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Fluorinated Pyrimidines

XLIV. Interaction of 5-Trifluoromethyl-2′-deoxyuridine 5′-Triphosphate with Deoxyribonucleic Acid Polymerases

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SUMMARY

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The interaction of 5-trifluoromethyl-2′-deoxyuridine 5′-triphosphate (F_3dThd-5′-PPP) with various DNA-directed DNA polymerases has been studied. This analogue exhibited competitive inhibition with dThd-5′-PPP for partially purified DNA polymerases from vaccinia virus-infected HeLa cells, uninfected HeLa cell cytoplasm and nuclei, and calf thymus. The inhibition produced by F_3dThd-5′-PPP of the incorporation of deoxycytidine 5′-triphosphate and deoxyadenosine 5′-triphosphate was noncompetitive or mixed. The K_m values for dThd-5′-PPP of vaccinia virus-induced, HeLa cell cytoplasmic, HeLa cell nuclear, and calf thymus DNA polymerases were 3.4, 4.6, 5.1, and 10 μM, respectively. The corresponding K_i values of F_3dThd-5′-PPP for these enzymes were 4.0, 8.5, 11.5, and 17 μM. F_3dThd-5′-PPP was incorporated into DNA by all these DNA polymerases. This incorporation was inhibited competitively by dThd-5′-PPP. The rates of F_3dThd-5′-PPP incorporation by these enzymes were slower than those of dThd-5′-PPP. The ratio of the incorporation rate of the analogue to that of dThd-5′-PPP was higher with vaccinia virus polymerase than with uninfected HeLa cell enzymes.

INTRODUCTION

5′-Trifluoromethyl-2′-deoxyuridine was synthesized in this laboratory as an analogue of thymidine (1), and is highly active in many biological systems. It effectively inhibits the growth of various mammalian cells in culture (2) and some tumors in mice (3). F_3dThd[3] is also active against herpes simplex and vaccinia viral keratitis

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[3] The abbreviations used are: F_3dThd, 5-trifluoromethyl-2′-deoxyuridine; F_3dThd-5′-PPP, 5-trifluoromethyl-2′-deoxyuridine 5′-triphosphate; dThd-5′-PPP, deoxythymidine 5′-triphosphate; dAdo-5′-PPP, deoxyadenosine 5′-triphosphate; dGuo-5′-PPP, deoxyguanosine 5′-triphosphate; dCyd-5′-PPP, deoxycytidine 5′-triphosphate; FdUrd, 5-fluoro-2′-deoxyuridine.

in rabbits (4) and inhibits the replication of vaccinia virus in HeLa cells (5). F_3dThd is clinically more effective and less toxic than 5-iodo-2′-deoxyuridine in the treatment of herpes simplex keratitis of the eye (6). The clinical effects of F_3dThd in patients with advanced breast cancer (7) and metabolic studies in mice and man have been also reported (8, 9).

F_3dThd exerts various effects on DNA synthesis. Thymidine kinase is inhibited by this analogue (10), and thymidylate synthetase is noncompetitively inhibited by its nucleotide, trifluoromethyl-2′-deoxyuridine 5′-monophosphate. (11). F_3dThd is also incorporated into the DNA of vaccinia virus (12), mammalian cells (13), and bacteriophage T4 (14). Fujiwara *et al.* (13) showed that, in HeLa and L5178Y cells blocked by an induced deoxythymidine deficiency, release with F_3dThd did not lead to cell division, although there was incorporation of F_3dThd into cellular DNA. In the cases of mammalian cells and vaccinia virus, the analogue-containing DNA had a smaller sedimentation coefficient than the normal DNA (12, 13).

In vaccinia virus-infected HeLa cells, early mRNA transcription was unaffected by F_3dThd, but late mRNA sedimented abnormally (15) and had sequences missing (16). Thus F_3dThd affects DNA replication in various ways. In this paper we present evidence that F_3dThd-5′-PPP inhibits DNA polymerases. Kinetic studies of the inhibition and incorporation into DNA of F_3dThd-5′-PPP by DNA-directed DNA polymerases are also presented.

MATERIALS AND METHODS

Chemicals

Nonradioactive nucleotides were obtained from Sigma Chemical Company. Radioactive nucleotides and calf thymus DNA (highly polymerized) were purchased from Schwarz/Mann. Phosphocellulose (P-1) and DEAE-cellulose (DE-23) were obtained from Whatman through H. Reeve Angel Company. Hydroxylapatite gel (Bio-Gel HT) was purchased from Bio-Rad. Pancreatic deoxyribonuclease was obtained from Worthington. F_3dThd-5′-PPP and [^{3}H]-F_3dThd-5′-PPP were synthesized in this laboratory (17).

DNA Polymerase Assay

DNA polymerase activity was assayed by a filter disc method (18), using Whatman No. 541 filter paper discs (2.3 cm in diameter). The composition of the reaction mixture is given in each table and figure, and the pH optimum for each enzyme was employed. Unless stated otherwise, 25 μl of the mixture were spotted onto a disc and the reaction was terminated by dropping the disc into cold 5% trichloracetic acid containing 1% $Na_4P_2O_7 \cdot 10H_2O$. The discs were washed twice with cold 5% trichloracetic acid, twice with ethanol, and once with ether, and were dried and placed in a toluene-based scintillation mixture for radioactive analysis. The absolute efficiency for tritium of our disc method was 20%.

For the [^{3}H]F_3dThd-5′-PPP incorporation studies, acid-insoluble radioactivity was assayed by a test tube method, since the specific radioactivity of the analogue was not high enough for the disc assay. The reaction was terminated by addition of cold trichloracetic acid (final concentration, 7.5%); 250 μg of calf thymus DNA and 2 mg of bovine serum albumin were added as carriers, and the precipitate was centrifuged, washed with cold 5% trichloracetic acid three times, and dissolved in 2 ml of Soluene 100 (Packard Instrument Company). The radioactivity was counted in a toluene-based scintillation mixture.

Activated and Denatured DNA

Activated DNA, used as a template in the assay of HeLa cell and vaccinia virus-induced DNA polymerases, was prepared from calf thymus DNA (25 mg/10 ml of 10 mM Tris-HCl buffer, pH 7.2) by treatment with 1 μg of pancreatic deoxyribonuclease in the presence of 50 μmoles of $MgCl_2$ at 37° for 25 min. The mixture was heated at 60° for 10 min (19).

For the assay of calf thymus DNA polymerase, calf thymus DNA (25 mg/10 ml of 10 mM Tris-HCl buffer, pH 7.2) was denatured in boiling water for 10 min and immediately cooled in an ice bath (18).

Protein Assay

Protein concentration was determined according to Lowry *et al.* (20). In some cases the biuret method was used (21).

Partial Purification of DNA Polymerases

All operations were performed at 0–4°, and all buffer solutions used for dialysis and chromatography contained 5 mM 2-mercaptoethanol, 1 mM EDTA, and 30% glycerol.

HeLa cell DNA polymerases were purified from partially synchronized cells according to Weisbach *et al.* (22), with several modifications. HeLa S3 cells were grown in suspension culture at 37° in minimal essential medium (Grand Island Biological Company) supplemented with 10% calf serum, 0.1% Pluronic F68, penicillin, and streptomycin. The cells were resuspended in the same medium at a concentration of 1×10^5 cells/ml. After incubation for 24 hr at 37°, FdUrd and uridine were added to the culture to block DNA synthesis specifically at concentrations of 1 and 10 μM, respectively. After 16 hr of additional incubation, the blockade was released by addition of 4 μM thymidine. The cells (1.3×10^9) were harvested 2 hr later, washed with cold NaCl solution, and suspended in 1 mM potassium phosphate buffer (pH 7.0) containing 10 mM NaCl, 5 mM $MgCl_2$, and 5 mM 2-mercaptoethanol. After swelling at 0° for 15 min, the cells were homogenized with a Dounce homogenizer. Disruption of cells was checked microscopically. The pellet obtained from the homogenate following sedimentation at $800 \times g$ for 5 min was washed with 1 mM potassium phosphate buffer (pH 7.0) containing 250 mM sucrose, 2 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 0.3% Triton N-101 (23). The nuclear fraction was obtained by centrifugation at $1000 \times g$ for 10 min. The washings and the supernatant fraction obtained from the first centrifugation were combined. The cytoplasmic fraction (70 ml) was obtained by centrifugation at $10,000 \times g$ for 20 min. To the nuclear fraction, which was suspended in 50 mM potassium phosphate buffer, pH 7.0, was added an equal volume of 4 M NaCl. The viscous suspension was centrifuged at $100,000 \times g$ for 4 hr. The pellet was resuspended in the same buffer and treated with NaCl in the same manner. The first and second supernatant fractions were combined and dialyzed against 100 volumes of 50 mM Tris-HCl, pH 7.5, for 4 hr with three changes of this buffer. The resulting precipitate was centrifuged at $10,000 \times g$ for 15 min. The supernatant fraction was dialyzed overnight against the same buffer. A clear nuclear extract (28 ml) was obtained by centrifugation at $10,000 \times g$ for 20 min. The same chromatographic purification procedure was applied to both cytoplasmic and nuclear extracts.

The crude extract was adsorbed on a DEAE-cellulose column (3×18 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.5. The column was eluted successively with 400 ml each of 50 mM Tris-HCl buffer (pH 7.5), 50 mM NaCl, 100 mM NaCl, and 250 mM NaCl dissolved in the same buffer. The nuclear extract gave a major peak in the 50 mM buffer, and a second peak in the 250 mM NaCl. The activity in this second peak represented cytoplasmic contamination, since almost no peak was present when there was good separation of nuclei. With the cytoplasmic extract, a major peak of DNA polymerase activity was eluted by 250 mM NaCl. In both nuclear and cytoplasmic extracts, very small peaks were observed in 50 mM and 100 mM NaCl.

The active fractions were combined and dialyzed against 50 mM Tris-HCl buffer, pH 7.2, instead of pH 8.9 (22). A phosphocellulose column (1.3×7 cm) was equilibrated with 50 mM Tris-HCl buffer, pH 7.2. To this column was applied the dialyzed active fraction from DEAE-cellulose. Elution was carried out with a linear NaCl gradient from 0 to 700 mM in the same buffer. The active fractions were collected and dialyzed against 50 mM Tris-HCl buffer, pH 8.5. The second phosphocellulose chromatography was performed in the same way, using a smaller column (1.3×2 cm). The peak of DNA polymerase activity was used for further experiments after dialysis. The specific activities of the nuclear and cytoplasmic enzyme preparations were 710 and 470 units/mg, respectively.

Vaccinia virus induces a new DNA

polymerase in HeLa cells. This enzyme was partially purified according to Citrella *et al.* (24). HeLa S3 cells, grown in suspension culture in F-14 medium supplemented with 10% calf serum, 0.1% Pluronic F68, and antibiotics, were harvested in the logarithmic phase and resuspended at 5×10^6 cells/ml (5×10^8 cells) in F-14 medium supplemented with 1% calf serum, 0.1% Pluronic F68, antibiotics, and 1 mM $MgCl_2$. Vaccinia virus [partially purified by sucrose gradient centrifugation (25)] was added at 10–25 plaque-forming units/cell. After adsorption at 37° for 1 hr, the cells were diluted to 5×10^5 cells/ml with 5% calf serum-supplemented F14 medium containing 10 μM FdUrd (26). The virus-infected cells were harvested after 11 hr of incubation at 37°. The cytoplasmic fraction from virus-infected cells was prepared by the same procedure as for uninfected HeLa cells.

DEAE-cellulose was equilibrated with 20 mM potassium phosphate buffer, pH 7.5, in a 2 × 25 cm column. The cytoplasmic fraction from virus-infected cells was dialyzed against the same buffer and adsorbed onto the column. Virus-induced and host cell DNA polymerases were eluted with a linear gradient from 20 to 300 mM potassium phosphate at pH 7.5. Citrella *et al.* (24) reported that the first peak of activity was the virus-induced enzyme and the second was HeLa cell cytoplasmic DNA polymerase; we obtained a similar result. The peak of virus-induced DNA polymerase, after dialysis against 50 mM potassium phosphate buffer, pH 7.5, was applied to a phosphocellulose column (1.3 × 10 cm) equilibrated with the same buffer. Elution was performed with a linear gradient from 50 to 500 mM potassium phosphate buffer, pH 7.5. The peak thus obtained was used for further experiments, and its specific activity was 720 units/mg.

Calf thymus DNA polymerase was purified according to Yoneda and Bollum (27) with the following modification. In the original procedure DEAE-cellulose chromatography was performed in 0.2 M phosphate buffer only to remove contaminating DNA. However, when the chromatography was carried out as described here, a contaminating terminal transferase and a different DNA polymerase were also removed. DEAE-cellulose was packed into a column 3 × 30 cm, and the dialyzed active fractions were adsorbed onto it after equilibration with the same buffer. Stepwise elution was carried out with 2.5 column volumes each of 20 mM Tris-HCl buffer, 20 mM NaCl, and 200 mM NaCl in the same buffer. A small peak appeared at the beginning, which contained terminal transferase and a small amount of a DNA polymerase. The major peak was eluted by 200 mM NaCl. In a small-scale purification, linear gradient elution was performed with the NaCl concentration varied from 0 to 300 mM. The active fractions eluted by 200 mM NaCl were pooled and dialyzed against 50 mM potassium phosphate buffer, pH 7.0, and were then adsorbed onto a phosphocellulose column (2 × 26 cm) equilibrated with the same buffer. DNA polymerase was eluted by a linear potassium phosphate gradient from 50 mM (pH 7.0) to 300 mM (pH 7.5). The active peak was dialyzed against 50 mM potassium phosphate buffer, pH 7.5. A hydroxylapatite gel column (2 × 8 cm) was prepared and equilibrated with the same buffer. The dialyzed solution was adsorbed onto this column. A peak of DNA polymerase was obtained by a linear potassium phosphate gradient from 50 to 500 mM. The active fractions were dialyzed and concentrated by ultrafiltration (Amicon, with a PM-10 membrane). The specific activity of the preparation was 2940 units/mg.

Unit of DNA Polymerase

One unit of DNA polymerase activity is defined as the incorporation of 1 pmole of [^{3}H]dThd-5′-PPP into an acid-insoluble product per minute at 37°.

RESULTS

Inhibition of DNA Polymerases by F_3dThd-5′-PPP

F_3dThd-5′-PPP inhibited all four partially purified DNA polymerases: HeLa cell, nuclear and cytoplasmic, vaccinia virus-induced, and calf thymus.

HeLa cell cytoplasmic DNA polymerase was inhibited by F_3dThd-5′-PPP. At increasing concentrations of the analogue at constant concentrations of dThd-5′-PPP and the other three deoxyribonucleoside triphosphates, the incorporation of [^{3}H]dThd-5′-PPP was decreased. In the absence of the analogue, 59 pmoles of [^{3}H]dThd-5′-PPP were incorporated at 30 min; in the presence of F_3dThd-5′-PPP at various concentrations from 6.25 to 50 μM, inhibition varied from 18% to 65%. Equimolar addition of F_3dThd-5′-PPP and dThd-5′-PPP gave 44% inhibition. The rates of incorporation were linear for 60 min.

Similar results were obtained with the HeLa cell nuclear, vaccinia virus-induced, and calf thymus DNA polymerases. Inhibition of incorporation of 20 μM [^{3}H]dThd-5′-PPP by the vaccinia enzyme was 20% with 6.25 μM, 44% with 12.5 μM, and 74% with 50 μM F_3dThd-5′-PPP. Equimolar concentrations of F_3dThd-5′-PPP and dThd-5′-PPP (20 μM each) gave 55% inhibition, which was a little higher than that of the cytoplasmic enzyme from uninfected HeLa cells.

With the HeLa cell nuclear and calf thymus DNA polymerases, inhibition at equimolar concentrations of F_3dThd-5′-PPP and dThd-5′-PPP was 23% and 35%, respectively.

Kinetic Studies

DNA polymerase from HeLa cell cytoplasm. The effect of F_3dThd-5′-PPP on the velocity of the incorporation of dThd-5′-PPP into DNA by HeLa cell cytoplasmic DNA polymerase in the presence of different concentrations of [^{3}H]dThd-5′-PPP and F_3dThd-5′-PPP is shown in Fig. 1, in which the data are plotted according to Lineweaver and Burk (28). The figure shows that the inhibition produced by F_3dThd-5′-PPP was competitive with dThd-5′-PPP. The K_m value for [^{3}H]dThd-5′-PPP obtained from this plot was 4.6 μM. A Dixon plot (29) gave 8.4 μM as the K_i for F_3dThd-5′-PPP (Fig. 2).

The effects of various concentrations of F_3dThd-5′-PPP on the incorporation of [^{3}H]dCyd-5′-PPP into DNA by the HeLa cell cytoplasmic polymerase are shown in Fig. 3. The inhibition was noncompetitive or mixed. The K_m for [^{3}H]dCyd-5′-PPP was 1.4 μM. Similarly, the inhibition of DNA polymerase produced by F_3dThd-5′-PPP in the presence of different concentrations of [^{3}H]dAdo-5′-PPP was noncompetitive or mixed. The K_m value for [^{3}H]dAdo-5′-PPP

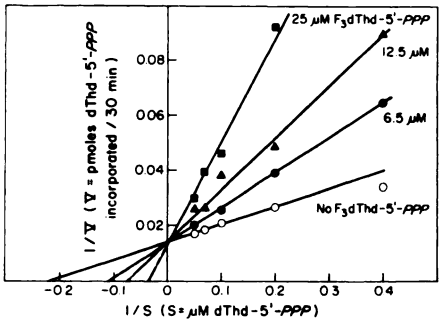

FIG. 1. *Effect of F_3dThd-5′-PPP on DNA polymerase from HeLa cell cytoplasm in the presence of different concentrations of dThd-5′-PPP (Lineweaver-Burk plot)*

The reaction mixture (125 μl) contained 6.25 nmoles each of dAdo-5′-PPP, dGuo-5′-PPP, and dCyd-5′-PPP, 25 μg of activated DNA, 1 μmole of $MgCl_2$, 125 nmoles of 2-mercaptoethanol, 5 μmoles of Tris-HCl buffer (pH 8.0), 2.3 units of enzyme, and the indicated concentrations of [^{3}H]dThd-5′-PPP (0.66 μCi) and F_3dThd-5′-PPP. The mixture was incubated at 37° for 30 min and assayed as described under MATERIALS AND METHODS.

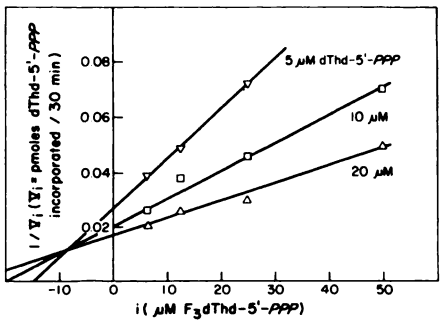

Fig. 2. *Effect of F_3dThd-5′-PPP on DNA polymerase from HeLa cell cytoplasm in the presence of different concentrations of dThd-5′-PPP (Dixon plot)*

The reaction mixture had the same composition as in Fig. 1. The mixture was incubated at 37° for 30 min and assayed as described under MATERIALS AND METHODS.

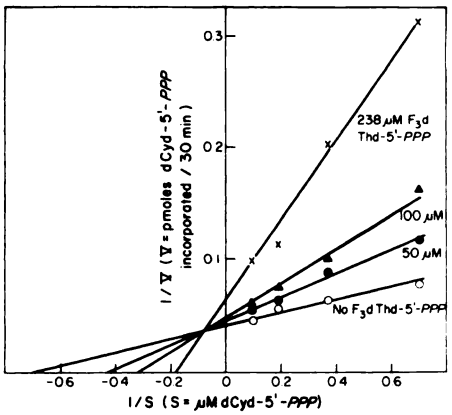

FIG. 3. *Effect of F_3dThd-5'-PPP on DNA polymerase from HeLa cell cytoplasm in the presence of different concentrations of dCyd-5'-PPP (Lineweaver-Burk plot)*

The reaction mixture (125 μl) contained 6.25 nmoles each of dAdo-5'-PPP, dGuo-5'-PPP, and dThd-5'-PPP, 25 μg of activated DNA, 1 μmole of $MgCl_2$, 125 nmoles of 2-mercaptoethanol, 5 μmoles of Tris-HCl buffer (pH 8.0), 2.3 units of enzyme, and the indicated concentrations of [^{3}H]dCyd-5'-PPP (0.64 μCi) and F_3dThd-5'-PPP. The mixture was incubated at 37° for 30 min and assayed as described under MATERIALS AND METHODS.

was 2.0 μM, as shown in Table 1. The concentrations of the inhibitors added in the experiments with [^{3}H]dCyd-5'-PPP and [^{3}H]dAdo-5'-PPP were much higher than those used with [^{3}H]dThd-5'-PPP.

DNA polymerase from HeLa cell nuclei. The effects of F_3dThd-5'-PPP on the velocity of the HeLa cell nuclear enzyme reaction in the presence of different concentrations of [^{3}H]dThd-5'-PPP were analyzed by a Lineweaver-Burk plot and found to be competitive. The K_m for [^{3}H]dThd-5'-PPP was 5.1 μM, and a Dixon plot showed that the K_i value for F_3dThd-5'-PPP was 11.5 μM (Table 1).

DNA polymerase induced in HeLa cells by vaccinia virus. The inhibition produced by F_3dThd-5'-PPP was competitive with [^{3}H]dThd-5'-PPP. The K_m for [^{3}H]dThd-5'-PPP was 3.4 μM, and the K_i for F_3dThd-5'-PPP was 4.0 μM (Table 1). F_3dThd-5'-PPP exerted noncompetitive or mixed inhibition of incorporation of [^{3}H]dCyd-5'-PPP. The K_m for [^{3}H]dCyd-5'-PPP was 0.67 μM. The inhibition of the virus-induced polymerase produced by this analogue was also noncompetitive or mixed with [^{3}H]dAdo-5'-

TABLE 1

Summary of kinetic experiments

Enzyme	pH of assay	Substrate	Inhibitor	K_m[a]	K_i[b]	Type of inhibition[a]
				μM	μM	
Vaccinia-induced polymerase	8.0	dThd-5'-PPP	F_3dThd-5'-PPP	3.4	4.0	Competitive
	8.0	dCyd-5'-PPP	F_3dThd-5'-PPP	0.67		Noncompetitive or mixed
	8.0	dAdo-5'-PPP	F_3dThd-5'-PPP	2.3		Noncompetitive or mixed
HeLa cell (nuclei) polymerase	8.0	dThd-5'-PPP	F_3dThd-5'-PPP	5.1	11.5	Competitive
Hela cell (cytoplasm) polymerase	8.0	dThd-5'-PPP	F_3dThd-5'-PPP	4.6	8.5	Competitive
	8.0	F_3dThd-5'-PPP	dThd-5'-PPP	6.5		Competitive
	8.0	dCyd-5'-PPP	F_3dThd-5'-PPP	1.4		Noncompetitive or mixed
	8.0	dAdo-5'-PPP	F_3dThd-5'-PPP	2.0		Noncompetitive or mixed
Calf thymus polymerase	7.0	dThd-5'-PPP	F_3dThd-5'-PPP	10	17	Competitive

[a] All K_m values and types of inhibition were obtained from Lineweaver-Burk plots (28).

[b] All K_i values were obtained from Dixon plots (29).

PPP; the K_m for [^{3}H]dAdo-5′-PPP was 2.3 μM (Table 1).

DNA polymerase from calf thymus. The inhibition produced by F_3dThd-5′-PPP was competitive with respect to [^{3}H]dThd-5′-PPP. The K_m and K_i values were 10 μM and 17 μM, respectively. The kinetic data obtained from all the enzyme preparations are summarized in Table 1.

Incorporation Studies

The incorporation of [^{3}H]F_3dThd-5′-PPP and of [^{3}H]dThd-5′-PPP into DNA by the vaccinia virus-induced DNA polymerase is shown in Fig. 4. The rates and ratios of incorporation of dThd-5′-PPP and F_3dThd-5′-PPP into DNA by DNA polymerases from HeLa cell cytoplasm and nuclei and calf thymus are given in Table 2.

The incorporation of [^{3}H]F_3dThd-5′-PPP was inhibited by dThd-5′-PPP. Figure 5 shows a Lineweaver-Burk plot of the inhibition by dThd-5′-PPP of [^{3}H]F_3dThd-5′-PPP incorporation into DNA by the HeLa cell cytoplasmic DNA polymerase; it was found to be competitive. The K_m for F_3dThd-5′-PPP was 6.5 μM, which is a little lower than its K_i value, as calculated from Fig. 4.

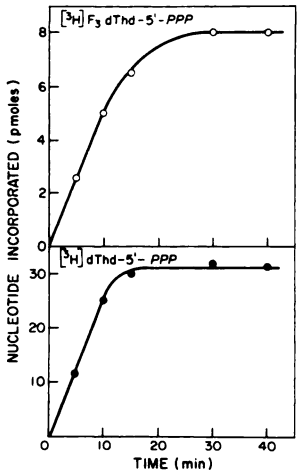

FIG. 4. *Incorporation of [^{3}H]F_3dThd-5′-PPP and of [^{3}H]dThd-5′-PPP into DNA by DNA polymerase induced by vaccinia virus*

The reaction mixture (250 μl) contained 25 nmoles each of dAdo-5′-PPP, dGuo-5′-PPP, and dCyd-5′-PPP, 50 μg of activated DNA, 2 μmoles of $MgCl_2$, 250 nmoles of 2-mercaptoethanol, 10 μmoles of Tris-HCl buffer (pH 8.0), 2.5 units of enzyme, and 12.5 nmoles of [^{3}H]F_3dThd-5′-PPP (0.414 μCi) or [^{3}H]dThd-5′-PPP (0.81 μCi). The mixture was incubated at 37° and assayed as described under MATERIALS AND METHODS.

DISCUSSION

Since it has been reported from this laboratory that F_3dThd is incorporated into the DNA of HeLa S3 cells, Leukemia L5178Y cells, and vaccinia virus, and that the presence of the analogue caused the production of fragments of DNA smaller than normal (12, 13), it was of interest to study DNA synthesis *in vitro* in the presence of F_3dThd-5′-PPP. We have shown in this study that F_3dThd-5′-PPP inhibits partially purified DNA-directed DNA polymerases from the nuclei and cytoplasm of HeLa cells, vaccinia virus-infected HeLa cells, and calf thymus. The kinetic data obtained with these DNA polymerases are summarized in Table 1. The inhibition of these four kinds of polymerases by F_3dThd-5′-PPP was competitive with respect to dThd-5′-PPP.

Our experiments show that [^{3}H]F_3dThd-5′-PPP can be incorporated into DNA instead of dThd-5′-PPP in cell-free systems, as expected from our studies in intact and vaccinia virus-infected cells. The rate of incorporation of F_3dThd-5′-PPP was much slower than that of dThd-5′-PPP. In addition, the incorporation of F_3dThd-5′-PPP was also inhibited competitively by dThd-5′-PPP. These data strongly suggest that F_3dThd-5′-PPP and dThd-5′-PPP occupy the same binding site on the DNA polymerase molecule, and that they compete with each other. However, the rate of formation of the phosphodiester bond between F_3dThd-5′-PPP and the hydroxyl group at the 3′-terminus of the DNA primer is much slower than that with dThd-5′-PPP. This result is very similar to the case of arabinofuranosylcytosine 5′-triphosphate (araCyd-5′-PPP), which is incorporated slowly into DNA by calf thymus DNA polymerase; this incorporation also produced termination of polynucleotide chain growth (30, 31). However, preliminary experiments suggest that incorporation of F_3dThd-5′-PPP does not lead to chain termination.[4]

[4] H. Tone, A. Sarrif, P. V. Danenberg, and C. Heidelberger, unpublished observations.

TABLE 2

Summary of rates of incorporation

Enzyme	Substrate	Rate of incorporation	Incorporation ratio: (F_3dThd-5′-PPP/dThd-5′-PPP) × 100
		pmoles/min/mg protein	
Vaccinia-induced polymerase	F_3dThd-5′-PPP	144	20
	dThd-5′-PPP	720	(100)
HeLa cell (nuclei) polymerase	F_3dThd-5′-PPP	32	5.1
	dThd-5′-PPP	630	(100)
HeLa cell (cytoplasm) polymerase	F_3dThd-5′-PPP	27	11
	dThd-5′-PPP	240	(100)
Calf thymus polymerase	F_3dThd-5′-PPP	46	1.3
	dThd-5′-PPP	3530	(100)

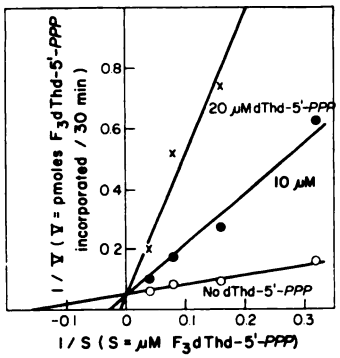

FIG. 5. *Effect of dThd-5′-PPP on incorporation of [^{3}H]F_3dThd-5′-PPP into DNA by DNA polymerase from HeLa cell cytoplasm in the presence of different concentrations of F_3dThd-5′-PPP (Lineweaver-Burk plot)*

The reaction mixture had the same composition as in Fig. 1 except that it contained the indicated concentrations of [^{3}H]F_3dThd-5′-PPP (36 μCi/mole) and dThd-5′-PPP. The mixture was incubated at 37° for 30 min and assayed as described under MATERIALS AND METHODS.

F_3dThd has been reported to be clinically the most effective drug known against vaccinia and herpes virus infections of the eye (6). Therefore it was also of interest to compare the effect of this analogue on the vaccinia virus-induced DNA polymerase with that on the uninfected HeLa cell enzyme. As summarized in Table 1, the inhibition of all enzymes is competitive between dThd-5′-PPP and F_3dThd-5′-PPP. However, there was a slight difference among the K_m and the K_i values of these enzymes. The ratio of the K_i value for F_3dThd-5′-PPP to the K_m value for dThd-5′-PPP is almost 2 in the HeLa cell and calf thymus enzymes, but in the vaccinia virus-induced polymerase it is close to 1. Therefore the analogue inhibits the virus-induced enzyme more than the cellular enzymes. Whether the same effect would be found with the herpes virus-induced DNA polymerase remains to be determined.

The rate of incorporation of F_3dThd-5′-PPP by the vaccinia virus-induced DNA polymerase is also higher than with the other enzymes (Table 2). However, an exact comparison among these enzymes is impossible because the purities of the enzyme preparations were different. Nevertheless, since the enzyme purities were similar, it may be permissible to compare the ratios of the rate of incorporation of the analogue with that of the normal substrate for each polymerase, because the ratio is probably not much affected by the purity of the enzyme. There was a considerable difference between the rate of incorporation of dThd-5′-PPP and that of F_3dThd-5′-PPP. The analogue was incorporated much more slowly than the normal substrate. The ratio of F_3dThd-5′-PPP incorporation to dThd-5′-PPP incorporation was different among the

DNA polymerases; in particular, the virus-induced polymerase had a higher ratio than other enzymes. This may explain the preferential toxicity of F_3dThd to vaccinia virus as compared to the cells in which it replicates.

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