The purine derivative, 9-(2-hydroxyethoxymethyl)guanine, has recently been shown to be a potent inhibitor of HS-1 and HS-II virus replication in vitro and in vivo (1, 2). Concomitant with the antiviral activity was the formation of the triphosphate derivative of acyclo-Guo. This was found almost exclusively in treated cells infected with virus and appeared to be the basis for the selective activity of the drug.

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1 The abbreviations used are: HS.1 virus, herpes simplex virus (type 1); acyclo-Guo, 9-(2-hydroxyethoxymethyl)guanine; acyclo-GM1 \(^\text{TM}\), the phosphorylated (side chain hydroxyl) product of acyclo-Guo; arabinosylcytosine, cytosine, 2',5'-arabinosine, guanine, and thymine are the \(\beta\)-D-arabinofuranosyl derivatives of the purine or pyrimidine bases; albumin, bovine serum albumin; PEI-cellulose, polyethyleneimine-imregnated cellulose; TLC, thin layer chromatography.

Recent studies with several pyrimidine nucleoside analogues (3-11) have indicated that inhibition of herpes simplex virus replication with low host cell toxicity may be attained because the virus contains the genetic information to induce a unique dThd kinase (12-16). The nucleoside analogues may be preferentially phosphorylated by the dThd kinase to toxic compounds only in infected cells.

Since phosphorylation of acyclo-Guo appeared to be necessary for its antiviral activity (2), it was of interest to determine the nature of the enzyme that phosphorylates this structural analogue of dGuo (Fig. 1). In addition, it was important to determine whether this enzyme was virus-coded and whether this phosphorylation was essential for the antiviral activity of acyclo-Guo. The studies indicate that acyclo-Guo is, in fact, phosphorylated by a dThd kinase that is virus-coded, and its antiviral activity depends on this phosphorylation. Some of these findings have been briefly described in a preliminary report (2).

**EXPERIMENTAL PROCEDURES**

**Materials**

Commercially Available These materials were obtained from the following sources: [methyl-\(^{14}\)C]deoxyguanosine (43 Ci/mol), [2, \(^{14}\)C]deoxyadenosine (30 Ci/mol), and [8-\(^{14}\)C]deoxythymidine (30 Ci/mol) from New England Nuclear, Boston, MA; [8-\(^{14}\)C]deoxyguanosine (51 Ci/mol) from Schwarz/Mann, Orangeburg, NY; phosphoenolpyruvic acid (14 Ci/mol), [8-\(^{14}\)C]AMP (41 Ci/mol), and [5, \(^{3}\)H]deoxythymidine (3000 Ci/mol) from Amersham, Arlington Heights, IL; nucleosides and nucleotides except where mentioned otherwise, di-thiothreitol, and Escherichia coli alkaline phosphatase (EC 3.1.3.1, 20 units/mg) from P-L Biochemicals, Milwaukee, WI; Tris, streptomycin sulfate, deoxymethylycystine, bovine serum albumin (Fraction V), beef heart lactate dehydrogenase (EC 1.1.1.27, type III, 620 units/mg), pyruvate kinase (EC 2.7.1.40, type II, 200 units/mg), and DNase I (EC 3.1.4.5, type DN-100, 1000 Kunitz units/mg) from Sigma Chemical Co., St. Louis, MO; phenylmethylsulfonyl fluoride from Calbiochem, La Jolla, CA; 5-bromo-2'-deoxyadenosine, 5-iododeoxyuridine, and enzyme grade ammonium sulfate from Schwarz/Mann, Orangeburg, NY; arabinosylcytosine from Pfanstiehl, Waukegan, IL; materials used for polyacrylamide gel electrophoresis from Bio-Rad. Richmond, CA; DEAE-cellulose paper (DE81) from Whatman, Clifton, NJ; thymidine phosphorylase (EC 2.4.2.4) from Wellcome Reagents, Research Triangle Park, NC; PEI-cellulose TLC plates from Brinkmann Instruments, Westbury, NY; xanthine oxidase (EC 1.2.3.2, 1 unit/mg) and RNase (EC 2.7.7.16, grade III, 90 Kunitz units/mg) from Gallard-Schlesinger, Carle Place, NY; Vero (CCL 81) and HeLa (CCL 2.2) cells from American Type Culture Collection, Rockville, MD.

Synthesized in these Laboratories—Arabinosylguanine, arabinosyl-2',5'-diaminopurine, and acyclo-Guo were prepared by reported procedures (1, 17). [8-\(^{14}\)C]Araclo-Guo (18 Ci/mol), [1-(2-hydroxyethoxymethyl)cytosine, and 1-2-(hydroxyethoxymethyl)thymyne were synthesized by Jeffrey Scharver, John Kelsey, and James Kelley by procedures (1, 17). [8-\(^{14}\)C]Acyclo-Guo (18 Ci/mol), 1-(2-hydroxyethoxymethyl)cytosine, and 1-2-(hydroxyethoxymethyl)thymyne were synthesized by Gerald Haggerty by the method of Horwitz et al. (18). An affinity gel with dThd bound to agarose was synthesized by Ronald Crouch using the method of Lee and Cheng (19).
A preparation of 5'-nucleoside phosphotransferase from *Serratia marcescens* (20) was used to synthesize authentic acyclo-Guo monophosphate (acyclo-GMP) (23). The cells (ATCC 14227) were grown to early stationary phase in Difco nutrient broth medium supplemented with 1% glucose, broken in a French pressure cell, and the resultant extract centrifuged at 5,000 × g to remove cell debris. This supernatant was incubated at about 24°C for 1 h with RNase and DNase (1 mg each per 20 ml) and centrifuged at 100,000 × g for 2 h. The precipitate was resuspended in 0.33% Triton X-100 containing 0.3% sodium deoxycholate, sonicated, and centrifuged as in the previous step. The supernatant was concentrated in an Amicon ultrafiltration cell using an XM-100 membrane.

The product was found to be >98% pure by high pressure liquid chromatography (9); it remained as a single spot during chromatography on cellulose with 1-propanol/water (7.3, RF = 0.59) and on PEI-cellulose with 2 M formic acid/2 M LiCl (1:1, RF = 0.72); its UV spectrum was the same as that of the starting reactant (acyclo-Guo; 0.1 M HCl, λmax = 255 nm, 277 nm (sh); pH 7, λmax = 252 nm, 272 nm (sh)); its base to phosphate (22) ratio was 1.00:0.99, and treatment with RNase and DNase completely modified the product to a compound that co-chromatographed with acyclo-Guo (cellulose TLC with 1-propanol/water, 7.3, RF = 0.53).

The preparation was kindly provided by Jack J. Fox, Sloan-Kettering Institute, Rye, NY; tetrahydrodouridine (NSC-112907) by Harry B. Wood, Jr., Drug Synthesis and Chemistry Branch, Sloan-Kettering Institute, Rye, NY; tetrahydrouridine (NSC-112907) by D. J. Bauer, Wellcome Research Laboratories, Beckenham, England; and other HS-I virus strains as reported previously (2).

**Enzyme Assays**

For all enzymes used in this study, a unit of activity was defined as that amount of enzyme that would catalyze the formation of 1 pmol of product/min at 37°C unless otherwise specified. Measurements of the activity of the HS-I virus dThd kinase (acyclo-Guo, dCyd) kinase were made with dThd as the nucleoside substrate. Protein concentration was determined by the Coomassie blue method (23, 24) with albumin as the protein standard.

**Standard Nucleoside Kinase DEAE Paper Assay**—Samples containing enzymes were assayed for nucleoside phosphorylating activities by incubating 40 to 50 μl of the sample in an 80- to 100-μl reaction mixture containing 155 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 10 mM ATP (neutralized with KOH), and 1 mM ³²P-labeled nucleoside (1 to 4 pmol/pmol, ethanol removed with dry N₂ before using) at 37°C. For reactions at pH 6.0, 135 mM potassium maleate was substituted for Tris-HCl. Reactions were initiated with the addition of enzyme and terminated after 1 to 60 min by spotting 20-μl aliquots onto Whatman DE81 paper. The DE81 sheets were handled in a manner similar to that of Altman and Lerman (25) except that 1.7 cm squares and a different rinsing procedure were used. For assays with acyclo-Guo, a 5-min rinse with water was followed by two rinses with a solution containing 70% ethanol, 2 mM ammonium acetate (pH 7.5), and 1 mM Guo, and one rinse with 95% ethanol. For dThd, the water rinse was followed by three rinses with 95% ethanol, and for dCyd the water rinse was followed by three more rinses with water and one with 95% ethanol. Toluene with 4 g of 2,5-diphenyloxazole and 0.1 g of dimethyl-1,4-bis[4-(methyl-5-phenyloxazolyl)]benzene per liter was used as the scintillation fluid. The reaction velocities were constant up to at least 1 h (less than 15% product formation). All reaction velocities reported were calculated from time courses of reactions with at least four time points. Comparisons of enzyme activity were made using this assay versus the coupled assays below or an assay employing a separation of reactant from product on TLC (PEI-cellulose and sequential development with water and either 0.5 M LiCl or 1 M LiCl/1 M formic acid (1:1) for dThd or acyclo-Guo, respectively). Quantitation with the latter assay was effected by cutting out product and reactant spots and determining radioactivity as above. The RF values for dThd, dCyd, and dTMP or its mono- or diphosphate fractions were 0.90, 0.51, 0.19 and 0.45, 0.14. The reaction rates measured under identical conditions by the two assay procedures were within experimental error of each other when acyclo-Guo was used as the phosphate acceptor.

**Nucleoside Kinase-coupled Assays**—When radioactive nucleoside was not available, nucleoside phosphorylation (and concomitant ADP generating) activity was monitored as the pyruvate kinase-catalyzed conversion of phosphoenolpyruvate (14C) to pyruvate. Reaction mixtures contained 1 mM nucleoside, 100 mM Tris, pH 7.5, 2.5 mM Mg-ATP, 50 mM KCl, 0.25 mM phosphoenolpyruvate (¹⁴C)pyruvate (50 cpm/pmol), about 30 units/ml of pyruvate kinase, 1 mg/ml of albumin, and purified nucleoside kinase. All reactions except nucleoside were mixed and preincubated for 5 min at 37°C. Reactions were started by adding nucleoside and were terminated by spotting 3 μl on PEI-cellulose plates. The plates were immediately developed in 1 M formic acid, 0.25 M LiCl, 0.065 M EDTA and scanned to determine the location of phosphoenolpyruvate. The RF values for phosphoenolpyruvate (RF = 0.57) and pyruvate (RF = 0.7) were not available, nucleoside phosphorylation (and concomitant ADP generating) activity at 37°C. The principle of the assay has been described (26). Reactions contained the following components: 100 mM Tris-HCl, pH 7.5; 1 mM Mg-ATP; 0.5 mM phosphoenolpyruvate; 0.1 mM NADH; 5 units/ml of pyruvate kinase; 10 units/ml of lactate dehydrogenase; 0.5 mg/ml of albumin; and nucleoside or nucleoside analog. Rates obtained in the absence of nucleoside (ATPase) were subtracted from the total rates for both coupled assays.

**Other Enzyme Assays**—Deamination of [³²P]dCyd (2 cpm/pmol) and of [³²P]dAdo (4 cpm/pmol) were assayed under the standard assay conditions but without ATP. The reaction rate was monitored over a period of 2 h. The reactants were separated from products by electrophoresis on paper with 20 mM NaHCO₃-Na₂CO₃, pH 10.5, at 27 V/cm for 25 min. The appropriate uv absorbing spots were cut out and counted as in the standard assay. Positive and negative controls were run with each assay. Hydrolysis of ATP to ADP by assayed with the spectrophotometric assay (above) with the omission of nucleoside. Oxidation of NADH was likewise assayed with the omission of ATP and nucleoside. Cleavage of inosine to hypoxanthine was assayed at 37°C (27, 28) by observing the change of absorbance at 290 nm. The assay solution contained 6 mM inosine, 100 mM potassium phosphate, pH 7.4, 0.2 unit of xanthine oxidase/ml, and purified enzyme. The molar extinction coefficient change for the reaction was 1.2 × 10⁵ M⁻¹ cm⁻¹. Nucleotide phosphorylase activity was measured with 14C]AMP (¹⁴C) or ¹⁴C]ATP (¹⁴C) in a reaction mixture containing 2 mM Mg-ATP, 90 mM Tris-HCl, pH 7.5, and purified enzyme. A recent communication by Chen and Prusoff (39) reported that dThd kinase from HS-I virus has an associated TMP phosphorylating activity. This is present in the purified enzyme studied here. Under the conditions used in the present studies, this activity was a substantial portion (<10%) of the total reaction.
Thymidine Kinase Phosphorylation of a Purine Antiviral

Aliquots of 5 μl were spotted on PEI-cellulose TLC plates at timed intervals. The plates were developed with isobutyl alcohol saturated with H2O (AMP RF = 0.01, adenosine RF = 0.45), and the spots containing nucleoside and nucleotide were cut out and quantitated (above). A positive control reaction contained alkaline phosphatase. Deoxythymidine cleavage was monitored at 37°C by incubating [5-3H]dThd (25 cpm/pmol) in the standard reaction mixture minus ATP but with 56 mM potassium phosphate. Samples (5 μl) of the reaction mixture were spotted on PEI-cellulose and quantitated as above (dThd RF = 0.78, deoxyribose RF = 0.28, deoxyribose-1-phosphate RF = 0.06).

Other Procedures

Polyacrylamide Gel Electrophoresis—A 100-μg sample from a 20 to 50% ammonium sulfate fraction (29) of HS-I virus (MacIntyre)-infected Vero cells was electrophoresed at pH 7.5 and 8.9 in a Buchler disc gel apparatus by the methods of Williams and Reisfield (30) and Davis (31) with 2.5 mA/gel. After electrophoresis, gels were sliced and incubated in 100 μl of 20 mM Tris, pH 7.5, 10% glycerol, and 2 mM Mg-ATP overnight at 4°C. These mixtures were assayed for dThd or acyclo-Guo phosphorylation for 3 h at 37°C with final concentrations of 50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 9 mM ATP, 0.7 mM [3H]nucleoside, 2 mM dithiothreitol, 7% glycerol, and 0.7 mg/ml of albumin. Reactions were terminated and processed as described above for the standard assay. Vero and HeLa S-3 cells were grown and infected as described previously (2). Unless noted otherwise, the cells were incubated with virus for 16 to 18 h (60 to 90% cytopathic effect) before being harvested for extraction. The cells were extracted by a modification of the procedure described by Cheng and Ostrander (29). The hypotonic buffer contained 1 mm dithiothreitol instead of mercaptoethanol, and the dThd was omitted. A protease inhibitor (32), phenylmethanesulfonyl fluoride, was added as a 10 mM solution in isopropl alcohol to the cytosol fractions in addition to the ICl and glycerol. The final concentrations of the inhibitor and the isopropl alcohol were 0.2 mm and 2% (v/v). These cytosol fractions were centrifuged for 1 h at 100,000 × g and the supernatants were assayed for enzyme activities and stored at −60°C. These are referred to as “100,000 × g supernatants.” The enzyme solution was kept at 0–4°C throughout the procedure unless indicated otherwise. One-tenth milliliter of a streptomyacin sulfate solution (20% w/v) was added to 2 ml of the 100,000 × g supernatant (about 4 × 107 units of host cell plus viral dThd kinase). This suspension was dialyzed for 3 h in 500 ml of 20 mM Tris, pH 7.5, 3 mM dithiothreitol, 10% glycerol (Buffer A) and centrifuged at about 27,000 × g for 20 min. The supernatant was added onto a column (0.7 × 8 cm) of agarose-dThd affinity gel equilibrated with Buffer A. The column was loaded at a flow rate of about 0.9 ml/h, eluted with 3 ml of Buffer A, and then eluted with 9 ml of 800 mM Tris, pH 6.8, 3 mM dithiothreitol, 10% glycerol. A linear gradient of dThd (0 to 0.2 mm, 40 ml total) in the same buffer plus a constant concentration of ATP (1 mM) and albumin (1 mg/ml) was used to elute (flow rate ~2 to 3 ml/h) the enzymes of interest.

The combined fractions that contained acyclo-Guo phosphorylating activity were dialyzed for about 18 h in two 500-ml volumes of (NH4)2SO4 (470 g/liter) containing 3 mM dithiothreitol. The precipitate was resuspended twice in fresh (NH4)2SO4 of the same concentration and then dissolved with Buffer A in one-fifth to one-half the volume of the original dialyzed material. This method removed dThd from the preparation. The recovery of acyclo-Guo kinase activity varied from about 75 to 85%. The specific activity of the enzyme preparation was not determined since albumin was present to stabilize the enzyme. The enzyme was stored at −80°C and was stable for several months under these conditions.

RESULTS

Product Formation and Identification

Acyclo[8-3H]Guo was incubated under the standard assay conditions with extracts of Vero cells infected with HS-I virus. A product was formed which was retained on DEAE cellulose paper after washing in the manner described above. Product formation was ATP- and Mg2+-dependent and was formed at a rate greater than 10-fold the rate with uninfected cell extract.

A reaction mixture that contained 11% product (DEAE paper assay) was deproteinized with perchloric acid. The retention time of the neutralized (KOH) product on high pressure liquid chromatography (21) coincided with that of authentic acyclo-GMP. The 254 to 280 nm absorbance ratio was typical of a 9-substituted guanine compound. The percentage of product calculated from the UV and from two radioactivity profiles were 9.6, 10.5, and 11.6%. The thin layer chromatography was also used to separate the radioactive substrate and product. A portion of the above reaction mixture was chromatographed on PEI-cellulose plates with ethanol/1 M ammonium acetate, pH 7.5 (7:3) as the eluent. About 12% of the radioactivity coincided with added acyclo-GMP (RF = 0.06) and the rest with acyclo-Guo (RF = 0.65). Treatment of another portion of the reaction mixture with E. coli alkaline phosphatase shifted 97% of the radioactivity from the nucleotide to the nucleoside spot.

After an extended incubation of uninfected Vero cell extract with acyclo-Guo (as above), a small amount of product was formed. This product was analyzed as above and with an additional TLC system (PEI-cellulose) which employed 63% ethanol/Na2B4O7 saturated solution (24°C)/5 M LiCl (3:1:1) as the eluent and separated acyclo-Guo (RF = 0.70), Hyp (0.65), Xan (0.53), uric acid (0.42), GMP (0.12), and ATP (0.09) from acyclo-GMP (0.22). The product was identified as the monophosphate of acyclo-Guo.

Levels of Acyclo-Guo Phosphorylating Activity in Cell Extracts

The results of some preliminary experiments suggested that the phosphorylation of acyclo-Guo with extracts from infected cells might be catalyzed by the enzyme that is described in the literature (12-16) as an HS-I virus-coded dThd (dCyd) kinase. In these preliminary experiments, extracts of infected Vero cells phosphorylated acyclo-Guo, dCyd, and sometimes dThd better than extracts of uninfected cells; dGuo phosphorylation was about the same in both kinds of extracts. The phosphorylating activity for acyclo-Guo was stabilized during heat treatment by Mg-ATP, albumin, and glycerol and was inhibited by ammonium sulfate in a manner similar to that reported by Cheng and Ostrander (29) for the viral-induced dThd kinase. It was also stabilized by TMP with magnesium and inhibited strongly by dThd.

For these reasons, dThd- and dCyd-phosphorylating activities as well as acyclo-Guo-phosphorylating activities were measured from several extracts of infected and uninfected cells (Table I). Four of the six strains of HS-I virus, in two different host cells, induced a kinase activity that phosphorylated acyclo-Guo with a 30- to 120-fold higher specific activity than that found in uninfected cells. Increases of dCyd-phosphorylating activity paralleled these increases. Measurements of virus-induced dThd phosphorylation were complicated by the appreciable and variable amount of host cell dThd kinase present. However, a study of the time course of infection (Fig. 2) revealed that dThd kinase measured at pH 6.0 (mostly viral enzyme activity (33)) increased in proportion to the other activities for at least 12 h. The levels of host cell dThd kinase appeared to decrease somewhat by 18 h of infection; this decrease, however, was variable (compare, e.g. the 18-h extract of Vero cells infected with a TK- virus (HS-I (tsA1)) in Table I). The data from the time course of infection were used to calculate the theoretical amount of acyclo-Guo that could be phosphorylated by the induced kinase (Table II). This calculation will be discussed below.

Infection of Vero cells with the KOS strain of HS-I virus...
Levels of nucleoside phosphorylation in cell extracts

Infections, extractions (16 to 18 h postinfection), and assay procedures are described under "Experimental Procedures." Addition of 50 mM NaF to the reaction mixtures did not increase the measured activities, nor did tetrahydrouridine (30 pg/ml) have an effect on the reaction velocity with 1 mM dCyd as substrate.

Extract source (virus/cell line)

- HS-I virus (Mac)/Vero
- HS-I virus (Mac)/HeLa
- HS-I virus (Clin)/Vero
- HS-I virus (H29)/Vero
- H29 virus (KOS)/Vero
- HS-I virus (tsAl)/Vero
- HS-I virus (H29R)/Vero
- Vaccinia/HeLa
- Vero (average of 3)
- HeLa (average of 2)

Nucleoside phosphorylation

- acyclo-Guo
- dThd
- dCyd

nmol/min/mg protein

- HS-I virus (Mac)/Vero 0.42 (47) 1.7 (1.0) 3.1 (48)
- HS-I virus (Mac)/HeLa 0.15 (30) 1.2 (0.6) N.D.
- HS-I virus (Clin)/Vero 0.34 (38) 1.9 (1.2) 0.99 (15)
- HS-I virus (H29)/Vero 0.42 (47) 2.7 (1.6) 2.1 (32)
- H29 virus (KOS)/Vero 1.1 (120) 5.0 (3.0) 4.9 (75)
- HS-I virus (tsAl)/Vero 0.008 (0.9) 1.3 (0.8) 0.073 (1.1)
- HS-I virus (H29R)/Vero 0.030 (3.3) 1.2 (0.7) 0.18 (2.8)
- Vaccinia/HeLa <0.002 (<0.4) 4.4 (3.1) N.D.
- Vero (average of 3) 0.009 (S.D., 0.006) 1.64 (S.D., 0.065) 0.048 (48)
- HeLa (average of 2) 0.005 (S.D., 0.003) 1.4 (S.D., 0.14) N.D.

Numbers in parentheses are ratios of (activity from infected cells)/(activity from uninfected cells).

Properties of the Acyclo-Guo-phosphorylating Enzyme

Electrophoresis—Polyacrylamide gel electrophoresis of a sample containing both acyclo Guo and dThd kinase activity was performed at pH 7.5 and 9.0. Gels were sliced and assayed for both activities. The results (Fig. 3) showed no significant separation of the proteins responsible for the two activities.

Purification—Virus-coded and host cell dThd kinase were separated from each other by using an affinity medium containing dThd linked to agarose (29). At least 95% of the protein was eluted prior to the initiation of the dThd gradient (Fig. 4A). Phosphorylating activity for dThd co-eluted with activities for acyclo-Guo and dCyd at pH 7.5 and 6.0.

Calculated turnover capacity of acyclo-Guo phosphorylation during the course of HS-I virus (H29) infection of Vero cells

<table>
<thead>
<tr>
<th>Time period after infection*</th>
<th>Average phosphorylation rate*</th>
<th>Amount of acyclo-GMP potentially formed</th>
<th>Concentration of acyclo-GMP potentially accumulated</th>
</tr>
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<tbody>
<tr>
<td>0-2 0.10 1.3 260</td>
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<td>2-4 0.40 5.87 1,440</td>
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<tr>
<td>8-10 0.78 12.6 7,820</td>
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</tbody>
</table>

*See "Experimental Procedures."

a Calculated turnover capacity of acyclo-Guo phosphorylation during the course of infection. The amount of acyclo-Guo phosphorylated was calculated as follows:

\[
\text{nmol/min/mg protein} \times \frac{\text{average protein concentration} \times \text{volume of extract}}{10^6 \text{ cells}} \times 200 \text{ pmol/nmol} \times \frac{10^6 \text{ cells}}{\text{nmol}} = \text{nmol} \times \text{mg protein}^{-1}
\]

b N.D., not determined.

c Estimating 1 nmol/10^6 cells = 200 pmol.

d From Fig. 2; (average rate, Hour 1) + (average rate, Hour 2)/2, etc. Concentration of acyclo-Guo = 1 mM.

e For 120-min period; average protein concentration from Fig. 2; estimate 6-ml extract per 1.5 x 10^6 cells.

Calculated turnover capacity of acyclo-Guo phosphorylation during the course of HS-I virus (H29) infection of Vero cells

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From Fig. 2; (average rate, Hour 1) + (average rate, Hour 2)/2, etc. Concentration of acyclo-Guo = 1 mM.

For 120-min period; average protein concentration from Fig. 2; estimate 6-ml extract per 1.5 x 10^6 cells.

Estimating 1 nmol/10^6 cells = 200 pmol.

Fig. 2. Nucleoside-phosphorylating activities of 100,000 x g supernatants during time course of infection (HS-I virus (H29)). A, protein concentrations of extracts are shown. B, infections, extractions, and assays were done as described under "Experimental Procedures." Activities with dThd were measured at pH 7.5 (□) and pH 6 (●). At pH 6, phosphorylation of dThd with Vero cell extract was about 11% of the rate at pH 7.5, but purified HS-I virus (H29) dThd kinase was about 120% of the rate at pH 7.5. The amount of dThd-phosphorylating activity due to Vero cell activity was calculated (-----) from the expression [1.2(rate from □) - (rate from ●)1/1.11. Activities with acyclo-Guo (△) and dCyd (□) were measured at pH 7.5.

Fig. 3. Polyacrylamide gel electrophoretic analysis of dThd and acyclo-Guo kinase activities from cytosol of HS-I virus (MacIntyre)-infected Vero cells. Activities with dThd and acyclo-Guo after electrophoresis at pH 7.5 are shown in Panels A and C, respectively; activities after electrophoresis at pH 8.9 are shown in Panels B and D. Procedures are described under "Experimental Procedures."
nor acyclo-Guo-phosphorylating activities could be eluted with 2 mM Mg·ATP in the absence of dThd.

The purified viral enzyme was tested for contaminating activities. The following activities were compared to the activity of dThd kinase in the same preparation: dCyd deaminating (<0.3%), dAdo deaminating (<0.3%), NADH oxidizing (<0.4%), inosine cleaving (<0.3%), dThd cleaving (<0.7%), AMP dephosphorylating (<0.7%), and ATP hydrolyzing (3 to 12%).

**Inhibitor and Substrate Activities of Some Nucleoside Analogues**—Binding of several compounds was evaluated by measuring the amount of inhibition that a test compound would cause when included in a reaction for assay of acyclo-[14C]Guo phosphorylation (Table III). The effectiveness of inhibition by several nucleosides (dThd ≥ 5 iododeoxyuridine > arabinosylthymine > 5-bromodeoxycytidine > dCyd > arabinosylcytosine) was essentially the same as found by Cheng (35) who used dThd as the radioactive substrate with the virus-coded dThd kinase. The nonsubstrate inhibitor, 5′-azido-2′,5′-dideoxythymidine, appeared to be a competitive inhibitor of the enzyme with either acyclo-Guo or dThd. A preliminary apparent K<sub>m</sub> value with acyclo-Guo (MgCl<sub>2</sub> and ATP at 2 mM) was about 0.1 mM. The kinetics at concentrations of phosphate acceptor much higher or lower than its apparent K<sub>m</sub> value when using acyclo-Guo or dThd was complex and is the subject of further study. The competitive nature of three of the substrates for the enzyme is also shown by the data in Table IV. The substrates were mutually inhibitory and could completely inhibit the activity with the other two substrates.

### Table III

**Nucleoside inhibition specificity and relative phosphorylation rates with HS-1 virus (MacIntyre) dThd kinase.**

<table>
<thead>
<tr>
<th>Nonradioactive nucleoside</th>
<th>% Inhibition</th>
<th>Relative phosphorylation rate</th>
</tr>
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<tbody>
<tr>
<td>dThd</td>
<td>100 (77)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thd</td>
<td>100</td>
<td>14</td>
</tr>
<tr>
<td>Arabinosylthymine</td>
<td>98 (67)</td>
<td>38</td>
</tr>
<tr>
<td>2′,3′-Dideoxythymidine</td>
<td>92</td>
<td>22</td>
</tr>
<tr>
<td>1-(2-Hydroxyethyl)methylthymine</td>
<td>45</td>
<td>12</td>
</tr>
<tr>
<td>5′-Azido-2′,5′-dideoxythymidine</td>
<td>96</td>
<td>&lt;3</td>
</tr>
<tr>
<td>5-Trifluoromethyldeoxyuridine</td>
<td>95</td>
<td>101</td>
</tr>
<tr>
<td>dGuo</td>
<td>39</td>
<td>5</td>
</tr>
<tr>
<td>Guo</td>
<td>74</td>
<td>12</td>
</tr>
<tr>
<td>Arabinosylguanine</td>
<td>37</td>
<td>&lt;3</td>
</tr>
<tr>
<td>9-(2-Hydroxyethyl)methylguanine (acyclo-Guo)</td>
<td>69</td>
<td>36</td>
</tr>
<tr>
<td>dCyd</td>
<td>64 (0)</td>
<td>190</td>
</tr>
<tr>
<td>Cyd</td>
<td>62</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Arabinosylcytosine</td>
<td>30 (0)</td>
<td>8</td>
</tr>
<tr>
<td>1-(2-Hydroxyethyl)methylcytosine</td>
<td>9</td>
<td>&lt;3</td>
</tr>
<tr>
<td>5-Bromodeoxycytidine</td>
<td>100 (50)</td>
<td>33</td>
</tr>
<tr>
<td>Arabinosyladenine</td>
<td>6</td>
<td>&lt;3</td>
</tr>
<tr>
<td>5-Iododeoxuridine</td>
<td>&lt;9 (16)</td>
<td>110</td>
</tr>
<tr>
<td>Arabinosyl-2,6-diaminopurine</td>
<td>&lt;2</td>
<td>&lt;3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Numbers in parentheses represent inhibition with 5 μM nonradioactive nucleoside present.

<sup>b</sup> Rate with dThd arbitrarily set at 100.

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*About 40 to 50 μM dThd (with 0.8 M Tris, pH 6.8) in the first of the two dThd kinase peaks. The ratio of activities in the pooled peak fractions was 1.0:0.5:1.5 with dThd/acyclo-Guo/dCyd as substrates. The second peak of dThd kinase eluted at about 100 to 110 μM dThd in a volume identical with that of the dThd kinase from uninfected Vero cells (Fig. 4D). This ratio of activities (concentrated, pooled fractions) was 3.0:1.0:1.0 for dThd/acyclo-Guo/dCyd. Chromatography without albumin resulted in an extensive loss of activity (Fig. 4B). Chromatography without ATP resulted in an identical shift of acyclo-Guo and dThd viral-induced activities to later elution at a higher concentration of dThd. These activities then partially co-eluted with host cell dThd kinase (Fig. 4C). This higher concentration of dThd was similar to the concentration used for elution of the viral enzyme from the same affinity media in a previous report (29). Neither dThd-
Table IV

<table>
<thead>
<tr>
<th>Substrate(s)</th>
<th>Concentration (μM)</th>
<th>% Inhibition of product</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyclo-Guo</td>
<td>100</td>
<td>260</td>
<td>99</td>
</tr>
<tr>
<td>Acyclo-Guo + dThd</td>
<td>100 + 300</td>
<td>&lt;1.2</td>
<td>99</td>
</tr>
<tr>
<td>Acyclo-Guo + dCyd</td>
<td>100 + 800</td>
<td>2.0</td>
<td>99</td>
</tr>
<tr>
<td>[3H]dThd</td>
<td>0.1</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>[3H]dThd + acyclo-Guo</td>
<td>0.1 + 600</td>
<td>0.036</td>
<td>98</td>
</tr>
<tr>
<td>[3H]dCyd + dCyd</td>
<td>0.1 + 6000</td>
<td>0.11</td>
<td>94</td>
</tr>
<tr>
<td>[14C]dCyd</td>
<td>10</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>[14C]dCyd + acyclo-Guo</td>
<td>10 + 6000</td>
<td>1.4</td>
<td>97</td>
</tr>
<tr>
<td>[14C]dCyd + dThd</td>
<td>10 + 10000</td>
<td>0.13</td>
<td>99</td>
</tr>
</tbody>
</table>

Other data in Table III are the relative reaction rates measured at a high concentration (1 μM) of nucleoside. Tri- and polyguanosine, arabinosylguanine, 5-bromodeoxyuridine, 5-iododeoxyuridine, and to some extent arabinosylcytosine were good inhibitors and good substrates. These compounds are known to be effective inhibitors of virus replication in tissue culture or in vivo (9). On the other hand, phosphorylations of the purine antiviral compounds, arabinosyl derivatives of 2,6-diaminopurine, guanine, and adenine (9, 17) were not detected.

DISCUSSION

The guanine derivative, acyclo-Guo, is a potent inhibitor of HS-I and HS-II virus replication in vitro and in vivo (1, 2). The data presented here indicate that acyclo-Guo is phosphorylated by the thymidine kinase (12-16) that is coded for by HS-I virus and which phosphorylates dCyd as well as dThd (29, 33-38). Proportionate increases of dThd- and acyclo-Guo phosphorylation (Fig. 2) suggest catalysis by host cell enzymes.

A subsequent question is whether acyclo-Guo and dThd are phosphorylated at the same catalytic site. Three alternative two-site models were considered and eliminated. These were 1) completely independent catalytic sites, 2) alternative sites of reaction but with one of the two sites inhibited by the other substrate, and 3) two sites, only one of which could catalyze the reaction of both substrates. Mutual inhibition by acyclo-Guo and dThd (Table IV) eliminated the first and second possibilities. Complete mutual inhibition eliminated the third possibility. Similar arguments are applicable to acyclo-Guo and dCyd or dThd and dCyd.

The amount of acyclo-Guo phosphates formed in tissue culture cells treated with acyclo-Guo was correlated with the levels of phosphorylating activity extractable from virus-infected cells (Table II). The potential amount of acyclo-Guo phosphates formed during a time course of infection was calculated. Vero cells were also infected with HS-I virus (H29) under identical conditions and incubated with 500 μM acyclo-Guo. The measured intracellular concentration of acyclo-Guo and acyclo-Guo nucleotides increased from 10 to 20 μM and from 9 to 122 μM, respectively, during the first 8 h. After this, the acyclo-Guo concentration dropped to about 2 μM, and the concentration of the nucleotides decreased gradually to 60 μM by 12 h. The "potential amount" of acyclo-Guo nucleotides formed (Table II) was 30- to 150-fold these amounts. If the average phosphorylation rate" in Table II is adjusted for the potential amount of intracellular acyclo-Guo found, the potential amount is still about 3 to 16 fold greater than the actual amount found. Thus, it would appear that the amount of acyclo-Guo phosphorylating activity observed was sufficient to account for the amount of acyclo-Guo nucleotides formed in these experiments.

The induction of the acyclo Guo phosphorylating activity appears to be necessary for the compound to be an effective inhibitor of virus replication. Vaccinia virus induces a dThd kinase that does not phosphorylate acyclo-Guo (Table I); the triphosphate of acyclo-Guo does not accumulate in vaccinia-infected cells treated with acyclo-Guo, nor does acyclo-Guo inhibit vaccinia replication (2). Two mutants of HS-I virus that were resistant to acyclo-Guo were selected only at 0.1 to 0.01 the amount of acyclo-Guo phosphorylation (H29R and tsA1, Table I) as their parent strains. Additionally, acyclo-Guo triphosphate formation and the antiviral effectiveness of acyclo-Guo could be reversed by dThd (2).

Other investigators have indicated that a correlation exists between the sensitivity of HS-I virus to inhibition by pyrimidine nucleoside analogues and the ability of these viruses to induce a dThd dCyd kinase (3-11). The purine derivative, arabinosyladenine, did not fit the correlation in those studies. In the present studies, it neither inhibited the HS-I virus dThd (acyclo-Guo) kinase well nor was a good substrate. Similar results were found with the antiviral compounds (17) arabinosylguanine and arabinosyl-2,6-diaminopurine (Table III). These purine derivatives are apparently activated by other enzymes such as adenosine kinase or deoxyguanosine kinase. On the other hand, the present studies indicate that at least one purine derivative, acyclo-Guo, can be phosphorylated (although much less efficiently than dThd), by the virus-coded pyrimidine deoxynucleoside kinase.

The picture that emerges is that of a virus-infected cell that readily phosphorylates acyclo-Guo in contrast to an uninfected cell. This may be a crucial difference between acyclo-Guo and several of the other anti-herpes compounds. The enzymatic basis for this difference is the presence of a virus-coded dThd kinase that has a broad enough specificity to phosphorylate the purine derivative. The fact that the vaccinia-coded dThd kinase does not appreciably phosphorylate the virus emphasizes the uniqueness of the enzyme from herpes simplex virus. In only certain virus-infected cells, then, can acyclo-Guo nucleotides accumulate and thus inhibit DNA polymerase or chain terminate DNA replication (or both) (2). It will be of interest to examine kinases induced by other viruses for their capacity to phosphorylate acyclo-Guo.

2. Assume K = [acyclo-Guo] Vmax/[Km + [acyclo-Guo]], K(K=acyclo-Guo) = 100 μM, and Vmax = average phosphorylation rate from Table II (1 mM acyclo-Guo).
3. Since the rate of phosphorylation of acyclo-GMP" is about the same with extracts from uninfected or infected cells, phosphorylation to the di- and triphosphate is thought to be catalyzed by host cell enzymes. P. Keller, unpublished observations.
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Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound, 9-(2-hydroxyethoxymethyl)guanine.

J A Fyfe, P M Keller, P A Furman, R L Miller and G B Elion