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Mode of Inhibition of Herpes Simplex Virus DNA Polymerase by Phosphonoacetate†

James C.-H. Mao* and Ellen E. Robishaw

ABSTRACT: Phosphonoacetate is a highly specific inhibitor of Herpes simplex virus-induced DNA polymerase. Sensitivity of herpesvirus type 1 or type 2 induced DNA polymerase to the drug was similar. However, DNA polymerases from other sources such as the host cells (Wi-38), *Micrococcus luteus*, and hepatitis B virus were highly resistant. In addition, *Escherichia coli* RNA polymerase and reverse transcriptase of Rous sarcoma virus were also insensitive to the drug. Enzyme kinetic studies showed that inhibition was noncompetitive with respect to deoxyribonucleotide triphosphates. The K_i value was about 0.45 μM. The apparent K_m values for dTTP, dATP, dCTP, and dGTP were 0.71, 0.75, 0.42, and 0.39 μM, respectively. The base composition of template has no profound effect on the extent of inhibition. The drug caused uncompetitive inhibition with respect to template which indicated that phosphonoacetate did not bind directly to template DNA. Results are presented which suggest that phosphonoacetate did not affect the formation of the enzyme-DNA complex but probably inhibited the elongation step of DNA polymerase reaction.

Sodium phosphonoacetate has been shown to suppress Herpes simplex virus (HSV)[1] replication in tissue culture (Overby et al., 1974) and in model animal systems (Shipkowitz et al., 1973). The former studies also demonstrated that this compound selectively inhibited viral DNA synthesis without effect on host cell DNA synthesis. This result was confirmed by a recent study (Mao et al., 1975), in which HSV-induced DNA-dependent DNA polymerase and normal cellular DNA polymerases were isolated and it was found that phosphonoacetate specifically inhibited HSV-induced DNA polymerase. However, the mode of action of phosphonoacetate was not elucidated.

Inhibition of DNA synthesis in vitro may occur at several different sites—the DNA template, the substrate, or the polymerase. Numerous antibiotics and synthetic compounds such as actinomycin, olivomycin, chromomycin, daunomycin, acridine, and ethidium bromide bind to DNA (Müller et al., 1971), thereby impairing the template function of DNA. These usually demonstrate little selective tox-

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[1] Abbreviation used is: HSV, Herpes simplex virus.

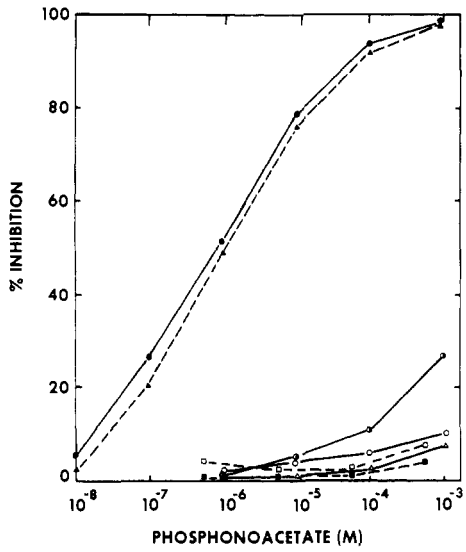

FIGURE 1: Specificity of phosphonoacetate to various DNA polymerases and RNA polymerase. DNA polymerases of HSV-1 (0.1 μg), of HSV-2 (0.1 μg), and of Wi-38 (10 μg) were assayed according to the procedures described under Enzyme Assay. *M. luteus* DNA polymerase (0.2 μg) and *E. coli* RNA polymerase (0.05 μg) were assayed by the previously published procedures (Aposhian and Kornberg, 1962; Burgess, 1969). Activated calf-thymus DNA was the template in the above assays. DNA polymerase of hepatitis B virus (0.1 mg) was assayed by the procedure of Kaplan et al. (1973), without addition of exogenous template. Reverse transcriptase of RSV (0.5 μg) was assayed by the procedure of Spiegelman et al. (1970), with poly(rA)-oligo-$(dT)_{12-18}$ as the primer-template. Percentage of inhibition was the extent of dTMP incorporation inhibited by phosphonoacetate after 30-min incubation at 37°C in comparison with the controls containing no drug: HSV-1 DNA polymerase (●); HSV-2 DNA polymerase (▲); Wi-38 DNA polymerase (◑); *M. luteus* DNA polymerase (○); hepatitis B virus DNA polymerase (△); *E. coli* RNA polymerase (■); and RSV reverse transcriptase (□).

icity. There are many nucleoside analogues such as halogenated nucleosides and arabinofuranosylcytosine which inhibit DNA synthesis at the substrate level (Prusoff, 1967; Momparler, 1969). This type of inhibitor may also be incorporated into host DNA, and the potential for genetic damage, infertility, or even carcinogenesis has been discussed (Welch, 1965; Prusoff and Goz, 1973). Compounds that inhibit DNA synthesis by a direct interaction with viral DNA polymerase should be promising candidates as therapeutic agents (Temin and Baltimore, 1972; Gallo, 1972).

In this paper, we have examined the mode of action of phosphonoacetate on purified DNA polymerase of HSV. Enzymes from both HSV type 1 and type 2 were studied. The study revealed that the action of phosphonoacetate differs from other known inhibitors of DNA polymerase in that it is a noncompetitive inhibitor with respect to substrates and an uncompetitive inhibitor with respect to the template.

Experimental Section

Materials. Sodium phosphonoacetate was synthesized from trimethyl phosphite and methyl bromoacetate (Nylén, 1924). Nucleoside triphosphates were purchased from Sigma Chemical Co. ^{3}H-Labeled nucleoside triphosphates were obtained from New England Nuclear Corp. or Schwarz/Mann (used after dilution to about 880 cpm/pmol). Calf-thymus DNA, *Micrococcus luteus* DNA, poly(dA-dT)·poly(dA-dT), *M. luteus* DNA polymerase (132 units/mg), and *Escherichia coli* RNA polymerase (870 units/mg) were purchased from Miles Laboratories. One unit of enzyme was defined as the amount catalyzing the incorporation of 1 nmol of deoxyribonucleotide or ribonucleotide into the acid-insoluble material during the 30-min incubation at 37°C. The molecular weight of calf-thymus DNA was determined by the sedimentation equilibrium method in an analytic ultracentrifuge equipped with a photoelectric scanner. This particular lot of calf-thymus DNA (batch No. 21, Miles Laboratories) is surprisingly homogeneous and has a molecular weight of 1.84×10^6. HSV [^{3}H]DNA (15 μCi/mg) was prepared as described previously (Overby et al., 1974). DNAs were activated by DNase I treatment (Aposhian and Kornberg, 1962). DNase I was obtained from Worthington Biochemical. Hepatitis B virus DNA polymerase was isolated as Dane particle (Dane et al., 1970) by the procedure described by Kaplan et al. (1973) with a specific activity of 0.15 unit/mg. Reverse transcriptase of Rous sarcoma was provided by P. P. Hung of Abbott Laboratories. HSV type 1 was obtained from American Medical Association Research Group and HSV type 2 (strain 333) was provided by R. G. Duff of Abbott Laboratories.

Tissue Culture and Purification of DNA Polymerases. Herpesvirus infected Wi-38 cells were grown as formerly reported (Overby et al., 1974), and harvested when 25% of the cells showed cytopathic effect of HSV. The DNA polymerase was isolated according to the procedure of Smith and Gallo (1972), which involved column chromatography on DEAE-cellulose and phosphocellulose. However, a buffer containing 20% glycerol instead of 10% was used. The final enzyme preparation has a specific activity of 280 units/mg for HSV type 1 and 313 units/mg for HSV type 2. Normal Wi-38 DNA polymerases were purified by the same procedure from logarithmic growth phase tissue culture (Mao et al., 1975). Chromatographic evidence indicated there were three DNA polymerases. However, a mixture of these three polymerases was used in this study. The specific activity of the normal cellular polymerases was 2.5 units/mg.

Enzyme Assay. HSV DNA polymerase was assayed in a reaction mixture of 0.2 ml containing 10 μ*M* dATP, dCTP, and dGTP and 2.5 μ*M* [^{3}H]dTTP which was appropriately diluted with unlabeled triphosphate to give 880 cpm per pmol, 10 μg of activated calf-thymus DNA, 50 m*M* Tris-HCl buffer (pH 8.0), 3 m*M* $MgCl_2$, 100 m*M* KCl, and 1 m*M* dithiothreitol. In the Wi-38 DNA polymerase assay, KCl was omitted and $MgCl_2$ was increased to 8 m*M* in order to optimize the reaction (Weissbach et al., 1971; Mao et al., 1975). *M. luteus* DNA polymerase was assayed according to the procedure of Aposhian and Kornberg (1962). *E. coli* RNA polymerase was assayed by the procedure of Burgess (1969). DNA polymerase of hepatitis B virus was assayed by the method of Kaplan et al. (1973). Reverse transcriptase of Rous sarcoma virus was assayed by the method of Spiegelman et al. (1970). The amount of enzyme used in each reaction was chosen to give a linear rate for at least 30 min at 37°C. The reaction was terminated by the addition of 3 ml of cold 5% trichloroacetic acid–0.01 *M* pyrophosphate. The acid-insoluble material was collected, washed on glass fiber disks (Reeve Angel 984-H), and counted by the liquid scintillation method.

Results

Specificity of Phosphonoacetate Inhibition. As shown in Figure 1, phosphonoacetate is a highly specific and potent inhibitor for HSV-induced DNA polymerase. Inhibition

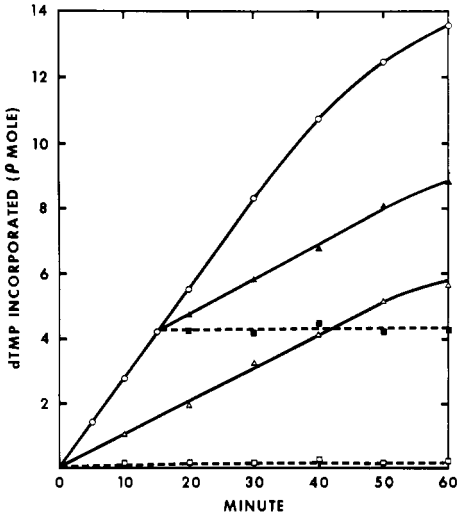

FIGURE 2: Time course of phosphonoacetate inhibition. The reaction conditions were described under Enzyme Assay. HSV-1 induced DNA polymerase, 0.03 μg, was used for each experimental point. Phosphonoacetate at a final concentration of 1×10^{-6} or 1×10^{-4} M was added at 0 (△,□) or 15 min after the initiation of the reaction (▲,■). A reaction mixture without addition of phosphonoacetate (○) was used as a control.

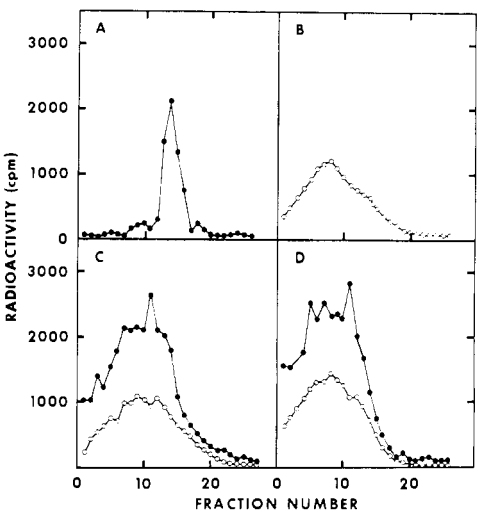

FIGURE 3: Effect of phosphonoacetate on the formation of DNA polymerase–DNA complex. Four samples containing (A) 0.4 μg of HSV-2 induced DNA polymerase, (B) 10 μg of HSV [^{3}H]DNA (7.3×10^4 cpm), (C) 0.4 μg of polymerase and 10 μg of [^{3}H]DNA, or (D) 0.4 μg of polymerase, 10 μg of [^{3}H]DNA, and 0.5 mM phosphonoacetate were incubated in a solution (0.3 ml) containing 50 mM Tris-HCl (pH 7.8), 100 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol. After incubation at 37°C for 5 min, samples were layered onto 10–30% linear glycerol density gradient made with 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, and 1 mM dithiothreitol. The gradients were centrifuged at 50000 rpm for 7.5 hr in an SW65 rotor. Twenty-six fractions were collected from the bottom of each tube. [^{3}H]DNA was located by precipitation of 50 μl of aliquot of 5% trichloroacetic acid (○). DNA polymerase activity in a 50-μl aliquot was determined by the standard assay as described under Enzyme Assay (●). The radioactivity contributed by [^{3}H]DNA was not subtracted.

could be detected at phosphonoacetate concentrations as low as 10^{-7} M. The sensitivity of HSV type 1 or type 2 induced DNA polymerase is essentially identical. On the other hand, DNA polymerases from human cells (Wi-38), from bacteria (*M. luteus*), and from hepatitis virus were not significantly inhibited at concentrations as high as 10^{-3} M. In addition, *E. coli* RNA polymerase and reverse transcriptase of Rous sarcoma virus were also resistant to this drug.

Time Course of Phosphonoacetate Inhibition. Phosphonoacetate (10^{-4} or 10^{-6} M) was added at zero time or 15 min after the initiation of DNA synthesis. At high concentrations, the drug inhibited the reaction completely and immediately; at low concentrations, the drug caused an instantaneous inhibition of identical extent whether the drug was added at the time of initiation or after the initiation of polymerization. It is evident that phosphonoacetate is effective even after the initiation of DNA synthesis (Figure 2).

Lack of Inhibition of the Formation of Enzyme–DNA Complex by Phosphonoacetate. HSV-induced DNA polymerase was incubated with activated HSV [^{3}H]DNA for 5 min at 37°C in the absence or presence of 0.5 mM phosphonoacetate. Samples then were sedimentated through linear glycerol gradients. Enzyme alone and HSV [^{3}H]DNA alone which served as controls were centrifuged in an identical manner. The sample which contained enzyme alone showed a sharp peak of polymerase activity in the middle part of the gradient (Figure 3A). The recovery of polymerase activity was 36%. The low recovery is probably due to the instability of the enzyme which could be partially denatured during the 7.5-hr centrifugation. Free [^{3}H]DNA was found to be rather heterogeneous but the peak position was located in the lower part of the gradient (Figure 3B). The heterogeneity of [^{3}H]DNA could result from DNase I digestion during the activation process or by radiation. When DNA polymerase was incubated with [^{3}H]DNA, the polymerase activity co-sedimented with DNA (Figure 3C). The recovery of polymerase activity was 83% which suggested that polymerase after complexing with DNA is more stable than the free enzyme. The presence of 0.5 mM phosphonoacetate which could inhibit polymerization completely, apparently, had no significant effect on the formation of the DNA–enzyme complex as shown in Figure 3D. The recovery of polymerase activity from the drug treated sample (Figure 3D) was not significantly different from that of the untreated sample (Figure 3C). This suggested that phosphonoacetate is a reversible inhibitor which dissociated from the enzyme–DNA complex during the centrifugation. The recovery of [^{3}H]DNA was about 85% in Figure 3B–D.

Effect of Substrate on Phosphonoacetate Inhibition. To characterize the nature of inhibition further, the effect of substrate concentration on phosphonoacetate inhibition was studied. In the experiment shown in Figure 4, the rate-limiting substrate was [^{3}H]dTTP; the other three triphosphates were in excess. Phosphonoacetate concentrations were 0, 1.1, and 5.5 μM. When the data were plotted by the method of Lineweaver and Burk (1934), straight lines could be drawn intersecting on the abscissa, indicating that the inhibition was noncompetitive with respect to dTTP. The apparent K_m for dTTP is 0.71 μM, while the K_i for phosphonoacetate is 0.47 μM.

To exclude the possibility that the results were due to the choice of labeled substrate, the experiments were repeated using, alternatively, radioactive dATP, dCTP, or dGTP as the rate-limiting substrates. In all cases, double reciprocal plots resulted on points through which a straight line could be drawn intersecting on the abscissa, again indicating noncompetitive inhibition. The apparent K_m and K_i values for dATP were 0.75 and 0.46 μM, for dCTP they were 0.42 and 0.45 μM, and for dGTP they were 0.39 and 0.48 μM, respectively.

Effect of Template DNA on Phosphonoacetate Inhibi-

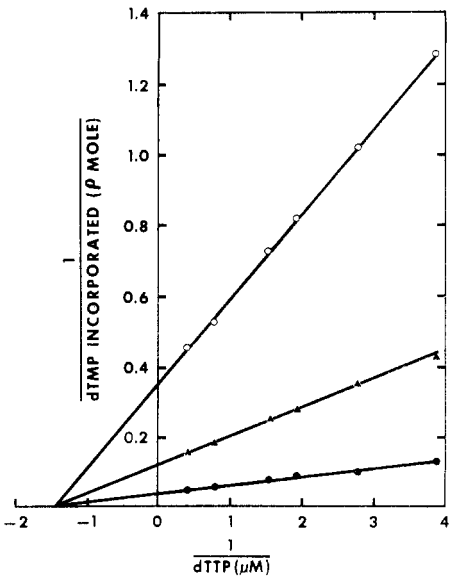

FIGURE 4: Effect of phosphonoacetate on reaction rate in the presence of different concentrations of dTTP. The reaction mixture (0.2 ml) contained 50 m*M* Tris-HCl buffer (pH 8.0), 3 m*M* $MgCl_2$, 100 m*M* KCl, 10 μM each of dATP, dCTP, and dGTP, 10 μg of activated calf-thymus DNA, 0.07 μg of HSV-2 DNA polymerase, and the indicated concentrations of [^{3}H]dTTP (880 cpm per pmol). Phosphonoacetate concentrations were 0 (●), 1.1 μM (▲), and 5.5 μM (○). The mixtures were incubated at 37°C for 30 min.

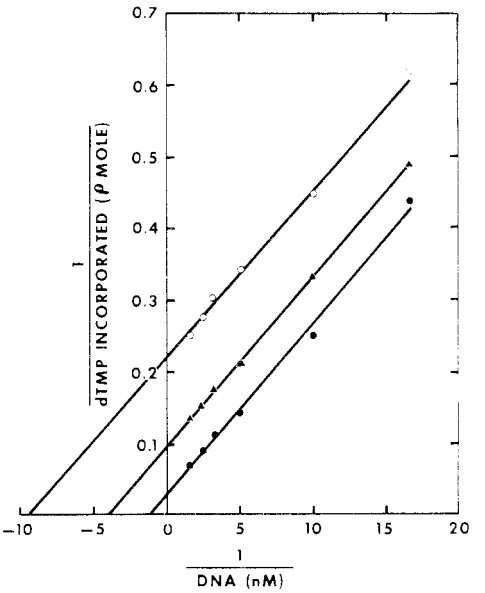

FIGURE 5: Effect of phosphonoacetate on reaction rate in the presence of different concentrations of primer template DNA. The reaction mixture (0.2 ml) contained 50 m*M* Tris-HCl buffer (pH 8.0), 3 m*M* $MgCl_2$, 100 m*M* KCl, 10 μM each of dATP, dCTP, and dGTP, 2.5 μM [^{3}H]dTTP (880 cpm per pmol), 0.07 μg of HSV-2 induced DNA polymerase, and the indicated concentrations of activated calf-thymus DNA. Phosphonoacetate concentrations were 0 (●), 1.1 μM (▲), and 3.3 μM (○). The mixture was incubated at 37°C for 30 min.

tion. The extent of inhibition by phosphonoacetate on three different activated DNAs, poly[d(A-T)], calf-thymus DNA which is 39% G + C, and *M. luteus* DNA which is 71% G + C, was tested. Results indicated that the base composition of template had no profound effect on the extent of inhibition. These data, together with the specificity of phosphonoacetate for HSV-induced DNA polymerase (Figure 1), suggested that the template is not the likely site of action of phosphonoacetate. To further rule out this possibility, the effect of activated calf-thymus DNA concentration on phosphonoacetate inhibition was studied. As shown in Figure 5, the double reciprocal plots of polymerization velocity vs. template concentration at various concentrations of phosphonoacetate yield families of parallel lines which indicate first that a high concentration of template cannot abolish inhibition and second that phosphonoacetate is an uncompetitive inhibitor with regard to the template. The apparent K_m value of activated calf-thymus DNA with HSV-induced DNA polymerase in the absence of phosphonoacetate was 8.3×10^{-10} *M*, while in the presence of 1.1 and 3.3 μM drug the apparent K_m values were decreased to 2.4×10^{-10} and 1.1×10^{-10} *M*, respectively. The K_i values calculated from the double reciprocal plot were 0.45 and 0.47 μM for two drug concentrations.

Effect of Mg^{2+} and K^+ Concentration on Phosphonoacetate Inhibition. Phosphonoacetate is an acidic compound with pK_a values of 2.6, 5.0, and 8.2 for the carboxylic and two phosphono groups, respectively. Conceivably, it can form salts with Mg^{2+} and K^+ ions, thereby reducing effective concentrations of Mg^{2+} and K^+ in the reaction mixture which might cause inhibition. However, the Mg^{2+} concentration in our assay was 3 m*M* and [K^+] was 100 m*M*. Phosphonoacetate even at a concentration of 0.1 m*M*, which could cause almost complete inhibition of polymerization, could not change Mg^{2+} or K^+ concentrations to a significant extent. Indeed, in the presence of 1 μM phosphonoacetate, neither varying the Mg^{2+} concentration from 1 to 24 m*M* nor varying K^+ concentrations from 30 to 200 m*M* altered the degree of inhibition significantly. It may be concluded that even if the drug decreased free Mg^{2+} or K^+ ions in the solution this was not the basis for enzyme inhibition.

Discussion

Phosphonoacetate is a very small molecule which exhibits surprisingly high specificity toward DNA polymerase induced by herpesvirus. Although DNA polymerases of HSV types 1 and 2 were equally sensitive, DNA polymerases of mammalian origin, bacteria, or certain other viruses were resistant to this drug. In all cases activated calf-thymus DNA was used as the template, except hepatitis B virus polymerase (Dane particle) which does not need exogenous template (Kaplan et al., 1973), and reverse transcriptase which used poly(rA)·oligo(dA) as template. These results suggested that phosphonoacetate is an enzyme-specific rather than a template-specific inhibitor.

Enzyme kinetic analysis (Figure 4) showed that phosphonoacetate exhibits a noncompetitive inhibition with respect to substrates. The apparent K_i values calculated from Lineweaver-Burk plots, using each deoxyribonucleoside triphosphate alternatively as the rate-limiting substrate, were in the range of 0.43–0.48 μM. Apparently this inhibitor had no preference for any one triphosphate. It is expected that since phosphonoacetate is not a competitive inhibitor of the substrates, unlike the antiviral drugs of nucleoside analogues, it will not incorporate into DNA.

Although phosphonoacetate is a very potent inhibitor of HSV-induced DNA polymerase, it apparently dissociates readily from the enzyme-DNA complex, as shown by the results from glycerol density gradient centrifugation which indicated that phosphonoacetate remained at the meniscus while the macromolecules migrated down (Figure 3D). This conclusion is consistent with the earlier report that phos-

phonoacetate-protected infected cells would resume virus replication after removal of the drug (Overby et al., 1974).

Several lines of evidence suggested the site of action of phosphonoacetate was not on template DNA. First, DNA synthesis mediated by templates of different base composition were equally sensitive to the drug. Second, DNA polymerases of various sources were assayed with the same template and only HSV enzyme was sensitive to the drug (Figure 1). Third, kinetic studies showed that phosphonoacetate was an uncompetitive inhibitor with respect to template (Figure 5). The K_i value estimated by varying template concentrations was 0.45–0.47 μM which could be considered identical with the K_i values obtained by varying substrate concentration. The families of parallel lines in the plot indicated that the extent of inhibition increased with template concentration. The opposite would be expected with a template inhibitor.

The polymerization reaction of DNA polymerase may be considered as a three-step process: (1) formation of enzyme–DNA complex; (2) initiation step; and (3) elongation step. The experiments concerning polymerase–DNA complex formation (Figure 3) showed that phosphonoacetate did not exert significant influence on this step. The time course study (Figure 2) showed that phosphonoacetate added 15 min after the start of the reaction was just as effective as when added at zero time. These data suggested that the elongation step in DNA synthesis is the step affected by the drug.

It has been pointed out by many researchers that antiviral drugs which interfered with template directly showed little selectivity (Temin and Baltimore, 1972; Gallo, 1972; Horwitz, 1974). Competitive inhibitors of substrates, such as nucleoside analogues, could be incorporated into cellular DNA thereby being potentially damaging to the host (Welch, 1965; Prusoff and Goz, 1973). The unique mode of action of phosphonoacetate, being a noncompetitive inhibitor with respect to substrates and having no direct interference with the template, gives rise to a new basis for antiviral chemotherapy.

Further observations from this study concern the comparative properties of HSV-induced DNA polymerase and cellular polymerases. The apparent K_m values of HSV DNA polymerase for dTTP, dATP, dCTP, and dGTP were 0.71, 0.75, 0.42, and 0.39 μM, respectively, which are one order of magnitude or more lower than reported K_m values of mammalian DNA polymerase (Momparler et al., 1973; Spardari and Weissbach, 1974). The apparent K_m value of HSV polymerase for template was 8.3×10^{-10} M which is again about one order of magnitude lower than that for mammalian DNA polymerase (Mao et al., 1975). The low K_m values of HSV enzyme for the substrate and template could provide a selective advantage for viral DNA synthesis in the infected cells. Furthermore, it is known that HSV DNA is rich in G + C. The particularly low K_m values for dGTP and dCTP could facilitate the incorporation of G + C into HSV DNA.

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