MOLECULAR PHARMACOLOGY, 20:415–422

Nucleoside Analogues with Clinical Potential in Antivirus Chemotherapy

The Effect of Several Thymidine and 2′-Deoxycytidine Analogue 5′-Triphosphates on Purified Human (α, β) and Herpes Simplex Virus (Types 1, 2) DNA Polymerases

JERRY L. RUTH AND YUNG-CHI CHENG

Department of Pharmacology, School of Medicine, and Drug Development Program, Cancer Research Center, University of North Carolina, Chapel Hill, North Carolina 27514

Received March 18, 1981; Accepted May 5, 1981

SUMMARY

RUTH, J. L., AND Y.-C. CHENG. Nucleoside analogues with clinical potential in antivirus chemotherapy: the effect of several thymidine and 2′-deoxycytidine analogue 5′-triphosphates on purified human (α, β) and herpes simplex virus (types 1, 2) DNA polymerases. *Mol. Pharmacol.* 20:415–422 (1981).

Many pyrimidine nucleoside analogues exhibit potent anti-herpesvirus activity. Analogues of current interest in several laboratories include 5-propyl-2′-deoxyuridine, *E*-5-propenyl-2′-deoxyuridine, *E*-5-(2-bromovinyl)-2′-deoxyuridine, *E*-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-thymine (2′-fluoro-araT), 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methylcytosine, and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine. To aid in establishing the mechanisms of action and basis for selectivities of these seven analogues, the 5′-triphosphates were prepared for testing with DNA polymerases; a general method for the direct chemical synthesis of nucleoside triphosphate from nucleoside is described. The effects of the analogue triphosphates were evaluated on the following four isolated DNA polymerases: virus-induced DNA polymerases from herpes simplex virus Type 1 (HSV-1) and Type 2 (HSV-2) infections, and human DNA polymerases α and β, using conditions optimal for each. Compounds were evaluated for (a) competitive inhibition with regard to both dTTP and dCTP as independently competing substrates; (b) ability to support DNA synthesis in the absence of normally competing substrate; and (c) the effect of analogue incubation on primer template capability of resultant DNA. Competitive inhibition results indicate that all seven analogue triphosphates (a) are good inhibitors of normal substrate utilization by DNA polymerase regardless of enzyme source, (b) have much higher apparent affinities (20- to 600-fold lower K_i) for HSV polymerases than for human polymerases, and (c) are equally inhibitory to both HSV-1 and HSV-2 DNA polymerases. For example, the apparent inhibition constant (K_i) of 2′-fluoro-araTTP was 0.048 μM for HSV-1 (K_m of dTTP = 0.14 μM), 0.060 μM for HSV-2 (K_m of dTTP = 0.18 μM), 1.2 μM for human polymerase-α (K_m of dTTP = 5.4 μM), and 18 μM for human polymerase-β (K_m of dTTP = 8.6 μM); the relative abilities of competitive inhibition (in order of decreasing binding affinity as reflected by increasing K_i) were *E*-5-(2-bromovinyl)-araUTP > 2′-fluoro-arabinoside triphosphates > *E*-5-(2-bromovinyl)-dUTP > *E*-5-propenyl-dUTP > 5-propyl-dUTP for all polymerases except human β. The analogues varied considerably in support of DNA synthesis in the absence of normally competing substrate, again with little difference between polymerases; for example, regardless of enzyme source, 5-propyl-dUTP in the absence of dTTP resulted in 60–70% DNA synthesis relative to dTTP, whereas *E*-5-(2-bromovinyl)-araUTP gave little or no DNA synthesis, suppressing polymerase activity below background levels. The relative ability to support DNA synthesis was generally *E*-5-propenyl-dUTP ≃ dTTP > *E*-5-(2-bromovinyl-dUTP) > 5-propyl-dUTP ≫ 2′-fluoro-arabinonucleoside triphosphates ≫ *E*-5-(2-bromovinyl)-araUTP. Incubation of analogue triphosphates and polymerase with activated DNA suggests that, with *E*-5-(2-bromovinyl)-araUTP as the exception, the analogues have little effect on the

This work was supported by American Cancer Society Grant CH-29 to Y.-C. Cheng.

0026-895X/81/020415-08$2.00/0

subsequent ability of product DNA to serve as primer template. *E*-5-Propenyl-dUTP exhibited behavior markedly the most similar to dTTP throughout these studies. Some general observations concerning structure-activity relationships are discussed.

INTRODUCTION

In the last decade, several pyrimidine nucleoside analogues have proven to be remarkably potent and selective in their activity against herpes simplex virus infections. Little has been known of the exact mechanism(s) of action for most of these analogues. However, several recent studies have documented the postinfection production of viral-encoded enzymes related to DNA replication, enzymes which are clearly distinct from host enzymes in many properties. Such viral-specific enzymes include deoxythymidine kinases (1, 2), DNA polymerases (3), and dUTP nucleotidohydrolases (4), all of which are now serving as exploitable targets for viral-selective chemotherapy. To date, the most well characterized of the three are the viral-induced deoxythymidine kinases, both from HSV-1[1] and HSV-2 infections, which can clearly utilize as preferential alternate substrates many of the analogues that human cytosolic thymidine kinase cannot (5–9).[2] This apparent viral-specific monophosphorylation is presumed to lead to cellular toxicity by action at subsequent metabolic targets (human or viral) such as DNA polymerases. This "selective alternate substrate" hypothesis has been described by several groups (5–9), and is supported by two other noted observations: HSV-1 mutants lacking the ability to induce deoxythymidine kinase are essentially unaffected by this class of compounds, and, in most cases, the potency of a given compound appears proportional to its ability to be differentially phosphorylated by isolated viral deoxythymidine kinase. The latter observation may also explain the decreased sensitivity of HSV-2 infections to many of these analogues relative to HSV-1, since the isolated HSV-2 kinase generally demonstrates less affinity for a given compound (5).[2]

Once analogue monophosphate is formed, normal cellular metabolism as endogenous substrate would dictate conversion to triphosphate. At this point, there appear to be at least two distinct possibilities for cellular toxicity: a direct inhibitory effect on the viral or human DNA polymerases, and/or an effect on DNA integrity/fidelity after incorporation. To date, the effects of this class of nucleoside analogue at the polymerase/DNA level have not been examined in detail.

Analogues which seem particularly promising for clinical use as antiherpetics include 5-propyl-2′-deoxyuridine (10), *E*-5-propenyl-2′-deoxyuridine (11), *E*-5-(2-bromovinyl)-2′-deoxyuridine (12, 13), *E*-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil (14), 2′-fluoro-araTTP (15), 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methylcytosine (15), and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodo-cytosine (15) (refer to structures as the corresponding 5′-triphosphates in Fig. 1). Each of these compounds belongs to the class of analogues that are preferentially monophosphorylated by HSV-induced deoxythymidine kinase. To aid in establishing their antiviral mechanism(s) of action, the 5′-triphosphate of each was chemically synthesized and its behavior was tested against both purified HSV-induced DNA polymerases from Type 1 and Type 2 infections, and purified human DNA polymerase-α and -β, using conditions optimal for each. The effects reported here represent the ability of each analogue to inhibit competitively the utilization of normal substrate by the DNA polymerase (inhibition constant, or K_i), the ability to support DNA synthesis in the absence of competing substrate, and the effect of analogue incorporation on DNA primer template efficiency.

MATERIALS AND METHODS

Compounds. The nucleoside analogue 5′-triphosphates were synthesized (as described in the next paragraph) from nucleosides obtained as follows (see Fig. 1 for structural formulae as the 5′-triphosphates): crystalline 5-propyl-2′-deoxyuridine and *E*-5-propenyl-2′-deoxyuridine were prepared as reported earlier (16, 17); *E*-5-(2-bromovinyl)-2′-deoxyuridine was provided by Drs. R. Walker and E. De Clerq (12, 13); *E*-5-(2-bromovinyl)-1-β-D-arabinofuranosyluracil was obtained through Dr. H. Machida (14); 2′-fluoro-araTTP, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methylcytosine, and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine were provided by Dr. J. Fox (15). Deoxynucleoside 5′-triphosphates (dATP, dGTP, dCTP, and dTTP), DTT, and BSA were purchased from Sigma Chemical Company (St. Louis, Mo.) and used without further purification. Tritiated deoxynucleoside-5′-triphosphates ([5-*methyl*-^{3}H]dTTP, [5-^{3}H]dCTP, and [8-^{3}H]dATP) as tetrasodium salts were purchased from ICN Chemical and Radioisotopes Division (Irvine, Calif.). Activated DNA was prepared by treating calf thymus DNA (from Sigma Chemical Company) with pancreatic DNase at room temperature for 15 min using the procedure of Baril *et al.* (18). For triphosphate synthesis, trimethyl phosphate and tributylamine were purchased from Aldrich Chemical Company, Inc. (Milwaukee, Wisc.), phosphorous oxychloride, DMF, and triethylamines were obtained from Fisher Scientific Company (Springfield, N. J.). Tris(tributylammonium)pyrophosphate was prepared by vigorous mixing of cold aqueous pyrophosphoric acid with 3 Eq of tributylamine followed by repeated extended lyophilizations, then two evaporations from pyridine and one from DMF. All other materials were of commercial reagent-grade purity. Concentration of standard nucleoside triphosphate solutions were measured directly by optical density. For use in the polymerase assays, analogue triphosphates were diluted to 200 μM (±2%) stock solutions at pH 7.5 by direct optical density using the following ε values: 5-propyl-dUTP, 9,000 at 267 nm (16); *E*-5-pro-

The abbreviations used are: HSV, herpes simplex virus; HSV-1, HSV Type 1; HSV-2, HSV Type 2; 2′-fluoro-araTTP, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)thymine 5′-triphosphate; DTT, dithiothreitol; BSA, bovine serum albumin; DMF, *N*,*N*-dimethylformamide; 2′-fluoro-araCTP, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)cytosine; HPLC, high-pressure liquid chromatography.

[2] J. L. Ruth and Y.-C. Cheng, unpublished results.

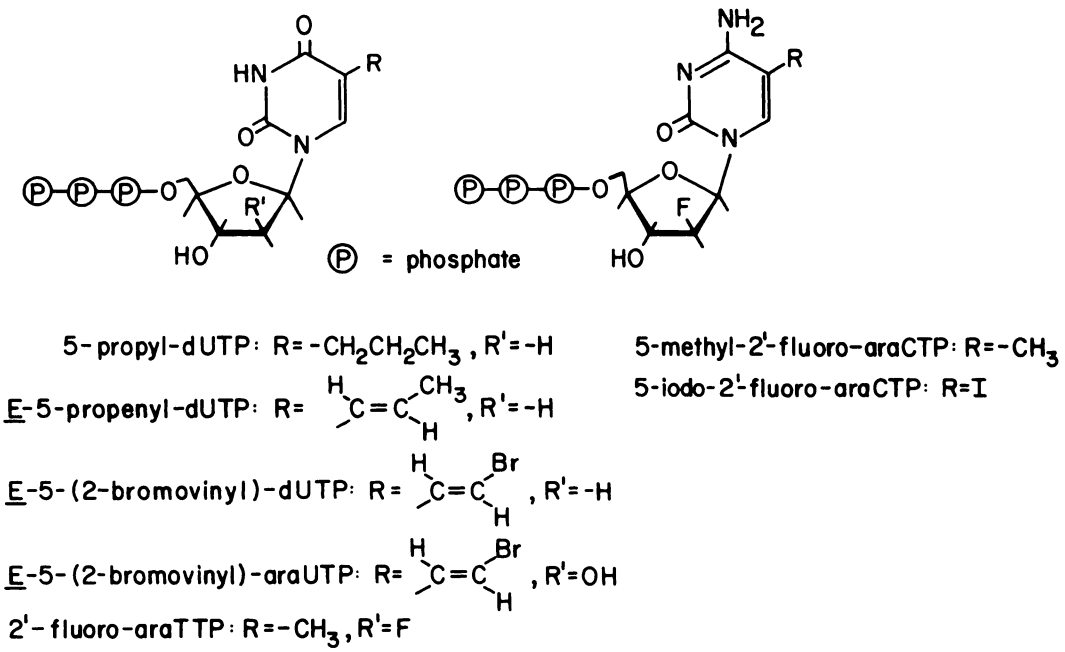

FIG. 1. *Structures of analogue 5'-triphosphates*

penyl-dUTP, 7,950 at 293 nm (16); *E*-5-(2-bromovinyl)-dUTP (12), and *E*-5-(2-bromovinyl)-araUTP, 11,660 at 296 nm; 5'-methyl-2'-fluoro-araCTP, 8,500 at 277 nm; 5-iodo-2'-fluoro-araCTP, 6,600 at 295 nm; and 2'-fluoro-araTTP, 9,600 at 266 nm (the latter three compounds were assumed to exhibit optical densities similar to those of their deoxynucleoside counterparts).

Synthesis and purification of analogue 5'-triphosphates. A general procedure for triphosphate synthesis was developed and used to prepare the seven analogue nucleotides. A known amount of nucleoside (15–80 mg, 0.04–0.3 mmole) was stirred in trimethyl phosphate (10 μl/mg of nucleoside) at −10°. Phosphorous oxychloride ($POCl_3$, 0.9 Eq)[3] was added, the reaction was stirred for 30 min, and a second 0.8 Eq of $POCl_3$ added. The reaction was allowed to warm to room temperature during stirring. At intervals, 2-μl aliquots of the reaction mixture were treated with excess aqueous hydroxide and assayed by analytical anion exchange HPLC using either a Whatman PXS 10/25 SAX or μ-Bondapak amine column eluting 0.03 N potassium phosphate, pH 6.6. After maximal formation (60–90%) of the intermediate nucleoside phosphodichloridate was observed (3–15 hr, strongly dependent on specific nucleoside), the reaction mixture was slowly added to excess tris(tributylammonium)pyrophosphate (5–8 Eq) in DMF (3–4 volumes relative to original reaction) at −10° with vigorous stirring. The reaction was allowed to warm to room temperature, and the mixture was assayed for triphosphate formation at frequent intervals by neutralization of 5-μl aliquots and chromatography by strong anion exchange HPLC (Whatman PXS 10/25 SAX column eluting 0.12 N potassium phosphate, pH 6.6). When formation of triphosphate appeared maximal (usually 20–60% of total nucleoside after 2–3 hr), the reaction was neutralized with cold excess aqueous triethylamine. The product was purified by ether extraction then column chromatography on Sephadex DEAE A-25 (formate) (30 ml of resin bed per millimole of nucleoside) eluting a gradient of 0.2–1.5 N triethylammonium formate. Fractions containing appropriately pure triphosphate (95–99% by HPLC) were combined, lyophilized to complete dryness, then relyophilized thoroughly from water to remove excess formate buffer. The nucleoside triphosphates were then redissolved in water, and brought to pH 7.5 with triethylamine; the yield was calculated by optical density at given wavelengths (see "Compounds"). On these synthetic scales, isolated yields of analogue triphosphate ranged from 8 to 46% (usually 20–35%) of theoretical from the nucleoside. In all cases, the observed UV absorption profile was unchanged from parent nucleoside. For the DNA polymerase assays, a small portion (50–100 optical density units) of the triphosphate was diluted to 1.0 mM at pH 7.5, and reassessed by analytical HPLC using reproducible electronic integration of the peak area to ensure integrity of the triphosphate (in all cases 96–99% triphosphate, 1–3% monophosphate, and trace nucleoside); the choice of UV detection at a wavelength appropriate to the specific analogue also allowed semiquantitative comparison of concentration. All triphosphates stored frozen at −20°. After completion of polymerase assays (20–30 thawings over 2 months), HPLC indicated no detectable decomposition.

Isolation of viral and human DNA polymerases. Enzyme preparations were provided by Mr. D. Derse in this laboratory. HSV-1 (KOS) and HSV-2 (333) DNA polymerases were highly purified from infected HeLa BU cells by DEAE-cellulose, phosphocellulose, and DNA cellulose column chromatography as detailed by Ostrander and Cheng (3). Human DNA polymerases were highly purified from HeLa S3 cells. The cells were homogenized, sonicated three times (20 sec each), and extracted by a buffer containing 200 mM potassium phosphate, pH 7.5.

[3] Initial monophosphorylation is an effective modification of the original approach by Yoshikawa *et al.* (19).

The supernatant was chromatographed on DEAE-cellulose eluting a feed buffer containing 200 mM potassium phosphate, pH 7.5, and the effluent was dialyzed to 50 mM and applied to a second DEAE-cellulose column. Elution with three column volumes of 50 mM buffer gave nonadsorbed protein-containing fractions which were pooled and used for isolation of DNA polymerase-β by a modification of the method of Stalker *et al.* (20). The column was then eluted with 200 mM buffer to bring off fractions containing DNA polymerase-α which was purified by modification of the method of Fisher and Korn (21). All enzymes were stored frozen at −70° in 50% glycerol (v/v) containing BSA, 1 mg/ml (no BSA present for polymerase-α).

Enzyme Assays

Competitive inhibition. Human DNA polymerase-α activity was assessed with respect to dTTP as competing substrate in a 100-μl assay mixture containing 70 mM Tris-HCl (pH 8.0); 8 mM $MgCl_2$; 70 μg of BSA; 0.7 mM DTT; 1–2% glycerol; 500 μM each of dATP, dGTP, and dCTP; 10 μM [^{3}H]dTTP (10 μCi/ml); 25 μg of activated calf thymus DNA; 150–200 units of enzyme[4]; and test compound at concentrations of 5.0, 10, and 40 μM. The mixture was incubated at 37° for 20 min. At precise intervals, acid-insoluble material was collected by precipitation of 50 μl of the assay mixture onto Whatman 3 MM paper discs with 10 ml/disc 5% trichloroacetic acid containing 10 mM tetrasodium pyrophosphate. Discs were washed three times (10 min per wash) with cold trichloroacetic acid/pyrophosphate solution, rinsed twice with 95% ethanol, and heat-dried thoroughly. Radioactive counts measured to less than 3% relative error by toluene-based liquid scintillation.

DNA polymerase-α activity with respect to dCTP as competing substrate was assessed in a similar manner by using 500 μM dTTP and 10 μM [^{3}H]dCTP (10 μCi/ml) in place of 500 μM dCTP and 10 μM [^{3}H]dTTP, respectively.

DNA polymerase-β activity was assayed in a manner similar to polymerase-α with inclusion of 100 mM KCl in the assay mixture, and using 200–250 units of enzyme.[4] It was assayed with independent respect to both dTTP and dCTP.

HSV-1 DNA polymerase activity with respect to dTTP as competing substrate was assessed in a 100-μl assay mixture containing 70 mM Tris-HCl (pH 8.0); 200 mM KCl; 4 mM $MgCl_2$; 70 μg of BSA; 0.7 mM DTT; 1–2% glycerol; 100 μM each dATP, dGTP, and dCTP; 1.0 μM [^{3}H]dTTP (5 μCi/ml); 13 μg of activated calf thymus DNA; 150–180 units of enzyme[4]; and test compound at concentrations of 0.50, 1.0, and 4.0 μM. The solution was incubated at 37° for 20 min; acid-insoluble radioactivity was then measured as with polymerase-α.

HSV-1 DNA polymerase activity with respect to dCTP as competing substrate was assessed in a similar manner using 100 μM dTTP and 1.0 μM [^{3}H]dCTP in place of 100 μM dCTP and 1.0 μM [^{3}H]dTTP, respectively.

HSV-2 DNA polymerase activity was assessed by using assay conditions similar to those used for HSV-1 polymerase, with independent respect to both dTTP and dCTP.

[4] One unit of polymerase is equal to the amount which will incorporate 1 pmole of [^{3}H]dTTP or [^{3}H]dCTP per hour using saturating conditions as described for the competitive inhibition studies.

Ability to support DNA synthesis in absence of dTTP or dCTP. The ability of an analogue triphosphate to support apparent DNA synthesis by human DNA polymerase-α in the absence of dTTP was assessed in 100-μl assay mixtures containing 70 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 70 μg of BSA, 0.7 mM DTT, 1–2% glycerol, 500 μM dGTP, 500 μM dCTP, 10 μM [^{3}H]dATP (10 μCi/ml), 25 μg of activated calf thymus DNA, enzyme, and 40 μM test compound. The solution was incubated at 37° for 20 min. At precise intervals, acid-insoluble material was assayed as before (see "Competitive Inhibition").

For polymerase-α in the absence of dCTP, the ability of analogue triphosphate to support apparent DNA synthesis was gauged in a similar manner by inclusion of 500 μM dTTP and exclusion of dCTP.

For DNA polymerase-β in the absence of dTTP or dCTP, the assays were similar to that for the α enzyme with inclusion of 100 mM KCl and using 30 μM test compound.

For both HSV-1 and HSV-2 DNA polymerases, the ability of the analogue triphosphate to support apparent DNA synthesis in the absence of dTTP or dCTP was assessed in a manner similar to that for DNA polymerase-α with the following changes: the amounts of $MgCl_2$ and activated DNA were one-half (4 mM and 13 μg, respectively), and 200 mM KCl was included.

Incubation. Incubation of human DNA polymerase-α with analogue triphosphate in absence of dTTP was carried out in 250-μl assay reaction mixtures containing 70 mM Tris-HCl(pH 8.0), 8 mM $MgCl_2$, 70 μg of BSA, 0.7 mM DTT, 1–2% glycerol, 400 μM dGTP, 400 μM dCTP, 8 μg of activated calf thymus DNA, enzyme, and 10 μM test compound. The solution was incubated for 40 min at 37°, covered, and immersed in water at 65° for 15 min to inactivate the enzyme. It was cooled at room temperature for 1 hr. Fifty microliters of [^{3}H]dATP and dTTP were added to final concentrations of 10 (10 μCi/ml) and 700 μM, respectively, in 250 μl of total assay volume. Fresh enzyme was added and the solution was incubated at 37°. At precisely indicated intervals (0, 10, 20, 40, and 80 min), 50 μl of the solution were withdrawn and absorbed onto a Whatman 3MM paper disc while the remainder continued to incubate over a time course. The disc was immediately treated in order to precipitate acid-insoluble materials as before.

Incubation of polymerase-α with analogue triphosphate in the absence of dCTP was carried out in a similar manner, using 400 μM dTTP in place of dCTP in the incubation medium and adding 600 μM dCTP after the incubation in place of 700 μM dTTP.

For human DNA polymerase-β incubation assays, the procedures were identical with those for polymerase-α with the inclusion of 100 mM KCl in the incubation.

For HSV-1 and HSV-2 DNA polymerase assays, the methods were similar to those used for polymerase-α with the following changes: the incubation mixture contained 200 μM each of dGTP and dCTP (or dTTP, as appropriate), 4 μg of activated DNA, 4 mM $MgCl_2$, 1.0 μM test compound, and 200 mM KCl.

RESULTS

Competitive inhibition. The abilities of 5-propyl-dUTP, *E*-5-propenyl-dUTP, *E*-5-(2-bromovinyl)-dUTP,

TABLE 1

Inhibition constants of analogue triphosphates for purified DNA polymerases

The 5′-triphosphates of several antiherpes compounds were tested against DNA polymerases isolated from various sources. All compounds were assayed concurrently against radiolabeled competing substrate (2–8 × K_m); assay conditions and enzyme purification are described under Materials and Methods. Values represent means of multiple trials in duplicate at three concentrations, giving 6–18 data points for each value with variations as standard deviation. Values given for K_m of dTTP and dCTP are as repeatedly established by this laboratory for these specific enzyme preparations.

Compound	Origin of DNA polymerase			
	HSV-1 (strain KOS)	HSV-2 (strain 333)	Human (HeLa)	
			α	β
	K_i μM			
With dTTP as competing substrate[a]				
K_m of dTTP[b]	0.14 ± 0.008	0.18 ± 0.04	5.4 ± 0.8	8.6 ± 0.4
5-Propyl-dUTP	0.24 ± 0.08	0.38 ± 0.09	19.7 ± 4.5	21 ± 4
E-5-Propenyl-dUTP	0.14 ± 0.03	0.12 ± 0.02	5.7 ± 0.8	8.7 ± 1.6
E-5-(2-Bromovinyl)-dUTP	0.068 ± 0.014	0.054 ± 0.09	3.6 ± 1.1	6.5 ± 1.3
E-5-(2-Bromovinyl)-araUTP	0.013 ± 0.004	0.021 ± 0.003	0.29 ± 0.07	12 ± 3
2′-Fluoro-araTTP	0.048 ± 0.018	0.060 ± 0.018	1.2 ± 0.3	18 ± 4
With dCTP as competing substrate[c]				
K_m of dCTP[b]	0.092 ± 0.005	0.11 ± 0.01	3.4 ± 1.8	2.4 ± 0.6
5-Methyl-2′-fluoro-araCTP	0.044 ± 0.010	0.058 ± 0.013	1.11 ± 0.25	4.7 ± 0.9
5-Iodo-2′-fluoro-araCTP	0.028 ± 0.006	0.041 ± 0.003	1.25 ± 0.1	5.2 ± 3.1
araCTP[b]	0.15	0.12	10	10
5-Iodo-dCTP[b]	0.12	0.08	—	—

[a] Compounds were also tested with respect to dCTP and were found to be noncompetitive.
[b] Included for comparison.
[c] Compounds were also tested with respect to dTTP and were found to be noncompetitive.

E-5-(2-bromovinyl)-araUTP, and 2′-fluoro-araTTP to inhibit the utilization of dTTP by cellular and virus-induced DNA polymerases are presented as apparent inhibition constants (K_i) in Table 1; 5-methyl-2′-fluoro-araCTP and 5-iodo-2′-fluoro-araCTP were also assayed as competitive inhibitors with respect to dTTP, but in all cases were found to be noncompetitive, as expected (for structures refer to Fig. 1). The abilities of 5-methyl-2′-fluoro-araCTP and 5-iodo-2′-fluoro-araCTP to inhibit the utilization of dCTP by the DNA polymerases are also presented in Table 1; the five uracil nucleoside triphosphates were also assayed and found to be noncompetitive with respect to dCTP, as expected. With the exception of 5-propyl-dUTP (which was mildly competitive), the analogue triphosphates were found to be good competitive inhibitors of both HSV DNA polymerases and human polymerase-α, with the K_i equal to or lower than the K_m of normal competing substrate; human polymerase-β was relatively less affected. Both viral-induced DNA polymerases were about equally inhibited by any given analogue, but were far more sensitive to all of the analogues than either of the human enzymes, with the viral K_i 20- to 80-fold lower than the K_i for polymerase-α, and 50- to 600-fold lower than the K_i for polymerase-β.

Ability to support DNA synthesis in the absence of competing substrate. The abilities of the analogue triphosphates to support DNA synthesis in the absence of competing substrates are summarized in Tables 2 and 3. The results clearly indicate that the 5-substituted-2′-deoxyuridine triphosphates [e.g., 5-propyl, *E*-5-propenyl- and *E*-5-(2-bromovinyl)-dUTP] support DNA synthesis to a great extent; the 2′-fluoro-arabinoside triphophates (e.g., 2′-fluoro-araTTP, 5-methyl-2′-fluoro-araCTP, and 5-iodo-2′-fluoro-araCTP) demonstrated little ability to support synthesis, the exceptions being significant DNA synthesis by both viral polymerases in the presence of either 2′-fluoro-araCTP analogue. *E*-5-(2-Bromovinyl)-araUTP was not supportive of DNA synthesis whatsoever in the absence of dTTP, and actually suppressed background activity in most instances.

TABLE 2

The ability of competing analogue triphosphates to support DNA synthesis in the absence of dTTP

The 5′-triphosphates of several antiherpes compounds were tested in the presence of DNA polymerases from various sources. All compounds were assayed concurrently against dTTP as standard. Assays contained 500 μM dGTP, 500 μM dCTP, 10 μM [^{3}H]dATP (10 μCi/ml), and 40 μM compound (30 μM with polymerase-β), using activated calf thymus DNA as primer template; specific assay conditions and enzyme purification are described under Materials and Methods. The values reported are percentages of apparent DNA synthesis relative to dTTP, and are the means of multiple trials in duplicate giving 4–12 data points for each, with variations as standard deviation.

Compound added	Origin of DNA polymerase			
	HSV-1 (strain KOS)	HSV-2 (strain 333)	Human (HeLa)	
			α	β
	relative % DNA synthesis			
dTTP	100	100	100	100
5-Propyl-dUTP[a]	64 ± 8	71 ± 0.5	65 ± 1.7	57 ± 2.5
E-5-Propenyl-dUTP	84 ± 0.7	83 ± 3	123 ± 4	86 ± 1.4
E-5-(2-Bromovinyl)-dUTP	72 ± 5	61 ± 5	99 ± 12	80 ± 3
E-5-(2-Bromovinyl)-araUTP	2 ± 0.2	1 ± 0.3	5 ± 1	34 ± 1.1
2′-Fluoro-araTTP	12 ± 5	13 ± 2	25 ± 6	37 ± 1.4
No additive (background)	7 ± 1	10 ± 0.5	18 ± 1	32 ± 3

[a] See ref. 22 for comparison in other systems.

Effect of incubation on primer template efficiency. Analogue triphosphates and DNA polymerases were in-

TABLE 3

The ability of competing analogue triphosphates to support DNA synthesis in absence of dCTP

The 5′-triphosphates of two antiherpes compounds were tested in the presence of DNA polymerases from various sources. Compounds were assayed concurrently against dCTP as standard in assays containing 500 μM dGTP, 500 μM dTTP, 10 μM [^{3}H]dATP (10 μCi/ml), and 40 μM compound (30 μM for polymerase-β), using activated calf thymus DNA as primer template. For specific assay conditions and enzyme purification see Materials and Methods. The values are reported as percentages of apparent DNA synthesis relative to dCTP, and are the means of multiple trails in duplicate giving 4–6 points for each with variations as standard deviation.

Compound added	Origin of DNA polymerase			
	HSV-1 (strain KOS)	HSV-2 (strain 333)	Human (HeLa)	
			α	β
	relative % DNA synthesis			
dCTP	100	100	100	100
5-Methyl-2′-fluoro-araCTP	38 ± 0.4	49 ± 1.2	24 ± 6	44 ± 2
5-Iodo-2′-fluoro-araCTP	41 ± 1.7	49 ± 1.4	30 ± 4	44 ± 4
No additive (background)	14 ± 0.5	19 ± 1.1	27 ± 3.5	48 ± 3

TABLE 4

Effect of incubation with analogue triphosphate on DNA primer template capability

The 5′-triphosphates of several antiherpes compounds were tested with DNA polymerases from various sources. All compounds were assayed concurrently against competing substrate as reference. Assays consisted of 40-min incubation of a minimum of activated DNA with saturating dGTP and dCTP (or dTTP when testing 2′-fluoro-araCTP analogue) with compound in the presence of the polymerase, followed by heat inactivation of enzyme and addition of 10 μM [^{3}H]dATP and 500 μM competing substrate; incorporation of label was plotted as a function of time after addition of fresh enzyme. Percentage efficiency relative to normal substrate was derived by comparison of rate with analogue present to rate with normal substrate present × 100%. See Materials and Methods for exact assay conditions and enzyme purification. Values are reported as means of duplicate trials at four time points (10, 20, 40, and 80 min).

Compound in incubation	Origin of DNA polymerase			
	HSV-1 (strain KOS)	HSV-2 (strain 333)	Human (HeLa)[a]	
			α	β
	Relative % efficiency (± 5%) of primer-template DNA			
5-Propyl-dUTP[b]	97	——[c]	98	100
E-5-Propenyl-dUTP	102	83	94	93
E-5-(2-Bromovinyl)-dUTP	101	95	100	95
E-5-(2-Bromovinyl)-araUTP	92	80	61[d]	79
2′-Fluoro-araTTP	87	——[c]	104	82
5-Methyl-2′-fluoro-araCTP	100	94	100	88
5-Iodo-2′-fluoro-araCTP	100	100	100	95

[a] Repetition with variable incubation and constant assay times gives similar results.

[b] See ref. 23[5] for comparison in other systems.

[c] Not tested.

[d] Refer to Fig. 2.

[5] D. Derse and Y.-C. Cheng, unpublished results.

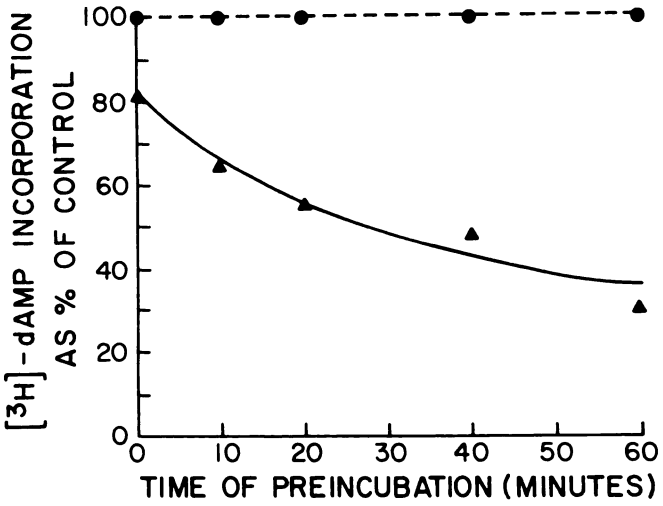

FIG. 2. *Effect of E-5-(2-bromovinyl)-araUTP incorporation on DNA primer template efficiency*

E-5-(2-bromovinyl)-araUTP (10 μM) or dTTP (10 μM) as control were incubated with 500 μM dGTP and dCTP in the presence of human DNA polymerase-α and activated DNA. After the indicated time of incubation, the reaction mixture was heated at 65° to inactivate the enzyme (see Materials and Methods). Incubation (40 min) with fresh enzyme after addition of 500 μM dTTP and 10 μM [^{3}H]dATP (10 μCi/ml) showed a decrease in DNA synthesis proportional to time of incubation with *E*-5-(2-bromovinyl)-araUTP (▲) relative to dTTP control (●); the 19% difference in activity at $t = 0$ is apparently due to direct inhibition of polymerase. In contrast, similar trials with *E*-5-propenyl-dUTP, *E*-5-(2-bromovinyl)-dUTP, and 2′-fluoro-araTTP gave no significant increasing inactivation.

cubated for constant times with a minimum of activated DNA to maximize incorporation, if any, of analogue. The primer template efficiency of the product DNA in subsequent assay with polymerase in the presence of saturating normal triphosphate was compared with unmodified control; results are summarized in Table 4. Whether or not they were incorporated, most analogues had little (less than 15–20%) effect on the ability of the modified DNA to serve as primer template. *E*-5-(2-Bromovinyl)-araUTP, however, appeared to cause a noticeable decrease in rate of DNA synthesis, particularly with polymerase-α. To ascertain the extent of effect of *E*-5-(2-bromovinyl)-araUTP, trials of similar design but using variable times of incubation showed a marked decrease in DNA primer template capability with increasing time of incubation in the absence of dTTP, as represented in Fig. 2.

DISCUSSION

The nucleoside precursors of the triphosphates examined here represent a class of potent antiviral pyrimidine nucleoside analogues, each with a structural modification at one or both of two distinct sites (C-5 and C-2′). Results summarized in Table 1 clearly indicate the mild-to-potent competitive inhibition of DNA polymerases by each of the analogue triphosphates. 5-Propyl-dUTP was the least inhibitory of the analogues, with $K_i \simeq 2\ K_m$ of dTTP. Good inhibition was exhibited by *E*-5-propenyl-dUTP and particularly by *E*-5-(2-bromovinyl)-dUTP; all three 2′-fluoroarabinofuranosylpyrimidine nucleotides were more potent, with a viral K_i of 3–6 × 10^{-8} M. However, the most potent inhibitory effect was demonstrated by

E-5-(2-bromovinyl)-araUTP, with K_i for viral polymerases of 2×10^{-8} M or less; *E*-5-(2-bromovinyl)-araUTP also exhibited a more marked inhibitory effect on human polymerase-α than the other analogues (refer to Table 5 and later discussion). The following two additional general observations are of particular note: (a) the compounds are clearly preferentially recognized by herpesvirus DNA polymerases, and (b) no compound shows significant differential inhibition of HSV-1 polymerase over HSV-2. All of the analogue triphosphates can thus be considered quite selective for the herpesvirus-induced DNA polymerases. However, despite the lack of distinct kinetic difference between isolated HSV-1 and HSV-2 polymerases, HSV-1 infections are generally much more sensitive to the parent nucleosides on a cellular level; presumably the preferential inhibition of virus-encoded DNA polymerase is not the sole factor governing antiviral activity, but may potentiate the selective effect initiated by the viral thymidine kinase (5–9).

The apparent affinity of the analogue triphosphate relative to normally competing substrate is represented by the value of K_m/K_i in Table 5. Comparison of K_m/K_i between the analogue triphosphates again offers a measure of relative potencies for the analogues, with an additional observation now more apparent: with the exception of human polymerase-β, the K_m/K_i ratio for a given analogue remains relatively constant. For example, K_m/K_i of 5-iodo-2′-fluoro-araCTP for HSV-1, HSV-2, and α polymerases are 3.3, 2.7, and 2.7, respectively. Thus, even though the absolute inhibition (as reflected by K_i) can vary significantly between polymerases of various origin, analogue competition with natural substrate (K_m/K_i) remains essentially constant for three of the enzymes, perhaps reflecting marked similarity in active sites. With polymerase-β, K_m/K_i similar to those of the viral and α enzymes are observed for the three deoxynucleotide analogues, but, unlike the other polymerases, modification to the sugar moiety significantly lessens affinity. From these similarities, several preliminary conclusions can be drawn concerning structure-activity relationships relative to naturally competing substrate: (a) the apparent binding affinity for DNA polymerases was decreased by C-5 propyl [and, presumably, longer alkyl (24)], was increased by C-5 2-bromovinyl and was essentially unaffected by the presence of C-5 propenyl, methyl, or iodo; (b) excluding human polymerase-β, the apparent binding affinity was increased significantly (2- to 4-fold) by the presence of 2′-hydroxyl or 2′-fluoro in the arabinose configuration (exception: the 2′-hydroxyl of araCTP actually decreased binding).

TABLE 5

Relative apparent binding of analogue triphosphates to DNA polymerases of various origins

Binding of analogue triphosphate to DNA polymerases relative to naturally competing substrate is expressed as K_m/K_i, where K_m is affinity of the competing substrate (dTTP or dCTP) and K_i is the apparent inhibition constant in the presence of saturating natural substrate, as in Table 1.

Compound	Origin of DNA polymerase			
	HSV-1 (strain KOS)	HSV-2 (strain 333)	Human (HeLa)	
			α	β
	K_m/K_i			
5-Propyl-dUTP	0.58	0.47	0.27	0.41
E-5-Propenyl-dUTP	1.0	1.5	0.95	0.99
E-5-(2-Bromovinyl)-dUTP	2.1[b]	3.3	1.5[b]	1.3[b]
E-5-(2-Bromovinyl)-araUTP	10.8	8.6	19	0.72
2′-Fluoro-araTTP	2.9	3.0	4.5	0.48
5-Methyl-2′-fluoro-araCTP	2.1	1.9	3.1	0.50
5-Iodo-2′-fluoro-araCTP	3.3	2.7	2.7	0.46
araCTP[a]	0.6	0.9	0.3	0.3
5-Iodo-dCTP[a]	0.8	1.4	——	——

[a] Included for comparison.

[b] See ref. 25.

To gauge the ability of an analogue triphosphate to serve as alternate substrate, the support of DNA synthesis in the absence of competing substrate (dTTP or dCTP) was measured by the inclusion of a saturating amount of kinetically independent [^{3}H]dATP. The results (refer to Tables 2 and 3) indicate that the three deoxynucleotides [5-propyl-, *E*-5-propenyl-, and *E*-5-(2-bromovinyl)-dUTP] were supportive of efficient DNA synthesis in the absence of dTTP; this was particularly true of *E*-5-propenyl-dUTP, since polymerase from any of the four sources recognized the analogue as a good alternate substrate (especially polymerase-α, which consistently exhibited activity 20–30% higher than in the sole presence of dTTP). 5-Propyl- (24)[6] and *E*-5-(2-bromovinyl)-dUTP were somewhat less effective, with the latter compound also showing a similar tendency to serve as a better alternate substrate for polymerase-α. In marked contrast, *E*-5-(2-bromovinyl)-araUTP did not act as alternate substrate for DNA elongation at all in the absence of dTTP, in effect suppressing synthesis below that observed as background. 2′-Fluoro-araTTP supported DNA synthesis very little, not more than 5% over background, whereas both 2′-fluoro-araCTP analogues (5-methyl- and 5-iodo-2′-fluoro-araCTP) apparently could be utilized to a limited extent (<50%) only by viral polymerases and not the human enzymes.

If an analogue known to be a competitive inhibitor of DNA polymerase does not support apparent DNA synthesis, one of two processes may be occurring: the analogue may be inhibitory but may not serve as alternate substrate, or it may be recognized/incorporated as an alternate substrate but may suppress apparent DNA synthesis by subsequent effect on primer template elongation. The former possibility is difficult to rule out completely without direct use of radiolabeled analogue. As a measure of the latter, the polymerase and analogue triphosphate were incubated with a minimum of activated DNA to maximize analogue incorporation. When the resultant DNA serves as primer template in subsequent assays with excess natural triphosphates present, any significant variation in rate from normal DNA synthesis should reflect the effect of the analogue after incorporation, i.e., on primer template capability. As is summarized in Table 4, most of the analogues had little, if any, effect. This was expected of the three dUTP analogues, since earlier results (Table 2) had indicated

[6] 5-Propyl-2′-deoxy-[2-^{14}C]uridine is phosphorylated *in vivo* and incorporated into the DNA of HSV-1-infected HeLa cells (unpublished results from this laboratory).

each could effectively serve as alternate substrate, including DNA elongation. The most notable effect on primer template was by *E*-5-(2-bromovinyl)-araUTP, especially when in the presence of polymerase-α. In subsequent experiments, variable incubation of *E*-5-(2-bromovinyl)-araUTP showed progressive inactivation of product primer template (refer to Fig. 2), indicating that *E*-5-(2-bromovinyl)-araUTP is incorporated but may suppress further DNA elongation by chain termination effects similar to those of araATP, araCTP, and 2′-azido-araCTP (23).[5] It should be noted here that both HSV-1- and HSV-2-induced DNA polymerases have integrally associated exonuclease activity (3, 23)[5]; as a result, analogues which may serve to affect primer template efficiency negatively after incorporation may be excised by the associated exonuclease, allowing at least temporary recovery of primer template capability. In this study, the three 2′-fluoroarabinosylnucleotide analogues may in fact be incorporated by viral polymerases, especially since both 2′-fluoro-araCTP analogues apparently support limited DNA synthesis by the viral enzymes, but excision by exonuclease could preclude detection of decreased primer template efficiency with this approach. This may also account for the less effective inactivation of viral polymerases by *E*-5-(2-bromovinyl)-araUTP. Note that the over-all effect on primer template by any of these analogues is the sum of two distinct properties: extent of incorporation and subsequent effects on elongation, both of which are also reflected to a degree by the previous alternate substrate studies (Tables 2 and 3).

Several preliminary conclusions can be drawn based on these structural effects: (a) the 5-substituted dUTP analogues [5-propyl-, *E*-5-propenyl-, and *E*-5-(2-bromovinyl)-dUTP] are recognized by DNA polymerases as alternate substrates, are incorporated, and have little or no effect on the primer template capability of the resultant DNA; (b) araUTP analogues [such as *E*-5-(2-bromovinyl)-araUTP] do not serve as efficient alternate substrates but can be incorporated and inhibit subsequent DNA elongation, most likely by chain termination; (c) 2′-fluoro-araCTP analogues (5-methyl- and 5-iodo-2′-fluoro-araCTP) are not recognized as alternate substrates to any extent except by viral polymerases and apparently do not affect DNA primer template capability significantly even if incorporated; and (d) 2′-fluoro-araTTP is largely unincorporated and may or may not exhibit some effect on subsequent primer template ability. Except as was noted for the two 2′-fluoro-araCTP analogues, there is no apparent selective utilization of any analogue as alternate substrate by the HSV-induced DNA polymerases relative to the human enzymes, nor are there significant differences between HSV-1 and HSV-2 polymerases. Similar testing of other antiviral nucleoside analogue triphosphates is in progress and should serve to verify further the structure-activity relationships discussed here.

ACKNOWLEDGMENTS

We thank Mr. David Derse of this laboratory for providing us with the purified DNA polymerases and much needed advice, and our colleagues Drs. Richard Walker, Eric De Clercq, Haruhiko Machida, and Jack Fox for providing many of the test compounds as nucleosides.

Note added in proof. K_m/K_i values derived for *E*-5-(2-bromovinyl)-dUTP agree well with values recently published.

REFERENCES

1. Cheng, Y.-C., and M. Ostrander. Deoxythimidine kinase induced in HeLa TK$^-$ cells by herpes simplex virus type 1 and type 2. II. Purification and characterization. *J. Biol. Chem.* **251**:2605–2610 (1976).
2. Cheng, Y.-C. Deoxythymidine kinase induced in HeLa TK$^-$ cells by herpes simplex virus type 1 and type 2. Substrate specificity and kinetic behaviors. *Biochim. Biophys. Acta* **452**:370–381 (1976).
3. Ostrander, M., and Y.-C. Cheng. Properties of herpes simplex virus type 1 and type 2 DNA polymerase. *Biochim. Biophys. Acta* **609**:232–245 (1980).
4. Caradonna, S., and Y.-C. Cheng. Induction of uracil-DNA glycosylase and dUTP nucleotidohydrolase activity in herpes simplex virus-infected human cells. *J. Biol. Chem.*, in press (1981).
5. Cheng, Y.-C., G. Dutschman, E. De Clercq, A. Jones, S. Rahim, G. Verhelst, and R. Walker. Differential affinities of 5-(2-halovinyl)-2′-deoxyuridines for deoxythymidine kinase of various origin. *Mol. Pharmacol.* **20**:000–000 (1981).
6. Elion, G., P. Furman, J. Fyfe, P. de Miranda, L. Beauchamp, and H. Shaeffer. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl) guanine. *Proc. Natl. Acad. Sci. U. S. A.* **74**:5716–5720 (1977).
7. Cheng, Y.-C. A rational approach to the development of antiviral chemotherapy: alternative substrates of herpes simplex virus type 1 and type 2 thymidine kinase. *Ann. N. Y. Acad. Sci.* **284**:594–598 (1977).
8. Prusoff, W., and P. Fischer. Basis for the selective antiviral and antitumor activity of pyrimidine nucleoside analogs, in *Nucleoside Analogues* (R. Walker, E. DeClercq, and F. Eckstein, eds.). Plenum Press, New York, 281–318 (1979).
9. Cheng, Y.-C. Strategy for the development of selective antiherpes virus agents based on the unique properties of virus-induced enzymes: thymidine kinase, DNase and DNA polymerase, in *Antimetabolites in Biochemistry, Biology and Medicine* (J. Skoda and P. Langen, eds.). Pergamon Press, Oxford, 263–274 (1979).
10. Cheng, Y.-C., B. Domin, R. Sharma, and M. Bobek. Antiviral action and cellular toxicity of four thymidine analogs: 5-ethyl-, 5-vinyl-, 5-propyl- and 5-allyl-2′-deoxyuridine. *Antimicrob. Agents Chemother.* **10**:119–122 (1976).
11. Cheng, Y.-C., S. Grill, J. Ruth, and D. Bergstrom. Anti-herpes simplex virus and anti-human cell growth activity of *E*-5-propenyl-2′-deoxyuridine and the concept of selective protection in antivirus chemotherapy. *Antimicrob. Agents Chemother.* **18**:957–961 (1980).
12. Jones, A., G. Verhelst, and R. Walker. Synthesis of the potent antiherpes agents (E)-5-(2-bromovinyl)-2′-deoxyuridine and related compounds. *Tetrahedron Lett.* **45**:4415–4418 (1979).
13. De Clercq, E., J. Descamps, P. De Somer, P. Barr, A. Jones and R. Walker. (E)-5-(2-Bromovinyl)-2′-deoxyuridine: a potent and selective antiherpes agent. *Proc. Natl. Acad. Sci. U. S. A.* **76**:2947–2951 (1979).
14. Sakata, S., S. Shibuya, H. Machida, H. Yoshino, K. Hirota, S. Senda, K. Ikeda, and Y. Mizuno. Synthesis and antiherpesviral activity of 5-C-substituted uracil nucleosides. *Nucl. Acids Res. Symposium Series*, **8**:s39–s42 (1980).
15. Watanabe, K., U. Reichman, K. Hirota, C. Lopez, and J. Fox. Nucleosides. 110. Synthesis and antiherpes virus activity of some 2′-fluoro-2′-deoxyarabinofuranosylpyrimidine nucleosides. *J. Med. Chem.* **22**:21–24 (1979).
16. Ruth, J., and D. Bergstrom. C-5 Substituted pyrimidine nucleosides. 1. Synthesis of C-5 allyl-, propyl-, and propenyl-uracil and cytosine nucleosides via organopalladium intermediates. *J. Org. Chem.* **43**:2870–2876 (1978).
17. Bergstrom, D., J. Ruth, and P. Warwick. C-5 substituted pyrimidine nucleosides. 3. Reaction of allylic chlorides, alcohols, and acetates with pyrimidine nucleoside-derived organopalladium intermediates. *J. Org. Chem.* **46**:1432–1441 (1981).
18. Baril, E., J. Mitchener, L. Lee, and B. Baril. Action of pancreatic DNase: requirements for activation of DNA as a template-primer for DNA polymerase α. *Nucl. Acids Res.* **4**:2641–2653 (1977).
19. Yoshikawa, M., T. Kato, and T. Takenishi. Phosphorylation. III. Selective phosphorylation of unprotected nucleosides. *Bull. Chem. Soc. Jpn.* **42**:3505–3508 (1969).
20. Stalker, D., D. Mosbaugh, and R. Meyer. Novikoff hepatoma deoxyribonucleic acid polymerases. Purification and properties of a homogenous β-polymerase. *Biochemistry* **15**:3114–3121 (1976).
21. Fisher, P., and D. Korn. DNA polymerase-α. Purification and structural characterization of the near homogenous enzyme from human KB cells. *J. Biol. Chem.* **252**:6528–6535 (1977).
22. Sagi, J., and L. Otvos. Modified polynucleotides. IV. Template activity of 5-alkyluracil-containing poly[d(A-r^{5}U)] copolymers for DNA and RNA polymerases. *Nucl. Acids Res.* **7**:1593–1601 (1979).
23. Derse, D., and Y.-C. Cheng. Comparative studies of the kinetic properties of herpes simplex virus type 1, type 2, human α, and β DNA polymerases. *Fed. Proc.* **39**:1956 (1980).
24. Sagi, J., R. Nowak, B. Zmuzka, A. Szemzo, and L. Otvos. A study of substrate specificity of mammalian and bacterial DNA polymerases with 5-alkyl-2′-deoxyuridine 5′-triphosphates. *Biochim. Biophys. Acta* **606**:196–201 (1980).
25. Allaudeen, H. S., J. W. Kozarich, J. R. Bertino, and E. De Clercq. On the mechanism of selective inhibition of herpesvirus replication by *E*-5-(2-bromovinyl)-2′-deoxyuridine. *Proc. Natl. Acad. Sci. U. S. A.* **78**:2698–2702 (1981).

Send reprint requests to: Dr. Jerry L. Ruth, Department of Pharmacology, School of Medicine, University of North Carolina, Chapel Hill, N. C. 27514.