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Characterization of the DNA Polymerases Induced by a Group of Herpes Simplex Virus Type I Variants Selected for Growth in the Presence of Phosphonoformic Acid*

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Five independently derived variants of a herpes simplex virus type I (HSV-1) strain were plaque purified from a virus population passaged in 1 mM phosphonoformic acid (PFA). The DNA polymerases induced by the parent and PFA-resistant viruses were purified and characterized. No differences were observed among the enzymes with respect to their chromatographic properties, specific activities, or polypeptides resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The variant enzymes exhibited levels of PFA resistance which ranged from 15- to 25-fold. Resistance to PFA was always associated with a similar degree of resistance to its congener phosphonoacetic acid, but cross-resistance to β-phenylphosphonoacetic acid was only seen with two of the five variant enzymes. PFA and pyrophosphate were mutually competitive in PP_i exchange reactions, but in DNA synthetic reactions the levels of resistance to PFA and PP_i were not equal. The apparent affinities of the enzymes for Mg^{2+} did parallel their affinities for PFA. K_m values of dNTPs were about 2-fold higher than the parent virus enzyme for all of the variant enzymes except one which was 4-fold higher. The processivity of polymerization was apparently unaffected by the enzyme changes related to PFA resistance although one variant enzyme had a lower value. Resistance among the variant enzymes to the triphosphates of 9-(2-hydroxyethoxymethyl)guanine and 2′,3′-dideoxyguanosine was directly related to the level of resistance to PFA. The data presented here indicated that (i) PFA resistance may result from several types of active site alterations, since the PFA-resistant enzymes were of three kinetically distinct types. Also, additional enzyme alterations, probably unrelated to PFA resistance, were detected in one enzyme. (ii) PFA and PP_i possess some different binding determinants within the active center of herpes simplex virus type I DNA polymerase. (iii) PFA and the triphosphates of 9-(2-hydroxyethoxymethyl)guanine and 2′,3′-dideoxyguanosine may have a common ultimate inhibitory mechanism.

PFA[1] is a potent and selective inhibitor of HSV replication *in vivo* and *in vitro* (1–5). PFA and its congener, PAA, are pyrophosphate analogues which have been shown to preferentially inhibit the HSV-induced DNA polymerase (2, 6–8). Viruses exhibiting decreased sensitivities to both of these agents have been isolated in cell culture where they occur at high frequency, since variants can be selected with relative ease (9–12). These variants induced DNA polymerase activities which were resistant to the direct action of PFA or PAA *in vitro* (10–13). It has been reported that plaque-purified isolates of HSV, selected for growth in the presence of a single concentration of PAA, exhibited a wide range of sensitivities to PAA in cell culture (12). The enzymic basis for the varied levels of resistance to PAA or PFA and the kinetic concomitants of resistance have not been reported. Previous *in vitro* studies of PAA-resistant HSV-induced DNA polymerase activities have generally employed partially purified preparations of the enzyme obtained from single drug-resistant virus isolates (11, 14). It is not entirely clear from such studies whether some of the altered kinetic properties of the drug-resistant DNA polymerases were a general consequence of drug resistance. It is possible that different types of alterations in the HSV DNA polymerase, each conferring resistance to PAA or PFA, could affect the other kinetic properties of the enzymes in quite different ways, making each enzyme unique. Furthermore, it is likely that some drug-resistant DNA polymerases possess additional biochemical changes which influence the kinetic behavior of the enzyme independent of the alterations which confer resistance to the agent used for selection.

To more clearly understand the molecular mechanisms of PFA inhibition and resistance in HSV-1, the DNA polymerases induced by a HSV-1 strain and five independently derived plaque-purified variants of this strain, selected for their ability to grow in the presence of a high concentration of PFA, were highly purified. Examination of the biochemical and kinetic properties of the purified enzymes revealed that the five PFA-selected virus-induced DNA polymerases were of three distinct types which differed in certain kinetic characteristics and in their sensitivities to various inhibitors.

EXPERIMENTAL PROCEDURES

Materials

All chemicals used were reagent grade or better. Deoxynucleoside 5′-triphosphates, calf thymus DNA, and bovine serum albumin were purchased from Sigma. [^{3}H]dTTP and [^{3}H]dGTP were from ICN Nutritional Biochemicals. [^{32}P]Pyrophosphate was obtained from New England Nuclear. All electrophoresis reagents were from Bio-Rad. Phosphonoformic acid, phosphonoacetic acid, and β-phenyl-

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[1] The abbreviations used are: PFA, phosphonoformic acid; HSV-1, herpes simplex virus type I; PAA, phosphonoacetic acid; β-phenyl-PAA, β-phenylphosphonoacetic acid; acyclovir, 9-(2-hydroxyethoxymethyl)guanine; acyclo-GTP, 9-(2-hydroxyethoxymethyl)guanine triphosphate; ddGTP, 2′,3′-dideoxyguanosine triphosphate; araHxTP, 9-β-D-arabinofuranosylhypoxanthine triphosphate.

phosphonoacetic acid were gifts of Astra Pharmaceuticals (Sweden). 2′,3′-Dideoxy-GTP was purchased from P-L Biochemicals. Acylco-GTP was a gift from Wellcome Research Laboratories, and 9-β-D-arabinofuranosylhypoxanthine 5′-triphosphate was generously provided by Dr. Jerry L. Ruth of Calbiochem-Behring. Pancreatic DNase I and micrococcal nuclease were obtained from Worthington. Native calf thymus DNA-cellulose was prepared according to Potuzak and Wintersberger (15). Activated calf thymus DNA was prepared by the method of Baril *et al.* (16) and then extensively dialyzed against 20 mM Tris-Cl, pH 8.0. Bovine serum albumin was extensively dialyzed and heat denatured at 60 °C for 3 h. 3′-Terminally labeled activated [^{3}H]dTMP-DNA (160 cpm/pmol) was prepared as previously described (17).

Methods

Cells and Virus—Vero cells were grown in RPMI 1640 medium supplemented with 5% calf serum (Gibco Laboratories). The KOS strain of HSV-1 was obtained from stocks maintained in this laboratory by low multiplicity passage in Vero cells.

Selection of HSV-1 Variants—Confluent monolayers of Vero cells were infected at a multiplicity of one plaque-forming unit per cell. The inoculum was replaced with medium supplemented with 1% calf serum and 1 mM PFA; after 6 days progeny virus was harvested. Viruses from individual wells were plaque purified by limiting dilution in the presence of 1 mM PFA, and working stocks were derived following low multiplicity passage in the absence of the drug.

Infection of HeLa S3 Cells—0.5-liter suspension cultures of HeLa S3 cells (10^6 cells per ml) were pelleted, resuspended in 20 ml of medium, and infected with virus at 10 plaque-forming units per cell; at 30 min postinfection the inoculum was removed and cells were resuspended in 100 ml of medium supplemented with 1% calf serum. At 12 h postinfection the cells were pelleted by centrifugation, rinsed twice with cold phosphate-buffered saline, and stored at −70 °C until their extraction.

HSV-1 DNA Polymerase Purifications—All of the following procedures were performed at 4 °C. All buffers contained 2 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 20% (v/v) glycerol, unless otherwise indicated, and were adjusted to pH 7.5 at 23 °C. The virus-infected cell pellet was resuspended in 10 ml of 0.3 M $KHPO_4$, 0.2% Triton X-100, 10% glycerol and disrupted by sonication. Cell debris was removed by centrifugation for 30 min at 15,000 rpm in a Sorvall RC-2B rotor. The supernatant was applied to a 10-ml column of DEAE-cellulose equilibrated with 50 mM $KHPO_4$ and eluted with 15 ml of 0.4 M $KHPO_4$. The column flow-through was collected and dialyzed overnight against 1 liter of 50 mM $KHPO_4$, 20% glycerol. This dialysate was applied to a 25-ml column of DEAE-cellulose equilibrated with 50 mM $KHPO_4$. The column was washed with 60 ml of 50 mM $KHPO_4$ and developed with a 120-ml linear gradient of 50 mM to 500 mM $KHPO_4$. HSV-1 DNA polymerase activity eluted as a broad peak centered around 0.2 M $KHPO_4$. Fractions eluted between 0.13 to 0.26 M $KHPO_4$ were pooled and dialyzed overnight against 1 liter of 0.1 M $KHPO_4$, 30% glycerol. This dialysate was loaded onto a 7.5-ml column of phosphocellulose equilibrated with 0.1 M $KHPO_4$. The column was washed with 40 ml of equilibration buffer and developed with a 100-ml linear gradient of 0.1 M to 0.5 M $KHPO_4$. HSV DNA polymerase activity eluted as a sharp peak between 0.16–0.19 M $KHPO_4$. Fractions containing the peak of HSV DNA polymerase activity were pooled and applied immediately to a 3-ml column of native DNA-cellulose equilibrated with 0.1 M $KHPO_4$. The column was washed with 20 ml of equilibration buffer, and the enzyme was eluted with a 100-ml linear gradient of 0.1 M to 0.8 M $KHPO_4$. DNA polymerase activity eluted as a sharp peak at 0.38 M $KHPO_4$. Fractions eluting between 0.37–0.39 M $KHPO_4$ were pooled and dialyzed for 4 h against 250 ml of 50 mM $KHPO_4$ plus 50% glycerol. The final recovery of HSV DNA polymerase activity with respect to the high speed supernatant was approximately 10%, with an average specific activity of 29,000 units/mg.

Enzyme Assays

All enzyme assays were performed at 37 °C.

HSV DNA Polymerase—Assays were performed as previously described (2, 17). The standard reaction mix contained in a volume of 0.1 ml: 50 mM Tris-Cl, pH 8.0, 3 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mg/ml of bovine serum albumin, 0.2 M KCl, 0.1 mM each of dATP, dGTP, and dCTP, 5 μM [^{3}H]dTTP, and 12.5 μg of activated calf thymus DNA. One unit of DNA polymerase activity is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of dTMP into DNA per h under these conditions.

HSV DNA Polymerase-associated 3′,5′-Exonuclease—Exonuclease assays were performed as previously described (17). The reaction mix of 0.2 ml contained the same components as the DNA polymerase assays except that dNTPs were absent and activated DNA was replaced by 3′-terminally labeled [^{3}H]dTMP-activated calf thymus DNA.

PP_i Exchange—[^{32}P]Pyrophosphate exchange into unlabeled dNTPs was monitored by the assay procedure described by Deutscher and Kornberg (18). Reaction mixtures (0.2 ml) contained 50 mM Tris-Cl, pH 7.5, 3 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 M KCl, 0.2 mg/ml of bovine serum albumin, 25 μg of activated DNA, 0.1 mM each dATP, dGTP, dCTP, and dTTP, and [^{32}P]PP_i (2–6 Ci/mol). Additional $MgCl_2$ was present at concentrations equal to PP_i to compensate for the metal ion chelation.

Processivity Determinations—DNA polymerase processivity was determined by the method of Bambara *et al.* (19) with micrococcal nuclease-digested calf thymus DNA as the competitive inhibitor. Reaction mixtures were identical with those described for DNA polymerase assays except that unlabeled dNTPs were present at 10 μM each and [^{3}H]dTTP was present at 5 μM (200 cpm per pmol); 50 μg of activated DNA and, when present, 30 μg of "inhibitor" DNA were used. Processivity was determined in the presence of the following combinations of limiting triphosphates: dTTP; dTTP and dGTP; dTTP, dGTP, and dATP. The "effective" template length on activated DNA available to HSV-1 (KOS) DNA polymerase was estimated by the procedure of Bambara *et al.* (19) as modified by Fisher *et al.* (20). Employing reaction conditions similar to those used to determine processivity, the effective template length on activated calf thymus DNA was approximately 32 nucleotides.

Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate—8% polyacrylamide slab gels 0.75 mm thick by 12 cm in length were cast and run in a Bio-Rad Protean slab gel electrophoresis cell using a 5% 2-cm long stacking gel. Gels were prepared and run essentially as described by Laemmli (21). Samples were concentrated by precipitation in 7% trichloroacetic acid at 4 °C, resuspended in 125 mM Tris-Cl, pH 6.8, 2% sodium dodecyl sulfate, 0.1 M β-mercaptoethanol, 30% (v/v) glycerol, followed by heating in a boiling water bath for 5 min. Gels were fixed and then stained in silver nitrate by the method of Merril *et al.* (22).

Other Methods—Protein concentrations were determined by the method of Bradford (23).

RESULTS

Variants of HSV-1 were readily obtained after passage of the parent virus in the presence of 1 mM PFA. Ten variants were plaque purified from independently selected populations and five of these, designated as PFA^r-1 to -5, were chosen for the following analyses of the virus-induced DNA polymerases. Details regarding the selection, frequency of occurrence, and other biological properties of the variants in cell culture will be presented elsewhere.[2]

HSV-1 DNA Polymerase Purifications—The infections of HeLa S3 cells with the parent and PFA-resistant viruses and the subsequent extractions and purifications of the HSV-induced DNA polymerases were performed under identical conditions, thus allowing comparisons of the amounts of activity induced and the chromatographic properties of the enzymes to be made. At 12 h postinfection with a multiplicity of 10 plaque-forming units per cell, all of the viruses induced approximately the same levels of DNA polymerase activity. No major differences were observed among the DNA polymerases during the purifications; the chromatographic behaviors, percentages of enzyme activity recovered at each purification step, and the final specific activities were similar for all of the enzymes. Representative purification data are shown in Table 1 for the parent HSV-1 DNA polymerase. PFA inhibition of each virus-induced DNA polymerase was monitored at each purification step; none of the enzymes exhibited a change in PFA sensitivity during the course of its purification. This is in contrast to a previous report (2) which indicated a change in

[2] K. F. Bastow, D. Derse, and Y.-C. Cheng, unpublished observation.

TABLE I
Purification of HSV-1 DNA Polymerase

Fraction	Volume	Protein	Activity[a]	Specific activity	Yield
	ml	*mg*	*units*	*units/mg*	%
Crude supernatant	15	79.5	18,000	226	100
First DEAE-cellulose	21	52.5	17,800	340	99
Second DEAE-cellulose	23	5.4	8,880	1,640	49
Phosphocellulose	10	0.27	4,070	15,100	23
DNA-cellulose	5.5	0.066	1,740	25,640	10

[a] Enzyme activity was determined in the standard HSV DNA polymerase assay as described under "Methods."

PFA sensitivity during the course of HSV-2 DNA polymerase purification. This discrepancy is difficult to rationalize due to the differences in biological materials and experimental methods employed.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—Polyacrylamide slab gel electrophoresis, in the presence of sodium dodecyl sulfate, of each virus-induced DNA polymerase is shown in Fig. 1*A*. Approximately 32 units of each enzyme (DNA-cellulose fraction) were applied to each gel lane. Three major polypeptide bands were resolved in the final DNA polymerase preparations with apparent molecular weights of 140,000, 88,000, and 62,000. The intensities of the 140,000 and 62,000 M_r bands were nearly identical for all of the DNA polymerases, whereas the intensity of the 88,000 M_r polypeptide did vary among the enzymes, but not in a manner which could be correlated with PFA sensitivity. A typical elution profile of DNA polymerase activity from the DNA cellulose column is shown in Fig. 1*B* for the parent virus enzyme; slab gel electrophoresis of the column fractions eluted across the peak of DNA polymerase activity is shown in Fig. 1*C*. It is evident here that the intensities of the 140,000 and 62,000 M_r polypeptides correlated with DNA polymerase activity, whereas the 88,000 M_r polypeptide was eluted slightly before these two. Thus, the variable intensity of this latter species among the enzymes was apparently due to slight differences in the pooling of DNA-cellulose fractions. It might be noted that there is a protein band which migrates slightly faster than the major stained polypeptide of 140,000 M_r shown in *lane 3* of Fig. 1*C*. The significance of this observation is currently under investigation.

PFA Inhibition of the Pyrophosphate Exchange Reaction—The kinetics of PFA inhibition of the HSV DNA polymerase-catalyzed synthetic reaction have previously been described (1, 2, 4). It was shown to be noncompetitive with respect to dNTPs and uncompetitive with respect to DNA. From these kinetic analyses and the structural similarity between PP_i and PFA, it has been presumed that they bind to the same site on HSV DNA polymerase. To examine their mutual binding site more closely, we have employed the PP_i exchange reaction catalyzed by the parent virus-induced DNA polymerase. In characterizing this reaction it was observed that the apparent K_m of PP_i was influenced by the pH of the reaction mixture. The kinetics of the reaction at several pH values is presented in the Lineweaver-Burk plot shown in Fig. 2*A*. As the pH of the reaction was elevated the apparent K_m of PP_i increased; apparent K_m values of PP_i were: pH 8.1, 5.0 mM; pH 7.6, 1.8 mM; pH 7.1, 0.6 mM. The PP_i exchange reaction catalyzed by *Escherichia coli* DNA polymerase I was similarly shown to be influenced by pH (18). The kinetics of PFA inhibition of the PP_i exchange reaction catalyzed by HSV-1 DNA polymerase at pH 7.6 is shown in Fig. 2*B*. PFA was found to be a competitive inhibitor with respect to PP_i; the apparent K_m and K_i values of PP_i and PFA were 1.8 mM and 1.3 μM, respectively. Although it could be concluded that PFA and PP_i bind to the same site on the HSV-1 DNA polymerase, it is more correct in view of the results discussed below to state that they share some common binding determinants and bind to the same form of enzyme.

Inhibition of DNA Synthetic Reactions by PP_i and PP_i Analogues—DNA synthetic reactions catalyzed by the various HSV-1 DNA polymerases were performed under conditions of saturating dNTP and DNA substrates. The apparent

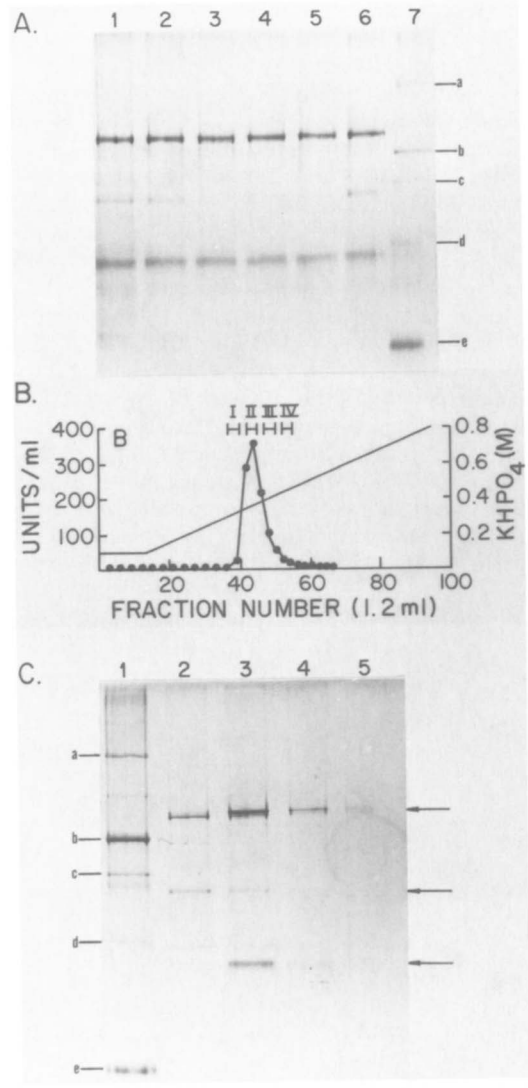

FIG. 1. **Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified HSV-1 DNA polymerases.** *A*, 8% polyacrylamide slab gel electrophoresis and silver staining were performed as described under "Methods." 32 units of each purified DNA polymerase, induced by the parent, PFA^r-1, PFA^r-2, PFA^r-3, PFA^r-4, and PFA^r-5 viruses, were applied, in order, to gel *lanes 1* through *6*. To *lane 7* was applied Bio-Rad molecular weight markers containing 0.25 μg of each protein. The markers, indicated in the margin, are: *a*, myosin (200,000 M_r); *b*, β-galactosidase (116,000 M_r); *c*, phosphorylase *b* (92,000 M_r); *d*, bovine serum albumin (66,000 M_r); *e*, ovalbumin (45,000 M_r). *B*, DNA-cellulose column profile of the parent HSV-1 DNA polymerase. The *brackets above the peak* of DNA polymerase activity represent fractions which were pooled for subsequent electrophoretic analysis. *C*, sodium dodecyl sulfate-slab gel electrophoresis of the pooled DNA-cellulose fractions shown in *B*. 80 μl of each sample were applied to the 8% polyacrylamide gel. *Lane 1* contains 0.25 μg of the marker proteins described in *A*. *Lanes 2* to *5* contained, in order: sample I (fractions 37–40), sample II (fractions 42–45), sample III (fractions (47–50), and sample IV (fractions 52–55). The *arrows* in the right-hand margin indicate the major protein bands present in *lane 3*.

K_i values of PP_i and the analogues, PFA, PAA, and β-phenyl-PAA, under these conditions, were determined from Dixon plots of $1/v$ *versus* (*I*).

The relative inhibitory potency of PFA against the purified parent and PFA-resistant virus-induced DNA polymerases is clearly revealed in Fig. 3, where the percentage of activity remaining is plotted *versus* the logarithm of the PFA concentration present. K_i values of PFA for each enzyme are summarized in Table II. As expected from the ability of the HSV-1 variants to replicate in the presence of PFA in cell culture, the purified DNA polymerases induced by these viruses were inhibited only at substantially higher PFA concentrations than were required to inhibit the parent virus enzyme. Interestingly, the DNA polymerases induced by the PFA-selected variants formed two groups defined by their degrees of resistance to PFA. The PFA^r-1 and PFA^r-5 DNA polymerases were approximately 10-fold more resistant to PFA, and the PFA^r-2, -3, and -4 enzymes were about 20-fold more resistant than the parental HSV-1 DNA polymerase. The different sensitivities to PFA exhibited among the PFA-selected virus-induced enzymes suggested that different changes in these enzymes had occurred, which conferred a particular degree of resistance to PFA.

Preliminary analyses of the effects of PP_i on DNA synthetic reactions revealed that the degree of inhibition was dependent on the pH of the reaction; the apparent K_i of PP_i was influenced by pH in the same way as its K_m was affected in the PP_i exchange reactions (Fig. 2*A*). K_i values of PP_i were, therefore, determined in synthetic reactions adjusted to pH 7.6. The K_i values of PP_i for the parent virus enzyme under these reaction conditions was 1.5 mM, which is very similar to the K_m value of 1.8 mM obtained in the PP_i exchange reaction. Unlike PP_i, the inhibition of DNA synthesis by PFA was independent of the pH of the reaction. Because PFA and PP_i appear to be structurally related and since PFA competitively inhibited the PP_i exchange reaction, one might predict that the K_i values of PP_i would vary among the enzymes in a manner similar to that observed with PFA. The apparent K_i values of PP_i, summarized in Table II, did not follow the pattern predicted for two compounds which bind in the same way to the same site on an enzyme. Although the K_i values of PFA were 10- and 20-fold higher among the variant DNA polymerases, the K_i values of PP_i were only 2.5 to 3 times higher compared to the parent virus enzyme. Furthermore, the two groups of

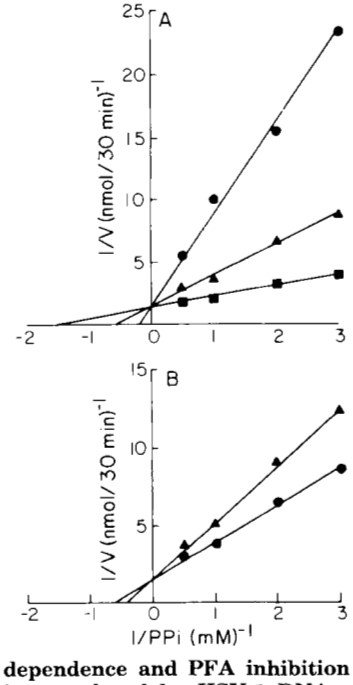

FIG. 2. **pH dependence and PFA inhibition of the PP_i exchange reaction catalyzed by HSV-1 DNA polymerase.** PP_i exchange reactions were performed as described under "Methods" using [^{32}P]PP_i (2–6 Ci per mol) at the indicated concentrations and 1.5 units of the parent HSV-1 DNA polymerase. Incubations were at 37 °C for 30 min. The data are presented in the form of Lineweaver-Burk plots, and lines were fitted by the method of least squares analysis. In *A*, the reactions were performed at pH 8.1 (●——●), pH 7.6 (▲——▲), pH 7.1 (■——■). The pH of the Tris-Cl buffers was adjusted at 37 °C. *B*, reactions were performed as in *A* at pH 7.6 and included PFA at 0 (●——●) and 0.5 μM (▲——▲).

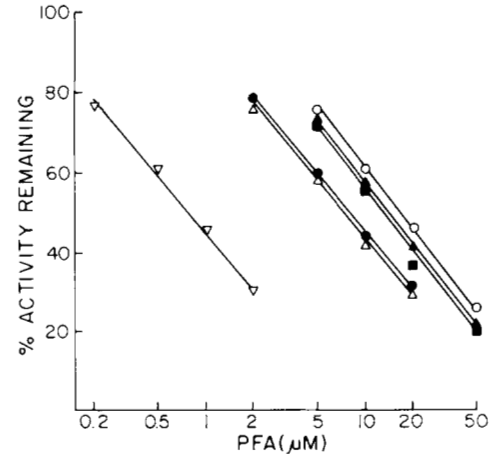

FIG. 3. **Inhibition of DNA synthetic reactions by PFA.** The standard DNA polymerase reaction mixture, described under "Methods," was incubated with the indicated concentrations of PFA and 1 unit of parent (▽——▽), PFA^r-1 (△——△), PFA^r-2 (▲——▲), PFA^r-3 (■——■), PFA^r-4 (○——○), or PFA^r-5 (●——●) DNA polymerase for 15 min at 37 °C. The data are plotted as the percentage of polymerase activity remaining *versus* the logarithm of the PFA concentration present. In the absence of PFA, 0.25 nmol of [^{3}H]dTMP was incorporated into DNA.

TABLE II

Apparent K_i values of PP_i, PFA, PAA, and β-phenyl-PAA for the HSV-1 DNA Polymerases

HSV-1 DNA polymerase	Apparent K_i values[a]			
	PP_i[b]	PFA[c]	PAA[d]	β-Phenyl-PAA[e]
	mM		mM	
Parent virus	1.5 ± 0.2	0.64 ± 0.01	0.97 ± 0.3	182 ± 8
PFA^r-1	3.7 ± 0.6	9.9 ± 1.8	7.4 ± 0.1	2147 ± 260
PFA^r-2	4.0 ± 0.2	14.6 ± 2.9	14.8 ± 0.4	162 ± 10
PFA^r-3	3.8 ± 0.3	12.6 ± 0.2	16.6 ± 0.4	178 ± 6
PFA^r-4	3.7 ± 0.4	17.4 ± 1.4	15.6 ± 0.1	225 ± 25
PFA^r-5	4.5 ± 0.3	9.6 ± 1.0	9.3 ± 0.2	1126 ± 52

[a] DNA polymerase reaction mixtures described under "Methods" contained 0.5 unit of HSV-1 DNA polymerase. Incubations were for 20 min at 37 °C. The apparent K_i values were obtained by plotting the data as $1/v$ *versus* (*I*), fitting straight lines by least square analysis, and calculating the *I* axis intercepts.

[b] PP_i was present in all reactions at 0, 0.5, 1.0, and 2.0 mM. Additional $MgCl_2$ was added to the reactions at concentrations equal to the added PP_i.

[c] PFA was present for the parent virus DNA polymerase at 0, 0.2, 0.5, 1.0, and 2.0 μM; for PFA^r-1 and PFA^r-5 DNA polymerases it was present at 0, 2, 5, 10, and 20 μM and at 0, 5, 10, 20, and 50 μM for the PFA^r-2, -3, and -4 DNA polymerases.

[d] PAA was added to give 0.5, 1.0, and 2.0 μM with the parent virus enzyme and was present at 5, 10, and 20 μM for all of the PFA-resistant DNA polymerases.

[e] β-Phenyl-PAA was present at 100, 200, and 500 μM in all of the DNA polymerase reactions.

variant DNA polymerases, defined by their degrees of resistance to PFA, did not differ with respect to PP_i. These data suggest that PFA and PP_i bind in different ways to the active center of HSV-1 DNA polymerase.

PAA is very similar to PFA in its potency and mechanism of action against HSV DNA polymerase (1, 4, 11). As the apparent K_i values of PAA shown in Table II reveal, the PFA-resistant DNA polymerases were cross-resistant to PAA, in agreement with other reports (11). The magnitudes of resistance to PAA among the variant DNA polymerases were very similar to those seen with PFA. Again two groups appeared among the variant enzymes; the PFA^r-1 and PFA^r-5 DNA polymerases formed the lower resistance group and the PFA^r-2, -3, and -4 DNA polymerases fell into the higher resistance group. β-Phenyl-PAA, previously shown to be a relatively weak inhibitor of HSV-1 DNA polymerase (4), was also examined. The pattern of inhibition of the DNA polymerases by β-phenyl-PAA was completely different than that observed with PFA and PAA. K_i values of this agent, presented in Table II, show that it was much less potent than either PFA or PAA against the parental HSV-1 DNA polymerase, having a K_i of 182 μM. Unexpectedly, the PFA^r-2, -3, and -4 DNA polymerases, which were the most resistant to PFA or PAA, were as sensitive to β-phenyl-PAA as the parent virus enzyme. The PFA^r-1 DNA polymerase, however, was about 12 times less sensitive to β-phenyl-PAA than the parent virus enzyme, a level of resistance comparable to that observed with respect to PFA and PAA. Although the PFA^r-5 and PFA^r-1 DNA polymerases appeared to be similar with respect to their PFA and PAA K_i values, the PFA^r-5 enzyme was about half as resistant to β-phenyl-PAA as the PFA^r-1 enzyme. Thus, β-phenyl-PAA inhibition revealed three distinct types of DNA polymerase activity within the group of PFA-resistant enzymes.

Reaction Optima and Cofactor Requirements—It was anticipated that the biochemical alterations which had conferred various degrees of PFA resistance among the variant DNA polymerases would be manifested in certain unique kinetic properties. If these could be correlated with the degrees of PFA resistance, insight would be gained regarding both the interaction of PFA with HSV-1 DNA polymerase and the role of its binding site in other catalytic functions of the enzyme. As an initial step in these studies the optimal reaction conditions for each enzyme were determined. *In vitro* DNA synthesis was maximal for all of the enzymes at pH 8.0 (adjusted at 37 °C) and in the presence of 0.2 M KCl. The stimulation of enzyme activity by KCl followed the same pattern for all of the enzymes; activities increased approximately 7-fold from 0 to 0.2 M KCl. The effects of varying the Mg^{2+} concentration on the DNA polymerase activities are shown in Fig. 4*A*. For clarity only the parent virus, PFA^r-1, and PFA^r-2 DNA polymerases are shown here. The data for the PFA^r-5 DNA polymerase were identical with PFA^r-1; the PFA^r-3 and PFA^r-4 enzymes behaved like the PFA^r-2 DNA polymerase. The differences among the enzymes with respect to varying Mg^{2+} concentrations are more clearly discerned when presented in the form of the double reciprocal plot shown in Fig. 4*B*. Although the absolute K_m values of Mg^{2+} cannot be determined from these data alone, it is evident that under these reaction conditions the relative apparent affinities for Mg^{2+} were lower among the PFA-resistant enzymes than the parent virus DNA polymerase. Furthermore, the differences in the apparent affinities for Mg^{2+} among the PFA-resistant enzymes paralleled their affinities for PFA, that is, the PFA^r-1 and PFA^r-5 enzymes had higher apparent affinities for PFA and Mg^{2+} than the PFA^r-2, -3, and -4 DNA polymerases.

K_m Values of Deoxynucleoside 5′-Triphosphates—Apparent K_m values of the deoxynucleoside triphosphates were determined for each DNA polymerase in reactions where the concentration of a single labeled dNTP was varied. The data obtained with variable [^{3}H]dGTP are presented in the form of Lineweaver-Burk plots shown in Fig. 5. For clarity, data are shown only for the parent virus, PFA^r-1, PFA^r-2, and PFA^r-5 DNA polymerases. The apparent K_m values of dGTP thus obtained for the HSV-induced DNA polymerases were: parent, 0.25 μM; PFA^r-1, 1.0 μM; PFA^r-2, 0.48 μM; PFA^r-3, 0.45 μM; PFA^r-4, 0.54 μM; PFA^r-5, 0.46 μM. All of the PFA-resistant DNA polymerases exhibited decreased apparent affinities for dGTP. The PFA^r-1 enzyme had the highest K_m value, which was four times higher than that of the parent virus DNA polymerase, while the other PFA-resistant enzymes had K_m values that were approximately 2-fold higher. Although the K_m values of dGTP were higher for the PFA-resistant, compared to the parent virus DNA polymerase, the K_m values among the PFA-resistant enzymes did not follow a pattern which could be correlated with their different sensitivities to PFA. Except for the PFA^r-1 enzyme, the decreased affinities for dGTP observed among the PFA-resistant enzymes appeared to be similar to their decreased apparent affinities for PP_i. To rule out the possibility that the various K_m values obtained above were unique to dGTP, K_m values of dTTP were also determined and found to be nearly identical with those of dGTP (data not shown).

Comparative Inhibitory Effects of Deoxynucleoside Triphosphate Analogues—It has previously been reported that HSV variants selected for their ability to replicate in the presence of PAA were less sensitive than the parent virus to the antiviral action of acyclovir in cell culture (14, 25). Furthermore, the partially purified DNA polymerases induced by

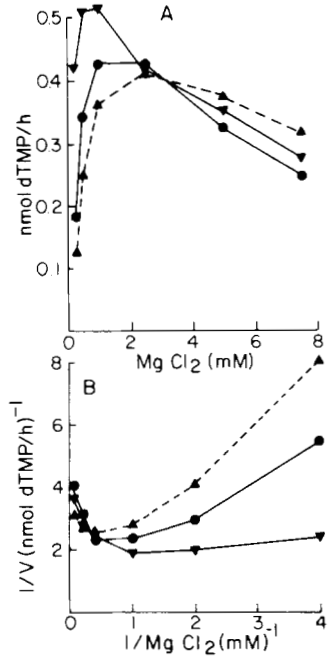

FIG. 4. **Magnesium ion requirement for DNA synthesis catalyzed by the HSV-1 DNA polymerases.** *A*, the standard DNA polymerase reaction mixture (without $MgCl_2$) described under "Methods" was incubated at 37 °C for 15 min with 0.5 unit of HSV-1 DNA polymerase and the indicated concentrations of $MgCl_2$. Reactions were performed with each DNA polymerase, but for clarity only the data for the wild type (▼——▼), PFA^r-1 (●——●), and PFA^r-2 (▲——▲) DNA polymerases are shown. The PFA^r-5 enzyme behaved identically with the PFA^r-1 enzyme, and the PFA^r-2, -3, and -4 DNA polymerases were alike. *B*, the data shown in *A* are presented in the form of a Lineweaver-Burk plot; *symbols* are the same as in *A*.

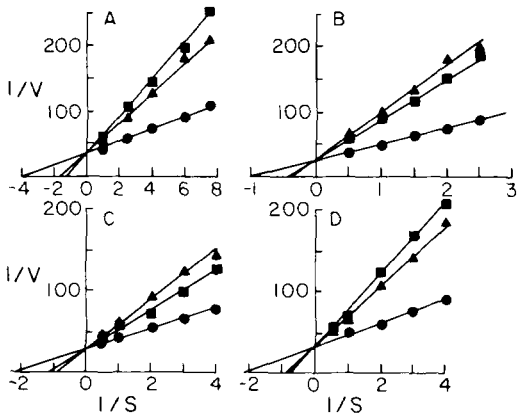

FIG. 5. **Competitive inhibition of the HSV DNA polymerases by acyclo-GTP and dideoxy-GTP.** Reactions were performed as described under "Methods" except that they contained 0.1 mM each dATP, dCTP, and dTTP, and [^{3}H]dGTP (400 cpm/pmol) at the indicated concentrations. The velocities are expressed as nanomoles of [^{3}H]dGMP incorporated into activated DNA per h. The data are presented in the form of Lineweaver-Burk plots, and *straight lines* were fitted by least squares analysis. *A*, parent virus DNA polymerase (0.03 unit) was incubated without inhibitors (●——●), with 0.004 μM acyclo-GTP (▲——▲), or 0.4 μM ddGTP (■——■). *B*, the PFA^r-1 DNA polymerase (0.04 unit) was incubated without drug (●——●), 0.05 μM acyclo-GTP (▲——▲), or 10 μM ddGTP (■——■). *C*, the PFA^r-2 DNA polymerase (0.04 unit) was incubated without drug (●——●), 0.1 μM acyclo-GTP (▲——▲), or 20 μM ddGTP (■——■). *D*, the PFA^r-5 DNA polymerase (0.04 unit) was incubated with no drug (●——●), 0.05 μM acyclo-GTP (▲——▲), or 10 μM ddGTP (■——■).

some PAA-resistant variants were cross-resistant to the triphosphates of acyclovir (24) and 2',3'-dideoxyguanosine (14). In order to place such observations in a more quantitative context and to determine how such cross-resistance between PP$_i$ and dNTP analogues could be explained, competitive inhibition analyses at fixed concentrations of acyclo-GTP and ddGTP and varied concentrations of [^{3}H]dGTP were performed with each HSV DNA polymerase. araHxTP was also included in these analyses since it has a modified sugar moiety, competitively inhibits dGMP incorporation into DNA,[3] and, unlike the other two dGTP analogues, possesses a 3'-hydroxyl moiety. The data for the parent virus, PFA^r-1, PFA^r-2, and PFA^r-5 DNA polymerases are shown in Fig. 5 in the form of Lineweaver-Burk plots. K_m values of dGTP and K_i values of the dGTP analogues obtained in this way for all of the virus-induced DNA polymerases are summarized in Table III. Acyclo-GTP was a potent inhibitor of the parent virus enzyme, having a K_i of 0.003 μM; the K_i of ddGTP was 0.2 μM, and araHxTP was a relatively poor inhibitor, having a K_i of 2.6 μM. The K_i values of acyclo-GTP for the DNA polymerases induced by the PFA-selected variants were 10- to 25-fold higher than for the parent virus enzyme, levels of resistance which closely paralleled those observed with PFA. That is, the K_i values of PFA and acyclo-GTP were approximately 10-fold higher for the PFA^r-1 and PFA^r-5 enzymes and about 25-fold higher for the PFA^r-2, -3, and -4 enzymes, compared to the parent virus DNA polymerase. K_i values of ddGTP were also much higher among the variant DNA polymerases than the parent virus enzyme. The relative sensitivities of the enzymes to ddGTP were qualitatively similar to acyclo-GTP in that the PFA^r-1 and PFA^r-5 DNA polymerases were less resistant than the group composed of PFA^r-2, -3, and -4. Quantitatively, the degrees of resistance to ddGTP observed for the variant DNA polymerases were greater than for acyclo-GTP. K_i values of ddGTP, relative to the parent virus enzyme, were 22 to 35 times higher for the PFA^r-5 and PFA^r-1 DNA polymerases, respectively, and about 100-fold higher for the PFA^r-2, -3, and -4 group of enzymes. Acyclo-GTP and ddGTP have been suggested to inhibit HSV DNA polymerase by similar mechanisms (26, 27); the similar patterns of sensitivities to ddGTP and acyclo-GTP observed among the enzymes here tend to support this.

[3] D. Derse and Y.-C. Cheng, unpublished observation.

AraHxTP competitively inhibited dGMP incorporation into DNA catalyzed by the HSV-1 DNA polymerases. The K_i values of araHxTP, shown in Table III, were elevated in all of the variant DNA polymerases compared to the parent virus enzyme, but not in a manner comparable to PFA or acyclo-GTP. Whereas the increases in the K_i values for the PFA^r-1 and PFA^r-5 DNA polymerases were similar with respect to acyclo-GTP and PFA, the PFA^r-5 DNA polymerase had about a 2-fold lower K_i of araHxTP than the PFA^r-1 enzyme. The PFA^r-1 through PFA^r-4 DNA polymerases had K_i values for araHxTP that were 8 to 10 times higher than the PFA-sensitive enzyme. It could be argued that the differences among the enzymes with respect to araHxTP were due to the inosine moiety in addition to the fraudulent sugar; however, competitive inhibition analyses using arabinofuranosylthymine 5'-triphosphate and variable concentrations of [^{3}H]dTTP revealed the same relative pattern of sensitivities as araHxTP.

Processivities and Relative Cycling Times—It was believed that the biochemical alterations which were present among the variant DNA polymerases might be expressed in their mechanisms of polymerization. Bambara *et al.* have described a method for determining the processivity of DNA polymerases based on cycling time perturbation (19). In addition to estimating the number of nucleotides incorporated into DNA per binding event, this method allows one to determine the relative "static" and "kinetic" affinities of a DNA polymerase for the primer-template during the course of DNA synthesis. The processivities and relative cycling times on activated calf thymus DNA for each HSV-1 DNA polymerase are shown in Table IV. The processivity of HSV-1 DNA polymerase, determined by this technique, has not previously been reported. The value of 10 ± 2 nucleotides obtained here appears comparable to the values of 11 ± 5 nucleotides reported for KB cell α-polymerase (20) and 14 ± 4 nucleotides for *Mycoplasma orale* DNA polymerase (28), but lower than the value of 22 nucleotides reported for *E. coli* DNA polymerase I (19). The relative cycling time (T_x/T_4) obtained here suggests that the static affinity of HSV-1 DNA polymerase for the primer

TABLE III

Apparent K_i values of acyclo-GTP, dideoxy-GTP, and AraHxTP for the HSV-1 DNA polymerases

HSV-1 DNA polymerase	dGTP K_m[a]	Acyclo-GTP K_i[a]	ddGTP K_i[a]	araHxTP K_i[b]
		μM		
Parent virus	0.25	0.003	0.20	2.6
PFA^r-1	1.02	0.028	6.9	22
PFA^r-2	0.48	0.072	22.4	31
PFA^r-3	0.45	0.065	21.4	28
PFA^r-4	0.54	0.085	23.5	28
PFA^r-5	0.46	0.030	4.3	12

[a] The K_m values of dGTP and the K_i values of ddGTP and acyclo-GTP were calculated from the data plotted in Fig. 5. The reaction conditions used in the PFA^r-3 and PFA^r-4 DNA polymerase-catalyzed reactions were identical with those used for the PFA^r-2 DNA polymerase reaction described in the legend to Fig. 5.

[b] K_i values of araHxTP were determined in reactions identical with those described in the legend to Fig. 5 for each enzyme. AraHxTP was present at 5 μM for the parent virus DNA polymerase and at 20 μM for all of the other enzymes.

TABLE IV

Processivities and relative cycling times of the HSV-1 DNA polymerases on activated calf thymus DNA

Processivity and relative cycling time (T_x/T_4) were determined by the method of Bambara *et al.* (19), using the three limiting dNTP reaction conditions described under "Methods." The values presented represent an average of values obtained from at least two determinations under the three reaction conditions.

HSV-1 DNA Polymerase	Processivity	T_x/T_4
Parent virus	10 ± 2.0	2.49 ± 0.20
PFA^r-1	6.5 ± 0.7	1.99 ± 0.24
PFA^r-2	9.4 ± 0.3	2.44 ± 0.12
PFA^r-3	10.4 ± 2.4	2.36 ± 0.26
PFA^r-4	8.6 ± 0.7	2.81 ± 0.25
PFA^r-5	10 ± 2.6	2.56 ± 0.29

TABLE V

Relative DNA polymerase and 3',5'-exonuclease activities of the HSV-1 DNA polymerases

DNA polymerase	Synthesis[a]	Hydrolysis[b]	Synthesis/hydrolysis
	pmol dTMP incorporated/min	*pmol [^{3}H]-dTMP released/h*	
Parent virus	323	120	2.7
PFA^r-1	332	255	1.3
PFA^r-2	415	189	2.2
PFA^r-3	284	135	2.1
PFA^r-4	280	108	2.6
PFA^r-5	364	126	2.9

[a] DNA polymerization reactions were performed under the standard reaction conditions described under "Methods." Incubations were for 15 min.

[b] 3',5'-Exonuclease assays were performed as described under "Methods" with the same amounts of each enzyme as were used in the parallel DNA polymerase assays. Incubations were for 30 min at 37 °C.

terminus is about 2.5 times greater than its kinetic affinity. The processivities and relative cycling times obtained for the PFA-selected variant DNA polymerases, with the exception of PFA^r-1, were not significantly different than the parent virus enzyme. The processivity of the PFA^r-1 DNA polymerase was 6.5 ± 0.7 nucleotides, and it also had an apparently decreased relative cycling time compared to the parent virus DNA polymerase. In general, however, it appeared that the processivity was independent of the changes which had conferred PFA resistance among the HSV-1 DNA polymerases.

3',5'-Exonuclease Activities—HSV DNA polymerase has been shown to possess a 3',5'-exonuclease activity (17, 29, 30). To determine whether this exonuclease activity was altered relative to the DNA polymerase activity among the enzymes, parallel synthetic and hydrolytic assays were performed. The relative activities are shown in Table V. It is apparent that the ratio of DNA synthesis to hydrolysis was similar for all of the DNA polymerases except PFA^r-1. The PFA^r-1 DNA polymerase exhibited a DNA polymerase to exonuclease ratio which was half that of the other enzymes.

DISCUSSION

Variants of herpes simplex virus type I which were able to replicate in the presence of PFA, an antiherpes virus agent which inhibits the virus-induced DNA polymerase, appeared readily.[2] Although the variants were selected for growth at a single PFA concentration, their DNA polymerases exhibited a range of sensitivities to PFA *in vitro*. Furthermore, the DNA polymerases induced by the five PFA-selected variants formed three major groups defined by certain kinetic parameters and by their sensitivities to various inhibitors. Because a range of PFA sensitivities existed among these DNA polymerases, it was possible for the first time to correlate the biochemical changes which conferred resistance to PFA with other altered properties of the enzymes.

The gross biological and biochemical properties of the variant DNA polymerases were apparently conserved. The growth of the parental and PFA-selected viruses in cell culture in the absence of PFA appeared identical, and none of the variants were temperature sensitive.[2] All of the virus-induced DNA polymerases behaved alike throughout the purification stages. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate resolved three major polypeptides in the final purified HSV-1 DNA polymerase fractions. The intensities of the silver-stained polypeptide bands of approximately 140,000 and 62,000 M_r were similar for all of the DNA polymerases, whereas the intensity of an 88,000 M_r protein varied among the enzymes. Electrophoresis of fractions across the peak of DNA polymerase activity eluted from the DNA-cellulose column revealed that, unlike the 140,000 and 62,000 M_r species which eluted with the peak of DNA polymerase activity, the 88,000 M_r polypeptide eluted slightly before this peak. The latter polypeptide thus appeared to be a contaminant of the HSV-1 DNA polymerases, present in variable amounts among the purified enzymes. The nature and origin of the 140,000 and 62,000 M_r species are not entirely clear at present. Previously, polyacrylamide gel electrophoresis and autoradiography of the HSV-1 DNA polymerase, purified by a procedure similar to that used here from cells labeled after infection with ^{14}C-amino acids showed a single virus-induced polypeptide with an apparent M_r of about 150,000 (31). This value correlates well with the molecular weights of the catalytic core of prokaryotic and eukaryotic replicative DNA polymerases (32). The labeling method used above, however, would not reveal host cell proteins which may copurify with the virus-induced DNA polymerase and perhaps function in virus DNA replication. Knopf observed at least four major protein-stained bands on dodecyl sulfate-polyacrylamide gel electrophoresis of HSV-1 DNA polymerase purified in a similar manner (30). Polypeptides of 144,000, 74,000, 62,000–66,000, and 29,000 M_r were resolved in the peak DNA-cellulose fraction. It is of interest to note that highly purified mammalian α-type DNA polymerases have been found to possess a high molecular weight protein associated with a number of lower molecular weight polypeptides whose functions are as yet unknown (33, 34). Estimates of the native molecular weight of HSV-1 DNA polymerase by glycerol or sucrose density gradient sedimentation yielded values ranging from 126,000 (2) to 142,000 (30). Although these values appear to correlate with the largest protein band observed by slab gel electrophoresis, they are based on the assumption that the enzyme is spherical. Thus, an accurate determination of the molecular weight of the native enzyme awaits further analyses.

It was anticipated that the biochemical alterations responsible for PFA resistance among the variant DNA polymerases had occurred at the PP_i binding site since PFA and PP_i are structurally related and PFA competitively inhibited the PP_i exchange reaction catalyzed by the parent HSV-1 DNA polymerase. It was previously shown that PAA, which behaved like PFA here, competitively inhibited the PP_i exchange reaction catalyzed by another herpes virus-induced DNA polymerase (35). However, it appeared that PFA and PP_i bind in different ways since their affinities for the parent virus DNA polymerase differed by about three orders of magnitude, and while affinities for PFA were 10 to 20 times lower with the variant DNA polymerases, their affinities for PP_i were only 2.5- to 3-fold lower, relative to the parent virus enzyme. Although the K_i values of PP_i were fairly uniform among the

variant enzymes, their apparent affinities for Mg^{2+} differed in a manner which was related to their apparent affinities for PFA. The group of variant enzymes which were about 20-fold resistant to PFA had the lowest apparent affinity for Mg^{2+} and the group of enzymes that were approximately 10-fold resistant to PFA exhibited an apparent affinity for Mg^{2+} that was intermediate between the parent and other PFA-resistant DNA polymerases. While all of the functions of the Mg^{2+} ion cofactor in the polymerization process are not known, it is clear that its chelation with the deoxynucleoside triphosphate substrate is required for the latter's binding to the enzyme and that the Mg^{2+}-PP_i chelate is released subsequent to nucleotide incorporation into DNA (36). The correlation of PFA and Mg^{2+} affinities observed here suggested that the active site alterations which conferred certain degrees of resistance to PFA also influenced Mg^{2+} binding and may thus define an area of the enzyme's PP_i binding site with which PFA strongly interacts.

That at least two different types of altered active sites existed among the variant DNA polymerases was inferred from the observed grouping of these enzymes with respect to their apparent affinities for PFA, PAA, and Mg^{2+}. However, the pattern of inhibition of the DNA polymerases by β-phenyl-PAA, which was previously shown to have a weak inhibitory effect on HSV DNA polymerase (4), was totally unlike that observed with either PFA or PAA. The variant DNA polymerases which were the most resistant to PFA or PAA were as sensitive to β-phenyl-PAA as the parent virus enzyme. However, the PFA^r-1 DNA polymerase was about as resistant to β-phenyl-PAA as to PAA or PFA; the PFA^r-5 and PFA^r-1 enzymes which behaved alike with respect to PFA, PAA, and Mg^{2+} differed by about 2-fold in their sensitivities to β-phenyl-PAA. This was the first clear indication that the PFA^r-1 and PFA^r-5 DNA polymerases were unique and that within this sample of five variants three distinct types of DNA polymerase activity were present. The cross-resistance between PFA and β-phenyl-PAA, exhibited by two of the variant enzymes but not by the other three, was enigmatic but might be rationalized in several ways. If it is assumed that PFA, PAA, and β-phenyl-PAA all inhibit the parent virus DNA polymerase by binding to the same determinants, then cross-resistance would be predicted. The sensitivity to β-phenyl-PAA seen with the PFA^r-2, -3, and -4 enzymes might then be the result of an interaction of the aromatic portion of the analogue with the altered enzyme moiety which conferred PFA resistance, that is a change in the mechanism of the drug was responsible for their sensitivities. Alternatively, it might be that PFA and β-phenyl-PAA bind to the parent HSV-1 DNA polymerase in quite different ways. Then, cross-resistance would not be predicted, but when observed could be due to unique changes which were related to PFA resistance; however, the possibility that more than one subsite has been altered on a variant DNA polymerase cannot be ignored. Probably the most important conclusion to be drawn here is that generalizations about cross-resistance, based on the relative sensitivity of a single drug-resistant virus isolate, could be erroneous. For example, had only the PFA^r-1 or PFA^r-2 enzymes been examined, one might have concluded that resistance to PFA conferred or did not confer, respectively, resistance to β-phenyl-PAA.

A model, based on studies with *E. coli* DNA polymerase I and perhaps applicable to other DNA polymerases, was advanced which proposed that the several different DNA polymerase activities, *i.e.* synthesis, hydrolysis, and PP_i exchange, are catalyzed by a single active center (18). It would thus follow that changes in the PP_i binding area of this active center might influence the binding of other substrates and perhaps alter some of the macroscopic functions of the enzyme. Indeed all of the PFA-selected HSV-1-induced DNA polymerases exhibited higher apparent K_m values for deoxynucleoside triphosphate substrates than the parent virus enzyme. The 2-fold increases in these K_m values, observed for most of the variant enzymes, appeared to parallel in magnitude their increased apparent K_i values of PP_i. The exception among the PFA-resistant DNA polymerases was that induced by PFA^r-1, which exhibited a 4-fold higher K_m value relative to the parent virus enzyme. Although the K_m values for all of the variant enzymes were higher than the parent virus enzyme, they did not vary in a manner related to their different apparent affinities for PFA. A previous study, using partially purified preparations of HSV DNA polymerase, showed no differences in the K_m values of dNTPs between PAA-sensitive and PAA-resistant isolates (14). The apparent K_m values obtained, however, were much higher than those observed here and elsewhere (2, 27). Unlike the affinities of certain substrates and inhibitors, the active site changes which conferred PFA resistance did not influence the processivities, relative cycling times, nor ratios of synthesis to hydrolysis, among the DNA polymerases. The single exception to this generalization was observed with the PFA^r-1 DNA polymerase which exhibited a decreased processivity and a lower ratio of synthesis to hydrolysis compared to the other enzymes. The uniqueness of the PFA^r-1 DNA polymerase is interesting; it was very similar to the PFA^r-5 enzyme with respect to its apparent affinity for PFA, PAA, and Mg^{2+}, but differed from all of the other variant enzymes in its higher dNTP K_m value, lower processivity, lower ratio of polymerase to exonuclease activity, and its resistance to β-phenyl-PAA. These latter characteristics may have been the consequence of a unique alteration directly related to PFA resistance. However, the data also suggest that this enzyme might possess a second active site alteration responsible for its unique kinetic properties but unrelated to its PFA resistance.

Several laboratories have reported that PAA resistance in HSV-1 segregated with acyclovir resistance (14, 25). A partially purified DNA polymerase induced by a PAA-resistant isolate of HSV-1 was shown to be less sensitive to the inhibitors acyclo-GTP (24) and dideoxy-GTP (14) than the parent virus-induced enzyme. Whether PAA or PFA resistance always confers acyclo-GTP resistance in HSV-induced DNA polymerases has not previously been proven. In view of the data presented here regarding cross-resistance between PFA and β-phenyl-PAA, such generalizations, based on single isolates, should be made cautiously. The PFA-selected variant DNA polymerases studied here all had decreased affinities for acyclo-GTP and ddGTP compared to the parent virus enzyme. Furthermore, the degrees of resistance to these analogues were directly related to the degrees of PFA resistance. These data strongly suggest that resistance to PFA confers resistance to acyclo-GTP; the basis for this cross-resistance, however, is puzzling. It was recently proposed that resistance to PAA is accompanied by a more stringent recognition by the triphosphate binding site of HSV-1 DNA polymerase, consequently prohibiting the incorporation of nucleotides lacking a 3′-OH moiety (14). That the triphosphate binding site of the PFA-resistant enzymes differed from the parent virus enzyme was revealed in the higher K_m values of the normal dNTPs for the former. However, the K_i values of acyclo-GTP and ddGTP were more clearly correlated with the K_i values of PFA than to the K_m values of dNTPs. For example, the PFA^r-1 DNA polymerase had a K_m of dGTP which was 2-fold higher than that observed with the PFA^r-5 enzyme, but both enzymes had similar affinities for PFA, acyclo-GTP, and ddGTP. An alternative explanation is that

PFA, acyclo-GTP, and ddGTP share a common ultimate inhibitory mechanism. PFA prevents HSV DNA polymerase release from, or translocation along the primer-template, by forming the dead-end polymerase-DNA-PFA complex. Acyclo-GTP, subsequent to its incorporation into DNA, also inhibits the release of HSV-1 DNA polymerase from the primer template (27), an effect like that observed with ddTTP on *E. coli* DNA polymerase I (37). In addition, ddTTP would not serve as a substrate in the PP_i exchange reaction nor could ddTMP-terminated DNA be hydrolyzed by the *E. coli* enzyme (18, 37). Similarly, 3′-terminal acyclo-GMP residues were resistant to the action of the HSV-1 DNA polymerase-associated 3′-exonuclease activity (27). Thus the cross-resistance between PFA and acyclo-GTP and ddGTP may be due to their mechanistic similarity, preventing DNA polymerase release from DNA. Although PFA resistance in HSV-1 DNA polymerase appears to be associated with resistance to acyclo-GTP, the converse does not necessarily follow. Because the inhibitory effect of acyclo-GTP on the DNA polymerase is manifested after its incorporation into DNA (27), it is conceivable that an active site alteration which decreased the affinity of the analogue for the enzyme or prohibited its incorporation into DNA would confer resistance to acyclo-GTP but not to PFA. An example of such an enzyme may be the DNA polymerase purified from an HSV-1 isolate, selected for growth in the presence of acyclovir (24); this enzyme was resistant to acyclo-GTP yet sensitive to PAA.

AraHxTP inhibition of the HSV-induced DNA polymerases was also examined since it is a dGTP analogue[3] and represents another class of DNA polymerase inhibitor. Unlike acyclo-GTP and ddGTP, araHxTP possesses a 3′-OH moiety and is not an absolute chain terminator. Like arabinofuranosyladenine triphosphate, it is incorporated into DNA by HSV-1 DNA polymerase, is very poorly elongated upon, and is readily excised by the HSV DNA polymerase-associated exonuclease activity[3] (17). All of the PFA-resistant enzymes examined here exhibited a decreased sensitivity to araHxTP compared to the parent virus enzyme. The relative magnitudes of resistance among the enzymes, however, were unlike those seen with PFA, acyclo-GTP, or ddGTP.

Since the antiviral action of PFA is associated with its inhibition of the virus-induced DNA polymerase, the emergence of PFA-resistant variants reflects the presence, in the population, of viruses which induce a unique DNA polymerase activity. That is, growth in the presence of PFA enriches a subpopulation of virus which specify altered DNA polymerase activities[2] (10). The existence of three kinetically unique activities within a sample of five PFA-selected virus-induced DNA polymerases leads to several conclusions regarding the virus and its DNA polymerase. It implies that resistance of the HSV-1 DNA polymerase to PFA may be achieved in different ways, through a variety of changes within the active site of the enzyme. This in turn may be related to the various degrees of PFA resistance observed among HSV variants in cell culture (10, 12) as well as to the relatively high frequency at which such variants emerge (9, 10). The differences in the kinetic properties observed within the small sample of variant enzymes studied here further suggest that the virus population is composed of a number of subpopulations which induce a variety of unique DNA polymerases, the basic catalytic functions of which must be conserved to maintain the virus in the population. This diversity may be an important adaptive and evolutionary property of HSV and is probably responsible for the interstrain differences observed among HSV isolates with respect to drug sensitivities. It is important to note that only a small group of DNA polymerases were examined here, induced by variants adapted for growth in the presence of a single agent at a single concentration. Thus, other selective conditions could yield variants which induce DNA polymerases with properties quite different than those observed here. Since a natural diversity in the DNA polymerases induced in a population of HSV exists, it is probable that some PFA-insensitive enzymes also possess alterations at other sites. Though unrelated to PFA binding, such alterations could affect the kinetic characteristics of these enzymes and thus prevent a valid correlation of PFA resistance with altered kinetic behavior or sensitivity to other drugs to be made. The PFA^r-1 DNA polymerase may be an example of this possibility. Whether the diversity observed among HSV-1 DNA polymerases is a general phenomenon characteristic of other HSV-induced enzymes seems likely. Studies of the deoxythymidine kinases induced by several drug-resistant isolates of HSV-1 have revealed that these enzymes exhibited a variety of kinetic alterations.[4]

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