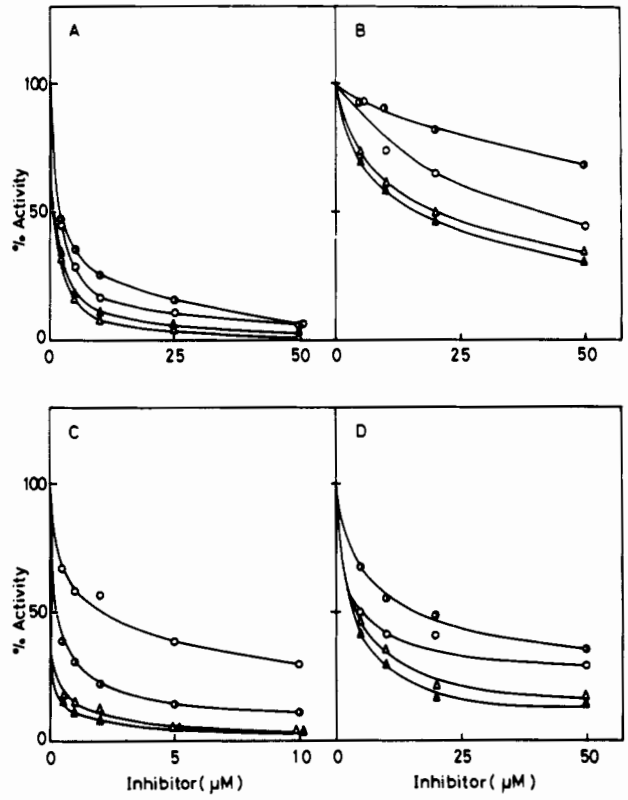

FIGURE 2: Effects of 5-halogenated aUTP's on the activities of various DNA polymerases. The enzymes used were DNA polymerases α (A), β (B), and γ (C) and RLV DNA polymerase (D). DNA polymerase activities were measured under the conditions described under Materials and Methods by determining incorporation of [^{3}H]dTMP with activated calf thymus DNA (A and B) or $(rA)_n\cdot(dT)_{12-18}$ (C and D). Incubation time was 30 min in (A) and 20 min in (B–D). Concentrations of inhibitors are indicated in the figures: aFUTP (○), aClUTP (●), aBrUTP (△), and aIUTP (▲). Specific activities of [^{3}H]dTTP were 400, 600, 6000, and 200 cpm/pmol in (A–D), respectively. One hundred percent values (picomoles) were 13.1 in (A), 33.8 in (B), 1.94 in (C), and 145.9 in (D).

lower than that of dTTP sufficed to inhibit the incorporation of dTTP, especially in the case of DNA polymerase α.

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 binds reversibly to the enzyme, the reaction was analyzed kinetically to examine the mode of inhibition and to determine kinetic constants. Typical examples on DNA polymerase α with aFUTP, DNA polymerase β with aClUTP, DNA polymerase γ with aBrUTP, and viral DNA polymerase with aIUTP are shown in Figure 3A–D. Inhibition of DNA polymerase α by aFUTP 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 aFUTP inhibited by competition with dTTP. The K_i of aFUTP for DNA polymerase α was 0.6 μM from a Dixon plot (Table III). The competition between dTTP and aFUTP indicates that both nucleotides bind to the same active site of DNA polymerase α. A competitive type of inhibition was also observed with the following combinations: DNA polymerase β and aClUTP (Figure 3B), DNA polymerase γ and aBrUTP (Figure 3C), and viral DNA polymerase and aIUTP (Figure 3D). However, the kinetics displayed in Figure 3B,D were not simply competitive but rather showed complexity, as indicated by the concave curves. The results imply that, in the presence of aClUTP or aIUTP, DNA polymerase β (Figure 3B) and viral DNA polymerase (Figure 3D) exhibit a coop-

Table III: Characterization of Inhibition of Various DNA Polymerases by 5-Halogenated aUTP's

DNA polymerase	template·primer	K_m for [^{3}H]-dTTP[a] (μM)	inhibition by aUTP[a] K_i (μM)	mode[b]	aFUTP K_i (μM)	mode	aClUTP K_i (μM)	mode	aBrUTP K_i (μM)	mode	aIUTP K_i (μM)	mode
α	activated calf thymus DNA	9.2	0.5	C	0.6	C	1.45	C	0.30	C	0.53	C
	$(dA)_n\cdot(dT)_{12-18}$	7.8	0.5	M	0.37	C	1.33	C	0.50	C	0.34	C
β	activated calf thymus DNA	8.3	5.5	C	26	C	60	C	13.5	C	13	C
	$(rA)_n\cdot(dT)_{12-18}$	45	4.5	C	3.4	C	10.5	C	3.7	C	1.5	C
	$(dA)_n\cdot(dT)_{12-18}$	12.9	1.4	C	1.1	C	2.0	C	1.0	C	0.7	C
γ	activated calf thymus DNA	0.29	18	C	27	C	1.0	C	1.3	C	1.3	C
	$(rA)_n\cdot(dT)_{12-18}$	0.18	0.33	C	0.25	M	0.10	C	0.022	C	0.015	C
	$(dA)_n\cdot(dT)_{12-18}$	0.31	1.0	C	6.0	M	0.6	C	0.3	C	0.15	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	72	C	120	C	18	C	5	C

[a] Quoted from our previous report (Ono et al., 1981). [b] C, competitive; M, mixed; NI, no inhibition.

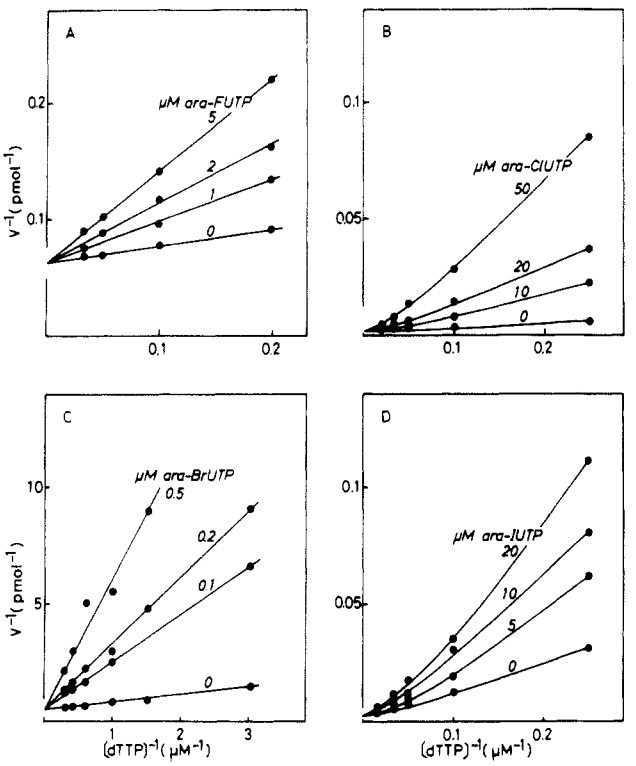

FIGURE 3: Analysis of inhibition of various DNA polymerases by 5-halogenated aUTP's. All reactions were carried out for 10 min under the assay conditions described under Materials and Methods. (A) Inhibition of DNA polymerase α by aFUTP (with activated calf thymus DNA as the template·primer); (B) inhibition of DNA polymerase β by aClUTP [$(dA)_n\cdot(dT)_{12-18}$]; (C) inhibition of DNA polymerase γ by aBrUTP [$(rA)_n\cdot(dT)_{12-18}$]; (D) inhibition of viral DNA polymerase by aIUTP [$(rA)_n\cdot(dT)_{12-18}$]. Concentrations of [^{3}H]dTTP and inhibitors are as indicated in the figures. Specific activity of [^{3}H]dTTP was 180 cpm/pmol in (A), (B), and (D) and 6000 cpm/pmol in (C). All figures represent double-reciprocal plots.

erative property among multiple dTTP-binding sites of these enzymes. In addition, the inhibitions of DNA polymerase α and viral DNA polymerase by aUTP [with $(dA)_n\cdot(dT)_{12-18}$ and activated DNA, respectively] and that of DNA polymerase γ by aFUTP [with $(rA)_n\cdot(dT)_{12-18}$ or $(dA)_n\cdot(dT)_{12-18}$] were not purely competitive but rather of the mixed type, because the straight lines intersected at a point to the left of the origin slightly above the $[dTTP]^{-1}$ axis (data not shown).

Summary of Effects of 5-Halogenated aUTP'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 the subsequent incorporation of [^{3}H]dTMP, were inhibited, in most cases competitively or in some cases partially so (mixed-type inhibition) by these compounds. Furthermore, except for DNA polymerases β and γ with activated calf thymus DNA and viral DNA polymerase with $(rA)_n\cdot(dT)_{12-18}$, the K_i's for these inhibitors were lower than the K_m's for dTTP. These results indicate that, in most cases, 5-halogenated aUTP's have a stronger affinity than dTTP for the substrate-binding site of DNA polymerases.

From the results in Table III, some interesting characteristics of the DNA polymerases can be cited: (1) aClUTP is less effective than other inhibitors to DNA polymerases α and β and to viral DNA polymerase. The K_i values are ordered as follows: aFUTP < aClUTP > aBrUTP ≥ aIUTP. (2) DNA polymerase γ is more sensitive to an inhibitor with a halogen of a higher atomic weight. The K_i values are ordered as follows: aFUTP > aClUTP > aBrUTP ≥ aIUTP. Thus, DNA polymerase γ has properties different from the other DNA polymerases in response to 5-halogenated aUTP's.

Discussion

Inhibitors of eukaryotic DNA polymerases are important not only in medical practice but also in determining the roles of the enzymes in complex DNA synthesizing processes. Among many types of DNA polymerase inhibitors, arabinosylnucleotides such as aCTP or aATP and dideoxynucleotides such as ddTTP have been most frequently used as potent competitive-type inhibitors. Among them, ddTTP was useful to discriminate DNA polymerase α from DNA polymerases β and γ, because this compound selectively inhibited the latter two enzyme species (Waqar et al., 1978; Edenberg et al., 1978). On the other hand, all four D-arabinosylnucleoside triphosphates (aATP, aCTP, aGTP, and aTTP) were as strong but less specific inhibitors of DNA polymerases than ddTTP (Matsukage et al., 1978a,b; Ono et al., 1979b).

In order to obtain more specific inhibitors of eukaryotic DNA polymerases, it was decided, first of all, to modify the structure of aTTP on the 5 position of the uracil nucleus. A series of 5-alkylated derivatives of aUTP were synthesized and tested for the inhibition of various DNA polymerases (Ono et al., 1981).

Successively, a number of 5-halogenated derivatives of aUTP were synthesized for the first time and shown to be strong inhibitors of all the DNA polymerases tested. The inhibitions by these compounds were, in all cases, competitive

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
F	0.43	0.22	0.092
Cl	0.41	0.72	0.603
Br	0.44	0.86	0.888
I	0.40	1.11	1.394

[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.

with respect to the incorporation of dTTP into DNA. Figures 1–3 shown the representative data, and the rest are summarized in Table III.

There are, in general, two major explanations for the inhibitions of DNA polymerases by arabinosylnucleotides: (a) arabinosyl compounds are incorporated into the elongating DNA, leading to inactive primer termini, or (b) these analogues inhibit DNA synthesis by competition at the triphosphate binding site of the DNA polymerase. However, our results in Figures 1 and 3 together with those in our previous reports (Ono et al., 1979b, 1981) indicate that very slight incorporation of arabinosyl compounds, if any, cannot explain the observed inhibitions by these compounds and that the inhibition is mainly due to competition between the triphosphate substrate and the analogue for the same binding site(s) on the enzyme molecule.

The DNA polymerases showed characteristic responses to these inhibitors, and the K_i values for the inhibitors varied depending on the template·primer. As shown in Table III, when activated calf thymus DNA was used as the template·primer, aUTP and aFUTP were strongly inhibitory against DNA polymerase α, and K_i/K_m was 0.054 and 0.064 for aUTP and aFUTP, respectively. The substitution by chlorine at the 5 position of aUTP gave an approximately 3-fold larger K_i value than with aUTP and aFUTP. This large K_i value decreased again when chlorine was replaced by bromine or iodine. With $(dA)_n\cdot(dT)_{12-18}$ as the template·primer, the same order of K_i values as those with activated DNA was obtained. These results suggest the importance of hydrophobic effects of the halogen substituents on the K_i value. The substitution with chlorine might have caused some steric interference between the analogue and the polymerase α molecule. This result resembles that of ethyl substitution in the 5-alkyl series of aUTP which was reported previously from our laboratories (Ono et al., 1981). Hansch et al. have previously 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-halogenated and 5-alkylated derivatives of aUTP studied.

In the case of DNA polymerase β with activated DNA, all the halogenated analogues gave 2–10-fold larger K_i values than that of aUTP. Therefore, it seems that the electron-withdrawing effect of the halogen atom is very crucial, and the halogen substitutions decreased the affinities of the analogues to the enzyme. However, when $(rA)_n\cdot(dT)_{12-18}$ or $(dA)_n\cdot(dT)_{12-18}$ was used as a template·primer, such inductive effects on the polymerase β disappeared completely. These results indicate that the recognition of the analogues by the enzyme changes depending on the template·primer. In addition to the inductive effect, some hydrophobic effects of aBrUTP and aIUTP were also observed with the β polymerase.

When the inhibitions of DNA polymerase γ by 5-halogenated aUTP's were analyzed with activated DNA, a great difference in K_i values was observed between aFUTP and aClUTP. This observation suggests the contribution of steric and hydrophobic interactions of aClUTP, aBrUTP, or aIUTP with the enzyme, because these compounds gave approximately 20–30-fold lower K_i values than aUTP and aFUTP. With $(rA)_n\cdot(dT)_{12-18}$ or $(dA)_n\cdot(dT)_{12-18}$ as a template·primer, the difference in K_i values between aFUTP and aClUTP was $^1/_3$–$^1/_{10}$; the difference was much larger when the K_i of aFUTP was compared to those of aBrUTP and aIUTP ($^1/_{10}$–$^1/_{40}$). Therefore, in the case of these synthetic template·primers, both steric and hydrophobic effects of the substituents seem to have changed the affinities of the analogues with the polymerase γ.

These analogues were largely inactive against reverse transcriptase when examined with activated DNA as the template·primer. This finding is similar to that of 5-alkylated analogues which we reported previously (Ono et al., 1981). When $(rA)_n\cdot(dT)_{12-18}$ was used as the template·primer, however, aUTP was found to be the most potent inhibitor among the compounds tested. aFUTP and aClUTP gave much larger K_i values than aUTP (35- and 80-fold, respectively). It is interesting that some recovery of inhibitory action was observed in the case of aBrUTP and aIUTP, suggesting that hydrophobic effects of the substituents increase the affinities of the compounds with reverse transcriptase. In contrast to 5-alkylated aUTP's, aUTP bearing iodine was shown to be moderately active against reverse transcriptase. This difference strongly suggests that not only an electron-withdrawing effect but also appropriate steric and hydrophobic effects of the iodine group have a greater inhibitory action on this polymerase [as indicated by the lower K_i/K_m value (0.2)] than those of the other 5-halogenated aUTP's.

We have now evaluated the inhibitory effects of all four 5-halogenated aUTP's on various DNA polymerases in comparison with those of 5-alkylated aUTP's. The results and the conclusions obtained in the present study are useful in designing further inhibitors for the DNA polymerases, and the numerous data will allow us to determine the "structure–activity relationships" of the arabinosylnucleotide inhibitors.

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Major High Mobility Group like Proteins of *Drosophila melanogaster* Embryonic Nuclei†

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ABSTRACT: Nuclei from *Drosophila melanogaster* embryos contain three major proteins which are extracted by 0.35 M NaCl and by 2% perchloric acid. One of these is histone H1, and we refer to the other two as A63 and A13 in accordance with their molecular weights determined by electrophoresis on sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gels (63 000 and 13 000, respectively). The molecular weight of A13, based on its amino acid composition, is approximately 10 000. The amino acid analyses of A63 and A13 show that both of these proteins have high proportions of acidic and basic amino acid residues, a property characteristic of the high mobility group proteins isolated from vertebrate tissues. While A13 comigrates with histone H2A on $NaDodSO_4$–polyacrylamide gels and with H2B on acid/urea gels, it can be readily resolved from the histones by Triton/acid/urea–$NaDodSO_4$ two-dimensional electrophoresis.

Four major acid-soluble nonhistone chromosomal proteins named high mobility group 1 (HMG-1),[1] HMG-2, HMG-14, and HMG-17 have been identified in both mammals (Sanders & Johns, 1974; Rabbani et al., 1978a) and birds (Rabbani et al., 1978b; Sterner et al., 1978). In addition, HMG-like proteins have been reported in trout (Watson et al., 1977; Marushige & Dixon, 1971; Wigle & Dixon, 1971), flies (Franco et al., 1977), yeast (Weber & Isenberg, 1980), and plants (Spiker et al., 1978).

While the precise function of the HMG proteins is not known, there are many indications that they function by participating in the basic nucleosome structure. Thus, HMG-14 and HMG-17 as well as trout H6 have been shown to be associated with the nucleosomes of transcriptionally competent chromatin (Levy-W. et al., 1979; Weisbrod & Weintraub, 1979; Weisbrod et al., 1980). HMG-14 and HMG-17 have been shown to confer DNase I sensitivity on the chick erythrocyte globin chromosomal domain (Weisbrod & Weintraub, 1979; Weisbrod et al., 1980) and to bind to "active" nucleosomes (Weisbrod & Weintraub, 1981). They also appear to partially inhibit histone deacetylases in vitro (Candido et al., 1980).

In the present paper, we report a characterization of proteins eluted from *Drosophila* embryonic nuclei by standard procedures known to extract HMG proteins. We find that *Drosophila* contains two major proteins having typical HMG chemical compositions and solubility characteristics. However, when compared to calf thymus and trout testis HMG proteins, these *Drosophila* proteins differ in both molecular weight and amino acid composition. Whether or not *Drosophila* contains analogues to HMG-1, HMG-2, HMG-14, and HMG-17 remains unclear, and the answer to this question must await a definition of these proteins based on functional characteristics as well as physical characteristics.

Experimental Procedures

Embryos. *Drosophila melanogaster* Oregon R embryos were collected 6–18 h after fertilization, washed, and stored at −70 °C until needed. All samples were handled at 0–4 °C, unless otherwise noted, and PMSF (0.2 mM) was present in all solutions to inhibit proteolysis.

Preparation of Nuclei. Nuclei were prepared by the method of Hewish & Burgoyne (1973) as modified by Mayfield et al. (1978). Purified nuclei were used immediately for the extraction of proteins.

Extraction of HMG-like Proteins. HMG-like proteins were extracted from homogenized nuclei (DNA at 10–15 mg mL^{-1}) by the addition of 70% PCA to a final concentration of 2%. After the suspension was gently stirred on ice for 30 min, it was centrifuged for 30 min at 15000*g*. The clear supernatant was neutralized to pH 7 with 50% KOH.

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[1] Abbreviations: PCA, perchloric acid; Cl_3CCOOH, trichloroacetic acid; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; HMG, high mobility group.

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