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Metabolism of 9-(1,3-Dihydroxy-2-propoxymethyl)guanine, a New Anti-herpes Virus Compound, in Herpes Simplex Virus-infected Cells*

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The metabolism of 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG), one of the most promising new anti-herpes virus compounds, in HeLa cells infected with herpes simplex virus type 1 was compared with that in the uninfected HeLa cells. In the virus-infected cells, the uptake of DHPG was enhanced and the major metabolites were found to be the mono-, di-, and triphosphate derivatives. The formation of these metabolites was dependent on the extracellular concentration of DHPG (0.5 to 5.0 μM). Virus-induced thymidine kinase was capable of phosphorylating DHPG to its monophosphate which could be further phosphorylated to the di- and triphosphate derivatives by the host cellular enzymes. Incorporation of the DHPG into DNA was observed in virus-infected cells. In contrast with 9-(2-hydroxyethoxymethyl)guanine, DHPG seemed not to serve as a chain terminator, but to be incorporated internally into DNA strands.

DHPG,[1] a newly synthesized guanosine analog, was found to have potent activity against HSV (1-3), varicella-zoster virus (1), cytomegalovirus (2, 3), and Epstein-Barr virus (3). DHPG is more potent and the spectrum of susceptible viruses to the compound is broader than that to acyclovir. Furthermore, HSV mutants, which are resistant to acyclovir because of altered thymidine kinase or DNA polymerase, were as sensitive to DHPG as the parental HSV (3). This unique spectrum of activity suggests the potential use of DHPG in the clinic for the treatment of herpesvirus infection. In order to have a better understanding of the mechanism of action of DHPG, it is important to explore its metabolism in virus-infected cells. This paper presents evidence of phosphorylation of DHPG, leading to the formation of DHPGTP, and of incorporation of the nucleoside analog into DNA.

MATERIALS AND METHODS

Chemicals and Enzymes—DHPG, DHPGMP, and [^{3}H]DHPG (16 Ci/mmol) were donated by Syntex (U.S.A.), Inc., Palo Alto, CA. Other nucleosides, nucleotides, and dithiothreitol were purchased from Sigma. Proteinase K and micrococcal nuclease were obtained from Boehringer Mannheim Gmbh. Spleen phosphodiesterase was from Worthington and *Escherichia coli* alkaline phosphatase was from Sigma.

Cells and Virus—Cells were grown at 37 °C in RPMI 1640 medium (GIBCO) containing 100 μg/ml of Kanamycin supplemented with 5% horse serum and 5% fetal calf serum for HeLa S_3 and Vero cells, respectively. HSV-1 (strain KOS) was maintained as described previously (4).

Preparation of HSV-1 Thymidine Kinase and Human Erythrocyte Lysate—A highly purified enzyme was prepared from HSV-1-infected HeLa BU (TK^-) cells using affinity column as described previously (5). Twenty-five ml of fresh heparinized blood was diluted with phosphate-buffered saline (0.14 M NaCl, 4 mM KCl, 0.94 mM Na_2HPO_4, 0.15 M KH_2PO_4). Erythrocytes were pelleted by centrifugation and 4 volumes of cold water were added to lyse the cells. After centrifugation at 12,000 × *g* for 20 min, supernatant was collected and solid ammonium sulfate was added to 70% saturation. Precipitate was collected by centrifugation, dissolved in 10 ml of 5 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, 10% glycerol, and used for phosphorylation of DHPGMP.

Preparation of Acid-soluble and -insoluble Fractions—HeLa S_3 cells in 25-cm^2 flasks were infected with 3 plaque-forming units/cell of HSV-1. Various concentrations of [^{3}H]DHPG were added at 0 h postinfection and the cells were harvested at 6 h and washed three times with phosphate-buffered saline. The cell pellets (5 × 10^6 cells) were extracted with 50 μl of 1.5 M perchloric acid at 0 °C for 30 min. The extract was neutralized with 1 N potassium hydroxide (77 μl) and 23 μl of 0.5 M potassium phosphate buffer (pH 7.4) was added. Precipitate was removed by centrifugation and the supernatant was used as the acid-soluble fraction. The precipitate from the perchloric acid extraction were washed twice with 1.5 N perchloric acid and designated as the acid-insoluble fraction.

Alkaline Hydrolysis of Acid-insoluble Fractions—The acid-insoluble fraction was dissolved in 100 μl of 1 N NaOH and incubated for 2 h at 37 °C. Four hundred μl of 5% trichloroacetic acid was added to the solution. The supernatant and an additional 200-μl wash of trichloroacetic acid were neutralized with KOH solution and counted in a liquid scintillation counter as the alkali-labile fraction. The pellet was redissolved in dimethyl sulfoxide and counted as the alkali-stable material.

HPLC Analysis—An anion exchange column, Partisil 10 SAX/25 (Whatman), was used in a solvent system with a gradient of potassium phosphate buffer (pH 6.6) from 0.03 to 0.15 M. The solvent system for a reverse phase column, C_8 (ALLTECH), was acetonitrile, 0.03 M acetic acid (1:5000, v/v).

Sodium Iodide and Cesium Sulfate Density Gradient Centrifugation—HSV-1-infected cells were washed with phosphate-buffered saline and lysed with 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.1 M NaCl, 0.5% Sarkosyl, 0.5% sodium dodecyl sulfate (250 to 300 μl). After 30 min at room temperature, proteinase K was added to the lysate (6 mg/ml) and the mixture was incubated overnight at 37 °C.

The mixture (300 μl) was directly layered on sodium iodide solution (4.7 ml) which contained 10 mM Tris-HCl (pH 7.9), 10 mM EDTA, 0.5 mg/ml of $NaHSO_3$, and 1.53 to 1.54 g/cm^3 of sodium iodide. Centrifugation was performed at 43,000 rpm for 48 h at 20 °C using a Beckman SW 50.1 rotor.

For cesium sulfate density gradient centrifugation, the mixture was extracted with ½ volume of phenol and the aqueous phase was further extracted with ether. One-tenth volume of 3 M sodium acetate buffer (pH 5.5) and 2.5 volume of ethanol were added to the aqueous phase

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[1] The abbreviations used are: DHPG, 9-(1,3-dihydroxypropoxymethyl)guanine; DHPGMP, DHPGDP, DHPGTP, 9-(1,3-dihydroxypropoxymethyl)guanine mono-, di-, and triphosphate, respectively; HSV-1, herpes simplex virus type 1; acyclovir, 9-(2-hydroxyethoxymethyl)guanine; HPLC, high performance liquid chromatography.

and the mixture was kept at −80 °C for 1 h. The precipitate was dissolved in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA and cesium sulfate was added to the solution to a density of 1.53 g/cm^3. Centrifugation was at 25,000 rpm for 60 h using the same rotor as above.

Gradient solution was fractionated from top to bottom with a Buchler Auto-Densi Flow pump-dripper. Density was calculated from refractive index measured with an American Optical ABBE refractometer.

RESULTS

Uptake of DHPG in Virus-infected and Mock-infected HeLa Cells—HeLa cells were infected with HSV-1 and various amounts of [^{3}H]DHPG were added at 0 h post-virus adsorption. The cells were harvested at 6 h post-virus adsorption. The uptake of DHPG into an acid-soluble and -insoluble fraction of these cells was analyzed. The results are presented in Table I. The more DHPG added, the more uptake into both fractions was observed, with the majority of [^{3}H]DHPG uptake taking place in the acid-soluble fraction. When 5 μM DHPG was added to mock-infected HeLa cells, the uptake of DHPG into the acid-soluble fraction was similar to that in infected cells exposed to 0.5 μM DHPG. The uptake of DHPG into the acid-insoluble fraction was about the same as that of infected cells exposed to between 1.0 and 2.0 μM DHPG. More than three-quarters of [^{3}H]DHPG in the acid-insoluble fraction could be rendered acid-soluble following treatment with 1 N KOH.

HPLC Analysis of DHPG Metabolites in Acid-soluble Fraction—The acid-soluble fraction of virus-infected and mock-infected HeLa cells after the treatment with [^{3}H]DHPG was analyzed by HPLC using a Partisil 10 SAX column system. The results are shown in Fig. 1. In addition to the radioactivity associated with the DHPG marker fraction (Fraction 5), three other metabolites of DHPG were found in either virus-infected or mock-infected cells. One of the metabolites (Fraction 20) was co-eluted with a DHPGMP marker. The amount of each metabolite formed in infected cells was dependent on the extracellular concentration of DHPG. In comparison with the virus-infected cells, very small amounts of metabolites could be found in the mock-infected cells and most of the radioactivity was in the DHPG fraction. When the extract from HSV-infected cells was treated with alkaline phosphatase for 4 h and then analyzed with the same HPLC system, the radioactivity associated with each of these metabolites was decreased with a corresponding increase of radioactivity associated with the DHPG fraction (Fig. 1*D*). When alkaline phosphatase-treated sample was subjected to the reverse phase C_8 HPLC column system, the majority of the radioactivity was found to be associated with DHPG.[2]

In order to identify these three metabolites further, each compound was isolated by HPLC and subjected to DEAE-Sephadex column chromatography in urea-containing buffer (Fig. 2). This allows the separation of nucleotides based on the number of ionic charges on phosphate groups. The metabolites associated with fractions 19 and 20 (Compound 1), 25 and 26 (Compound 2), and 52 to 55 (Compound 3) in Fig. 1 eluted slightly faster than GMP, GDP, and GTP, respectively. These results indicate that Compounds 1, 2, and 3 were mono-, di-, and triphosphate derivatives of DHPG.

In Vitro Formation of Metabolites—Highly purified HSV-1 thymidine kinase was incubated with [^{3}H]DHPG in the presence of ATP. The major metabolite formed co-eluted with DHPGMP on HPLC/Partisil 10 SAX system as shown in Fig. 3. [^{3}H]DHPGMP was isolated and then incubated with human erythrocyte lysate in the presence of ATP. As shown in Fig. 4, it was converted to metabolites which gave the same retention times as those of *in vivo* metabolites, Compounds 2 and 3, respectively. This conversion was inhibited by GMP, but not by TMP, UMP, or AMP,[2] suggesting that the enzyme responsible for the conversion of DHPGMP to DHPGDP is GMP kinase. The decrease of Compound 2 and the increase of Compound 3 with longer incubation suggested that Compound 2 is the precursor of Compound 3 (Fig. 3, *B* and *C*).

Incorporation of DHPG into DNA—HSV-1-infected cells were treated with 2 μM [^{3}H]DHPG for 8 h and the cell lysate was subjected to sodium iodide density gradient centrifugation. Although this method allows separation of virus DNA (density ≃ 1.52) from host cell DNA (density ≃ 1.50), they were not distinguishable for [^{3}H]DHPG-labeled DNA, which distributed into the fractions between the two densities (Fig. 5*A*).

TABLE I

Concentration-dependent uptake of [^{3}H]DHPG by HSV-infected HeLa S_3 cells

HeLa S_3 cells were infected with HSV-1 (KOS) at 3 plaque-forming units/cells. [^{3}H]DHPG (1.6×10^3 cpm/pmol) was added at 0 h postinfection. Cells were harvested 6 h postinfection. Alkali-labile and alkali stable acid-insoluble radioactivity was determined as described under "Materials and Methods."

[^{3}H]DHPG	Acid-soluble	Acid-insoluble	
		Alkali-labile	Alkali-stable
μM		*pmol/10^6 cells*	
0.5	2.63	0.08	0.03
1	5.10	0.16	0.05
2	7.70	0.36	0.08
5	17.0	0.80	0.20
5[a]	2.19	0.24	0.03

[a] Uninfected HeLa S_3 cells.

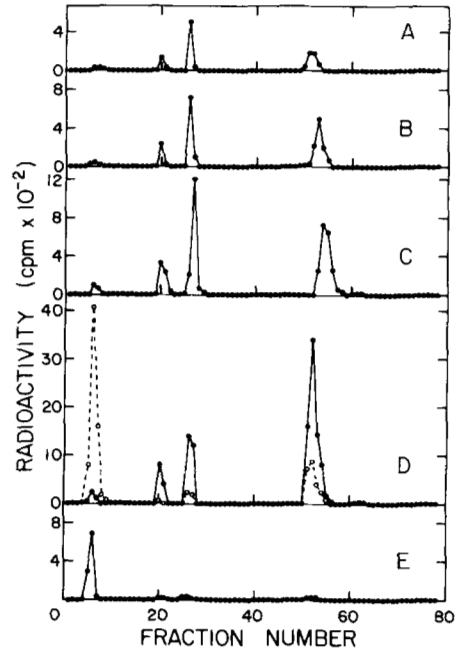

FIG. 1. **HPLC analysis of DHPG metabolites in HSV-infected and mock-infected HeLa cells.** Acid-soluble fraction was prepared from HSV-infected cells treated with 0.5 (*A*), 1 (*B*), 2 (*C*), and 5 (*D*) μM [^{3}H]DHPG and from uninfected cells treated with 5 μM [^{3}H]DHPG (*E*) and applied on a Partisil 10 SAX column as described under "Materials and Methods." Acid extract from HSV-infected cells treated with 5 μM [^{3}H]DHPG was incubated with alkaline phosphatase for 4 h at 37 °C and analyzed (*D*,o). The radiospecificity of [^{3}H]DHPG used was 1.6×10^3 cpm/pmol. The sample injected was equivalent to the amount extracted from 5×10^5 cells.

[2] Y.-C. Cheng, S. P. Grill, G. E. Dutschman, K. Nakayama, and K. F. Bastow, unpublished observations.

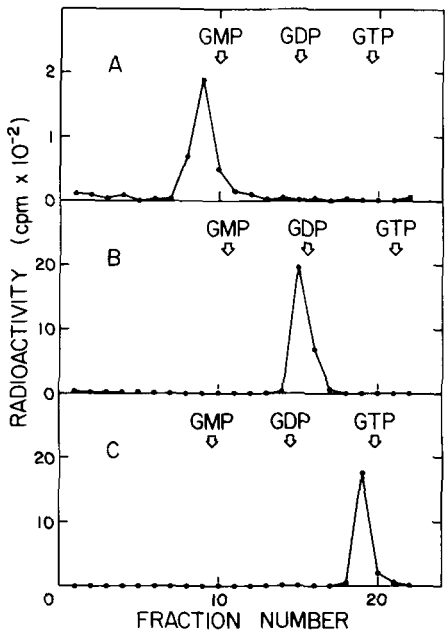

FIG. 2. **DEAE-Sephadex column chromatography of [^{3}H] DHPG metabolites.** An aliquot of Compound 1 (*A*), 2 (*B*), or 3 (*C*) (as designated in the text), together with 100 nmol each of GMP, GDP, and GTP, were applied on a DEAE-Sephadex column (0.3 × 25 cm) equilibrated with 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 7 M urea. Elution was performed with a NaCl gradient (30 ml) from 0 to 150 mM in the same buffer as above. One-ml fractions were collected. After absorbance at 260 nm was measured to locate the elution position of GMP, GDP, and GTP (*arrows*), each fraction was mixed with 10 ml of ACS scintillant (Amersham) and counted in a liquid scintillation counter to determine the elution of the [^{3}H]DHPG metabolites.

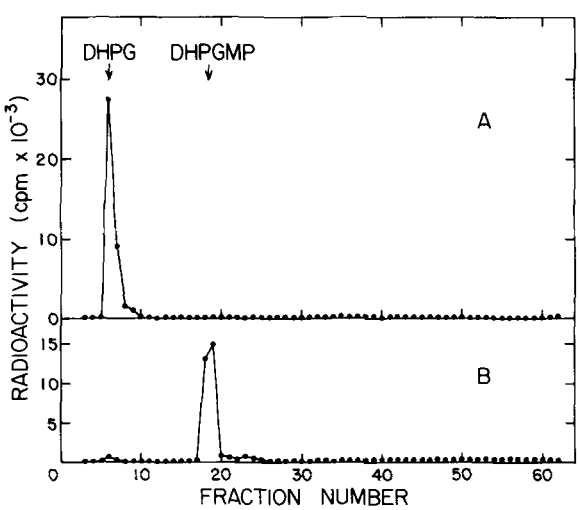

FIG. 3. ***In vitro* phosphorylation of [^{3}H]DHPG with HSV-1 thymidine kinase.** [^{3}H]DHPG (0.26 μCi, 7.3 nmol) was incubated at 37 °C in a mixture (100 μl) containing 4.0 mg/ml of bovine serum albumin, 12.0 mg/ml of creatine phosphate, 0.4 mg/ml of creatine phosphokinase, 67 mM Tris-HCl buffer (pH 7.5), 3 mM sodium fluoride, 0.6 mM dithiothreitol, 9 mM ATP-Mg^{2+}, and 4 units of HSV-1 thymidine kinase. Acid-soluble fractions at 0 h (*A*) and 2 h (*B*) of incubation were analyzed by HPLC using Partisil 10 SAX as described under "Materials and Methods." Elutions of standard DHPG and DHPGMP were indicated by *arrows*.

It is quite possible that [^{3}H]DHPG-labeled DNA has different density from normal DNA, which makes the virus and host DNA indistinguishable by density.

When these fractions were treated with alkali, a portion of radioactivity became acid-soluble and the radioactivity in the

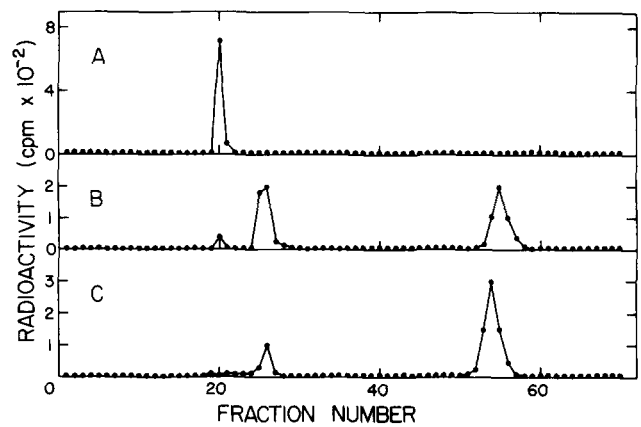

FIG. 4. ***In vitro* formation of di- and triphosphate derivatives from [^{3}H]DHPGMP with human erythrocyte lysate.** [^{3}H] DHPGMP was prepared with HSV-1 thymidine kinase and purified by HPLC as described in the legend to Fig. 3. Aliquots of [^{3}H] DHPGMP were incubated with 10 units of HSV-1 thymidine kinase for 5 h (*A*) or 80 μl of human erythrocyte lysate for 5 h (*B*) and 17 h (*C*) in the reaction mixtures described in the legend to Fig. 3. Reaction was terminated by the addition of perchloric acid and acid-soluble fraction was analyzed by HPLC using Partisil 10 SAX column as described under "Materials and Methods."

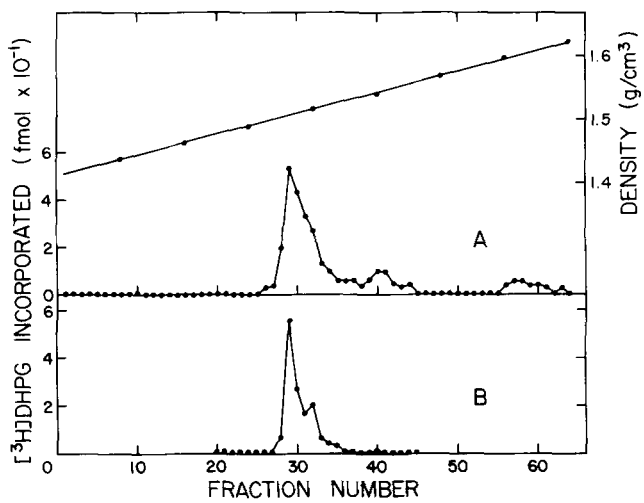

FIG. 5. **Sodium iodide density gradient centrifugation by [^{3}H]DHPG-labeled nucleic acid.** HeLa S_3 cells in a 25-cm^2 flask were infected with HSV-1 and incubated in medium (2 ml) containing [^{3}H]DHPG (53 μCi, 9.3 nmol) for 8 h. Extraction of nucleic acid and centrifugation were as described under "Materials and Methods." Seventy-five μl of each fraction were collected. Twenty-five μl of each fraction were absorbed into fiber glass discs (Whatman GF/A) and the discs were washed three times with ice-cold 5% trichloroacetic acid and once with ethanol. When dry, the discs were counted in ACS scintillant (*A*). Five μl of 5 N NaOH was added to another 25 μl of each fraction. The mixtures were incubated at 37 °C for 2 h, absorbed into discs, and washed as above (*B*).

alkali-stable form resulted in two peaks with densities 1.50 and 1.52, respectively (Fig. 5*B*).

Nucleic acid extracted from [^{3}H]DHPG-treated infected cells was analyzed by cesium sulfate density gradient centrifugation. Most of the radioactivity was associated with DNA (Fig. 6*A*). The radioactive DNA was digested with micrococcal nuclease and spleen phosphodiesterase and the products were analyzed by HPLC/Partisil 10 SAX. This method allows us to distinguish terminally incorporated nucleoside, which gives nucleoside, from internally incorporated nucleoside, which gives nucleoside 3′-monophosphate. As shown in Fig. 6*B*, there was little DHPG produced, while a significant amount

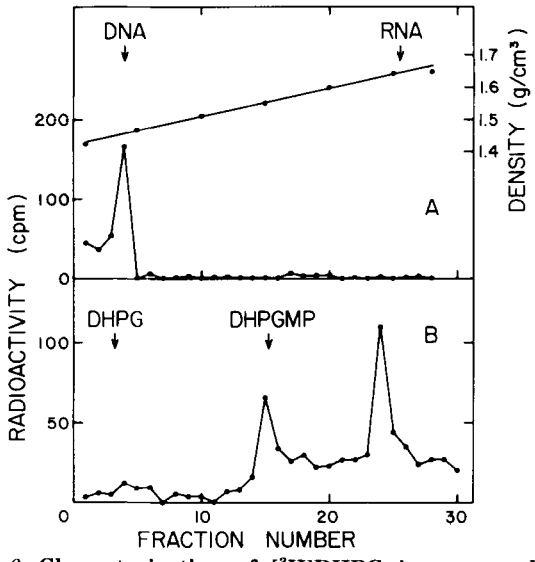

FIG. 6. **Characterization of [^{3}H]DHPG incorporated into DNA.** *A*, cesium sulfate density gradient centrifugation of [^{3}H] DHPG-labeled nucleic acid. Incubation of HSV-infected cells with [^{3}H]DHPG was performed as described in the legend to Fig. 5. Other details are described under "Materials and Methods." One hundred sixty μl of each fraction was collected and 15 μl each was counted for radioactivity. *B*, HPLC analysis of spleen phosphodiesterase digests of [^{3}H]DHPG-labeled DNA. Fractions 3 and 4 from cesium sulfate gradient centrifugation were combined and dialyzed against 10 mM Tris-HCl (pH 7.4), 1 mM EDTA. DNA was precipitated with ethanol and dissolved in the above buffer (20 μl). After heat denaturation, the labeled DNA was treated with 0.6 unit of micrococcal nuclease in a total volume of 50 μl (10 mM Tris-HCl, pH 8.8, 2 mM $CaCl_2$) for 30 min at 37 °C. Added to the mixture were 1 μl of 50 mM EDTA, 3 μl of 1 M sodium acetate buffer (pH 6.5), 3 μl of 0.1 M $MgCl_2$, and 2.2 μl of 10 mg/ml spleen phosphodiesterase (0.1 unit). Incubation was at 37 °C for 60 min. Protein was precipitaed by the addition of 2 volumes (120 μl) of 1 M perchloric acid. After centrifugation in a microfuge, supernatant was collected and neutralized with 4 M potassium hydroxide solution. Precipitate was removed by centrifugation and supernatant was lyophilized. The DNA digest was dissolved in water and an aliquot was applied on a Partisil 10 SAX column together with standard DHPG and DHPGMP (*arrows*).

of radioactivity was associated with DHPGMP and other material, which was possibly dinucleotide resulting from incomplete digestion. These results indicate that DHPG was internally incorporated into DNA and therefore does not act as pure chain terminator for DNA synthesis.

DISCUSSION

Like several other selective anti-HSV nucleoside analogs (6–15), DHPG could be preferentially phosphorylated in HSV-infected cells. The primary metabolic scheme is shown in Fig. 7. In spite of two symmetrical —CH_2OH groups at carbon 2 of the propoxyl group of DHPG, the phosphorylation of DHPG could take place at one of those two —OH groups by virus-induced thymidine kinase. Two stereoisomers at carbon 2 could be anticipated. It is probable that only one stereoisomer of DHPGMP which could be utilized for the further metabolism was formed since all the DHPGMP was converted to DHPGDP and DHPGTP by the erythrocyte lysate. However, the possibility that the enzyme(s) responsible for further phosphorylation could not distinguish the two stereoisomers requires further experimentation. It seems likely that the isomer formed should have a steric conformation similar to dGMP, but regardless of the outcome of this issue, the host cells apparently have an efficient system to carry out further phosphorylation as indicated in Fig. 1*E*. There was no accumulation of DHPGMP in mock-infected

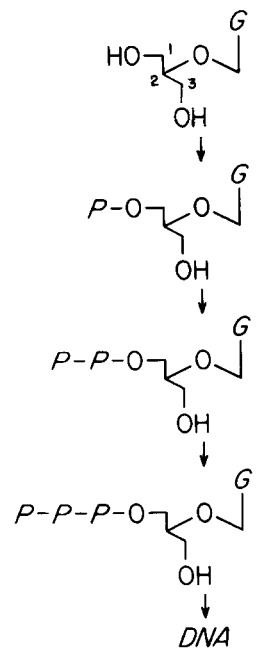

FIG. 7. **Possible metabolic pathways of DHPG.** *G* and *P* represent guanine and phosphate group, respectively.

cells, although DHPG could be found in the acid-insoluble fraction. This implies that a cell enzyme could perform the initial phosphorylation of DHPG but in a relatively inefficient manner since other human herpesviruses are less susceptible to DHPG than HSV and these viruses do not induce virus-specific thymidine kinase (1–3). The host enzymes responsible for the phosphorylation of DHPGMP to DHPGDP could be GMP kinases since GMP could inhibit this process. A similar suggestion was also made recently (16). Although there are several GMP kinase isozymes (17), it is not clear whether there is a preference for a particular GMP kinase isozyme in this process.

The amount of accumulated DHPGTP in infected cells treated with DHPG at concentrations of 0.5, 1, 2, and 5 μM was 0.5, 1.2, 2.8, and 8.4 pmol/10^6 cells, respectively. The amount of DHPG incorporated into the acid-insoluble fraction was found to be less than 5-propyl-dUrd (13). This suggested the posssibility that DHPG is acting as a chain terminator such as acyclovir or possibly a pseudochain terminator such as arabinosyl adenine (19). A detailed study of the mode of DHPG (18) on virus DNA replication is in progress. Since a portion of DHPG incorporated into the acid-insoluble fraction is alkali-labile, the possibility is raised that either DHPG could be incorporated into RNA which is alkaline-labile or into DNA with an alkali-labile linkage. Using the Cs_2SO_4 isopycnic centrifugation technique, all the radioactivity of [^{3}H]DHPG in the nucleic acid from the infected cells was found to be with DNA. DHPG was also demonstrated to be incorporated into the internucleotide chain of DNA. The unpublished results of this laboratory using an *in vitro* DNA synthesis system catalyzed by HSV DNA polymerase further confirms the incorporation of DHPG into the internucleotide chain of DNA. The alkaline lability of DHPG in the internucleotide DNA chain or at the terminal of DNA is currently under investigation.

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