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Utilization of 5-Fluoro-2′-deoxyuridine Triphosphate and 5-Fluoro-2′-deoxycytidine Triphosphate in DNA Synthesis by DNA Polymerases α and β from Calf Thymus[1]

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ABSTRACT

Chemically synthesized 5-fluoro-2′-deoxyuridine 5′-triphosphate and 5-fluoro-2′-deoxycytidine 5′-triphosphate were used efficiently as substitutes for DNA synthesis catalyzed by DNA polymerases α or β from calf thymus. 5-fluoro-2′-deoxyuridine 5′-triphosphate and 5′-fluoro-2′-deoxycytidine 5′-triphosphate were incorporated into DNA in place of deoxythymidine 5′-triphosphate and deoxycytidine 5′-triphosphate, respectively. The incorporated pyrimidine analogs supported further elongation of DNA. The apparent Km's for 5-fluorodeoxyuridine 5′-triphosphate in the reaction of DNA polymerases α and β were 4.3 and 15.4 μM, while those for 5-fluorodeoxycytidine 5′-triphosphate with DNA polymerases α and β were 7.7 and 8.8 μM, respectively, which are comparable to Km's for natural substrates. These results suggest the new possibility that the fluorinated pyrimidines are incorporated into DNA via their triphosphate forms to exhibit their cytostatic actions.

INTRODUCTION

FdUrd[3] is known as a potent carcinostatic agent (8, 9), and FdCyd was found to be the only fluorinated pyrimidine among 35 kinds of the related analogs which was more potent than was FdUrd (9). Furthermore, FdCyd showed a relatively high therapeutic index against mouse leukemia B82 (9). In comparable studies with FdUrd, FdCyd exhibited a different tumor spectrum of activity against a wide variety of experimental tumors. It is noteworthy that FdCyd proved to be effective against leukemia B815Y and B815, which were resistant to FdUrd (1).

The mode of carcinostatic action of FdUrd may be explained by the inhibition of thymidylate synthetase via its 5′-monophosphate (FdUMP). However, other possibilities including the incorporation into nucleic acid remain to be studied. On the other hand, the mode of action of the carcinostatic activity of FdCyd has not been studied systematically, whereas it has been attributed to the inhibition of thymidylate synthetase via FdUMP, a metabolite which is common to that of FdUrd. It was reported, however, that the inhibition of the cytidine deaminase with tetrahydrouridine increased remarkably the carcinostatic activity of FdCyd (2). This finding suggests the possibility that FdCyd is incorporated into DNA after the phosphorylation to FdCTP.

From the viewpoint of the possible incorporation of these fluorinated pyrimidine analogs into DNA, we tested whether the triphosphate form of these analogs can be used as substrates in the reaction of mammalian DNA polymerases. The triphosphate forms of the analogs have been newly synthesized by chemical procedures.

MATERIALS AND METHODS

Preparation of FdCTP. FdCyd and FdUrd were synthesized by the methods described in the literature (5, 10). FdCyd (90 mg, 0.37 mmol) in triethyl phosphate (1.5 ml) was reacted with phosphorus oxychloride (51 μl, 0.55 mmol) under cooling at −10°. The reaction mixture was stirred for 2.5 hr at room temperature and then mixed with distilled water (2 ml) and chloroform. The chloroform layer was washed 6 times with distilled water by shaking. The combined aqueous phase was extracted again with chloroform. The aqueous solution was neutralized with sodium bicarbonate and diluted with water to the final volume of 200 ml. The solution was applied to a column of DEAE-cellulose (bicarbonate form) (2.9 x 26.5 cm). The column was eluted with a linear gradient from water (700 ml) to 0.4 M triethylammonium bicarbonate (pH. 8.0, 700 ml), and the desired 5′-monophosphate was eluted at 0.16 to 0.20 M. Combined fractions were dried by evaporation under diminished pressure. The monophosphate yield was 0.22 mmol (60%) (λ_{max} in water, 280 nm; ϵ = 8000).

FdCMP (23 μmol) in anhydrous dimethylformamide (1 ml) was added to *N,N′*-carbonyldiimidazole (20 mg, 0.12 mmol), and the mixture was stirred at room temperature for 3 hr. Methanol (3.9 μl) was added to the reaction mixture for decomposition of the excess reagent. After 30 min of stirring at room temperature, the solution was added to tri-*n*-butylammonium pyrophosphate (0.06 mmol) in dimethylformamide. After 4, 6, and 7 hr, the same amount of the pyrophosphate was further added and stirred for 11 hr. The reaction mixture was evaporated to dryness under the reduced pressure. The residue was dissolved in 50 ml of distilled water and applied to a DEAE-cellulose column (1.7 x 13 cm, bicarbonate form). Elution was carried out by a linear gradient from water (250 ml) to 0.5 M triethylammonium bicarbonate (pH 8.0, 250 ml). The fractions containing triphosphate were combined and evaporated in a vacuum. The nucleotide was further purified by charcoal treatment, preparative paper electrophoresis, and preparative paper chromatography. The yield was 11.7 μmol (50%) (λ_{max} in water, 279 nm; ϵ = 7950).

Preparation of FdUTP. From FdUrd (100 μmol), its triphosphate was obtained in a yield of 20% by the same phosphorylation process as described for FdCTP.

Chemical Properties of FdUTP and FdCTP. Paper electrophoresis was performed in 15 mM triethylammonium bicarbonate (pH 7.8) at 700 V for 40 min for 5′-triphosphate. Paper chromatography was carried out on a Toyo No. 51A paper sheet in Solvent A [ethanol:0.5 M ammonium acetate, pH 7.5 (5:2, v/v)], Solvent B [ethanol:0.5 M

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[3] The abbreviations used are: FdUrd, 5-fluoro-2′-deoxyuridine; FdCyd, 5-fluoro-2′-deoxycytidine; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; FdCTP, 5-fluoro-2′-deoxycytidine 5′-triphosphate; FdCMP, 5-fluoro-2′-deoxycytidine 5′-monophosphate; FdUTP, 5-fluoro-2′-deoxyuridine 5′-triphosphated; NTP, deoxyribonucleoside triphosphate; FUra, 5-fluorouracil.

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ammonium acetate, pH 7.5 (1:1, v/v)], and Solvent C (isobutyric acid: NH_4OH:water (50:1:29, v/v] using a descending technique.

Paper Electrophoretic Mobilities. FdCTP, R_{dCMP} = 0.93; FdUMP, R_{dTMP} = 1.21; FdCTP, R_{dCTP} = 1.04; and FdUTP, R_{dTTP} = 0.98.

R_F Values of Nucleotides in Various Solvent Systems. Solvent A: FdCMP, 0.19; FdUMP, 1.21. Solvent B: FdCTP, 0.37; FdUTP, 0.43. Solvent C: FdCMP, 0.44; FdUMP, 0.36; FdCTP, 0.21; FdUTP 0.17.

Phosphate Analysis. FdCTP: calculated ϵ (P) = 2650; found ϵ (P) = 2860. FdUTP: calculated ϵ (P) = 2500; found ϵ (P) = 2860.

Enzymes. DNA polymerase α was purified from calf thymus as described previously (18). The specific activity of the DNA polymerase α (6.5S) was 3000 to 4000 nmol dNTP incorporation per 60 min per mg protein under the assay conditions described below. It sedimented at about 6.5S. DNA polymerase β was purified from calf thymus as described previously (17). The specific activity of DNA polymerase β used in this study was approximately 20,000 nmol dNTP incorporation per 60 min per mg protein with poly(rA)·$(dT)_{10}$ under the assay conditions described previously (16).

Chemicals. [*methyl*-^{3}H]dTTP and other labeled compounds were purchased from New England Nuclear (Boston, Mass.). Unlabeled dNTP's were from Boehringer (Mannheim, German Federal Republic). Activated calf thymus DNA was prepared as described by Yoshida *et al.* (14).

Assay of DNA polymerase α. The reaction mixture (62.5 μl) contained 40 mM potassium phosphate (pH 7.2); 10 mM 2-mercaptoethanol; 8 mM $MgCl_2$, 0.1 mM each of dATP, dCTP, dGTP, and [^{3}H]dTTP (40,000 cpm nmol); and 10 μg of activated DNA and DNA polymerase α fraction. When [^{3}H]dCTP (40,000 cpm/nmol) was used as a labeled precursor, unlabeled dCTP was omitted and [^{3}H]dTTP was replaced with unlabeled dTTP. Incubation was carried out at 37° for 30 min.

Assay of DNA polymerase β. The reaction mixture (62.5 μl) contained 40 mM Tris-HCl (pH 9.0); 80 mM NaCl; 0.1 mM each of dATP, dCTP, dGTP, and [^{3}H]dTTP (40,000 cpm/nmol); and 10 μg of activated DNA and DNA polymerase β fraction. When [^{3}H]dCTP was used, [^{3}H]dTTP was replaced by unlabeled dTTP, and unlabeled dCTP was omitted. Incubation was carried out at 37° for 30 min. Acid-insoluble radioactivity was measured as described previously (14).

RESULTS

Incorporation of FdUTP into DNA. Chart 1 shows that FdUTP can be utilized as a substitute for dTTP by DNA polymerases α and β from calf thymus. DNA polymerases of mammalian cells can catalyze the certain amount of polymerization of DNA with only 3 kinds of dNTP's (limited synthesis) using activated DNA as a template (16). The addition of FdUTP increased the incorporation 2.5-fold in the absence of dTTP with DNA polymerase α and 2-fold with DNA polymerase β. The maximal activities of both DNA polymerases α and β with FdUTP were approximately 50% of controls with complete reaction mixture containing dTTP. The apparent Km's of DNA polymerases α and β for FdUTP were 4.34 and 15.4 μM, respectively (Table 1). As shown in Chart 2*A*, FdUTP inhibited the incorporation of [^{3}H]dTMP into DNA in the reaction of DNA polymerase α, although it did not inhibit the incorporation of [^{3}H]dGMP. The mode of inhibition was competitive with respect to dTTP (Chart 2*B*). Similar results were obtained with DNA polymerase β (data not shown).

Incorporation of FdCTP into DNA. Chart 3 shows that FdCTP can substitute for dCTP in the reaction of both DNA polymerases α and β and support the elongation of DNA as was observed with FdUTP (Chart 1). The addition of FdCTP stimulated the limited synthesis 3-fold in the absence of dCTP with DNA polymerase α and 2.7-fold with DNA polymerase β. The activities of DNA polymerase α and β with FdCTP were 80 and 100%, respectively, of the control with the complete reaction mixture. The apparent Km's of DNA polymerases α and β for FdCTP were 7.7 and 8.8 μM, respectively (Table 1). As shown in Chart 4, the incorporation of [^{3}H]dCMP was inhibited by FdCTP while that of [^{3}H]dGMP was not inhibited. FdCTP inhibited the incorporation of [^{3}H]dCMP competitively with respect to dCTP. These observations indicate that both FdUTP and FdCTP can be uitlized as substrates in the reaction of either DNA polymerase α or β in place of their counterparts, dTTP and dCTP, respectively.

Table 1

K_m values of DNA polymerases α and β for FdUTP, FdCTP, and their counterparts

	K_m (μM)	
	DNA polymerase α	DNA polymerase β
FdUTP	4.3	15.4
dTTP	5.3	5.5
FdCTP	7.7	8.4
dCTP	5.5	8.8

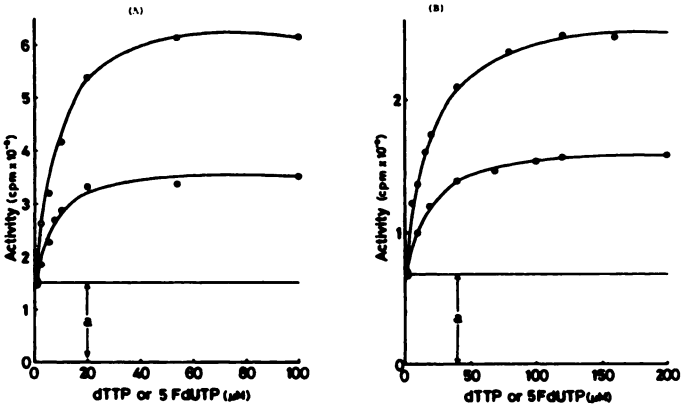

Chart 1. Substitution of dTTP with FdUTP in the reactions of DNA polymerases α (*A*) and β (*B*). DNA polymerases α and β were assayed by using [^{3}H]dGTP as a labeled precursor in the presence of 3 other unlabeled dNTP's (○) or in the presence of FdUTP in place of dTTP (●). The composition of the other reaction mixture was the same as that described in "Materials and Methods." *a*, activity of limited synthesis of DNA polymerase α or β in the presence of dATP, dCTP, and [^{3}H]dGTP.

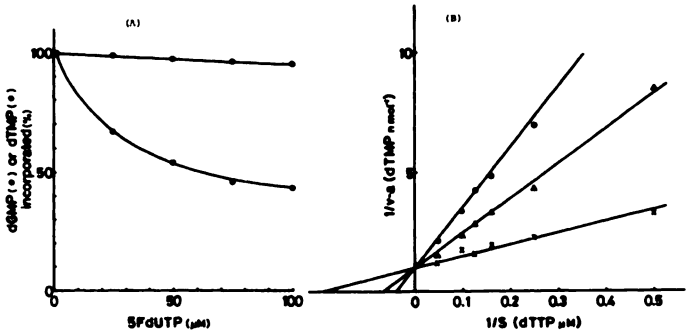

Chart 2. *A*, inhibition of the incorporation of [^{3}H]dGMP or [^{3}H]dTMP by FdUTP. DNA polymerase α was assayed by using either [^{3}H]dGTP (○) or [^{3}H]dTTP (●) as a labeled precursor. FdUTP was added at the concentrations indicated. (*B*, competitive inhibition of the incorporation of [^{3}H]dTMP by FdUTP in the reaction of DNA polymerase α. The reaction was carried out by using [^{3}H]dTTP as a labeled precursor by the methods described in "Materials and Methods" except that the concentration of [^{3}H]dTMP was changed as indicated. FdUTP was added at concentrations of 0 (×), 20 (△), and 50 (●) μM.

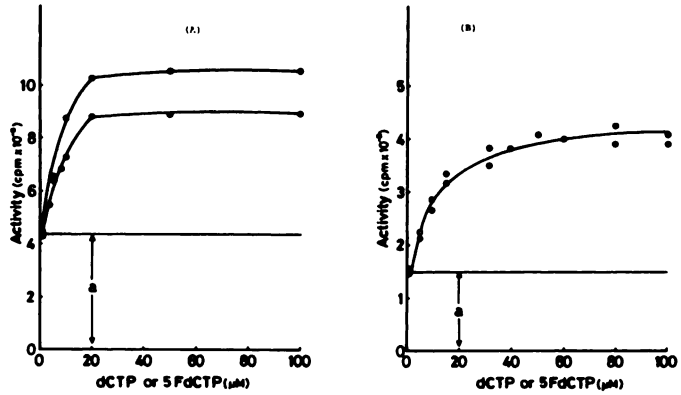

Chart 3. Substitution of dCTP with FdCTP in the reaction of DNA polymerase α (*A*) and β (*B*). DNA polymerases α and β were assayed by using [^{3}H]dGTP as a labeled precursor for the assay of DNA polymerase α and β in the presence of 3 other dNTP's (○) or in the presence of FdCTP in place of dCTP (●). Other assay conditions were the same as that described in "Materials and Methods." *a*, activity of limited synthesis in the presence of dATP, dTTP, and [^{3}H]dGTP.

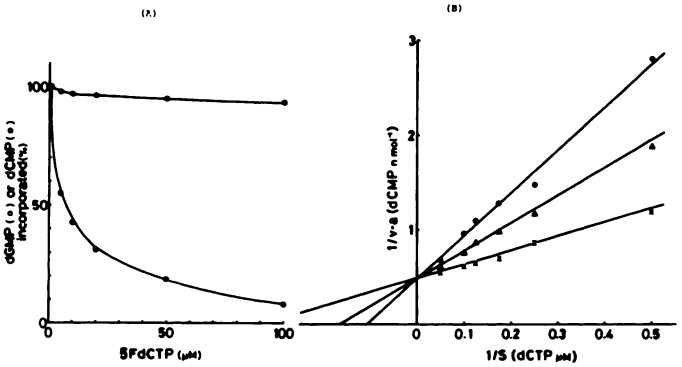

Chart 4. *A*, inhibition of the incorporation of [^{3}H]dGMP or [^{3}H]dCMP by FdCTP. [^{3}H]dGMP (○) or [^{3}H]dCMP (○) was used as a labeled precursor for the assay of DNA polymerase. FdCTP was added at the concentrations indicated. *B*, competitive inhibition of the incorporation of [^{3}H]dCTP by FdCTP in the reaction of DNA polymerase α. [^{3}H]dCTP was used as a labeled precursor at the concentrations indicated. FdCTP was added at 0 (×), 20 (△), and 50 (●) μM.

DISCUSSION

The kinetic analyses of the DNA polymerase reaction with FdUTP and FdCTP strongly suggested that these 2 analogs are utilized by both DNA polymerases α and β from calf thymus and are incorporated into DNA. These results are not unexpected because the fluorine is similar in size to the hydrogen atom, especially when dUTP have been proven to be a good substrate for DNA polymerases (4, 15).

However it has been reported that neither FUra nor FdUrd are incorporated into DNA, while uracil is easily incorporated into RNA *in vivo* (11, 12). This situation is analogous to that of deoxyuridine; its triphosphate form, dUTP, can be used efficiently as a substrate for the DNA polymerase reaction (4, 15), whereas uracil is not found in the normal DNA. Recently, Goulian *et al.* (7) showed that a detectable amount of dUTP is incorporated into DNA of mammalian cells treated with methotrexate which inhibits thymidylate synthetase. It is conceivable, therefore, that FdUMP may be also incorporated into DNA and will impair its function in replication or transcription if nucleotide metabolism would be modulated to allow the accumulation of FdUTP.

A part of the cytostatic effect of FUra or FdUrd comes from the inhibition of thymidylate synthetase via FdUMP (8). In addition, FUra is reported to be incorporated into mRNA which leads to the altered function for translation (6). It is also reported that FUra causes the abnormal rRNA (12, 13). Recently, Spiegelman *et al.* (11) succeeded in improving the antitumor activity of FUra by increasing its incorporation into RNA by treating cells with thymidine. Another possibility for this drug is suggested, *i.e.*, the incorporation into DNA, probably by a combination with other carcinostatic agents to induce another kind of metabolic modulation.

FdCyd has been shown to have a potent carcinostatic activity *in vivo* for leukemias L1210, B82, and B174 (1). It was found that FdCyd was more carcinostatic than was FdUrd *in vitro* for Ehrlich ascites carcinoma cells (9). The mode of carcinostatic action of FdCyd has been attributed to the inhibition of thymidylate synthetase by FdUMP generated from FdCyd by phosphorylation followed by deamination. Cooper and Geer (3) reported that 5-halogenated deoxycytidine could be phosphorylated by deoxycytidine kinase, and the apparent K_m of deoxycytidine kinase for FdCyd was lower than that for deoxycytidine (3). They reported, however, that the carcinostatic effect of FdCyd was increased 5-fold by the inhibition of cytidine deaminase with tetrahydrouridine. This finding suggests that the carcinostatic action of FdCyd cannot be attributed solely to the inhibition of thymidylate synthetase via FdUMP. Taken together with our finding that FdCTP can be used by DNA polymerase, it is possible that FdCyd is incorporated into DNA after being phosphorylated to its triphosphate form.

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