DNA POLYMERASES OF MAREK'S DISEASE HERPESVIRUS AND HERPESVIRUS OF TURKEYS: CHARACTERIZATION AND MECHANISM OF INHIBITION BY PHOSPHONOACETATE

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Susan Singley Leinbach

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John A Boezi
Major professor

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ABSTRACT

DNA POLYMERASES OF MAREK'S DISEASE HERPESVIRUS AND HERPESVIRUS OF TURKEYS: CHARACTERIZATION AND MECHANISM OF INHIBITION BY PHOSPHONOACETATE

By

Susan Singley Leinbach

Infection of duck embryo fibroblasts (DEF) by Marek's disease herpesvirus (MDHV) led to the induction of a novel DNA polymerase. Infection of DEF by herpesvirus of turkeys (HVT) also led to the induction of a novel DNA polymerase. The properties of these enzymes have been examined. One unique property of herpesvirus-induced DNA polymerases is their sensitivity to the compound phosphonoacetate. Studies to elucidate the mechanism of phosphonoacetate inhibition have been performed and a model to explain this inhibition has been developed.

The MDHV-induced and HVT-induced DNA polymerase activities were partially purified from extracts of infected DEF by DEAE-cellulose chromatography and/or phosphocellulose chromatography and were characterized. The properties of these enzymes were virtually identical. Both required 1-2 mM $MgCl_2$ and 200-250 mM KCl for maximal activity. When 200 mM KCl was added to DNA polymerization reaction mixtures, DNA polymerase activity was 10- to 60-fold greater than in its absence. Both induced DNA polymerases were

inhibited by phosphonoacetate such that in the presence of 1-3 μM phosphonoacetate 50% inhibition of DNA polymerization occurred. Both enzymes were determined to be DNA-dependent DNA polymerases and to have sedimentation coefficients in the presence of 0.25 M KCl of about 7S. These properties distinguished the MDHV-induced and HVT-induced DNA polymerases from the DNA polymerases of uninfected DEF.

A phosphonoacetate-resistant mutant of HVT (HVT_{PA}) was isolated. It was able to replicate in the presence of 1.6 mM phosphonoacetate while wild type HVT (HVT_{WT}) was unable to replicate in the presence of 0.3 mM phosphonoacetate. The properties of the DNA polymerase induced by infection of DEF by HVT_{PA} were compared with those of the wild type induced DNA polymerase. The HVT_{PA}-induced DNA polymerase had an apparent inhibition constant for phosphonoacetate of about 10-fold greater than the HVT_{WT}-induced DNA polymerase. *In vitro* thermal inactivation studies demonstrated that the HVT_{PA}-induced DNA polymerase was more temperature-sensitive than the wild type enzyme. Other catalytic properties of the two enzymes were similar. These results suggest that the HVT-induced DNA polymerase is viral coded and that inhibition by phosphonoacetate occurs by direct interaction with the herpesvirus-induced DNA polymerase.

The inhibition of the HVT-induced DNA polymerase by phosphonoacetate was examined in detail using steady state enzyme kinetics. In the DNA polymerization reaction phosphonoacetate was a noncompetitive inhibitor when the four dNTPs were the variable substrate and activated DNA concentration was 200 μg per ml. Phosphonoacetate was also a noncompetitive inhibitor when activated DNA was the variable substrate and the concentration of each of the four dNTPs

was 2.5 μM. When the concentration of each of the four dNTPs was raised to 100 μM, inhibition by phosphonoacetate became nearly uncompetitive. Phosphonoacetate was a competitive inhibitor of pyrophosphate in the dNTP-pyrophosphate exchange reaction catalyzed by the HVT-induced DNA polymerase. In the DNA polymerization reaction multiple inhibition analysis of phosphonoacetate and pyrophosphate demonstrated that the two were mutually exclusive inhibitors. The inhibition patterns obtained for pyrophosphate in the polymerization reaction were similar to those obtained for phosphonoacetate. These results indicate that phosphonoacetate inhibits the herpesvirus-induced DNA polymerase by binding to the pyrophosphate binding site of the enzyme.

Rate equations were derived for kinetic mechanisms which described phosphonoacetate inhibition as occurring at the pyrophosphate binding site of the DNA polymerase. The mechanism which was consistent with all the phosphonoacetate kinetic inhibition data was the alternate product inhibition model. In this case the binding of phosphonoacetate to the pyrophosphate binding site of the enzyme would create an alternate reaction pathway. Phosphonoacetate as an alternate product of the DNA polymerization reaction would promote an exchange reaction in which a nucleotide analogue of phosphonoacetate would be generated as an alternate substrate.

The alternate product inhibition model predicted that the nucleotide analogue of phosphonoacetate should be a substrate for the DNA polymerization reaction and a competitive inhibitor of dNTP and should be generated in the reaction mixture during phosphonoacetate inhibition. Deoxythymidine 5'-phosphophosphonoacetate

was synthesized and was tested as an alternate substrate. It could not substitute for dTTP in activated DNA polymerization reactions. It was a noncompetitive inhibitor when the four dNTPs were the variable substrate in the DNA polymerization reaction. No nucleotide analogue of phosphonoacetate was generated when [^{3}H]phosphonoacetate was incubated in the presence of HVT-induced DNA polymerase, activated DNA, and the four dNTPs.

Therefore, no direct evidence was obtained in support of the alternate product inhibition model for phosphonoacetate inhibition. Nevertheless, the conclusion from the kinetic inhibition studies remains that phosphonoacetate inhibits the herpesvirus-induced DNA polymerase by binding to the pyrophosphate binding site of the enzyme. These studies also indicate that the herpesvirus-induced DNA polymerase has a high apparent affinity for phosphonoacetate. Apparent inhibition constants for phosphonoacetate were 1-2 μM while apparent inhibition constants for pyrophosphate were 1-2 mM.

Twenty analogues of phosphonoacetate were tested as inhibitors of the HVT-induced DNA polymerase. Only 2-phosphonopropionate was an inhibitor although its apparent inhibition constant was about 50 times greater than that of phosphonoacetate. Thus the structural requirements for inhibition at the pyrophosphate binding site of herpesvirus-induced DNA polymerases are very rigorous.

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By

Susan Singley Leinbach

A DISSERTATION

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DEDICATION

To my parents and husband Ed, who supported me with patience and understanding in my goal to become a biochemist.

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This work is the result of a collaboration between Dr. John Boezi and Dr. Lucy Lee of the USDA, ARS, Regional Poultry Laboratory. I wish to express my sincere thanks to Dr. Boezi and Dr. Lee for their continued assistance and guidance during my graduate studies. My thanks also goes to the members of my guidance committee: Dr. Fritz Rottman, Dr. Leland Velicer, Dr. Allan Morris, and Dr. William Deal.

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LIST OF ABBREVIATIONS

MDHV	Marek's Disease Herpesvirus
HVT	herpesvirus of turkeys
EBV	Epstein-Barr virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HCMV	human cytomegalovirus
DEF	duck embryo fibroblasts
CEF	chick embryo fibroblasts
PA	phosphonoacetate
PP_i	pyrophosphate
dNTP	deoxynucleoside triphosphate
dNMP	deoxynucleoside monophosphate
dTMP-PA	deoxythymidine 5' phosphophosphonoacetate
DEAE	diethylaminoethyl
Tris	tris-(hydroxymethyl)-methylamine

INTRODUCTION

Marek's disease is a highly contagious lymphoproliferative disease of chickens whose etiological agent is a herpesvirus, Marek's disease herpesvirus (MDHV). In lymphoid tumor cells of the infected chicken, MDHV genomes are present but no intracellular viral antigens or virus particles are produced. In the feather follicle epithelial cells of the infected chicken, productive infection occurs and cell-free infectious MDHV is produced. Thus, different virus-cell interactions exist within the MDHV-infected chicken. In each type MDHV DNA replicates. The mechanism by which MDHV DNA replicates in these different cells is central to understanding the molecular basis for different virus-cell interactions between herpesviruses and their hosts. Virus-cell interactions similar to those found for MDHV have also been found for the Epstein-Barr virus which causes the human lymphoproliferative disease infectious mononucleosis and is found closely associated with another human lymphoproliferative disease, Burkitt's lymphoma.

Evidence will be presented in this dissertation that, in productive infections, herpesvirus DNA is replicated by novel induced-DNA polymerases. Study of such DNA polymerases is important for two major reasons. First, with the knowledge of the properties of the herpesvirus-induced DNA polymerases, a search can be made in herpesvirus-derived tumors for the same enzyme to determine if the

herpesvirus DNA replicates by the same mechanism as in productive infections. The MDHV system is an excellent one for such a study because productive infections are readily established in tissue culture and tumors are readily established in chickens. Second, with the knowledge of the properties of the herpesvirus-induced DNA polymerase, a search can be made for a specific inhibitor of the enzyme which could have potential for controlling and perhaps eliminating herpesvirus infections. Such a compound, phosphonoacetate, does exist and has been shown to be effective in the treatment of herpes simplex virus infections in rabbits and mice. Elucidation of the mechanism of inhibition of phosphonoacetate at the site of the herpesvirus-induced DNA polymerase might provide information for the development of other anti-herpesvirus compounds as well as information about the structure of herpesvirus-induced DNA polymerases.

This dissertation describes research concerning the DNA polymerases of Marek's disease herpesvirus and herpesvirus of turkeys, a related virus, and the mechanism of inhibition of phosphonoacetate.

It begins with a literature review which describes: (1) the basic characteristics of Marek's disease and MDHV, (2) the basic characteristics of vertebrate DNA polymerases and of herpesvirus-induced DNA polymerases in other systems, and (3) studies by others with herpesviruses and phosphonoacetate.

Following the literature review, the results of work done with MDHV- and HVT-induced DNA polymerases are presented. Before this work was initiated, Boezi et al. had published a characterization

of a DNA polymerase induced by infection of duck embryo fibroblasts by MDHV, strain GA. The properties of this enzyme were different from those previously reported for the herpes simplex-induced DNA polymerase. Therefore, duck embryo fibroblasts infected by MDHV, strain BC-1, and duck embryo fibroblasts infected by herpesvirus of turkeys were examined for the presence of a more typical herpesvirus-induced DNA polymerase. Such an activity was found in these cells. The characterization of these enzymes is described. Much of this work was presented by Dr. Lucy Lee at the 3rd International Congress for Virology.

One property common to all herpesvirus-induced DNA polymerases is their sensitivity to phosphonoacetate. The next section of this dissertation describes results obtained from a steady state kinetic analysis of phosphonoacetate inhibition of the HVT-induced DNA polymerase undertaken in an effort to elucidate the mechanism of phosphonoacetate inhibition. A model describing the mechanism of phosphonoacetate inhibition consistent with the kinetic data was developed. This work has been published (Leinbach, S. S., J. M. Reno, L. F. Lee, A. F. Isbell, J. D. Boezi. 1976. *Biochemistry 15*: 426-430). This proposed model was tested directly after a nucleotide analogue of phosphonoacetate was synthesized and labelled phosphonoacetate of high specific radioactivity was prepared. A preliminary report of the results of these experiments was presented at the 67th Annual Meeting of the Federation of American Societies for Experimental Biology (Leinbach, S. S., L. F. Lee, and J. A. Boezi. 1976. *Federation Proceedings 35*: 1186).

The sensitivity of herpesviruses to phosphonoacetate was used by Dr. Lucy Lee to isolate a mutant of HVT which grew in the

presence of phosphonoacetate. The DNA polymerase induced by this mutant was characterized for its catalytic, structural and kinetic properties. These were compared with those of the DNA polymerase induced by wild-type HVT. This work has been submitted to *Virology* for publication.

Finally, using those properties of the MDHV-induced DNA polymerase which describe it uniquely, a chicken lymphoblastoid cell line transformed by MDHV was examined for the presence of the MDHV-induced DNA polymerase.

LITERATURE REVIEW

Marek's Disease

Marek's disease is a highly contagious lymphoproliferative disease of chickens whose etiological agent is a herpesvirus, Marek's disease herpesvirus (MDHV) (1-5). Unlike other neoplastic diseases, Marek's disease can be prevented with a live vaccine (6).

Two forms of Marek's disease have been observed to exist in the chicken: the classical form and the acute form (2). In the classical form, lymphoid cells infiltrate, proliferate and cause lesions in peripheral and autonomic nerves. The result is a paralysis of that part of the body innervated. In the acute form, lymphoid tumors develop in one or several organs, including gonad, liver, kidney, lung, muscle, and skin. These tumors are composed of lymphoblasts, small and medium lymphocytes, and Marek's disease cells, which are degenerate blast cells having little nuclear detail and a vacuolated cytoplasm.

The extent of expression of the MDHV genome varies in the cells of different tissues of the infected chicken (7). In tumors MDHV DNA is present (8) but no virus particles or intracellular antigens are observed. In the bursa of Fabricius and the thymus an abortive infection exists. Cellular necrosis, intranuclear inclusion bodies, and virus antigens are observed, but virus maturation does not occur. In the feather follicle epithelium a productive infection occurs. Infectious cell-free enveloped virus is produced.

Although Marek's disease was first described in 1907 (1), unequivocal proof of MDHV as its etiological agent has just recently been established because of the cell-associated nature of the virus. When tumor cells from MDHV-infected chickens were used to infect cultured chick or duck embryo fibroblasts, cytopathic effects and noninfectious unenveloped herpesvirus particles developed in the cultures. When these infected culture cells were inoculated into susceptible chickens, the chickens developed Marek's disease (4,5). Thus, a good correlation was established between the presence of the herpesvirus in cell culture and the ability of these cultures to produce Marek's disease in chickens. When the feather follicle epithelium was discovered to be the site of MDHV replication and dissemination in the chicken, infectious MDHV was isolated and was used successfully in producing Marek's disease in susceptible chickens (10,11). Most recently, the demonstration of the presence of MDHV genomes in tumors of diseased chickens has identified MDHV as the etiological agent of Marek's disease (8).

Although MDHV has been established as the etiological agent of Marek's disease, the mechanism by which MDHV induces proliferation of lymphoid cells is still unclear. Payne proposed that Marek's disease lymphomas might be caused by direct transformation of lymphoid cells (intrinsic mechanism) or by an immune response of lymphoid cells to viral antigens expressed in other cells (extrinsic mechanism) (12). Several lines of evidence support the intrinsic mechanism for MDHV-induced proliferation and suggest that T lymphocytes are the target cells of MDHV infection: (1) Marek's disease tumors were shown by membrane immunofluorescence to be composed of 77% T cells and 11% B cells (13); (2) thymectomy of susceptible

chickens decreased the number of birds with lymphomas (12); (3) the recently established lymphoblastoid cell lines derived from Marek's disease lymphomas carry T cell surface antigens (14,15). The final proof that MDHV causes Marek's disease by transformation of T cells requires the demonstration of *in vitro* transformation of normal chicken lymphoid cells by MDHV. However, it is becoming apparent that two types of T cells are involved in Marek's disease: those which are transformed and those which participate in immune surveillance against lymphoma development (16).

Three types of live vaccines are used to protect chickens from Marek's disease: an avirulent strain of MDHV, an attenuated strain of MDHV, and herpesvirus of turkeys (6,16). Herpesvirus of turkeys (HVT) is antigenically related to MDHV but is nonpathogenic. It is used commercially in the vaccination of chickens. Although vaccination protects chickens from contracting Marek's disease when challenged with MDHV, MDHV replicates in the feather follicle epithelium of the chicken. This suggests that the vaccine acts as an antitumor agent rather than as an antiviral agent (17).

Marek's Disease Herpesvirus

Marek's disease herpesvirus is considered to be a member of the genus *Herpesvirus* because: (1) its nucleocapsid is 100-110 nm in diameter, is an icosahedron composed of 162 hollow capsomeres and is surrounded by a lipid membrane (16); (2) its DNA is a linear duplex with a molecular weight of $103 \pm 5 \times 10^6$ daltons and a base composition of 46 mole% GC (18,19); (3) its main site of development is the nucleus of the cell (9). Marek's disease herpesvirus is a group B, cell-associated, herpesvirus.

Marek's disease herpesvirus may be grown in culture in duck embryo fibroblasts (DEF), chick embryo fibroblasts (CEF) and chicken kidney cells (9). Infection of these cells with MDHV results in the formation of plaques of altered cells. Plaque morphology and size vary for each cell type and virus strain, but in all cases small rounded refractile cells and giant multinucleated cells are observed. In culture MDHV is highly cell associated. Since cell-free infectious virus does not exist in MDHV-infected cultures, virus transfer from cell to cell is probably achieved via temporary intercellular bridges. Evidence for this comes from the fact that ultraviolet-inactivated Sendai virus increased the efficiency of MDHV infection (20).

Herpesvirus of turkeys may also be readily grown in avian cell cultures. It is not so highly cell associated as MDHV and produces low levels of cell-free infectious virus (16).

Comparative Aspects of Marek's Disease and Epstein-Barr Virus-Induced Human Proliferative Diseases

Epstein-Barr virus has been established as the causative agent of the human lymphoproliferative disease, infectious mononucleosis. It is also found in close association with Burkitt's lymphoma, another human lymphoproliferative disease (21). A number of parallels may be drawn between these diseases and Marek's disease (12).

Infectious mononucleosis is a self-limiting lymphoproliferative disease. In certain cases Marek's disease becomes a self-limiting disease where lesions in nerves and organs regress. The organ distribution of lymphomas in Marek's disease and Burkitt's lymphoma is similar. In each case it is unknown why proliferation of lymphoid cells initiates or terminates. Perhaps this stimulation of lymphoid

cells depends on viral replication at extralymphoid sites (extrinsic mechanism). If immunity develops and limits viral replication, lymphoid cell stimulation would cease and lesions could regress.

In chickens with Marek's disease, MDHV replicates at a single site in the chicken, in nonlymphoid epidermal cells. Epstein-Barr virus is regularly found in the throat washings obtained from patients with infectious mononucleosis. This finding suggests that a cell type of the salivary glands, perhaps epithelial cells, is a site of replication of EBV (22).

Finally, in Marek's disease, a chicken contracts the classical form or the acute form of the disease depending on a number of variables, including strain of virus, susceptibility of host and age of host. With Epstein-Barr virus, infectious mononucleosis could be considered as a classical form of disease and Burkitt's lymphoma as an acute form of disease. Therefore, perhaps the same variables which determine the pathology of MDHV infections also determine the pathology of EBV infections.

Until recently, comparative research between MDHV and EBV was difficult because of the lack of tumor derived cell lines in the Marek's disease system. However, four such lines have now been reported (14,23,24).

The MSB-1 cell line derived from an ovarian tumor of a chicken infected with MDHV, strain BC-1, has been characterized extensively (24,25). The cells grow in suspension at 41° with a rapid doubling time (8-12 hr). They display T cell surface markers and the morphology of typical lymphoblasts. The cells contain MDHV DNA (26). The MSB-1 cell line is a producer cell line. Virus-specific antigens and naked nucleocapsids typical of herpesviruses are

produced in a low percentage of the cell population. The percentage of these positive cells seems to increase with age in subculture. Infection of DEF or CEF with MSB-1 cells produced typical plaques. Inoculation of chickens with MSB-1 cells caused typical lesions. No C-type virus particles or antigens were found associated with the cells. All of these observations indicate that these cells were transformed by MDHV.

One important difference between these MDHV-derived cell lines and EBV-derived cell lines is that the former are of T cell origin and the latter are of B cell origin (15).

Vertebrate DNA Polymerases

Before herpesvirus-induced DNA polymerases will be discussed, the properties of vertebrate DNA polymerases will be reviewed, since herpesvirus-induced DNA polymerases must be distinguished from these. Three general classes of DNA polymerases are found in cells of all vertebrates: DNA polymerase α, DNA polymerase β, and DNA polymerase γ (27). The Greek letters designate their order of discovery. A mitochondrial DNA polymerase has also been reported (28,29) but it is not pertinent to this discussion. Each of these enzymes has been reported from numerous sources, as has been reviewed by Weissbach (30). In this discussion the general properties of these enzymes will be noted.

DNA Polymerase α

DNA polymerase α is the vertebrate high molecular weight DNA polymerase which, when disaggregated, exhibits a sedimentation coefficient of 6-8S. In its native state it exists as a dimer. The monomeric molecular weight for the enzyme from calf thymus and

from rat liver and spleen was reported to be 53,000-54,000 daltons (31). That from human KB cells was 87,000 daltons (32). DNA polymerase α exhibits anomalous gel filtration behavior probably due to assymetry of the molecule (31). DNA polymerase α is an acidic protein. Isoelectric points for this polymerase from human lymphocytes, human KB cells, and calf thymus fall in the range of 4.5-5.5 (33,34,35). In growing cells DNA polymerase α may often comprise 80-90% of the total DNA polymerase activity (30).

DNA polymerase α has often been referred to as the "cytoplasmic DNA polymerase" because it has been found predominantly in the cytoplasmic fraction when cells are subjected to normal extraction procedures. This localization of the enzyme may be an artifact, however. Lynch et al. report that when rat liver is homogenized in 0.3 M sucrose and 4 mM $CaCl_2$, only 1.4% of the total DNA polymerase α is found in the cytoplasmic fraction (36). If the homogenization solution contained Tris buffer and/or KCl, α polymerase leached out of the nuclei. If glycerol was used to prepare the nuclei, approximately 85% of α polymerase activity was found in the nuclear fraction. Herrick et al. have also demonstrated 85% of α polymerase activity to be associated with nuclei by subjecting mouse L cells to cytochalasin-B enucleation (37).

DNA polymerase α is particularly sensitive to high ionic strength (44). Significant inhibition of enzyme activity can be observed with 25 mM sodium chloride. DNA polymerase α is also strongly inhibited by reagents which block sulfhydryl groups (30). Pyrophosphate inhibits α polymerase but, at best, a minimal dNTP-pyrophosphate exchange reaction occurs (32,45). The polymerase

from KB cells promotes an exchange reaction which is only about 0.8% of the polymerase activity (32).

No nuclease activity has been found associated with the most highly purified preparations of DNA polymerase α. For α polymerase from KB cells, the limit of detection for nuclease activity was 0.003% of the polymerase activity based on production of acid soluble radioactive nucleotide fragments (32). It did not excise mispaired primer termini. When a highly sensitive endonuclease assay, which measured the conversion of supercoiled SV40 DNA to SV40 DNA with single strand nicks, was used to assay the most highly purified fraction of DNA polymerase α from regenerating rat liver, no nuclease activity was detected (38).

DNA polymerase α utilizes gapped duplex DNA (activated DNA) at a high rate. Spadari and Weissbach have reported that α polymerase from HeLa cells can utilize DNA primed with natural RNA (39). It cannot use ribohomopolymers as templates.

A number of investigators have reported that DNA polymerase α appears to be heterogeneous (31,35,40). In each case several peaks of activity were obtained by DEAE-cellulose or DEAE-Sephadex chromatography.

DNA Polymerase β

DNA polymerase β is the vertebrae low molecular weight DNA polymerase which, when disaggregated, exhibits a sedimentation coefficient of 3-4S. It is a single polypeptide of molecular weight 43,000-45,000 daltons in KB cells (41) and 32,000 daltons in Novikoff hepatoma cells (42). At low ionic strength, β polymerase forms aggregates comparable in size to α polymerase (41).

Consequently, a mistaken structural relationship was first reported for these two polymerases. DNA polymerase α and DNA polymerase β are also not antigenically related (43). DNA polymerase β is a basic protein. Isoelectric points for the enzyme from human lymphocytes, KB cells, calf thymus and Novikoff hepatoma cells are in the range of 8.5-9.5 (33,41,42,44). The primary intracellular location of DNA polymerase β is nuclear. In growing cells it comprises about 5-15% of the total DNA polymerase activity.

The sensitivity of DNA polymerase β to high ionic strength varies with the source of the enzyme. Activities in the presence of 0.2 M sodium chloride may range from 0.5-4-fold of those in its absence (44). DNA polymerase β is insensitive to sulfhydryl group inhibitors (30). It has no detectable nuclease activities associated with it and consequently does not excise misplaced primer termini (41). Although β polymerase is inhibited by pyrophosphate, no dNTP-pyrophosphate exchange has been demonstrated (45,46).

DNA polymerase β requires the four dNTPs for maximal activity but shows activity in the absence of one or more dNTPs. This is probably a reflection of the fact that activated DNA may contain at least 10^{13} 3'OH termini per μg of DNA (30). DNA polymerase β utilizes activated DNA at a high rate and can also utilize poly(A)-oligo$(dT)_{12-18}$ to a lesser extent (47). It cannot use ribonucleotide primers.

Chang has conducted a phylogenetic survey for DNA polymerase β activity using its low molecular weight and insensitivity to N-ethylmaleimide as primary identifying characteristics (48). This type of polymerase has a widespread occurrence in multicellular animals beginning with the sponge, the most primitive animal surveyed.

This type of polymerase was not found in prokaryotes, protozoa or plants.

DNA Polymerase γ

DNA polymerase γ utilizes poly(A)-oligo$(dT)_{12-18}$ at a higher rate (5-10 times) than activated DNA. It does not utilize natural RNA (49). It is not antigenically related to reverse transcriptase of the RNA tumor viruses (49,50). DNA polymerase γ has a molecular weight of approximately 110,000 daltons, is an acidic protein, and requires sulfhydryl-groups for maximum activity (30). In growing cells it comprises about 1-2% of the total DNA polymerase activity (51). In HeLa cells two forms of DNA polymerase γ are resolved by phosphocellulose or hydroxyapatite chromatography (49). Both can use poly(A) as a template but only one can use poly(C), poly(I) and poly(U). They also exhibit differential sensitivities to $(NH_4)_2SO_4$ and ethanol. With DNA polymerase γ apparent Michaelis constants for dNTPs are 10-fold lower than those of DNA polymerase α and DNA polymerase β.

Physiological Function of Vertebrate DNA Polymerases

In attempts to assign physiological functions to these DNA polymerases, their levels have been monitored in cells in different stages of growth. The levels of DNA polymerase activities were measured when mouse L cells and baby hamster kidney cells were in stationary phase or lag phase (52,53). In each case, in log phase the levels of DNA polymerase α were 5- to 12-fold higher than in stationary phase. The levels of DNA polymerase β remained unchanged. Likewise, in regenerating rat liver DNA polymerase α levels increased

6- to 7-fold 48 hours after hepatectomy whereas DNA polymerase β levels remained constant (54).

The levels of DNA polymerase activities in synchronized HeLa cells have also been studied. Spadari and Weissbach measured DNA polymerase activities in crude nuclear and cytoplasmic extracts. They observed a rise in DNA polymerase γ levels in the early part of S phase, a steady rise in cytoplasmic α polymerase throughout S phase and no change in DNA polymerase β levels (55). Chiu and Baril measured nuclear DNA polymerase activities which had been partially purified by phosphocellulose chromatography (56). They observed a 7- to 10-fold increase in DNA polymerase α levels in G_1 and early S and a subsequent decline in this activity in late S and G_2. No changes in DNA polymerase β levels were observed. No reproducible variation in DNA polymerase γ was observed. Addition of cycloheximide to cultures prior to G_1 eliminated the rise in α polymerase levels which suggested that this rise was a result of *de novo* protein synthesis.

In another study Bertazzoni et al. measured DNA polymerase levels in phytohemagglutinin-stimulated, ultraviolet-irradiated human lymphocytes (57). DNA polymerase α and β activities were measured in crude extracts or after sucrose gradient centrifugation of crude extracts. In these cells a wave of DNA replication was observed which reached maximum levels after five days of stimulation. Two waves of repair synthesis were observed which reached maximum levels after three days and seven days of stimulation. The levels of DNA polymerase α paralleled the levels of DNA replication. A 20-fold increase in α polymerase activity was observed after five days of stimulation. The levels of DNA polymerase β increased more

slowly and to a lesser extent and seemed to parallel the second wave of repair synthesis. After seven days of stimulation, maximum levels were obtained which were approximately 7-fold greater than those of the unstimulated cells. From these results these investigators suggested a correlation between DNA polymerase α activity and DNA replication and a correlation between DNA polymerase β activity and repair synthesis.

Thus, there is evidence from several laboratories which suggests that DNA polymerase α may be one of the proteins involved in DNA replication since DNA polymerase α levels change with the proliferative state of the cell and it can use RNA-primed DNA as a template. The physiological functions of DNA polymerase β and DNA polymerase γ are less clear, but DNA polymerase β may be involved in DNA repair synthesis.

Herpesvirus-Induced DNA Polymerases

Herpesvirus-induced DNA polymerases have been reported for herpes simplex, Marek's disease virus and cytomegalovirus infections. The herpes simplex-induced DNA polymerase was the first reported and has been most extensively studied. The properties of each of these polymerases will be presented and compared using the herpes simplex-induced DNA polymerase as the prototype.

Herpes Simplex-Induced DNA Polymerase

Herpes simplex virus is a general group of herpesviruses which can be divided into two groups, type 1 and type 2, on the basis of antigenic and biological differences (58). Man is their only natural host. Herpes simplex virus-1 is spread primarily by the oral-respiratory route, while herpes simplex virus-2 appears to be

venereally transmitted. The lesions produced by these viruses occur in tissues derived from embryonic ectoderm. The skin, oral cavity, vagina, conjunctiva and nervous system are the most frequent sites of infection.

Herpes simplex virus undergoes productive infections *in vitro* and in active infections *in vivo* (58). Virus can readily be isolated from culture and infected tissues. Herpes simplex virus establishes latent infections in the sensory cells of the trigeminal ganglion of man and experimental animals.

The first evidence for the existence of an induced DNA polymerase in HSV infections was obtained when Keir and Gold measured DNA polymerase activity in extracts of HSV-1-infected and uninfected baby hamster kidney cells (59). DNA polymerase levels in infected cells increased two hours after infection, remained at these levels until eight hours after infection and then declined sharply. The maximum increase in specific activity of DNA polymerase was about 7-fold at four hours after infection. The majority of this induced DNA polymerase activity was found in the nuclear fraction of the infected cells (59-60).

Subsequent characterizations of this induced DNA polymerase activity were performed using infected cells four hours after infection since deoxyribonuclease I activity had not yet reached maximum values at this time. When crude extracts of HSV-1-infected and uninfected baby hamster kidney cells were compared, the DNA polymerase activity in infected cells was found to be more heat stable, to utilize native DNA as a template more effectively, and to have different Mg^{2+} requirements than the DNA polymerase activity in uninfected cells (61). Moreover, the DNA polymerase activity in

extracts from HSV-1 infected baby hamster kidney cells (BHK21/C13) or HSV-1 infected human epithelioid carcinoma cells (HEp2) was stimulated 3-fold when 100 mM ammonium sulfate was added to standard reaction mixtures. The DNA polymerase activity in uninfected extracts was completely inhibited by this salt concentration (62). Furthermore, antiserum prepared against HSV-infected cells inhibited the DNA polymerase activity of infected cells but not of uninfected cells. Thus, a DNA polymerase activity was present in HSV-1 infected cells which was different catalytically and structurally from the DNA polymerase activity in uninfected cells. A similar DNA polymerase activity was also found in HSV-2 infected BHK21/C13 cells (63).

Weissbach et al. extended these studies by partially purifying the HSV-induced DNA polymerase from infected HeLa cells (64). Crude extracts of HSV-1 infected and mock infected HeLa cells were first assayed for DNA polymerase activity. As was previously observed in other cells, HSV-1 infection of HeLa cells produced a large increase in DNA polymerase activity. It increased linearly for at least eight hours after infection. This activity was stimulated when 150 mM potassium sulfate was added to standard reaction mixtures. At four hours after infection the specific activity of DNA polymerase in infected cells, when assayed in the presence of 150 mM potassium sulfate, was 60 times higher than in mock-infected cells. Approximately 71% of this activity was found in the nuclear fraction of infected cells. When assays were performed in the absence of potassium sulfate, no difference in specific activity of host DNA polymerases was observed between HSV-infected and mock-infected cells.

The HSV-induced DNA polymerase was purified by DEAE-cellulose and phosphocellulose chromatography (64). The most highly purified

HSV-induced DNA polymerase fraction was purified approximately 1330-fold over the crude cell homogenate fraction. The HSV-induced DNA polymerase was resolved into two activities by DEAE-cellulose chromatography. No differences in structural or catalytic properties could be found between these two activities, although only one was completely resolved from HeLa DNA polymerase α. Both peaks of HSV-induced DNA polymerase activity were resolved from DNA polymerase β, which does not adsorb to DEAE-cellulose. Phosphocellulose chromatography then resolved the HSV-induced DNA polymerase from the induced deoxyribonuclease activity.

The properties of the most highly purified HSV-induced DNA polymerase fraction were examined (64). It was stimulated 25- to 50-fold by 150 mM potassium sulfate, ammonium sulfate, or potassium phosphate. HeLa DNA polymerase activities were inhibited by 90% at these concentrations. It had a molecular weight of 180,000-200,000 daltons. It was inhibited 95% by 50 μM N-methylmaleimide. In template-primer studies the HSV-induced DNA polymerase was determined to be a DNA-dependent DNA polymerase which could use poly(dC)·oligo$(dG)_{12}$ at a higher rate than activated DNA. A nuclease activity was associated with the most highly purified fraction of the enzyme. It solubilized [^{3}H](dG·dC) or [^{3}H](dA-T) at about 5% of the rate of the polymerization of activated DNA in the presence of 150 mM potassium sulfate. Finally, this partially purified HSV-induced DNA polymerase was completely inhibited by antiserum prepared against HSV-infected cells (70).

Thus, there is good evidence which supports the existence of an induced DNA polymerase in HSV infections. The single property that readily established the existence of the induced DNA polymerase and distinguished it from the host DNA polymerases was its stimulation by

salt. In the early studies a 3-fold stimulation by 100 mM ammonium sulfate of HSV-induced DNA polymerase in crude extracts was observed (62). When the HSV-induced DNA polymerase was purified by DEAE-cellulose and phosphocellulose chromatography, it was stimulated 25- to 50-fold by 150 mM potassium sulfate or ammonium sulfate (64). This difference in salt stimulation may be related to the different degrees of purity of the enzyme. A similar discrepancy exists for the levels of HSV-induced DNA polymerase found in infected cells. Weissbach et al. observed 60 times more DNA polymerase activity in infected cells than in mock-infected cells (64), while Keir and Gold noted only seven times more DNA polymerase activity in infected cells (59). This difference probably reflects the fact that Weissbach et al. assayed DNA polymerase activity in the presence of 150 mM potassium sulfate with activated DNA while Keir and Gold assayed it in the absence of salt with denatured DNA. In both cases, however, the HSV-induced DNA polymerase was found predominantly in the nuclear fraction of infected cells.

The existence of a herpesvirus-induced DNA polymerase had been demonstrated. However, the origin of this activity was unknown. It could be viral coded. It could be a derepressed host DNA polymerase or it could be a modified host DNA polymerase. Studies with temperature-sensitive mutants of HSV-1 indicate that the HSV-induced DNA polymerase is viral coded (65). When human embryonic lung cells were infected with temperature-sensitive mutants from complementation groups C and D at the nonpermissive temperature (39°), no DNA polymerase activity was induced while normal levels of DNA polymerase activity were induced at the permissive temperature (34°). When cells infected with those mutants were maintained at 34° and then shifted to 39°,

HSV-induced DNA polymerase activity declined to less than 25% of the activity present at the time of the shift three hours after the shift. However, *in vitro* thermal inactivation studies with crude extracts from mutant-infected cells and from wild type-infected cells demonstrated no difference in temperature sensitivity between the mutant-induced DNA polymerase and the wild type-induced DNA polymerase.

Marek's Disease Herpesvirus-Induced DNA Polymerase

An induced DNA polymerase in MDHV-infected duck embryo fibroblasts has been reported by Boezi et al. (66). When DNA polymerase activities from extracts of MDHV-infected DEF were fractionated by phosphocellulose chromatography, a peak of activity was observed which was not present when DNA polymerase activities from uninfected DEF were fractionated by phosphocellulose chromatography. This activity exhibited very different properties from those observed for the HSV-induced DNA polymerase. It was completely inhibited by 100 mM ammonium sulfate. It had a sedimentation coefficient of 5.9S, which corresponds to a molecular weight of 100,000 daltons for a globular protein. It was insensitive to 1 mM p-hydroxymercuribenzoate. Template primer studies indicated that it was a DNA-dependent DNA polymerase, but it could only utilize activated DNA and not poly(dC)·oligo$(dG)_{12-18}$ or poly(dA)·oligo$(dT)_{12-18}$.

Cytomegalovirus-Induced DNA Polymerase

Cytomegalovirus is a herpesvirus so named because of the distinctly enlarged cells (cytomegaly) containing intranuclear and cytoplasmic inclusions produced in infection (67). The human cytomegalovirus (HCMV) causes a variety of syndromes, including

postperfusion syndrome, cytomegalovirus-mononucleosis and cytomegalic inclusion disease of infancy. Congenital infection with HCMV may be fatal or asymptomatic. In the latter case viruria exists, and in later life extensive abnormalities, particularly neurological ones, may develop. Patients undergoing immunosuppressive therapy or extensive blood transfusions appear to be particularly susceptible to HCMV infections. Evidence suggests that HCMV may establish latent infections.

In culture HCMV establishes a productive infection in human fibroblasts. An abortive infection is established in guinea pig embryo cells. No viral particles are formed, but virus-specific early antigens are synthesized and cellular DNA synthesis is stimulated (68).

Two laboratories have reported the induction of a DNA polymerase activity when WI-38 human diploid fibroblast cells are infected with HCMV. In one case the activity was analyzed in crude extracts (68). In the second case the activity was partially purified (69).

Hirai et al. measured DNA polymerase activity in extracts of HCMV-infected and uninfected WI-38 cells at various times after infection (68). Induction of DNA polymerase activity occurred in infected cells at about 20 hours after infection and reached a maximum stimulation of about 6-fold at 30 hours. The major increase in DNA polymerase levels occurred in the nuclear fraction of the infected cells. Levels of HCMV DNA increased at about 48 hours after infection. The induced DNA polymerase activity was stimulated 3-fold in the presence of 100 mM ammonium sulfate. This salt concentration inhibited the WI-38 DNA polymerase activities by 75%. When infected cells were cultured in the presence of cycloheximide,

no induction of DNA polymerase activity was observed, suggesting that *de novo* protein synthesis was required for induction.

When DNA polymerase activity was measured in extracts of HCMV-infected guinea pig cells at 48 hours after infection, a 3- and 6-fold stimulation of DNA polymerase activity was observed in the nuclear and cytoplasmic fractions, respectively; however, this activity was not stimulated in the presence of 100 mM ammonium sulfate (68). This result suggests that the HCMV-induced DNA polymerase may not be a necessary enzyme in abortive infections but more sensitive assays are necessary to establish this point.

Huang has fractionated the DNA polymerase activities in extracts of HCMV-infected and uninfected WI-38 cells by DEAE-cellulose chromatography followed by phosphocellulose chromatography (69). All levels of DNA polymerase activities in HCMV-infected cells were substantially higher than those in uninfected cells. Moreover, a new peak of DNA polymerase activity in infected cells was found after DEAE-cellulose chromatography and particularly after phosphocellulose chromatography. This DNA polymerase activity was stimulated approximately 1.5-fold by 30-60 mM ammonium sulfate. No stimulation of host DNA polymerase activities occurred in the presence of these salt concentrations. Sedimentation coefficients of 9.3S, 8.2S and 5.3S were found after glycerol density gradient sedimentation velocity centrifugation of the HCMV-induced DNA polymerase in the absence of salt. This result suggests that the enzyme was aggregated. The HCMV-induced DNA polymerase was inhibited 96% by 0.25 mM p-hydroxymercuribenzoate. It did not appear to be a zinc metalloenzyme. Template-primer studies indicated that it was a DNA-dependent DNA polymerase; poly(dA)·oligo$(dT)_{12-18}$ was utilized at a higher rate than activated DNA or poly(dC)·oligo$(dG)_{12-18}$.

Thus, a HCMV-induced DNA polymerase activity exists. Its activity is stimulated in the presence of salt as was also observed for the HSV-induced DNA polymerase. However, the amount of stimulation observed in the presence of ammonium sulfate appeared to be substantially lower for the HCMV-induced DNA polymerase than for the HSV-induced DNA polymerase. After DEAE-cellulose and phosphocellulose chromatography, the HCMV-induced DNA polymerase was stimulated 1.5-fold in the presence of 30-60 mM ammonium sulfate, whereas the HSV-induced DNA polymerase purified in the same manner was stimulated 25- to 50-fold in the presence of 150 mM ammonium sulfate (69,64).

No genetic evidence is available to determine if the HCMV-induced DNA polymerase is viral coded as is the case for the HSV-induced DNA polymerase. However, evidence suggests that *de novo* protein synthesis is required for HCMV DNA polymerase induction (68).

The HCMV-induced DNA polymerase as well as the HSV-induced polymerase is predominantly a nuclear enzyme. It is interesting to note, however, that the HCMV-induced DNA polymerase activity was first observed 20 hours after infection while the HSV-induced DNA polymerase activity was first observed two hours after infection (68). Thus, the amount of time required for these two herpesviruses to establish productive infections is very different.

Phosphonoacetate and Herpesvirus Infections

Using a random testing of compounds with a tissue culture screen, workers at Abbott Laboratories discovered that phosphonoacetate (Figure 1) was an effective inhibitor of the replication of herpes simplex virus types 1 and 2 (71). This antiviral activity was consistently observed in tissue culture at a concentration of 500 µM.

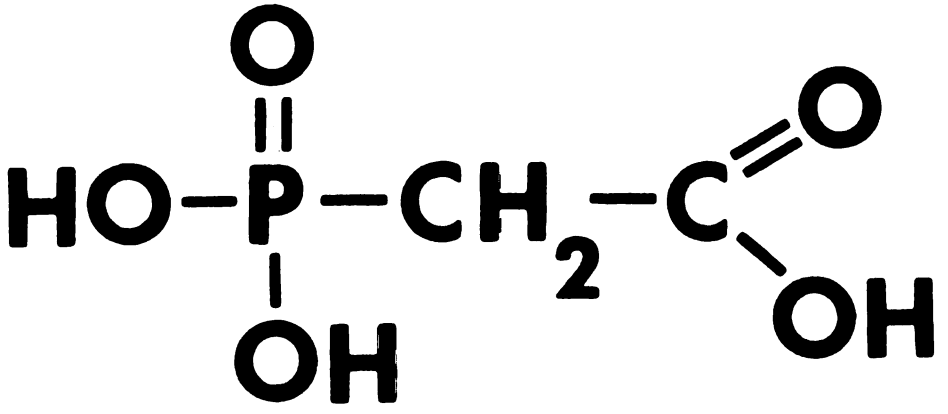

Figure 1. Structure of phosphonoacetic acid.

Since this initial observation, the workers at Abbott and others have studied the effects of phosphonoacetate on herpesvirus in tissue culture, biochemical, and animal studies.

Effect of Phosphonoacetate on Herpesvirus Infections in Tissue Culture

Overby et al. first characterized the inhibitory effect of phosphonoacetate on herpesvirus infections in tissue culture (72). Infection of WI-38 cells with HSV-1 resulted in rounded, clumped, multinucleated cells. However, when HSV-1-infected WI-38 cells were cultured in the presence of 500 μM phosphonoacetate, cytopathic effects were not observed. The infected cells could not be distinguished from uninfected WI-38 cells. This suggested that phosphonoacetate prevented the replication of HSV-1.

Studies were then performed to determine at what stage of infection phosphonoacetate inhibited (72). Phosphonoacetate appeared to have no effect on adsorption, penetration, or release of virus. When phosphonoacetate was removed from the culture medium of HSV-1-infected WI-38 cells, cytopathic effects reappeared. Thus, the viral genomes were still present and functional in the infected cells. No difference in RNA and protein synthesis, as measured by [^{3}H]uridine and [^{14}C]amino acid incorporation, was observed between PA-treated and untreated infected cells. However, a reduction in DNA synthesis, as measured by [^{14}C]thymidine incorporation, was observed in PA-treated infected cultures. This inhibition of DNA synthesis was specific for viral DNA synthesis as determined by isolation and buoyant density centrifugation of DNA from infected cultures grown in the presence and absence of phosphonoacetate. At 12 hours postinfection two peaks of [^{14}C]thymidine, corresponding in density to HSV-1 DNA

and cellular DNA, were found in CsCl gradients for untreated cells. Only one peak of [^{14}C]thymidine, corresponding in density to cellular DNA, was found in CsCl gradients for PA-treated cells.

The effect of phosphonoacetate on uninfected WI-38 cells was examined (72). Phosphonoacetate was not toxic to contact-inhibited WI-38 cells. No morphological changes were observed when confluent WI-38 cells were incubated in the presence of 1 mM phosphonoacetate for two days. No effect was noted on DNA, RNA, or protein synthesis. Phosphonoacetate was toxic to growing WI-38 cells, however. When 500 μM phosphonoacetate was present in the culture medium, a 40% decrease in the number of cells in culture was observed. Partial inhibition of DNA synthesis was also observed at this concentration of phosphonoacetate (73). No toxic effects were noted in growing cells when 50 μM phosphonoacetate was present in the culture medium (72).

Phosphonoacetate also inhibited the replication of other herpesviruses in culture by inhibiting viral DNA synthesis. Those herpesviruses studied were: human cytomegalovirus, mouse cytomegalovirus, herpesvirus saimiri (73), Marek's disease herpesvirus, herpesvirus of turkeys and owl herpesvirus (74). Thus, phosphonoacetate appears to be a universal inhibitor of herpesvirus DNA synthesis.

Phosphonoacetate as an Inhibitor of Herpesvirus-Induced DNA Polymerases

Mao et al. first demonstrated that phosphonoacetate specifically inhibits herpesvirus-induced DNA polymerases (75). When HSV-1-induced DNA polymerase or HSV-2-induced DNA polymerase was assayed in the presence of 1 μM phosphonoacetate, enzyme activity was

inhibited by 50% (75,77). The HCMV-induced DNA polymerase was inhibited similarly by phosphonoacetate (73).

The sensitivity of other DNA polymerases to phosphonoacetate was examined. The DNA polymerases from WI-38 cells were relatively resistant to phosphonoacetate inhibition (73,75). When WI-38 DNA polymerases, partially purified by phosphocellulose chromatography, were assayed in the presence of 300 μM phosphonoacetate, only 10-15% inhibition of enzyme activity resulted (75). DNA polymerase β from HeLa cells was not inhibited by 500 μM phosphonoacetate. However, DNA polymerase α and γ from HeLa cells were inhibited by 50% when assayed in the presence of 30 and 400 μM phosphonoacetate, respectively. The vaccinia virus-induced DNA polymerase was inhibited by 50% when assayed in the presence of 30 μM phosphonoacetate (76). *Micrococcus luteus* DNA polymerase, hepatitis B virus DNA polymerase and Rous sarcoma virus reverse transcriptase were not significantly inhibited by 100 μM phosphonoacetate (77). Thus, phosphonoacetate is a specific inhibitor of herpesvirus-induced DNA polymerases because the herpesvirus enzymes are inhibited by phosphonoacetate concentrations which are at least 30 times lower than those which inhibit other DNA polymerases.

Phosphonoacetate did not inhibit the herpesvirus-induced DNA polymerases because of the formation of a PA-DNA complex (73,75). The specificity of phosphonoacetate for these polymerases precludes this possibility. Moreover, phosphonoacetate inhibition was not decreased when DNA concentrations were increased in DNA polymerization reaction mixtures. Finally, the presence of phosphonoacetate in HSV-1-induced DNA polymerization reaction mixtures did not prevent the formation of an enzyme-DNA complex as determined by glycerol

density gradient sedimentation velocity centrifugation of these reaction mixtures (77). These results suggest that phosphonoacetate inhibits by direct interaction with herpesvirus-induced DNA polymerases.

Animal Studies with Phosphonoacetate

The first studies using phosphonoacetate as an antiherpesvirus agent in animals, as reported by Shipkowitz et al., involved herpes simplex virus infections of mice and rabbits (71). Mice were infected with HSV-2 by topical application to denuded skin. The resulting herpes dermatitis infection caused death in untreated mice. However, if phosphonoacetate was administered orally or topically to infected mice two hours after infection, mortality was significantly reduced. Rabbits were infected with HSV-1 by topical application to the eyes. The resulting herpes keratitis infection caused corneal lesions in untreated rabbits. However, if an ointment of phosphonoacetate was topically applied to the eyes of the infected rabbits two hours after infection, a reduction in lesions was observed.

The effectiveness of phosphonoacetate in the treatment of herpes dermatitis in mice and herpes keratitis in rabbits has been confirmed by others (78,79,80). Moreover, phosphonoacetate suppressed clinical disease and inhibited herpes simplex virus replication in animals with established herpesvirus infections.

When chickens were infected with Marek's disease herpesvirus, phosphonoacetate was not effective in inhibiting the replication of MDHV or in preventing the development of lymphomas when phosphonoacetate was administered intra-abdominally before MDHV inoculation (74).

The initial successes obtained with phosphonoacetate in the treatment of herpes simplex infections in mice and rabbits led the

investigators from Abbott Laboratories to postulate that phosphonoacetate might be useful in the treatment of herpesvirus infections in man, such as herpesvirus dermatitis and herpesvirus keratitis (71). It was also thought that phosphonoacetate might be effective against herpes encephalitis in man since it greatly reduced mortality of mice infected with herpesvirus. Moreover, phosphonoacetate might be useful in preventing sometimes fatal herpesvirus infections in patients undergoing immunosuppression therapy.

Effect of Phosphonoacetate on a Cell Line Transformed by Epstein-Barr Virus

Epstein-Barr virus is a herpesvirus for which no completely permissive culture system exists, i.e., no cell type supports the efficient replication of EBV. If there is no good source of replicating EBV, there is no good source of material to use to determine if an EBV-induced DNA polymerase exists. There are, however, several well characterized human lymphoblastoid cell lines transformed by EBV. These cell lines have been cultured in the presence of phosphonoacetate in an attempt to determine the mechanism of EBV DNA replication (81,82). The results obtained with one cell line, the Rajii cell line, will be discussed.

The Raji cell line is a transformed lymphoblastoid cell line derived from a tumor biopsy from a patient with Burkitt's lymphoma (83). It is a nonproducer cell line. Raji cells contain approximately 50 EBV genomes per cell but express only a single viral antigen, EBV-specific nuclear antigen, and produce no virus particles. Replication of resident EBV genomes is under cellular control mechanisms and occurs only during early S phase (84). If Raji cells are superinfected

with EBV, however, host cell DNA synthesis is completely inhibited and productive replication of EBV DNA occurs (81).

The effect of phosphonoacetate on the EBV content in Raji cells before and after superinfection was determined. When Raji cells were grown in the presence of 1 mM phosphonoacetate for three days or in the presence of 0.5 mM phosphonoacetate for three weeks, there was no change in the number of resident EBV genomes as determined by hybridization techniques (81,82). Thus, the EBV DNA present in these transformed cells replicates by a mechanism which is insensitive to phosphonoacetate as might be expected since resident EBV DNA replication is under cellular control.

When Raji cells were superinfected, the amount of EBV DNA present in these cells increased to such levels that it could be detected in CsCl gradients if the superinfected cells were labelled with [^{3}H]thymidine. When Raji cells were superinfected and then cultured in the presence of 0.5 mM phosphonoacetate, EBV DNA could not be detected in CsCl gradients. Thus, productive EBV DNA replication in the absence of cellular controls is sensitive to phosphonoacetate. In light of the fact that the known herpesvirus-induced DNA polymerases are sensitive to phosphonoacetate, the sensitivity of productive EBV DNA replication to phosphonoacetate suggests that a herpesvirus-induced DNA polymerase replicates EBV DNA in these superinfected cells. However, this postulated herpesvirus-induced DNA polymerase does not appear to replicate EBV DNA in transformed cells.

MATERIALS AND METHODS

Materials

Phosphocellulose (P-11) and DEAE-cellulose (DE52) were purchased from Reeve Angel. Whatman no. 40 acid-washed chromatography paper was from Fisher Scientific Co. Analtech, Inc. was the source of precoated cellulose thin layer chromatography plates. Nitrocellulose membrane filters (type B-6) were obtained from Schleicher and Schuell, Inc. Calf thymus DNA, dithiothreitol, unlabelled nucleotides, poly(dA)·oligo$(dT)_{12-18}$, poly(rA)·oligo$(dT)_{12-18}$, poly(dC)·oligo$(dG)_{12-18}$, and poly(rC)·oligo$(dG)_{12-18}$ were purchased from P-L Biochemicals, Inc. Poly d(A-T) and poly(dA) were from Miles Laboratories, Inc. International Chemical and Nuclear Corp. was the source of [^{3}H]-deoxynucleoside triphosphates. New England Nuclear was the source of [^{32}P]sodium pyrophosphate. Deoxythymidine 5'phosphomorpholidate, bovine serum albumin and beef heart lactate dehydrogenase were purchased from Sigma Chemical Co. Snake venom phosphodiesterase I, bacterial alkaline phosphatase, and pancreatic DNase I (RNase free) were obtained from Worthington Biochemical Corp. *Escherichia coli* DNA polymerase I was purchased from Boehringer Mannheim.

Dowex 1 (BioRad AG 1-X2, 200-400 mesh) was a gift from Dr. Fritz Rottman of this department. Nonidet P-40 was a gift from Dr. Leland Velicer of the Department of Microbiology and Public Health. Avian myeloblastosis virus reverse transcriptase was a gift from Dr. J. Beard, Life Science Research Laboratories.

Phosphonoacetate, disodium salt, was a gift from Abbott Laboratories. Phosphonoacetic acid, phosphonopropionate, the trimethyl ester of phosphonoacetate, phenylphosphonoacetate, 2-aminophosphonoacetate, 2-methyl-2-phosphonopropionate and 2-phosphonopropionate were generously provided by Dr. A. F. Isbell of Texas A&M University. The monocyclohexylamine salt of phosphonoacetamide, N-methyl phosphonoacetamide and N-(phosphonoacetyl)-L-aspartate were gifts from Dr. George Stark of Stanford University. Acetonyl phosphonate, the monomethyl ester of acetonyl phosphonate and the monomethyl ester of acetyl aminophosphate were gifts from Dr. Ronald Kluger of the University of Toronto. Phosphonoacetaldehyde was prepared by Mr. John Reno of this laboratory. Other reagents were from the usual commercial sources.

[^{3}H]Phosphonoacetic acid (20 Ci/mmol) was prepared by New England Nuclear using an acid catalyzed exchange reaction developed by Mr. Steve Saxe of this laboratory. Anhydrous phosphonoacetic acid (2 mg) was dissolved in carrier-free [^{3}H]water (3 µl) and was stirred gently for 5 hr at 100°. Excess [^{3}H]water was removed, *in vacuo*, using water as a solvent. The radioactive material prepared in this manner was determined to be a homogeneous solution of phosphonoacetic acid when analyzed by electrophoresis, paper chromatography in solvent system I (Table 4), thin layer cellulose chromatography, DEAE-cellulose (HCO_3^-) chromatography and Dowex 1 (Cl^-) chromatography. No radioactive material vaporized when a solution of [^{3}H]phosphonoacetic acid was vaporized to dryness, which indicated no [^{3}H]water remained in the preparation.

Activated calf thymus DNA was prepared by a modification of the procedure of Schlabach et al. (85). Calf thymus DNA (approximately

200 mg) was dissolved in 10 mM Tris-HCl (pH 8), 50 mM NaCl, 0.5% sodium dodecyl sulfate (approximately 125 ml) with stirring and was extracted with one volume of freshly distilled, water-saturated phenol. After extensive dialysis against three changes (6 l each) of 10 mM Tris-HCl (pH 8), 50 mM NaCl and one change of 10 mM Tris-HCl (pH 8), 50 mM KCl, the concentration of the DNA solution was determined spectrophotometrically ($E_{260}^{1\%}$=200). A solution of $MgCl_2$ was added to a final concentration of 5 mM. Additional buffer was used if needed so that the final DNA concentration was 1 mg per ml. Pancreatic DNase I (RNase free) was added to a final concentration of 0.2 U per ml. Incubation was at 37° for 25 min. The reaction was terminated by incubation at 65° for 10 min. The DNA solution was again extracted with one volume of water-saturated phenol, dialyzed extensively against 10 mM Tris-HCl (pH 8) and stored at -20°.

Preparation and Growth of Cells

Cultures of HVT-infected DEF and uninfected DEF were grown in the laboratory of Dr. Lucy Lee. Cultures of MDHV-infected DEF, uninfected CEF and MSB-1 cells were grown in the laboratory of Dr. Keyvan Nazerian.

Duck embryo fibroblasts were prepared from 13-day-old embryos. Chicken embryo fibroblasts were prepared from 11-day-old embryos. The embryos were decapitated, washed with a sterile solution of phosphate-buffered saline, macerated, and trypsin treated as described by Soloman et al. (86). The cells were suspended in culture medium at a concentration of 10^6 per ml, and 20-ml samples of the suspension were seeded onto 150 mm-diameter Falcon plastic tissue culture dishes. The culture medium consisted of 40 volumes of medium 199, 50 volumes

of nutrient medium F10, 5 volumes of tryptose phosphate broth, 3 volumes of 2.8% sodium bicarbonate, 4 volumes of inactivated calf serum, 100 units of penicillin per ml, 100 µg of streptomycin per ml, and 10 µg of aureomycin per ml (87). Cultures were incubated at 37° in a humidified atmosphere containing 5% carbon dioxide. After 48 hr, the culture medium was removed. For the preparation of uninfected cells, 20 ml of fresh culture medium was added to each of the dishes and incubation was continued for another 24 hr. The cells were then scraped from the tissue-culture dishes, washed with phosphate-buffered saline, quick frozen and stored at -80°. For the preparation of infected cells, 20 ml of fresh culture medium containing HVT or MDHV-infected DEF at a concentration of 2×10^5 per ml (2×10^4 PFU per ml) was added to the dishes. After 24 hr of incubation, the culture medium was replaced with fresh medium. Cytopathic effect due to viral infection of the cells was confluent 24 hr later. The infected cells were then scraped from the tissue culture dishes, washed with phosphate-buffered saline, quick frozen and stored at -80°.

Cultures of HVT-infected DEF were also grown in roller bottles (10 x 40 cm) by the same procedure except that DEF were seeded at a concentration of 4×10^6 per ml in 50 ml culture medium and rotated at 0.1 rpm at 37° in the absence of carbon dioxide; 4×10^5 per ml of HVT-infected DEF were used for infection.

A phosphonoacetate-resistant mutant of HVT was isolated by Dr. Lucy Lee by growing HVT in secondary DEF cultures in medium 199 and medium F10 supplemented with 4% heat-inactivated calf serum and increasing concentrations of phosphonoacetate. The concentration of phosphonoacetate in the medium was increased from 0.3 to 1.6 mM.

Each concentration was used for three to four passages of virus. The virus (HVT_{PA}) from the 30th passage was resistant to 1.6 mM of phosphonoacetate and was cloned in the absence of phosphonoacetate.

The MSB-1 lymphoblastoid cell line was grown in RPMI-1640 medium (Flow Laboratories) supplemented with 10% bovine fetal calf serum at 41° in a 5% carbon dioxide humidified atmosphere. Cells were seeded in Falcon plastic petri dishes at an initial concentration of 5×10^5 cells per ml and were subcultured every two to three days.

Purification of DNA Polymerases

All procedures were performed at 0-4°. Frozen HVT-infected duck embryo fibroblasts (20 g wet weight) were suspended in buffer A (50 mM Tris-HCl [pH 7.5], 25 mM KCl, 5 mM $MgCl_2$, and 0.25 M sucrose) containing 0.2% nonidet P-40. Cytoplasmic and nuclear extracts were prepared by a modification of the procedure of Chang et al. (88). A cell homogenate was prepared using a Dounce homogenizer and was centrifuged at 1,000 x g for 5 min.

The supernatant fraction was centrifuged at 10,000 x g for 10 min, made 5 mM in 2-mercaptoethanol, and centrifuged at 100,000 x g for 60 min. The supernatant fraction from the 100,000 x g centrifugation was termed the cytoplasmic extract.

The nuclear pellet from the 1,000 x g centrifugation step was suspended in buffer A, centrifuged, and then resuspended in buffer A containing 1% Triton X-100 (v/v). After 15 min the nuclei were collected by centrifugation and washed three times with buffer A (crude nuclear fraction). The final nuclear pellet was suspended in buffer A containing 5 mM 2-mercaptoethanol and 0.2 M potassium phosphate (pH 7.5). After gently stirring for 30 min to extract the nuclear

DNA polymerase activities, the suspension was centrifuged at 100,000 x g for 60 min. The resulting supernatant fraction was termed the nuclear extract.

The nuclear extract was dialyzed against buffer B (25 mM Tris-HCl [pH 8], 100 mM KCl, 5 mM 2-mercaptoethanol, and 20% glycerol [v/v]) and was applied to a phosphocellulose column. The phosphocellulose column (1.4 x 8 cm) had been washed with 5 ml of buffer B containing 1 mg per ml of bovine serum albumin to improve recoveries from the column and then equilibrated with buffer B. After washing the column with 20 ml of buffer B, the DNA polymerase activity was eluted with a linear gradient of 100 ml of 0.1 to 1.0 M KCl in buffer B. Fractions of 2 ml each were collected. A single peak of HVT-induced DNA polymerase activity eluted at about 0.35 M KCl as determined by conductivity measurements. The fractions containing the majority of the HVT-induced DNA polymerase activity were pooled (phosphocellulose fraction) and dialyzed against buffer C (0.02 M potassium phosphate [pH 7.5], 0.5 mM dithiothreitol, and 20% [v/v] glycerol). The dialyzed fraction was applied to a DEAE-cellulose column (0.6 x 10 cm) which had been washed with 5 ml of buffer C containing 1 mg per ml of bovine serum albumin and with 5 ml of 0.75 M potassium phosphate in buffer C and then equilibrated with buffer C. After sample application, the column was washed with 20 ml of buffer C and was eluted with a linear gradient of 30 ml of 0.02 to 0.4 M potassium phosphate in buffer C. Fractions of 0.5 ml each were collected. The HVT-induced DNA polymerase activity eluted as a single peak of activity at about 0.14 M potassium phosphate. The fractions containing the majority of the DNA polymerase activity were pooled and stored at -20° after addition of bovine serum albumin,

dithiothreitol, and glycerol to give final concentrations of 2 mg per ml, 0.5 mM and 50% (v/v), respectively. After six weeks at -20°, 75% of the activity of the HVT-induced DNA polymerase remained.

The MDHV-induced DNA polymerase was extracted from MDHV-infected DEF and was purified by DEAE-cellulose chromatography as described above.

Duck embryo fibroblast DNA polymerase α from cytoplasmic extracts and DNA polymerase β from nuclear extracts were likewise purified through DEAE-cellulose columns.

When less than 5 g wet weight of cells were used as starting material, bovine serum albumin was added to nuclear extracts to a final concentration of 1 mg per ml to stabilize the DNA polymerase activities.

Assay of DNA Polymerases

The standard reaction mixture employed for the HVT-induced DNA polymerase contained, in 200 µl: 50 mM Tris-HCl (pH 8), 2 mM $MgCl_2$, 200 mM KCl, 1 mM dithiothreitol, 500 µg per ml of bovine serum albumin, 200 µg per ml of activated calf thymus DNA, 20 µM [^{3}H]dNTP (300-400 cpm per pmol), 100 µM each of the other dNTPs, and DNA polymerase. Incubation was at 37° for 30 min.

Assay conditions were such that the rate of DNA polymerization was linear with time and with the amount of DNA polymerase added. One unit of DNA polymerase activity was defined as that amount of activity which directed the incorporation of 1 nmole of [^{3}H]dNTP into acid-insoluble material in 30 min at 37°.

In experiments in which product inhibition by pyrophosphate was examined, supplementary $MgCl_2$ was added to reaction mixtures

along with pyrophosphate to compensate for the chelation of Mg^{2+} by pyrophosphate. The concentration of $MgCl_2$ to be added to reaction mixtures in addition to the standard 2 mM $MgCl_2$ concentration was calculated using the equations described by Moe and Butler (89). It was determined that equimolar amounts of both pyrophosphate and $MgCl_2$ were to be added to the reaction mixtures.

The standard reaction mixture employed for the MDHV-induced DNA polymerase contained all the same components as the HVT-induced DNA polymerase assay except that 1 mM $MgCl_2$ was used instead of 2 mM $MgCl_2$.

The standard reaction mixture employed for duck and chicken DNA polymerase assays contained in 200 µl: 50 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 1 mM dithiothreitol, 500 µg per ml of bovine serum albumin, 200 µg per ml of activated calf thymus DNA, 20 µM [^{3}H]dNTP (300 to 400 cpm per pmol), 100 µM each of the other three dNTPs, and DNA polymerase. Incubation was at 37° for 30 min.

The standard reaction mixture employed for *E. coli* DNA polymerase I contained all the same components as the duck and chicken DNA polymerase assays except that 5 mM $MgCl_2$ was used instead of 10 mM $MgCl_2$.

The standard reaction mixture employed for the AMV reverse transcriptase assay contained in 200 µl: 50 mM Tris-HCl (pH 8), 0.5 mM $MnCl_2$, 50 mM KCl, 1 mM dithiothreitol, 100 µM [^{3}H]dTTP (80 cpm per pmol), and 0.5 optical density units (at 260 nm) per ml of poly(rA)·oligo$(dT)_{12-18}$. Incubation was at 37° for 30 min.

All DNA polymerase reactions were terminated by addition of 10% trichloroacetic acid-1% sodium pyrophosphate (2 ml). Bovine serum albumin (50 µl of a 5 mg per ml solution) was then added as carrier.

After 5 min at 0°, reaction mixtures were centrifuged at 10,000 x g for 2 min. The supernatant solution was removed and discarded. The pellet was suspended in 0.35 ml of 0.5 N NaOH. After readdition of cold 10% trichloroacetic acid-1% sodium pyrophosphate and after 5 min at 0°, the precipitated material was collected on a nitrocellulose filter. The filter was then washed with three portions of cold 10% trichloroacetic acid-1% sodium pyrophosphate, dried, and monitored for radioactivity by liquid scintillation spectrometry. The scintillation fluid (5 ml) contained 4 g of 2,5-bis[2-(5-t-butylbenzoxazoyl)]thiophene per liter of toluene.

Assay of the dNTP-Pyrophosphate Exchange Reaction

The reaction mixture contained in 200 µl: 50 mM Tris-HCl (pH 8), 1 mM dithiothreitol, 200 mM KCl, 1 mM $MgCl_2$, 500 µg per ml bovine serum albumin, 200 µg per ml activated calf thymus DNA, 0.1 mM each of the four deoxyribonucleoside triphosphates, HVT-induced DNA polymerase, and various concentrations (0.14-1.1 mM) of [^{32}P]-labelled sodium pyorphosphate (specific radioactivity of approximately 100 cpm per pmol). In addition to the 1 mM $MgCl_2$ routinely added to the reaction mixture, supplementary $MgCl_2$, in an amount equimolar to the sodium pyrophosphate added to the reaction mixture, was used.

Incubation was at 37° for 30 min. The assay measuring the conversion of [^{32}P]-labelled pyrophosphate to a Norit-adsorbable form was performed as described by Deutscher and Kornberg (90). For HVT-induced DNA polymerase, the pyrophosphate exchange reaction was shown to be dependent on added enzyme, activated calf thymus DNA, Mg^{2+} ions, and deoxyribonucleoside triphosphates. When assayed

using 1.1 mM [^{32}P]-labelled sodium pyrophosphate, the reaction was found to be linear with time for at least 120 minutes and was directly proportional to the amount of HVT-induced DNA polymerase added to the reaction mixture. The rate of the dNTP-pyrophosphate exchange reaction was about 25% of the rate of the DNA polymerization reaction.

Analytical Methods

Protein concentrations were determined by the method of Lowry et al. (91) with bovine serum albumin as the standard.

Lactate dehydrogenase was assayed spectrophotometrically at 340 nm by following the oxidation of NADH in the presence of pyruvate. The sedimentation coefficient of beef heart lactate dehydrogenase was taken as 7.4S (92).

The presence of a deoxyribonucleoside triphosphatase activity in the HVT-induced DNA polymerase preparations was determined by incubating an excess of HVT-induced DNA polymerase in standard reaction mixtures at 37° for 30 min. Aliquots (10 µl) were spotted on Whatman No. 40 acid-washed chromatography paper. Paper chromatograms were developed in solvent system I (Table 4) and were then cut into 2 x 1 cm pieces. These pieces were extracted with water (1 ml) mixed with Bray's scintillation fluid (10 ml) (93) and monitored for radioactivity using liquid scintillation spectrometry.

The presence of an inorganic pyrophosphatase activity was determined by measuring the conversion of [^{32}P]inorganic pyrophosphate to inorganic phosphate in standard reaction mixtures containing 1 mM [^{32}P]inorganic pyrophosphate (100 cpm/pmol) by the method of Martin and Doty (94).

The presence of deoxyribonuclease activity in HVT-induced DNA polymerase preparations was determined by incubating standard reaction mixtures containing [^{3}H]poly d(A-T) (approximately 200 cpm per pmol) and no labelled dNTP or activated DNA with HVT-induced DNA polymerase and by analyzing for: (1) conversion of acid-insoluble [^{3}H] material to acid-soluble [^{3}H] material or (2) conversion of [^{3}H] polymer to [^{3}H]dTMP by descending paper chromatography in isobutyric acid-conc. ammonium hydroxide-water (66:1:33) (95).

Total phosphorus determinations of samples in solution were made using the method of Ames and Dubin (96). Samples were ashed in the presence of 10% $Mg(NO_3)_2$ in alcohol and reacted with a 0.36% ammonium molybdate, 0.86 N sulfuric acid, 1.4% ascorbic acid solution. All the glassware used in these determinations was rinsed with concentrated nitric acid and recycled.

Alkaline phosphatase digestions of thymidine nucleotides employed reaction mixtures containing in 1 ml: 0.3 mM thymidine nucleotide, 50 mM Tris-HCl (pH 8.5) and 1.25 U bacterial alkaline phosphatase. Incubation was at 45° for 2 hr. Reaction mixtures were diluted with 5 mM ammonium bicarbonate and applied to a micro DEAE-cellulose (HCO_3) column (1 x 2.5 cm). Columns were washed with 5 mM ammonium bicarbonate (6 ml) and 300 mM ammonium bicarbonate (8 ml). Fractions of 2 ml were collected and absorbance at 267 nm measured. A thymidine nucleotide was sensitive to alkaline phosphatase if it did not adsorb to the DEAE-cellulose (HCO_3^-) column.

Snake venom phosphodiesterase I digestions of thymidine nucleotides employed reaction mixtures containing 10 mM thymidine nucleotide, 30 mM Tris-HCl (pH 8.9), 30 mM magnesium acetate, and 2 U per ml snake

venom phosphodiesterase I. Incubation was at 37° for 30 min. Digestions were terminated by placing the reaction tubes on ice. Aliquots of 10 µl were spotted on Whatman No. 40, acid washed paper. Chromatograms were developed in solvent system I (Table 4). Reaction products were identified by ultraviolet quenching and a phosphate spray reagent (97).

Thin layer chromatography on cellulose plates was performed in ethanol-29% aqueous ammonia-water (6:1:3) (98).

Kinetic Analysis

Initial velocity patterns, inhibition patterns, kinetic constants and reaction mechanisms were defined according to the nomenclature of Cleland (99,100; Appendices A and C). Each assay was run in duplicate. The data for the double reciprocal plots were evaluated using a computer program based on the method of Wilkinson (101). For evaluation of apparent inhibition constants, replots of the intercepts and slopes of the double reciprocal plots were analyzed using linear least-squares analysis.

RESULTS

Marek's Disease Herpesvirus-Induced DNA Polymerase

Identification of an Induced DNA Polymerase from Marek's Disease Herpesvirus-Infected Duck Embryo Fibroblasts

The first evidence that infection of DEF by MDHV, strain BC-1, led to the induction of a salt-stimulated DNA polymerase activity similar to the HSV-induced DNA polymerase activity was obtained when extracts of infected and uninfected DEF were tested in the MDHV-induced DNA polymerase assay (+200 mM KCl) and the duck DNA polymerase assay (-KCl) (Table 1). When nuclear and cytoplasmic extracts of MDHV-infected DEF were assayed by the MDHV-induced DNA polymerase assay, the levels of DNA polymerase activity were at least ten times greater than those in nuclear and cytoplasmic extracts of uninfected DEF. When nuclear and cytoplasmic extracts of MDHV-infected and uninfected DEF were assayed by the duck DNA polymerase assay, similar levels of DNA polymerase activity were found.

The nuclear extract of MDHV-infected DEF was used to partially purify and characterize the MDHV-induced DNA polymerase since it contained substantially more MDHV-induced DNA polymerase activity than duck DNA polymerase activity.

The DNA polymerase activities from a nuclear extract of MDHV-infected DEF were fractionated by DEAE-cellulose chromatography (Figure 2, upper panel). The MDHV-induced DNA polymerase activity

Table 1. Intracellular distributions of DNA polymerase activities in duck embryo fibroblasts

Sample	Units per g wet weight cells: MDHV-Induced DNA Polymerase Assay	Units per g wet weight cells: Duck DNA Polymerase Assay
MDHV-infected DEF		
nuclear extract	20-80	2-10
cytoplasmic extract	3-30	30-40
DEF		
nuclear extract	2	5
cytoplasmic extract	0.3	40

Nuclear and cytoplasmic extracts were prepared as described in Materials and Methods. Both the MDHV-induced DNA polymerase assay and the duck DNA polymerase assay contained 50 mM Tris-HCl (pH 8), 1 mM dithiothreitol, 500 µg per ml bovine serum albumin, 200 µg per ml activated calf thymus DNA, 20 µM [^{3}H]dNTP, and 100 µM each of the other dNTPs. In addition, the MDHV-induced DNA polymerase contained 1 mM $MgCl_2$ and 200 mM KCl. The duck DNA polymerase assay also contained 10 mM $MgCl_2$ and no KCl (66).

was the only major DNA polymerase activity found. It eluted from the DEAE-cellulose column as a single peak at about 0.1 M potassium phosphate. Fractionation of the DNA polymerase activities from a nuclear extract of uninfected DEF by DEAE-cellulose chromatography revealed that the MDHV-induced DNA polymerase activity was not present in the nuclear extract of these cells and that the levels of duck DNA polymerase activities were approximately 20-fold less than the level of MDHV-induced DNA polymerase activity (Figure 2, lower panel). The peak MDHV-induced DNA polymerase fractions were pooled and used to characterize the enzyme.

Figure 2. DEAE-cellulose chromatography of DNA polymerase activities from nuclear extracts of MDHV-infected duck embryo fibroblasts (upper panel) and uninfected duck embryo fibroblasts (lower panel). Nuclear extracts from 2.0 g wet weight of MDHV-infected DEF and 1.2 g wet weight of uninfected DEF were prepared and subjected to DEAE-cellulose chromatography as described in Materials and Methods. Samples (25 µl) of the fractions were assayed for DNA polymerase activities by the MDHV-induced DNA polymerase assay (dotted line) and the duck DNA polymerase assay (solid line). Potassium phosphate concentrations were determined by conductivity measurements. DNA polymerase activities were expressed as pmol [^{3}H]dTMP incorporated into DNA in 30 min per g wet weight of cell.

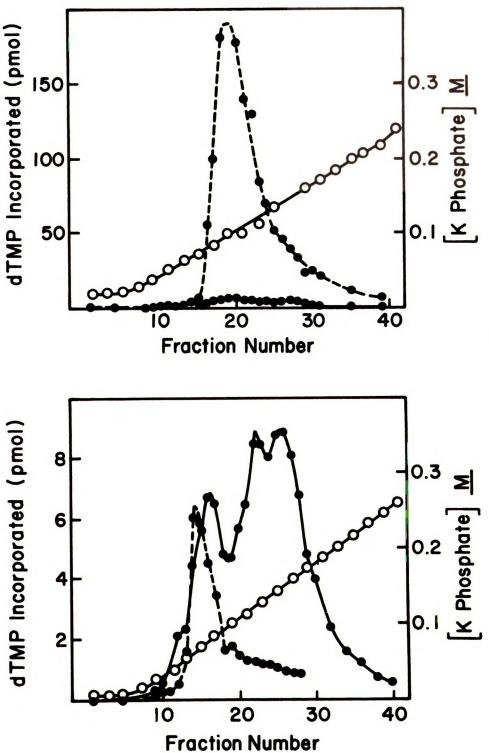
dTMP Incorporated (pmol)
150
100
50
0.3
0.2
0.1
[K Phosphate] M
10
20
30
40
Fraction Number
dTMP Incorporated (pmol)
8
6
4
2
0.3
0.2
0.1
[K Phosphate] M
10
20
30
40
Fraction Number

Figure 2

Characterization of the MDHV-Induced DNA Polymerase

The general reaction requirements of the MDHV-induced DNA polymerase activity were examined. The MDHV-induced DNA polymerase activity required Mg^{2+} in activated DNA reaction mixtures (Figure 3, upper panel). In the absence of $MgCl_2$, negligible DNA polymerization occurred. In the presence of 1 mM $MgCl_2$, maximum DNA polymerization occurred. When Mg^{2+} was replaced with Mn^{2+}, maximum DNA polymerization occurred in the presence of 0.05 mM $MnCl_2$, but this concentration of $MnCl_2$ was only 33% as effective as 1 mM $MgCl_2$ (Figure 3, lower panel).

The MDHV-induced DNA polymerase activity required KCl in activated DNA reaction mixtures (Figure 4). Maximum DNA polymerization occurred in the presence of 200 to 250 mM KCl. In the presence of 200 mM KCl, DNA polymerization was 10- to 60-fold greater than in its absence depending on the enzyme preparation. Stimulation of DNA polymerization was also observed with other salts. Maximum stimulation was observed with 200 mM NH_4Cl, 110 mM $(NH_4)_2SO_4$, and 110 mM K_2SO_4. However, with these salt concentrations stimulation was only 0.6 of that with 200 mM KCl (data not shown). Thus, stimulation was more complicated than a simple ionic strength effect.

The MDHV-induced DNA polymerase activity was dependent on the presence of dithiothreitol in activated DNA reaction mixtures. When the dithiothreitol concentration was reduced from 1.1 mM to 0.1 mM, DNA polymerization was reduced by 40%. When 1 mM p-hydroxymercuribenzoate was added to reaction mixtures, DNA polymerization was inhibited by 98% (data not shown).

Figure 3. Effect of $MgCl_2$ concentration (upper panel) and $MnCl_2$ concentration (lower panel) on the activity of the MDHV-induced DNA polymerase. Reaction mixtures were prepared for the MDHV-induced DNA polymerase assay as described in Materials and Methods except that $MgCl_2$ or $MnCl_2$ was added to the final concentrations indicated.

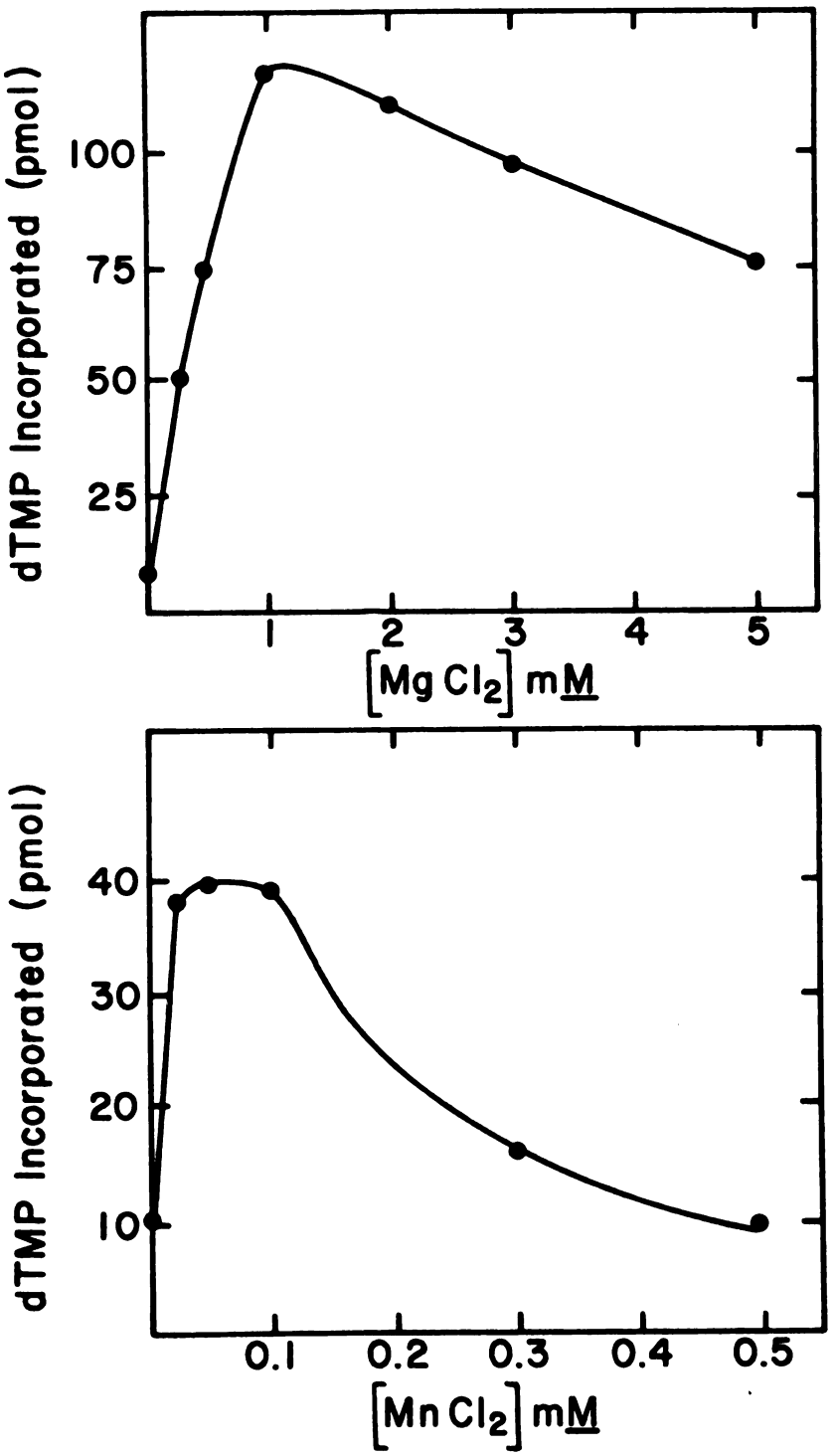
dTMP Incorporated (pmol)
100
75
50
25
1
2
3
4
5
[Mg Cl2] mM
dTMP Incorporated (pmol)
40
30
20
10
0.1
0.2
0.3
0.4
0.5
[Mn Cl2] mM

Figure 3

Figure 4. Effect of KCl concentration on the activity of the MDHV-induced DNA polymerase. Reaction mixtures were prepared for the MDHV-induced DNA polymerase assay as described in Materials and Methods except that KCl was added to the final concentrations indicated.

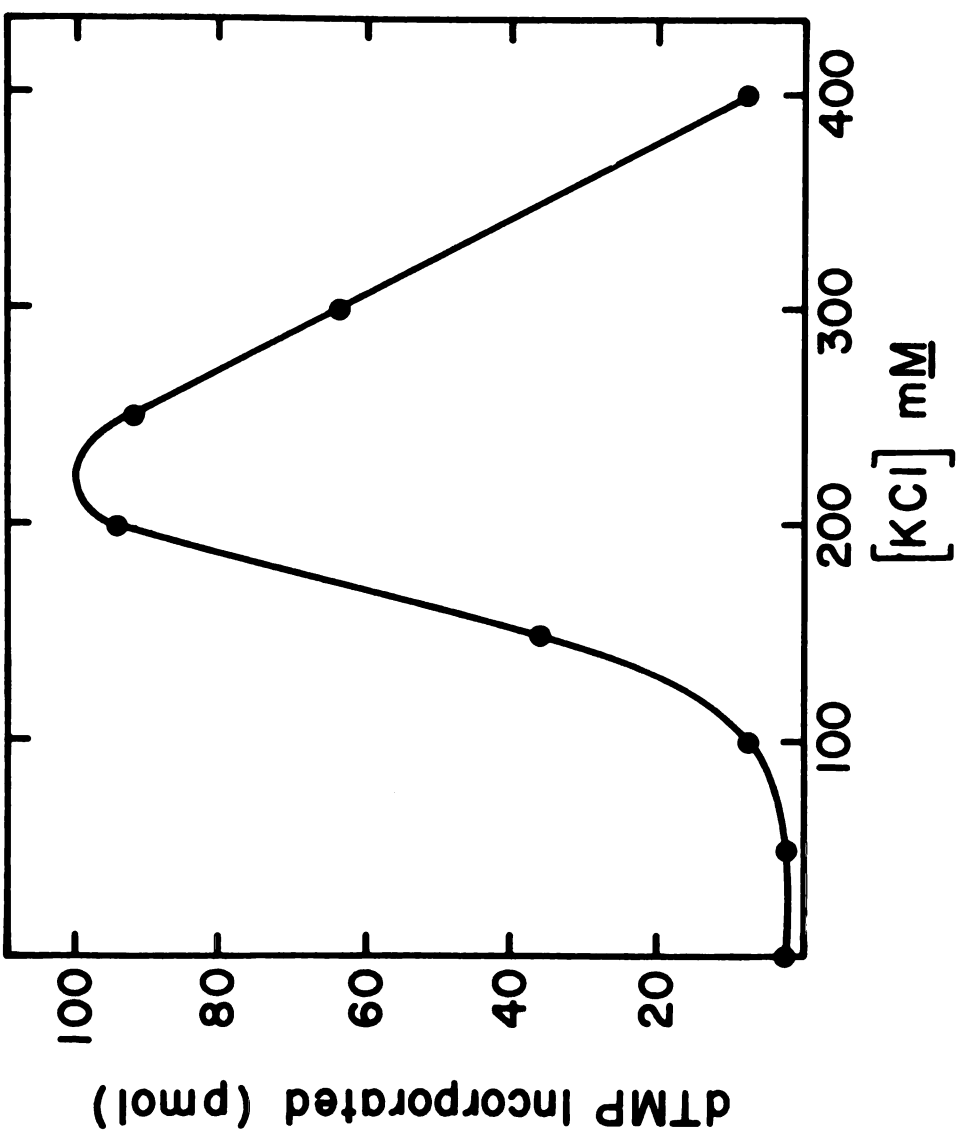

Figure 4

The MDHV-induced DNA polymerase activity required deoxynucleoside triphosphates in activated DNA reaction mixtures. When dATP, dCTP and dGTP were absent from reaction mixtures, [^{3}H]dTMP incorporation was only 12% of that in their presence (data not shown).

The MDHV-induced DNA polymerase activity required exogenous template-primer in reaction mixtures. Template-primer specificities of the polymerase were examined (Table 2). The MDHV-induced DNA polymerase did not utilize double-stranded or single-stranded calf thymus DNA. The oligomer-homopolymers poly(dA)·oligo(dT)$_{12-18}$ and poly(dC)·oligo(dG)$_{12-18}$ were utilized effectively. No significant polymerization was observed with poly(rA)·oligo(dT)$_{12-18}$, poly(rC)·oligo(dG)$_{12-18}$ and poly(dA). Thus, the MDHV-induced DNA polymerase is a DNA-dependent DNA polymerase and not DNA polymerase γ (49), reverse transcriptase (102,103) or terminal nucleotidyl transferase (104,105).

The sedimentation coefficient of the MDHV-induced DNA polymerase was determined by sedimentation velocity centrifugation. The MDHV-induced DNA polymerase sedimented as a single symmetrical peak in a 10 to 30% glycerol gradient (Figure 5). The reference protein lactate dehydrogenase was centrifuged in the same gradient. Based on the sedimentation coefficient of lactate dehydrogenase, the MDHV-induced DNA polymerase exhibited a sedimentation coefficient of 7.0 $\pm$ 0.2S. For a typical globular protein, a sedimentation coefficient of 7.0S would correspond to a molecular weight of 130,000 daltons.

Table 2. Template-primer specificities of MDHV-induced DNA polymerase

Template-primer	dNTP Substrates	[^{3}H]dNMP
Activated calf thymus DNA	[^{3}H]dTTP,dGTP,dCTP,dATP	100(100)
Native calf thymus DNA	[^{3}H]dTTP,dGTP,dCTP,dATP	1
Denatured calf thymus DNA	[^{3}H]dTTP,dGTP,dCTP,dATP	3
Poly(dA)·oligo(dT)$_{12-18}$	[^{3}H]dTTP	300
Poly(rA)·oligo(dT)$_{12-18}$	[^{3}H]dTTP	3
Poly(dC)·oligo(dG)$_{12-18}$	[^{3}H]dGTP	80
Poly(rC)·oligo(dG)$_{12-18}$	[^{3}H]dGTP	3
Poly(dA)	[^{3}H]dGTP	3

The composition of the reaction mixtures containing calf thymus DNA was as described in Materials and Methods. The composition of the reaction mixtures containing olgio-homopolymers was: 50 mM Tris-HCl (pH 8), 0.5 mM $MgCl_2$, 150 mM KCl, 1 mM dithiothreitol, 500 µg per ml bovine serum albumin, 20 µM [^{3}H]dTTP (400 cpm per pmol) or 20 µM [^{3}H]dGTP (300 cpm per pmol), 0.2 optical density units at 260 nm per ml of poly(dC)·oligo(dG)$_{12-18}$ or 0.5 optical density units at 260 nm per ml of the other template-primers and DNA polymerase. Incubation was at 37° for 30 min. The picomoles of [^{3}H]dTMP incorporated into activated DNA are given in parentheses.

Figure 5. Glycerol gradient centrifugation of MDHV-induced DNA polymerase. An aliquot of the DEAE-cellulose fraction of MDHV-induced DNA polymerase was dialyzed against a buffered solution containing 50 mM Tris-HCl (pH 7.5), 250 mM KCl and 0.5 mM dithiothreitol. Samples containing MDHV-induced DNA polymerase and lactate dehydrogenase (0.2 ml) were layered on 4.8 ml linear 10 to 30% glycerol gradients prepared in the above buffered soluion and containing 0.5 mg per ml of bovine serum albumin. Gradients were centrifuged for 16 hr at 4° in a Beckman SW 50.1 rotor. After centrifugation fractions (0.16 ml) were collected from the bottom of the centrifuge tube. Samples (25 µl) of the glycerol gradient fractions were assayed for DNA polymerase activity using the MDHV-induced DNA polymerase assay. The arrow indicates the peak position of the reference protein lactate dehydrogenase (LDH). The recovery of MDHV-induced DNA polymerase activity was approximately 70%.

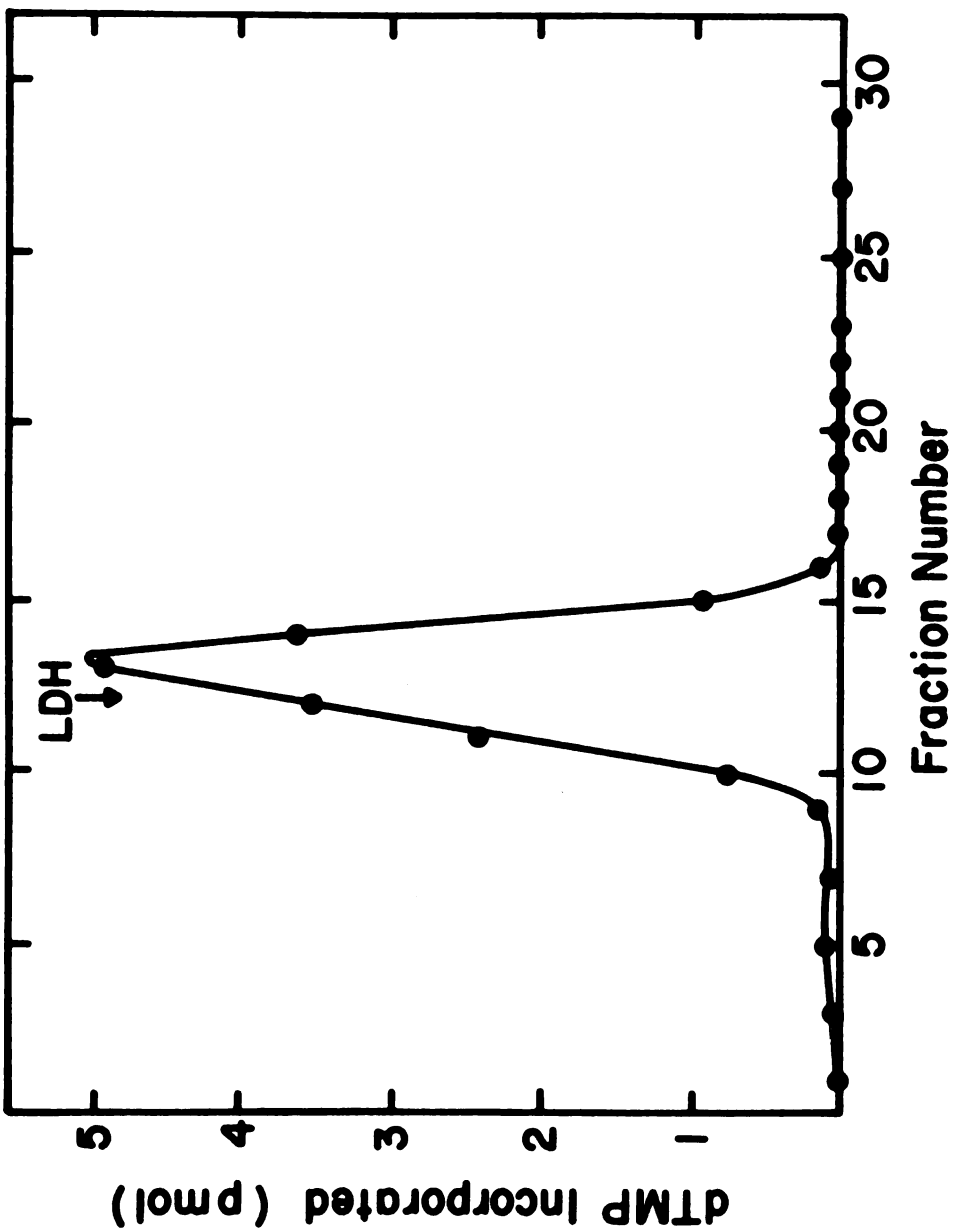
dTMP Incorporated (pmol)
1
2
3
4
5
LDH
5
10
15
20
25
30
Fraction Number

Figure 5

Herpesvirus of Turkeys-Induced DNA Polymerase

Purification of the HVT-Induced DNA Polymerase

When extracts from HVT-infected DEF were examined for an induced DNA polymerase, an activity was found in amounts similar to that found in extracts of MDHV-infected DEF. When the DNA polymerase activities from nuclear extracts of HVT-infected DEF were fractionated by DEAE-cellulose chromatography, two peaks of HVT-induced DNA polymerase activity eluted from the column at about 0.08 M and 0.13 M potassium phosphate. These two activities were present in approximately equal amounts and were identical with respect to $MgCl_2$ optimum, KCl optimum, template specificity, sedimentation coefficient and phosphonoacetate sensitivity (data not shown). This result is different from that obtained for the MDHV-induced DNA polymerase where a single peak of activity eluted from the DEAE-cellulose column at about 0.1 M potassium phosphate.

The HVT-induced DNA polymerase was subjected to more extensive purification. DNA polymerase activities from nuclear extracts of HVT-infected DEF were fractionated by phosphocellulose chromatography. The HVT-induced DNA polymerase eluted from the column as a single peak of activity at about 0.35 M KCl. The peak fractions of HVT-induced DNA polymerase activity were pooled, dialyzed and subjected to DEAE-cellulose chromatography. The HVT-induced DNA polymerase eluted from the column as a single peak of activity at about 0.14 M potassium phosphate. A summary of such a purification may be found in Table 3. The phosphocellulose fraction had a specific activity of 58 units per mg protein and represented a 30-fold purification over the

Table 3. Purification of HVT-induced DNA polymerase

Fraction	Total Protein (mg)	Total Units	Recovery (%)	Specific Activity
Homogenate	920	1580	100	2
Crude nuclear	90	780	50	9
100,000 x g Nuclear supernatant	40	500	30-	12
Phosphocellulose	5	290	20	58
DEAE-cellulose	1	100	6	100

HVT-induced DNA polymerase was purified from 20 g (wet weight) of HVT-infected DEF grown in roller bottles as described in Materials and Methods.

homogenate fraction; DEAE-cellulose chromatography resulted in an additional 1.7-fold purification of the HVT-induced DNA polymerase.

No detectable deoxyribonucleotide triphosphatase activity or inorganic pyrophosphatase activity and little or no deoxyribonuclease activity was found in the phosphocellulose enzyme fraction when tested using the standard HVT-induced DNA polymerase assay conditions. This fraction of HVT-induced DNA polymerase activity was used for many of the kinetic studies to be reported.

Characterization of the HVT-Induced DNA Polymerase

The phosphocellulose fractions and the DEAE-cellulose fractions of the HVT-induced DNA polymerase were characterized. In each case, for the activated DNA polymerization reaction, maximum activity was obtained in the presence of 2 mM $MgCl_2$ and 200-250 mM KCl. On the

average, a 10-fold stimulation of DNA polymerization resulted when 200 mM KCl was added to reaction mixtures. In template-primer studies the HVT-induced DNA polymerase was able to utilize poly(dA)·oligo$(dT)_{12-18}$ and poly(dC)·oligo$(dG)_{12-18}$ effectively when compared with its ability to utilize activated calf thymus DNA. It could not utilize poly(rA)·oligo$(dT)_{12-18}$ and poly(rC)·oligo$(dG)_{12-18}$. The sedimentation coefficient of the HVT-induced DNA polymerase was 7.0 $\pm$ 0.2S as determined by glycerol gradient sedimentation velocity centrifugation in the presence of 0.25M KCl (data not shown). Thus, the properties of the HVT-induced DNA polymerase were similar to those of the MDHV-induced DNA polymerase.

Apparent Michaelis constants for activated DNA and dNTP were determined for the HVT-induced DNA polymerase from initial velocity studies (Figure 6; Appendices A and C). With dNTP as variable substrate and activated DNA as changing fixed substrate, the apparent Michaelis constant for activated DNA was determined to be 7.3 $\pm$ 0.2 µg per ml from the replot of intercept against reciprocal activated DNA concentration. With activated DNA as variable substrate and dNTP as changing fixed substrate, the apparent Michaelis constant for dNTP was determined to be 2.1 $\pm$ 0.5 µM from the replot of intercept against reciprocal dNTP concentration (data not shown).

Inhibition of HVT-Induced DNA Polymerase by Phosphonoacetate

Mao et al. (75) have reported that phosphonoacetate is an effective inhibitor of the herpes simplex-induced DNA polymerase. Phosphonoacetate was also an effective inhibitor of the DNA polymerases induced by MDHV and HVT. The addition of 1 to 3 µM phosphonoacetate to reaction mixtures resulted in about a 50% decrease in the rate of

Figure 6. Initial velocity pattern for HVT-induced DNA polymerase with the four dNTPs as the variable substrate and activated DNA as the changing fixed substrate. Activated DNA concentrations were (●) 2.5 µg per ml, (○) 5.0 µg per ml, and (Δ) 25 µg per ml. Initial velocities were expressed as pmol of [^{3}H]dCMP incorporated into DNA per 30 min. The specific radioactivity of [^{3}H]dCTP was 2000 cpm per pmol. Equimolar concentrations of each of the four dNTPs were present in each reaction mixture. Replots of the slopes (○) and intercepts (●) are shown in the left panel.

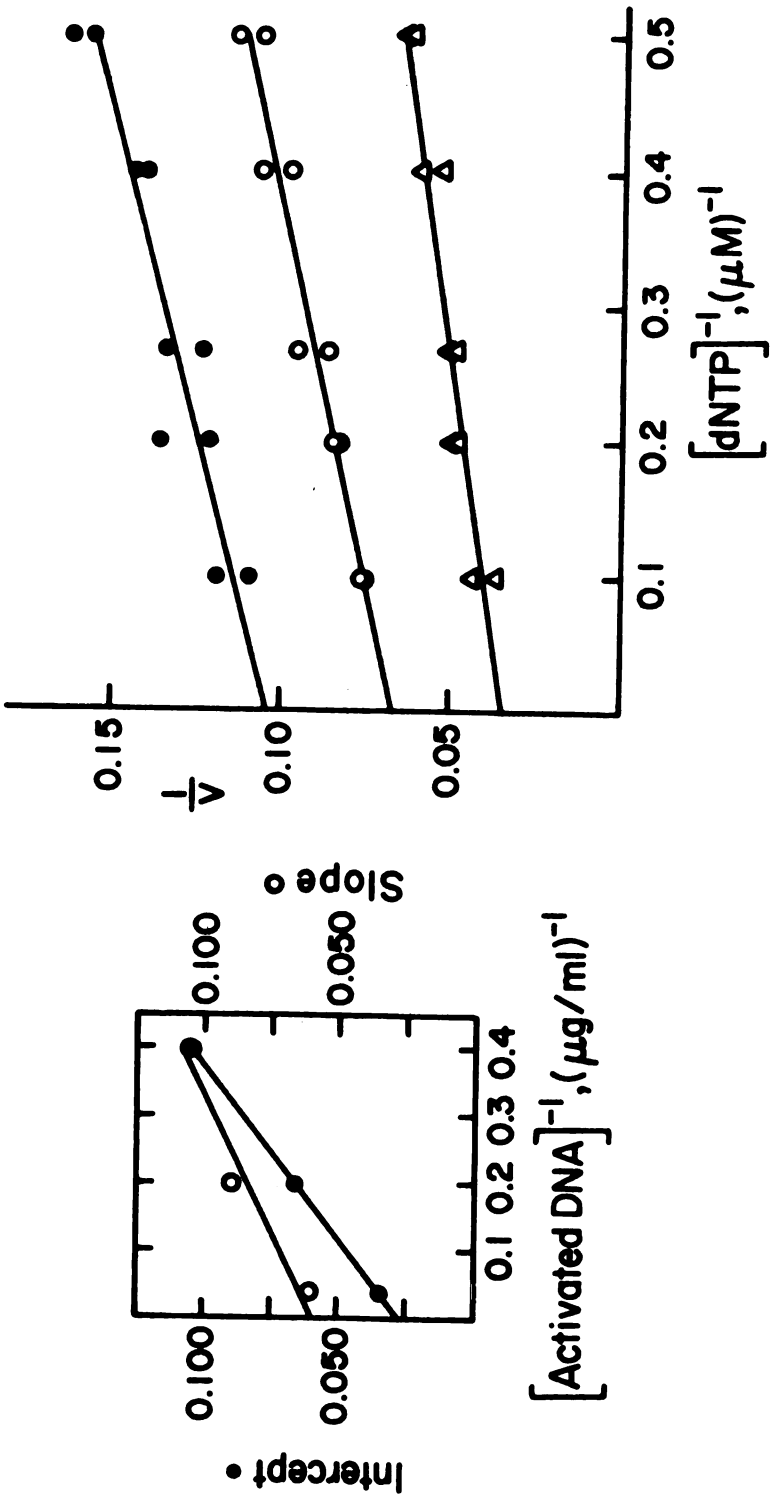
0.15
0.10
0.05
1/v
0.1
0.2
0.3
0.4
0.5
[dNTP]⁻¹,(μM)⁻¹
Slope o
0.100
0.050
0.1 0.2 0.3 0.4
[Activated DNA]⁻¹,(μg/ml)⁻¹
Intercept •
0.100
0.050

Figure 6

the DNA polymerization reaction catalyzed by these enzymes (Figure 16). Either in the presence or absence of phosphonoacetate, the rate of the DNA polymerization reaction was linear for at least 1 hr. Phosphonoacetate is a reversible inhibitor of the HVT-induced DNA polymerase. It could be dialyzed away from the enzyme with full recovery of enzyme activity.

Phosphonoacetate Inhibition Patterns for the DNA Polymerization Reaction Catalyzed by HVT-Induced DNA Polymerase

Phosphonoacetate gave linear noncompetitive inhibition with the four dNTPs as the variable substrate and with activated DNA at a saturating concentration of 200 µg per ml (Figure 7; see Appendix A for explanation of nomenclature). The apparent inhibition constant (K_{ii}) determined from the replot of the vertical intercepts against phosphonoacetate concentration was 1.4 $\pm$ 0.6 µM. The apparent inhibition constant (K_{is}) determined from the replot of the slopes against phosphonoacetate concentration was 1.0 $\pm$ 0.2 µM.

With activated DNA as the variable substrate, and the four dNTPs at 2.5 µM each, phosphonoacetate gave linear noncompetitive inhibition (Figure 8). A replot of the vertical intercepts yielded a K_{ii} of 1.5 $\pm$ 0.2 µM and a replot of the slopes yielded a K_{is} of 2.5 $\pm$ 0.6 µM. Phosphonoacetate also gave linear noncompetitive inhibition with activated DNA as the variable substrate and with the four dNTPs at 100 µM each. K_{ii} was determined to be 1.5 $\pm$ 0.2 µM and K_{is} was about 9 $\pm$ 3 µM. The higher K_{is} value seen at 100 µM dNTP than at 2.5 µM dNTP indicated that the phosphonoacetate inhibition pattern was more nearly uncompetitive at the higher concentration of dNTP than it was at the lower concentration.

Figure 7. Double reciprocal plots with the four dNTPs as the variable substrate and phosphonoacetate as inhibitor. Activated DNA concentration was 200 µg per ml. Phosphonoacetate concentrations were 0 (●), 0.55 µM (○), 1.65 µM (□) and 2.75 µM (Δ). Initial velocities were expressed as pmol of [^{3}H]dCMP incorporated into DNA per 30 min. Specific radioactivity of [^{3}H]dCTP was 2000 cpm per pmol. Equimolar concentrations of each of the four dNTPs were present in each reaction mixture. Replots of the slopes (○) and intercepts (●) as a function of phosphonoacetate concentration are shown in the left panel.

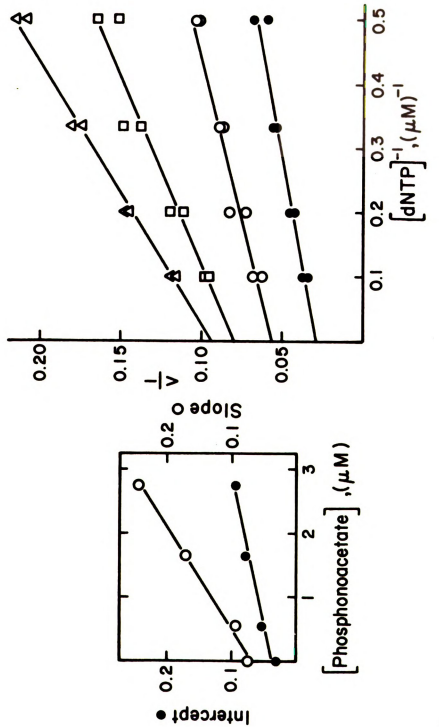
0.20
0.15
0.10
0.05
1/v
0.1
0.2
0.3
0.4
0.5
[dNTP]⁻¹, (μM)⁻¹
Slope ○
0.2
0.1
1
2
3
[Phosphonoacetate], (μM)
Intercept ●
0.2
0.1

Figure 7

Figure 8. Double reciprocal plots with activated DNA as the variable substrate and phosphonoacetate as inhibitor. The four dNTPs were at 2.5 μM each. Phosphonoacetate concentrations were 0 (●), 0.55 μM (○), 1.1 μM (□) and 2.2 μM (Δ). Initial velocities were expressed as pmol of [^{3}H]dCMP incorporated into DNA per 30 min. Specific radioactivity of [^{3}H]dCTP was 1000 cpm per pmol. Replots of the slopes (○) and intercepts (●) as a function of phosphonoacetate concentration are shown in the left panel.

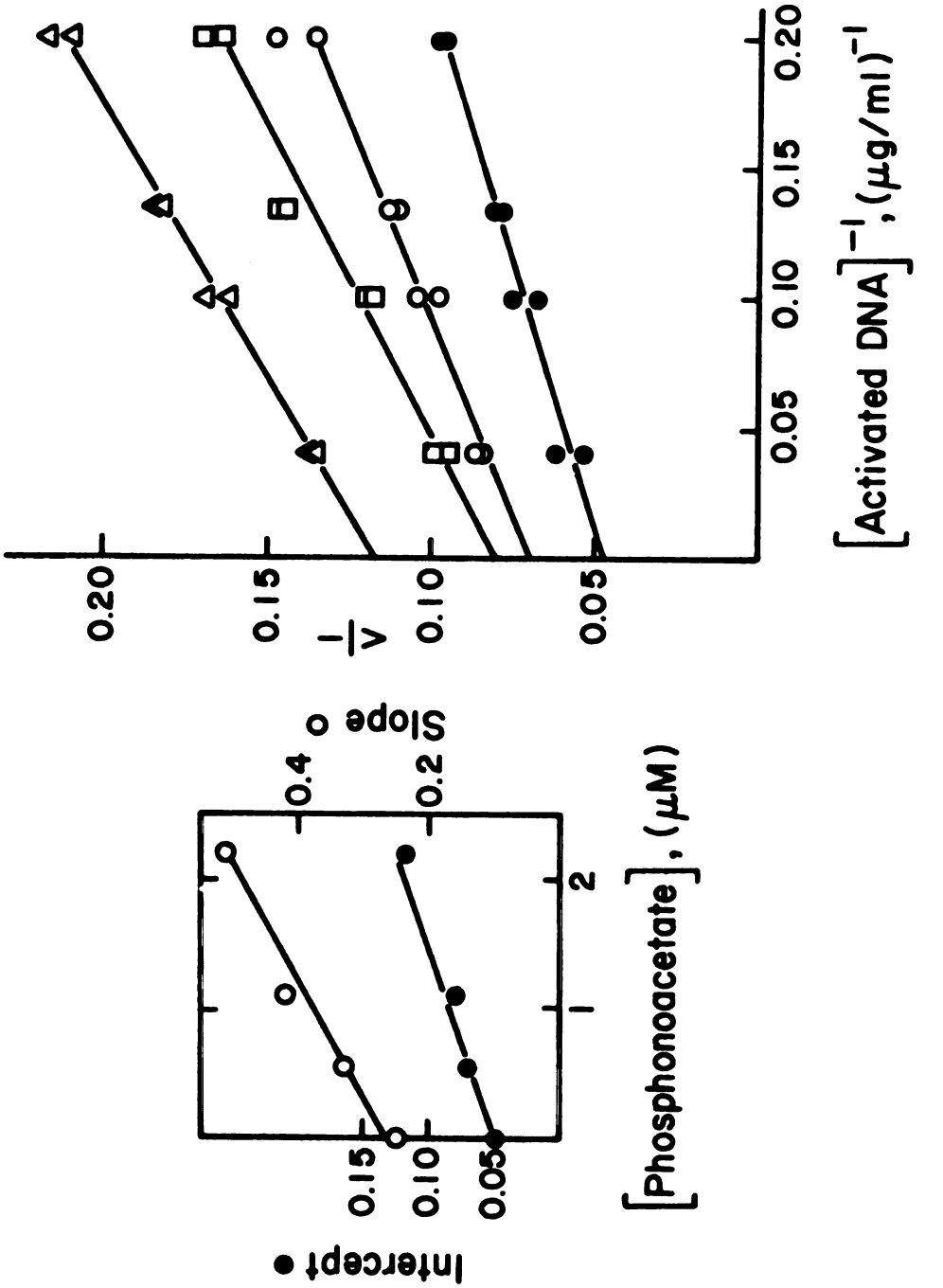
0.20
0.15
0.10
0.05
1/v
0.05
0.10
0.15
0.20
[Activated DNA]⁻¹, (μg/ml)⁻¹
Slope
0.4
0.2
Intercept
0.15
0.10
0.05
1
2
[Phosphonoacetate], (μM)

Figure 8

Phosphonoacetate Inhibition Pattern for the dNTP-Pyrophosphate Exchange Reaction Catalyzed by HVT-Induced DNA Polymerase

Since phosphonoacetate and pyrophosphate have structural features in common, it was suspected that phosphonoacetate might be inhibiting the DNA polymerization reaction by interacting with the polymerase at the pyrophosphate binding site. If so, phosphonoacetate should be a competitive inhibitor of pyrophosphate in the dNTP-pyrophosphate exchange reaction. This was the case (Figure 9). The apparent K_{is} value for phosphonoacetate was 1.3 $\pm$ 0.4 µM. The apparent Km value for pyrophosphate was 0.24 $\pm$ 0.04 mM.

Multiple Inhibition Analysis of Phosphonoacetate and Pyrophosphate in the DNA Polymerization Reaction Catalyzed by HVT-Induced DNA Polymerase

The results of the dNTP-pyrophosphate exchange reaction indicated that phosphonoacetate interacts with the polymerase at the pyrophosphate binding site. Additional evidence for this conclusion was obtained from multiple inhibition analysis of the DNA polymerization reaction (106,107). The concentration of phosphonoacetate was varied in the presence of fixed concentrations of pyrophosphate when dNTP concentrations were 2.5 µM each and activated DNA concentration was 200 µg per ml. A plot of $\frac{1}{V}$ against phosphonoacetate concentration resulted in a series of parallel lines (Figure 10). This result indicated that phosphonoacetate and pyrophosphate are mutually exclusive inhibitors and, therefore, bind at the same site on the DNA polymerase (Appendix D). When dNTP concentrations were 20 µM each and activated DNA concentration was 200 µg/ml, phosphonoacetate and pyrophosphate were also found to be mutually exclusive inhibitors of the HVT-induced DNA polymerase (data not shown).

Figure 9. Double reciprocal plots of the dNTP-pyrophosphate exchange reaction with pyrophosphate as the variable substrate and phosphonoacetate as inhibitor. Activated DNA was at 200 µg per ml and the four dNTPs were at 100 µM each. Phosphonoacetate concentrations were 0 (●), 2 µM (○) and 3 µM (□). Initial velocities were expressed as pmol [^{32}P]pyrophosphate converted to Norit-adsorbable form per 30 min.

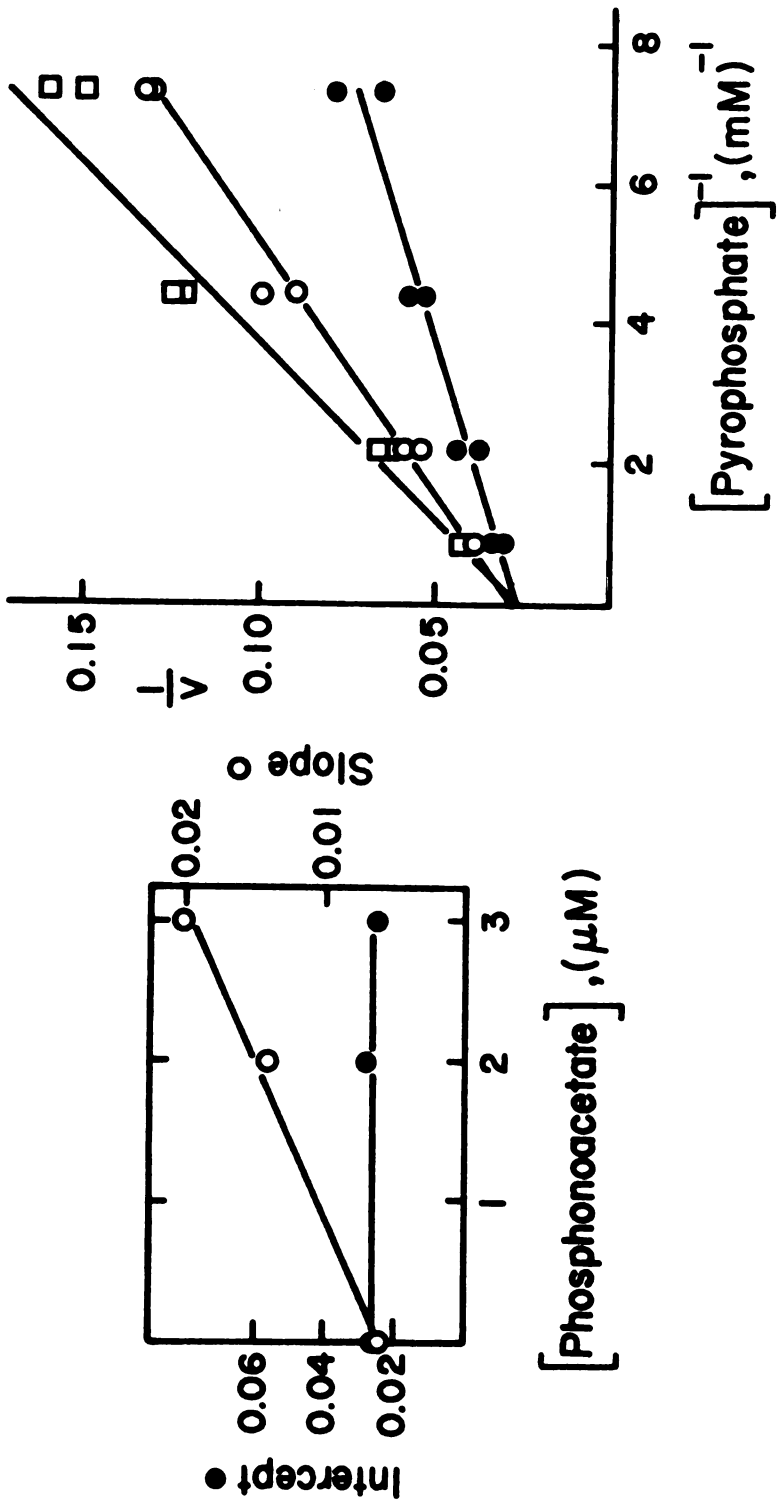
0.15
0.10
0.05
1/v
2
4
6
8
[Pyrophosphate]⁻¹,(mM)⁻¹
Slope o
0.02
0.01
Intercept •
0.06
0.04
0.02
1
2
3
[Phosphonoacetate],(μM)

Figure 9

Figure 10. Multiple inhibition of the HVT-induced DNA polymerase by phosphonoacetate and pyrophosphate. Activated DNA was at 200 µg/ml and the four dNTPs were at 2.5 µM each. Pyrophosphate concentrations were 0 (●), 0.2 mM (○), 0.4 mM (□) and 0.6 mM (Δ). Initial velocities were expressed as pmol [^{3}H]dTMP incorporated into DNA per 30 min. Specific radioactivity of [^{3}H]dTTP was 2000 cpm per pmol.

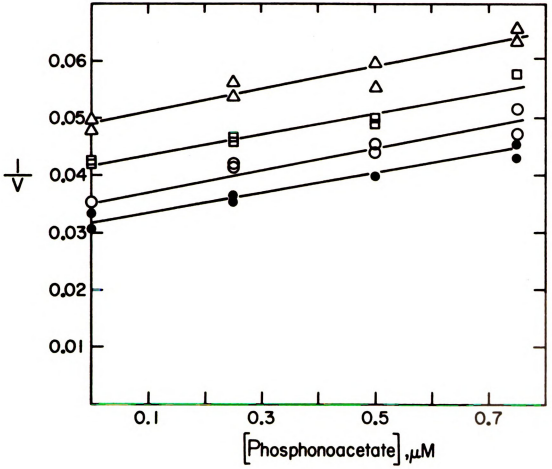

Figure 10

Pyrophosphate Inhibition Patterns for the DNA Polymerization Reaction Catalyzed by HVT-Induced DNA Polymerase

For comparison to the phosphonoacetate inhibition patterns and apparent K_i values, inhibition studies using pyrophosphate, a product inhibitor, were performed. With the four dNTPs as the variable substrate, pyrophosphate gave linear noncompetitive inhibition. K_{ii} was 1.3 $\pm$ 0.2 mM and K_{is} was 0.7 $\pm$ 0.2 mM. With activated DNA as the variable substrate and with the four dNTPs at 2.5 M each, pyrophosphate gave linear noncompetitive inhibition. K_{ii} was 0.95 $\pm$ 0.05 mM and K_{is} was 1.7 $\pm$ 0.3 mM. Pyrophosphate gave linear uncompetitive inhibition with activated DNA as the variable substrate and with the four dNTPs at 20 μM each. K_{ii} was about 0.9 $\pm$ 0.1 mM.

Effect of $MgCl_2$ Concentration on Inhibition of HVT-Induced DNA Polymerase by Phosphonoacetate

In the pyrophosphate inhibition studies of the HVT-induced DNA polymerase, as in parallel studies with *E. coli* DNA polymerase I, it is assumed that $MgPP_i$ is the inhibiting species (108). Since phosphonoacetate binds to the pyrophosphate binding site of the enzyme, the effect of $MgCl_2$ concentration on phosphonoacetate inhibition of the HVT-induced DNA polymerase was determined (Figure 11). As the $MgCl_2$ concentration in standard reaction mixtures containing 1 μM phosphonoacetate increased, the amount of enzyme inhibition also increased until maximum inhibition was reached at 2 to 5 mM $MgCl_2$. This result is consistent with a magnesium-phosphonoacetate complex as the inhibiting species. It was unnecessary to add supplementary $MgCl_2$ to reaction mixtures containing phosphonoacetate,

Figure 11. Effect of $MgCl_2$ concentration on inhibition of HVT-induced DNA polymerase by phosphonoacetate. Reaction mixtures contained in 200 µl: 50 mM Tris-HCl (pH 8), 200 mM KCl, 1 mM dithiothreitol, 500 µg per ml bovine serum albumin, 200 µg per ml activated calf thymus DNA, 20 µM [^{3}H]dCTP (400 cpm per pmol), 100 µM each of dATP, dGTP, dTTP, HVT-induced DNA polymerase, 0 or 1 µM phosphonoacetate and $MgCl_2$ concentrations as indicated. For each $MgCl_2$ concentration the ratio of activity in the presence of 1 µM phosphonoacetate to activity in the absence of phosphonoacetate X 100 was calculated. This value was subtracted from 100 to give percent inhibition.

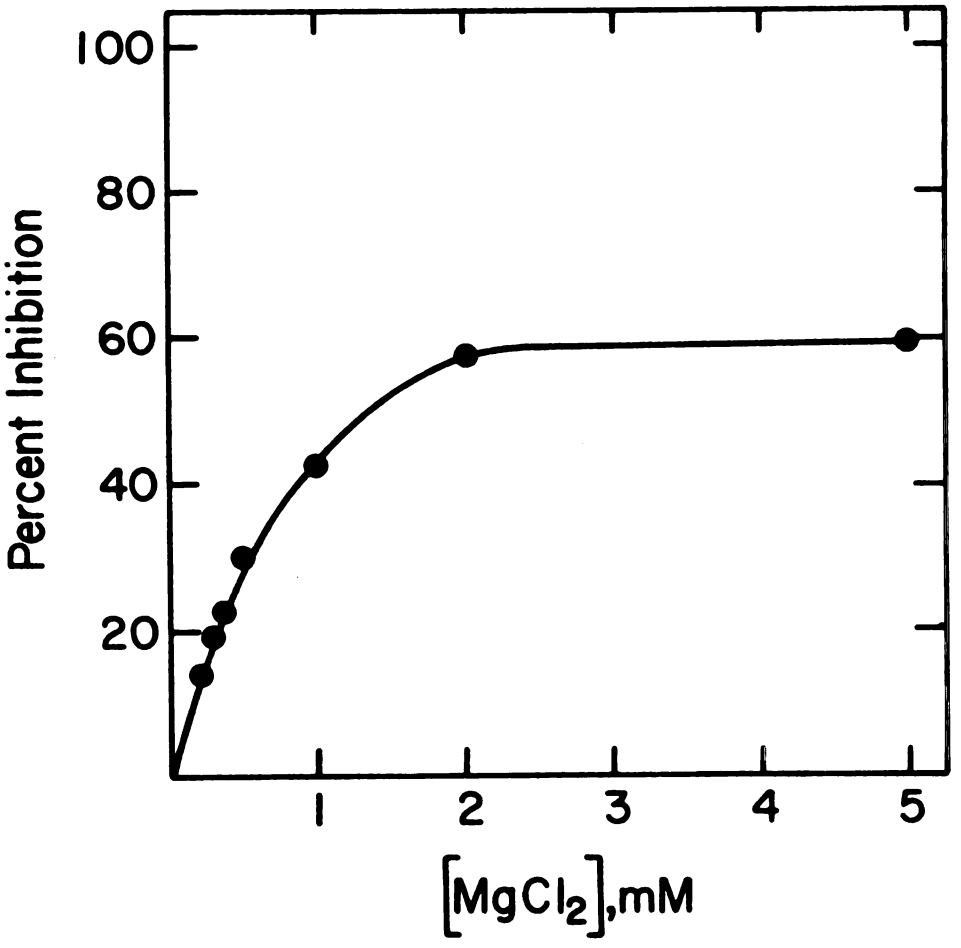

Figure 11

since phosphonoacetate was present in μM concentrations and $MgCl_2$ was present in mM concentrations.

Inhibition by Structural Analogues of Phosphonoacetate

When tested at a concentration of 200 μM, the following analogues of phosphonoacetate produced no significant inhibition of either the polymerization reaction or of the dNTP-pyrophosphate exchange reaction catalyzed by HVT-induced DNA polymerase: methylene diphosphonate, malonate, phosphoglycolate, sulfoacetate, carbamyl phosphate, phosphonoacetaldehyde, phosphonopropionate, amino methyl phosphonate, α-amino ethyl phosphonoate, trimethyl ester of phosphonoacetate, α-phenyl phosphonoacetate, 2-amino phosphonoacetate, acetonyl phosphonate, monomethyl ester of acetonyl phosphonoacetate, monomethyl ester of acetyl aminophosphate, N-methyl phosphonoacetamide, and 2-methyl-2-phosphonopropionate. 2-Phosphonopropionate was an inhibitor of the polymerization reaction and of the pyrophosphate exchange reaction. As determined in the dNTP-pyrophosphate exchange reaction, the apparent K_{is} for 2-phosphonopropionate was about 50 μM. Therefore, 2 phosphonopropionate was about 50-fold less effective as an inhibitor than phosphonoacetate.

Two other analogues of phosphonoacetate also inhibited the HVT-induced polymerization reaction. When 30 μM N-(phosphonoacetyl)-L-aspartate was added to standard reaction mixtures, the DNA polymerization reaction was inhibited by 50%. When 60 μM phosphonoacetamide, monocyclohexylamine salt, was added to standard reaction mixtures, the DNA polymerization reaction was inhibited by 50%. However, thin layer chromatography of these compounds on cellulose plates indicated that the N-(phosphonoacetyl)-L-aspartate preparation was contaminated

with phosphonoacetate and that the phosphonoacetamide preparation contained two unidentifiable components (data not shown). Therefore, the identity of these inhibiting compounds is unresolved.

The Effect of Phosphonoacetate on the DNA Polymerization Reaction Catalyzed by Other DNA Polymerases

DNA polymerase α, but not β, from uninfected duck embryo fibroblasts, was inhibited by phosphonoacetate. The inhibition patterns with the α polymerase for the DNA polymerization reaction were similar to those produced with the HVT-induced polymerase, but the apparent K_i values were 10 to 20 times greater. DNA polymerase β, when tested at a phosphonoacetate concentration of 200 μM, was not significantly inhibited. Likewise, neither *E. coli* DNA polymerase I nor AMV reverse transcriptase was significantly inhibited.

Investigation of Phosphonoacetate as an Alternate Product Inhibitor of the HVT-Induced DNA Polymerization Reaction

Phosphonoacetate was a competitive inhibitor of pyrophosphate in the dNTP-pyrophosphate exchange reaction. In the polymerization reaction multiple inhibition studies with phosphonoacetate and pyrophosphate indicated that the two were mutually exclusive inhibitors. Inhibition patterns for phosphonoacetate and pyrophosphate were similar. Therefore, phosphonoacetate inhibits the HVT-induced DNA polymerase by binding to the pyrophosphate binding site of the enzyme. By binding at this site phosphonoacetate could act as a dead end inhibitor or as an alternate product inhibitor. As a dead end inhibitor phosphonoacetate would simply bind reversibly to the pyrophosphate binding site. As an alternate product inhibitor, phosphonoacetate would reverse the DNA polymerization reaction via

an alternate reaction pathway. In so doing it would generate a nucleotide analogue of itself which would be an alternate substrate in the polymerization reaction. In an attempt to provide direct evidence for phosphonoacetate as an alternate product inhibitor, a nucleotide analogue of phosphonoacetate, deoxythymidine 5'phosphophosphonoacetate, was synthesized. If phosphonoacetate were an alternate product inhibitor, then as an alternate substrate this compound should: (1) serve as a substrate in the polymerization reaction, (2) act as a competitive inhibitor of dTTP, and (3) be generated during phosphonoacetate inhibition. These predictions were tested.

Synthesis of Deoxythymidine 5'Phosphophosphonoacetate

Deoxythymidine 5'phosphophosphonoacetate was synthesized by reaction of deoxythymidine 5'phosphomorpholidate with phosphonoacetate in a modification of the procedure for synthesis of $\alpha[^{32}P]$adenosine 5'triphosphate (109). Phosphonoacetic acid (1.75 mmol) was dissolved in water (1.5 ml); anhydrous pyridine (9 ml) was added followed by tri-n-octylamine (5.5 mmol). This solution containing the tri-n-octylammonium salt of phosphonoacetate was evaporated to dryness. The phosphonoacetate was dried by three evaporations with anhydrous pyridine (5 ml each) and two with anhydrous benzene. Deoxythymidine 5'phosphomorpholidate (.5 mmol) was also dried by evaporations with anhydrous pyridine and anhydrous benzene. The deoxythymidine 5'phosphomorpholidate was dissolved in anhydrous dimethyl sulfoxide (7 ml) and was added to the phosphonoacetate. The reaction mixture was stirred for 1 hr at room temperature under nitrogen, was then diluted with water (70 ml) and was applied to a DEAE-cellulose

(HCO_3^-) column (2.5 x 60 cm) at 4°. The column was washed with 5 mM triethylammonium bicarbonate (pH 7.5) until all unadsorbed ultraviolet-adsorbing material had washed through. The column was eluted with a 3.5 l linear gradient of 5 to 350 mM triethylammonium bicarbonate (pH 7.5). Three peaks of ultraviolet-adsorbing material were found and were identified as unreacted deoxythymidine 5'phosphomorpholidate (39%), dTMP (1%) and dTMP-PA (60%), in order of elution. Unreacted phosphonoacetate eluted in a position between dTMP and dTMP-PA.

Fractions containing dTMP-PA were pooled and lyophilized. Residual triethylammonium bicarbonate was removed by five evaporations with methanol (20 ml each). The final residue was dissolved in methanol (5 ml) and was precipitated as the sodium salt by addition of sodium iodide (1.2 ml of a 1 M solution in acetone) and cold acetone (30 ml). The precipitate was washed three times with cold acetone (15 ml) and was dried *in vacuo*. The resulting powder was stored at -20°.

If the reaction time was increased, the 60% yield of dTMP-PA decreased with a proportional increase in the yield dTMP.

This synthesis was performed in collaboration with Barbara Duhl-Emswiler of the Chemistry Department.

Structural Properties of Deoxythymidine 5'Phosphophosphonoacetate

The deoxythymidine 5'phosphophosphonoacetate preparation was determined to be homogeneous by paper chromatography in two solvent systems and by electrophoresis (Table 4). The stability of dTMP-PA was monitored under several different conditions: (1) incubation in 10 mM Tris-HCl (pH 8) at 37° for six days, (2) incubation in 0.01 N HCl at room temperature for 24 hr, and (3) incubation in a standard

DNA polymerization reaction mixture with an excess of HVT-induced DNA polymerase at 37° for 4 hr. In each case analysis by paper chromatography in solvent system I indicated that dTMP-PA had not been degraded.

Table 4. Characterization of deoxythymidine 5'phosphophosphonoacetate by paper chromatography and electrophoresis

Compound	RF I	RF II	Relative Electrophoretic Mobility
dTMP-PA	0.23	0.28	0.86
dTTP	0.11	0.16	1.0
dTDP	0.19	0.18	0.86
dTMP	0.34	0.23	0.64
dTMP-morpholidate	0.73	0.56	0.36
PA	0.19	-	1.11

Descending chromatography with Whatman No. 40 acid washed paper was used with solvent systems (I) ethyl alcohol-1 M ammonium acetate (pH 7.5) (5:2) and (II) isopropyl alcohol-conc. ammonium hydroxide-water (7:1:2) (110). Whatman No. 40 acid washed paper was used for electrophoresis on a water-cooled flat-plate apparatus with 0.05 M triethylammonium bicarbonate (pH 7.5), 35 V per cm for 45 min. Compounds were detected by ultraviolet quenching and/or a phosphate spray reagent (97).

Deoxthymidine 5'phosphophosphonoacetate was determined to have the structure shown in Figure 12 based on the following: It exhibited an adsorption maximum for thymine at 267 nm. It had a molecular weight of 550 $\pm$ 10 based on the molar extinction coefficient for thymine ($E = 9.6 \times 10^3$ at 267 nm). The theoretical molecular weight for Na_3 dTMP-PA$\cdot 2H_2O$ would be 546. It contained 2.0 $\pm$ .1 phosphorus atoms per thymine. It was degraded by snake venom phosphodiesterase

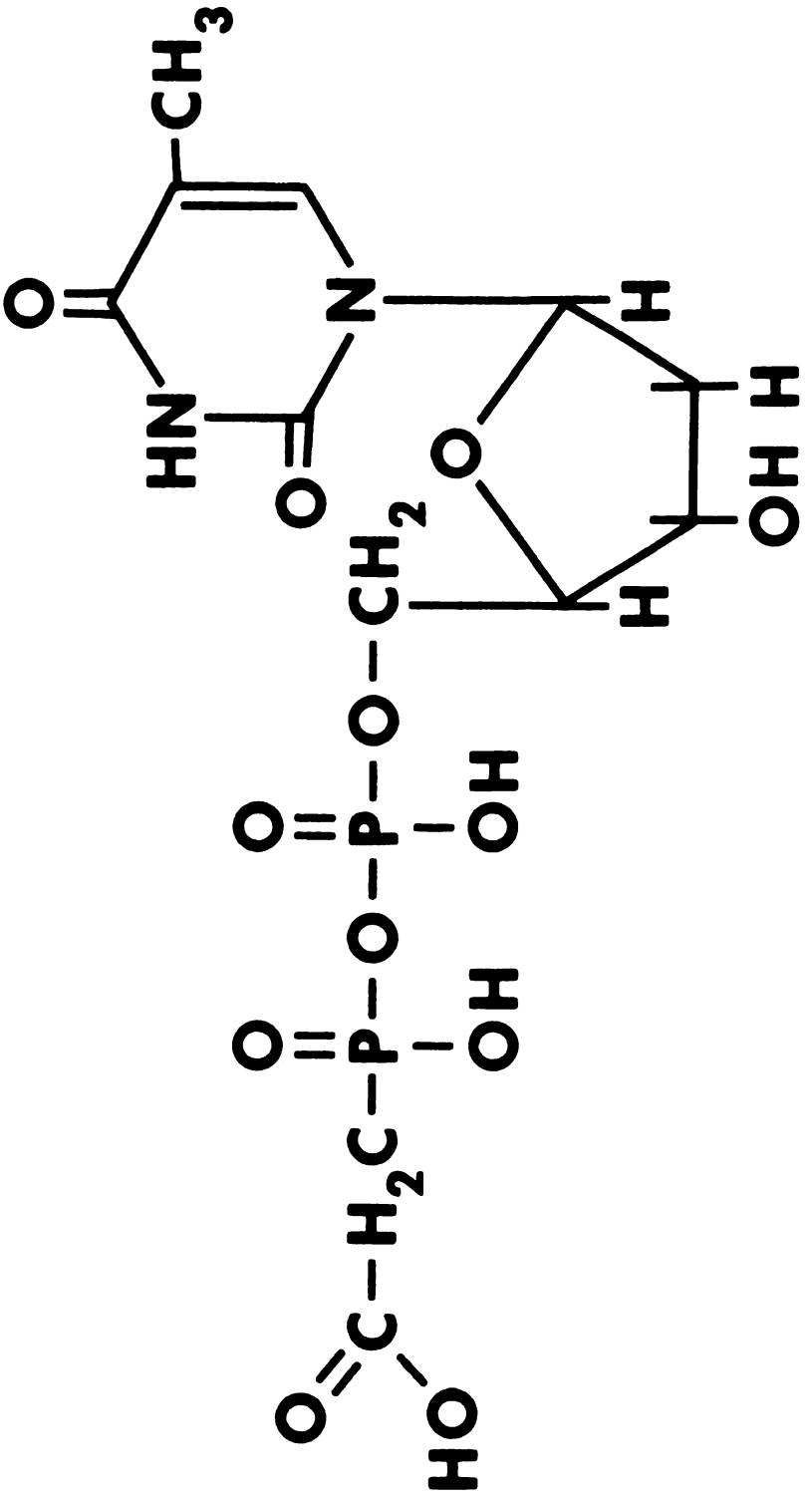

Figure 12. Structure of deoxythymidine 5'phosphophosphonoacetic acid.

I into dTMP and PA. The dTMP and PA components of dTMP-PA were also identified by comparison of proton decoupled ^{13}C nuclear magnetic resonance spectra of dTMP-PA, dTMP and PA (Figure 13). Alkaline phosphatase did not degrade dTMP-PA indicating that no terminal phosphate ester existed in this compound. Evidence for the existence of a free carboxyl group in dTMP-PA was obtained from the presence of a carboxyl doublet at 176.4 and 176.9 ppm in the ^{13}C NMR spectrum and from the presence of a carboxylate anion stretch at 1580 cm^{-1} in the infrared spectrum.

Deoxythymidine 5'Phosphophosphonoacetate as a Substrate of the HVT-Induced DNA Polymerization Reaction

In order to determine if dTMP-PA could serve as a substrate in place of dTTP in the activated DNA polymerization reaction, HVT-induced DNA polymerase was assayed in standard reaction mixtures in the absence of dTTP, in the presence of increasing concentrations of dTMP-PA, and in the presence of a saturating amount of dTTP (Table 5). In the absence of dTTP 12.1 pmol of [^{3}H]dCMP was incorporated. Addition of increasing concentrations of dTMP-PA to reaction mixtures did not enhance this incorporation, even when 640 µM dTMP-PA had been added. In fact, inhibition was observed. Addition of 100 µM dTTP increased [^{3}H]dCMP incorporation by 3-fold. These results suggest that dTMP-PA cannot replace dTTP as a substrate in the DNA polymerization reaction. The inhibition that was observed when increasing concentrations of dTMP-PA were added to reaction mixtures was probably a result of contaminating phosphonoacetate as explained below.

Figure 13. Proton decoupled ^{13}C nuclear magnetic resonance spectra of phosphonoacetate (top panel), dTMP (middle panel), and dTMP-PA (lower panel). Solutions of 0.71 M phosphonoacetate (pH 8.2), 0.83 M dTMP (pH 8.3), and 0.14 M dTMP-PA (pH 8.6) were prepared in D_2O and were analyzed at 12° using a Brucker WP60 60 MHZ ^{13}C nuclear magnetic resonance spectrometer.

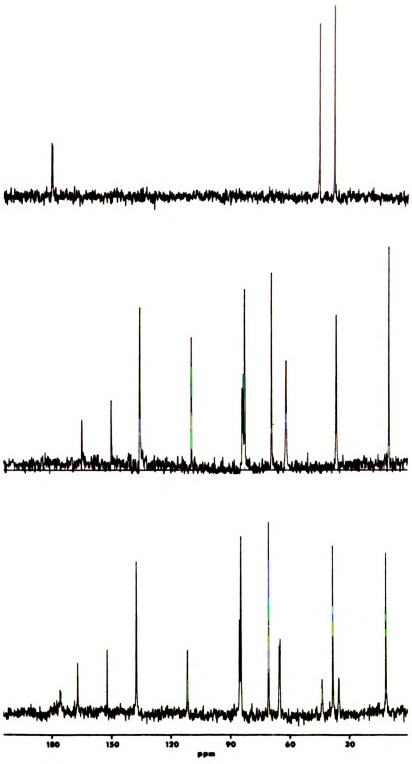

Figure 13

Similar results were also obtained in an activated poly d(A-T) polymerization reaction. Incorporation of [^{3}H]dAMP in the absence of dTTP was not enhanced by addition of increasing concentrations of dTMP-PA to reaction mixtures.

Table 5. Ability of the HVT-induced DNA polymerase to utilize dTMP-PA in an activated DNA polymerization reaction

dTMP-PA, μM	dTTP, μM	pmol [^{3}H]dCMP Incorporated
0	0	12.1
40	0	12.5
80	0	12.0
160	0	11.4
320	0	11.0
640	0	8.7
0	100	37.0

All reaction mixtures contained in 200 μl: 20 μM [^{3}H]dCTP (350 cpm per pmol), 100 μM each dATP, dGTP, 2 mM $MgCl_2$, 200 mM KCl, 50 mM Tris-HCl (pH 8), 1 mM dithiothreitol, 500 μg per ml bovine serum albumin, 200 μg per ml activated calf thymus DNA, HVT-induced DNA polymerase and the indicated concentrations of dTMP-PA or dTTP.

Deoxythymidine 5'Phosphophosphonoacetate as an Inhibitor of the HVT-Induced DNA Polymerization Reaction

With dNTP as variable substrate and activated DNA at a concentration of 200 μg per ml, dTMP-PA gave linear noncompetitive inhibition (Figure 14). Apparent inhibition constants, K_{ii} and K_{is}, were calculated to be 52 μM and 120 μM, respectively, from intercept and slope replots. If dTMP-PA could bind at the dNTP binding site of the DNA polymerase, it would be a competitive inhibitor of dTTP. This result was not observed.

Figure 14. Double reciprocal plots with the four dNTPs as variable substrate and dTMP-PA as inhibitor. Activated DNA was at 200 µg per ml. Deoxythymidine 5'phosphophosphonoacetate concentrations were: 0 (●), 20 µM (○) and 60 µM (Δ). Initial velocities were measured as pmol [^{3}H]dTMP incorporated into DNA per 30 min. Specific radioactivity of [^{3}H]dTTP was 1000 cpm per pmol.

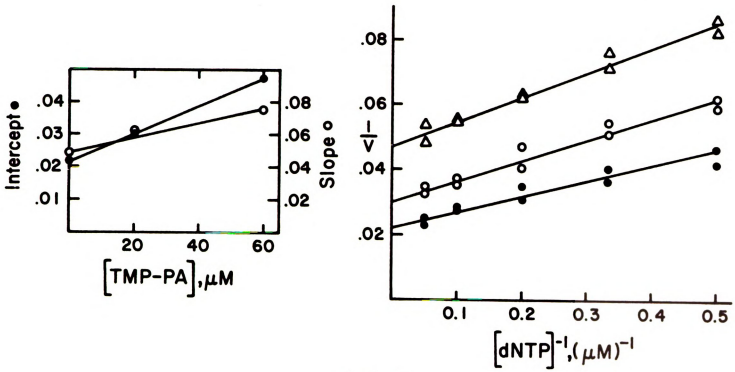
Intercept •
.04
.03
.02
.01
Slope ○
.08
.06
.04
.02
20
40
60
[TMP-PA], μM
1/V
.08
.06
.04
.02
0.1
0.2
0.3
0.4
0.5
[dNTP]⁻¹, (μM)⁻¹

Figure 14

The noncompetitive inhibition observed for dTMP-PA was probably due to a small amount of contaminating PA in the dTMP-PA preparation. Phosphonoacetate gave linear noncompetitive inhibition with dNTP as the variable substrate and activated DNA at a concentration of 200 µg per ml. Apparent inhibition constants, K_{ii} and K_{is}, were 1.5 µM and 1.0 µM, respectively. Therefore, a 1 to 3% molar contamination of the dTMP-PA preparation by phosphonoacetate would have been sufficient to produce the observed inhibition pattern. When dTMP-PA was rechromatographed on a DEAE-cellulose (HCO_3^-) column and the peak fraction was used in the same kind of inhibition experiment, linear noncompetitive inhibition was again observed. However, K_{ii} and K_{is} values had increased from 52 µM and 120 µM to 525 µM and 540 µM, respectively.

When dTTP was the variable substrate and dATP, dGTP, and dCTP were each 100 µM and activated DNA was 200 µg per ml, dTMP-PA was also a noncompetitive inhibitor.

Generation of Deoxythymidine 5'Phosphonoacetate in HVT-Induced DNA Polymerization Reactions Inhibited by Phosphonoacetate

The existence of dTMP-PA in polymerization reactions inhibited by phosphonoacetate was investigated using [^{3}H]phosphonoacetate of high specific activity. Reaction mixtures containing 2.7 µM [^{3}H]-phosphonoacetate (6000 cpm per pmol), 100 µM each of the four dNTPs and 200 µg per ml activated DNA were incubated in the presence and absence of HVT-induced DNA polymerase. After incubation the reaction mixtures were analyzed by Dowex-1 chromatography (Figure 15). For the reaction mixture which contained enzyme, a single peak of radioactivity corresponding to phosphonoacetate eluted from the column.

Figure 15. Analysis of the effect of [^{3}H]phosphonoacetate on HVT-induced DNA polymerization reactions by Dowex-1 (Cl^-) chromatography. Reaction mixtures (0.2 ml) contained 2.7 μM [^{3}H]phosphonoacetate (6000 cpm per pmol), 100 μM each dATP, dCTP, dGTP, dTTP, 200 μg per ml activated calf thymus DNA, 50 mM Tris-HCl (pH 8), 2 mM $MgCl_2$, 200 mM KCl, 1 mM dithiothreitol, 500 μg per ml bovine serum albumin and were incubated at 37° for 30 min in the presence or absence of HVT-induced DNA polymerase. Incubations were terminated by addition of EDTA to a final concentration of 20 mM. Reaction mixtures were diluted with water (2 ml) and applied to a Dowex-1 (Cl^-) column (0.6 x 10 cm) at 4°. The column was washed extensively with water and eluted with a 200 ml linear gradient of 0 to 0.2 M LiCl in 0.01 N HCl. Fractions of 2 ml were collected. One milliliter of alternate column fractions was added to 8 ml of a triton-toluene based scintillation fluid (1 l triton-X100, 2 l toluene, 16.5 g 2,5diphenyloxazole and 0.3 g 1,4bis[2-(4-methyl-5-phenyloxazolyl)]benzene) and was monitored for radioactivity using liquid scintillation spectrometry. Concentrations of LiCl were determined by conductivity measurements. The elution position of dTMP-PA was determined by Dowex-1 (Cl^-) chromatography of a reaction mixture containing 4 mM dTMP-PA, 2.7 μM [^{3}H]phosphonoacetate, 2 mM $MgCl_2$, 200 mM KCl and 50 mM Tris-HCl (pH 8). Recoveries from the Dowex-1 (Cl^-) column were 80% or better.

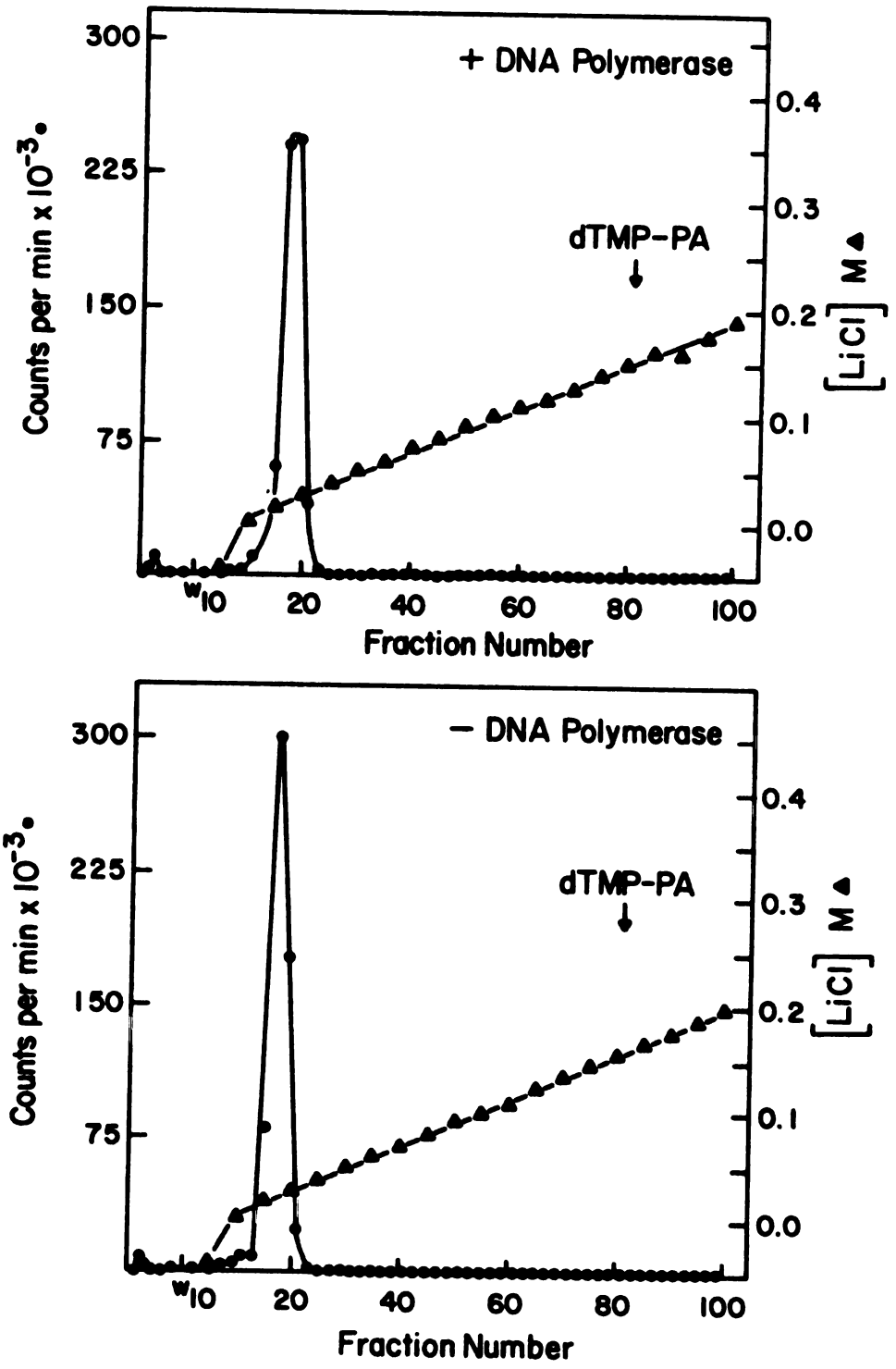
+ DNA Polymerase
dTMP-PA
Counts per min x 10^{-3}
[LiCl] M
Fraction Number
300
225
150
75
0.4
0.3
0.2
0.1
0.0
w10
20
40
60
80
100
− DNA Polymerase
dTMP-PA
Counts per min x 10^{-3}
[LiCl] M
Fraction Number

Figure 15

No radioactive material eluted from the column in the region where dTMP-PA eluted. A similar result was obtained for the control reaction mixture which contained no enzyme. The phosphonoacetate concentration used in this experiment was one which was used in the kinetic studies on phosphonoacetate inhibition. The DNA polymerization reaction was inhibited by 67%. This analysis could have detected an exchange reaction whose rate was 3% of the rate of the polymerization reaction. Thus, no evidence was obtained which demonstrated that deoxythymidine 5'phosphophosphonoacetate was generated during inhibition of the HVT-induced DNA polymerase by phosphonoacetate.

Characterization of a Phosphonoacetate-Resistant HVT-Induced DNA Polymerase

A phosphonoacetate resistant mutant of HVT (HVT_{PA}) was isolated by Dr. Lucy Lee (manuscript submitted for publication). The mutant virus replicates in the presence of 1.6 mM of phosphonoacetate whereas the replication of the wild type virus was completely inhibited by 0.3 mM. The mutant virus replicates as well as the wild type virus at 37°, but much less well at 41°. Since phosphonoacetate specifically inhibits the wild type HVT-induced DNA polymerase, it was of interest to examine the properties of the PA-resistant HVT-induced DNA polymerase.

Nuclear extracts from PA-resistant HVT-infected duck embryo fibroblasts were prepared and fractionated by phosphocellulose chromatography. The viral-induced DNA polymerase, HVT_{PA}-induced DNA polymerase, eluted from the phosphocellulose column as a single peak at about 0.35 M KCl. The peak DNA polymerase fractions were pooled and were used to define the properties of the enzyme.

The sensitivity of the HVT_{PA}-induced DNA polymerase to PA was determined (Figure 16). When added to the standard reaction mixture, 17 μM PA inhibited the HVT_{PA}-induced DNA polymerase by 50%; 1.2 μM PA inhibited the HVT_{WT}-induced DNA polymerase by 50%. The apparent inhibition constant (K_i) for PA of the HVT_{PA}-induced DNA polymerase was determined and compared with that for the HVT_{WT}-induced DNA polymerase (Table 6). The apparent inhibition constant for PA for the HVT_{PA}-induced DNA polymerase was about 10-fold greater than that for the HVT_{WT}-induced DNA polymerase.

Table 6. Kinetic constants of PA-resistant HVT-induced DNA polymerase and of wild-type HVT-induced DNA polymerase

Kinetic constant	HVT_{PA} DNA polymerase	HVT_{WT} DNA polymerase
$K_{i\ PA}$	18 ± 2 μM	1.4 ± 0.6 μM
$K_{i\ PP_i}$	2.5 ± 0.5 mM	1.3 ± 0.2 mM
K_{DNA}	7.2 ± 0.2 μg/ml	7.3 ± 0.2 μg/ml
K_{dNTP}	5.5 ± 0.6 μM	2.1 ± 0.5 μM

K_i values for phosphonoacetate (PA) and for pyrophosphate (PP_i) were determined as K_{ii} with dNTP as the variable substrate and with activated DNA at a concentration of 200 μg per ml. K_{DNA} and K_{dNTP} values were determined in initial velocity studies.

Evidence has been presented that PA inhibits the herpesvirus-induced DNA polymerase by binding at the pyrophosphate binding site of the enzyme. Therefore, an apparent inhibition constant for pyrophosphate for the HVT_{PA}-induced DNA polymerase was determined and compared with that of the HVT_{WT}-induced DNA polymerase (Table 6). $K_{i\ PP_i}$ for HVT_{PA}-induced DNA polymerase was about 2-fold greater

Figure 16. Inhibition of the HVT_{PA}-induced DNA polymerase (O) and the HVT_{WT}-induced DNA polymerase (●) by phosphonoacetate. V represents pmol [^{3}H]dTMP incorporated into DNA per 30 min at 37° in the absence of phosphonoacetate. V_i represents dTMP incorporation in the presence of phosphonoacetate. The phosphonoacetate concentration which inhibits the enzyme activity by 50% is that concentration for which V/V_i is 2.

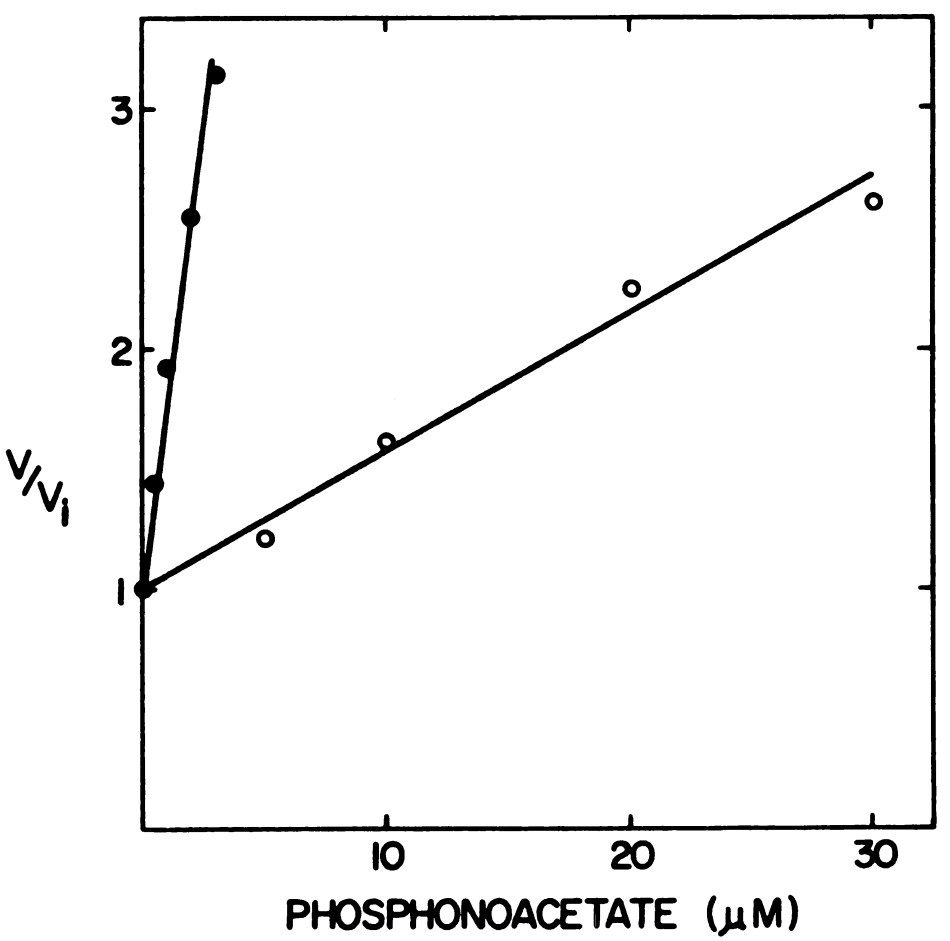

Figure 16

than $K_{i\ PP_i}$ for the wild type enzyme, indicating that the mutant enzyme has a decreased affinity for pyrophosphate.

Apparent Michaelis constants for activated DNA and dCTP were determined (Table 6). No difference in K_{DNA} for the two enzymes was seen. The apparent Michaelis constant for dNTP (K_{dNTP}) was about 2.5-fold greater for the HVT_{PA}-induced DNA polymerase than for the HVT_{WT}-induced DNA polymerase.

The decreased affinity of the HVT_{PA}-induced DNA polymerase for PA suggested that the structure of the enzyme had changed. A change in structure might result in a change in the temperature sensitivity of the enzyme. Therefore, the thermal inactivation of the HVT_{PA}-induced DNA polymerase and the HVT_{WT}-induced DNA polymerase was examined. As shown in Figure 17, the thermal inactivation of both enzymes followed first order reaction kinetics. The activity of the HVT_{PA}-induced DNA polymerase decayed with a half life of about 10 min. The activity of the HVT_{WT}-induced DNA polymerase decayed with a half life of about 17 min. The greater temperature sensitivity of the mutant enzyme as compared with the wild type enzyme was also seen in crude nuclear extracts.

Although HVT_{PA}-induced DNA polymerase and HVT_{WT}-induced DNA polymerase showed different sensitivities to PA and heat, other properties of the two enzymes were similar. Both enzymes eluted from phosphocellulose at about the same KCl concentration. Both exhibited similar optima for $MgCl_2$ and KCl. The sedimentation coefficient for both enzymes was 7.0 ± 0.2S as determined by glycerol gradient sedimentation velocity centrifugation in the presence of 200 mM KCl, 2 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8) (data not shown).

Figure 17. Thermal inactivation of the HVT_{PA}-induced DNA polymerase (O) and the HVT_{WT} DNA polymerase (●) at 52°. Aliquots of pooled phosphocellulose fractions of HVT-induced DNA polymerase were incubated at 52°. Incubation buffer contained 2 mg per ml bovine serum albumin, 0.5 mM dithiothreitol, 15 mM Tris-HCl (pH 8), 0.2 M KCl, and 50% glycerol (v/v). At the indicated time intervals, 10 µl aliquots of enzyme were removed to test tubes on ice. After 2 min on ice, DNA polymerase was assayed by addition of warmed reaction components to the test tubes. Incubation was at 37° for 30 min.

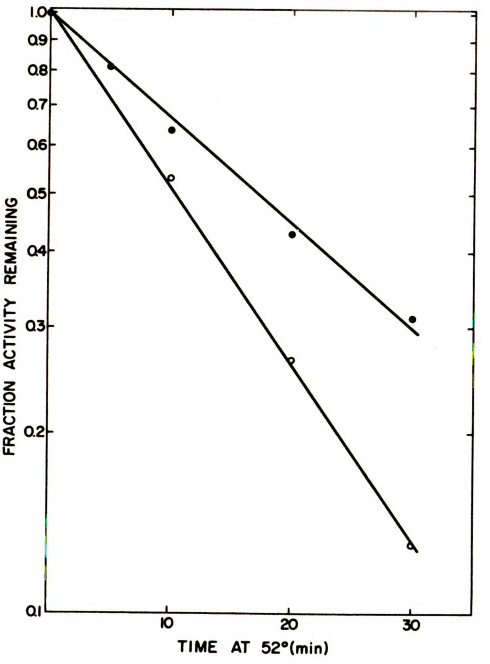
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
FRACTION ACTIVITY REMAINING
10
20
30
TIME AT 52°(min)

Figure 17

Presence of MDHV-Induced DNA Polymerase in the MSB-1 Lymphoblastoid Cell Line

The productive infection of DEF with MDHV results in the induction of a novel DNA polymerase. Tumors from chickens with Marek's disease carry the MDHV genome (8; Leinbach, unpublished results). Therefore, it is of interest to determine if the MDHV-induced DNA polymerase is present in these transformed lymphoid cells to replicate the MDHV genome. As a first approach to this problem, the MSB-1 lymphoblastoid cell line was examined for the presence of the MDHV-induced DNA polymerase.

The MSB-1 lymphoblastoid cell line is a continuous transformed cell line derived from a spleen tumor of a chicken infected with MDHV, strain BC-1 (23). It carries MDHV genomes (26; Leinbach, unpublished results). In 2-day-old cultures approximately 1 to 2% of the cell population produces viral antigens and virus particles. In cultures kept for seven days, 15 to 20% of the cells contain viral antigens and virus particles. Those cells spontaneously producing viral antigens and virus particles eventually degenerate and die, whereas other nonproducer cells actively grow and divide (25).

Nuclear extracts of MSB-1 6-day-old and 2-day-old cultures were examined by DEAE-cellulose chromatography for DNA polymerase activities. Because only small quantities of MSB-1 cells were available for this analysis, the MDHV-induced DNA polymerase was determined to be present in these cells if the following criteria were met: an activity identified by the MDHV-induced DNA polymerase assay eluted from DEAE-cellulose at about 0.1 M potassium phosphate and was inhibited by 50% when 1 to 3 μM phosphonoacetate was added to the MDHV-induced DNA polymerase assay.

The DNA polymerase activities from a nuclear extract of uninfected chicken embryo fibroblasts were fractionated by DEAE-cellulose chromatography to demonstrate that the MDHV-induced DNA polymerase activity was not present in chicken cells (Figure 18). The MDHV-induced DNA polymerase assay revealed no activity which eluted from the DEAE-cellulose column at about 0.1 M potassium phosphate.

When DNA polymerase activities from nuclear extracts of 6-day-old MSB-1 cells were examined by DEAE-cellulose chromatography, a major DNA polymerase activity was found by the MDHV-induced DNA polymerase assay which eluted from the column at about 0.1 M potassium phosphate (Figure 19, left panel). This activity (fractions 16-17) was inhibited by 50% when 3 μM phosphonoacetate was added to the MDHV-induced DNA polymerase reaction mixture. These results suggest that the MDHV-induced DNA polymerase is present in these 6-day-old MSB-1 cells. The level of activity of the MDHV-induced DNA polymerase in the nuclear extracts of these 6-day-old MSB-1 cells is only 2% of the level of activity of the MDHV-induced DNA polymerase in the nuclear extracts of MDHV-infected DEF, however (compare Figure 19 with Figure 2).

When DNA polymerase activities from nuclear extracts of 2-day-old MSB-1 cells were examined by DEAE-cellulose chromatography, only a minor DNA polymerase activity was found by the MDHV-induced DNA polymerase assay which eluted from the column at about 0.1 potassium phosphate (Figure 19, right panel). The peak fraction of this activity, fraction 16, was tested for sensitivity to phosphonoacetate. When 8 μM phosphonoacetate was added to MDHV-induced DNA polymerase reaction mixtures, 50% inhibition of the DNA polymerase activity was obtained. This sensitivity to phosphonoacetate was less than expected for the MDHV-induced DNA polymerase. This could be explained, however,

Figure 18. DEAE-cellulose chromatography of DNA polymerase activities from nuclear extracts of uninfected CEF. Nuclear extracts from 0.6 g wet weight of uninfected CEF were prepared and subjected to DEAE-cellulose chromatography as described in Materials and Methods. Samples (25 µl) of the fractions were assayed for DNA polymerase activities by the MDHV-induced DNA polymerase assay (●) and the chicken DNA polymerase assay (○). Potassium phosphate concentrations (—) were determined from conductivity measurements of alternate fractions. DNA polymerase activities were expressed as pmol [^{3}H]dTMP incorporated into DNA in 30 min per g wet weight of cells.

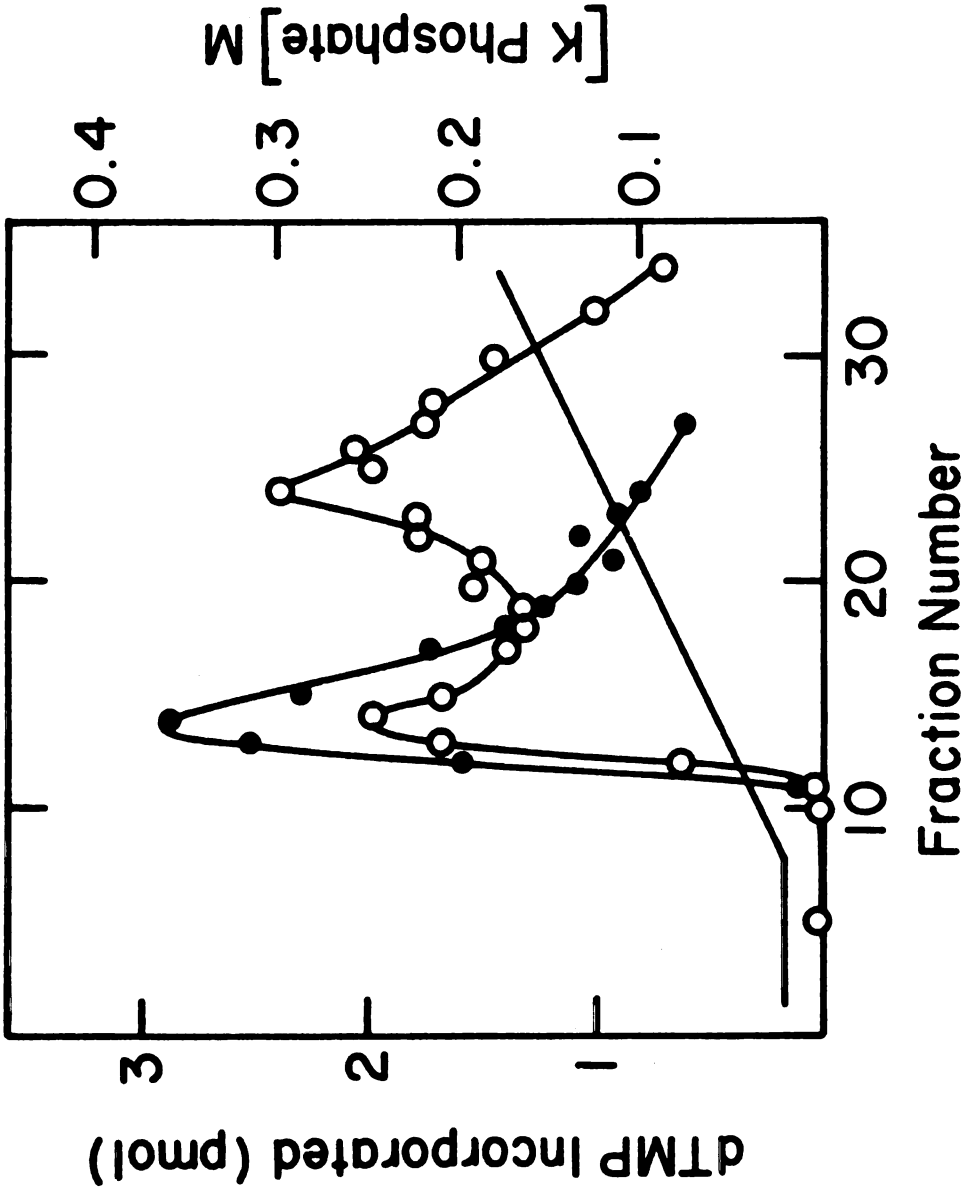
dTMP Incorporated (pmol)
1
2
3
Fraction Number
10
20
30
[K Phosphate] M
0.1
0.2
0.3
0.4

Figure 18

Figure 19. DEAE-cellulose chromatography of DNA polymerase activities from nuclear extracts of MSB-1 cells. Nuclear extracts from 0.8 g of 6-day-old MSB-1 cells, passages 38 and 39, were prepared and subjected to DEAE-cellulose chromatography as described in Materials and Methods (left panel). Nuclear extracts from 1.3 g of 2-day-old MSB-1 cells, passage 49, were prepared and subjected to DEAE-cellulose chromatography (right panel). Samples (25 µl) of the fractions were assayed for DNA polymerase activities by the MDHV-induced DNA polymerase assay (●) and the chicken DNA polymerase assay (○). Potassium phosphate concentrations (——) were determined from conductivity measurements of alternate fractions. DNA polymerase activities were expressed as pmol [^{3}H]dNMP incorporated into DNA in 30 min per g wet weight of cells.

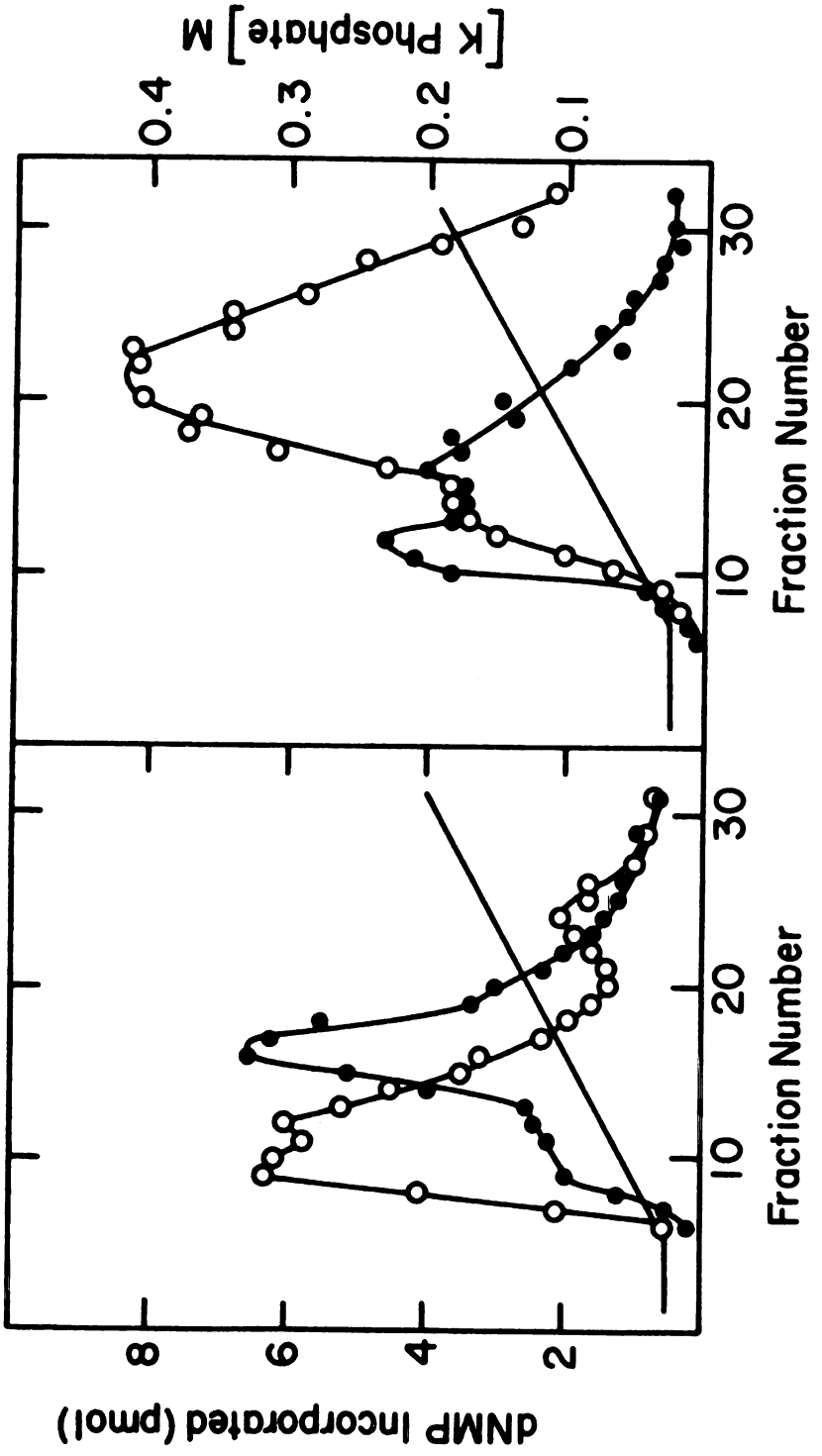
dNMP Incorporated (pmol)
2
4
6
8
10
20
30
Fraction Number
10
20
30
Fraction Number
0.1
0.2
0.3
0.4
[K Phosphate] M

Figure 19

if fraction 16 contained, in addition, the MDHV-induced DNA polymerase, a DNA polymerase with a substantially lower sensitivity to phosphonoacetate than that of the MDHV-induced DNA polymerase. In fact, when fraction 16 was assayed by the chicken DNA polymerase assay, 50% inhibition of this DNA polymerase activity was obtained when 40 μM phosphonoacetate was added to the reaction mixtures. This DNA polymerase activity probably existed in nuclear extracts from 2-day-old cultures and not in those from 6-day-old cultures because the physiology of the two cultures differed greatly. The 2-day-old cultures contained a greater number of viable cells than did the 6-day-old cultures. Therefore, the MDHV-induced DNA polymerase is probably also present in 2-day-old MSB-1 cells, although more conclusive evidence is needed to firmly establish this point.

DISCUSSION

Characterization of DNA Polymerases of Marek's Disease Herpesvirus and Herpesvirus of Turkeys

Infection of duck embryo fibroblasts by MDHV, strain BC-1, led to the induction of a novel DNA polymerase. Infection of duck embryo fibroblasts by HVT also led to the induction of a novel DNA polymerase. The properties of these two enzymes were very similar as might be expected since the two viruses are closely related. Both exhibited maximum activity in the presence of 1-2 mM $MgCl_2$ and 200-250 mM KCl. Both were inhibited by 50% in the presence of 1-3 M phosphonoacetate. Both were DNA-dependent DNA polymerases and had sedimentation coefficients in the presence of 0.25 KCl of about 7S.

The single major difference between these two enzymes was in their chromatographic behavior on DEAE-cellulose columns. The MDHV-induced DNA polymerase eluted as a single peak of activity at about 0.1 M potassium phosphate. The HVT-induced DNA polymerase eluted as two peaks of activity at about 0.08 M and 0.13 M potassium phosphate. These two HVT-induced DNA polymerase activities were structurally and catalytically identical so that their significance remains unexplained. If the HVT-induced DNA polymerase was chromatographed first on a phosphocellulose column and then on a DEAE-cellulose column, only a single DNA polymerase activity was found. This enzyme fraction was purified approximately 60-fold over the whole cell homogenate.

The MDHV- and HVT-induced DNA polymerases could be distinguished from the DNA polymerases of duck embryo fibroblasts by DEAE-cellulose chromatography, by Mg^{2+} optima, by KCl sensitivity and by phosphonoacetate sensitivity. DNA polymerase α of DEF eluted from DEAE-cellulose columns at about 0.14 M potassium phosphate, required 10 mM $MgCl_2$ for maximum activity, was inhibited by 80% in the presence of 200 mM KCl and was inhibited by 50% in the presence of 16 μM phosphonoacetate. DNA polymerase β of DEF did not adsorb to DEAE-cellulose, required 10 mM $MgCl_2$ for maximum activity, was inhibited by 10% in the presence of 200 mM KCl and was not inhibited by 200 μM phosphonoacetate (data not shown). All these properties are different from those of the MDHV- and HVT-induced DNA polymerases as described above. Moreover, the induced DNA polymerase activities in MDHV- and HVT-infected DEF were 10-40 times higher than the DNA polymerase activities in uninfected DEF.

The properties of the DNA polymerases induced by HVT and MDHV, strain BC-1, are very different from the properties of the DNA polymerase induced by MDHV, strain GA (66). This latter polymerase exhibited maximum activity in the presence of 10 mM $MgCl_2$, was totally inhibited by 100 mM $(NH_4)_2SO_4$, was insensitive to phosphonoacetate and had a sedimentation coefficient of 5.2S in the presence of 0.25 M KCl. It is now apparent, however, that this DNA polymerase was not a herpesvirus-induced DNA polymerase but a DNA polymerase of a contaminant of the MDHV-infected cells used to inoculate the DEF. When aureomycin was routinely added to the tissue culture medium of cultures of DEF infected with MDHV, strain GA, this DNA polymerase disappeared. When DNA polymerase activities from nuclear extracts of these cells were fractionated by phosphocellulose chromatography, and

were assayed with DNA polymerase reaction mixtures containing 2 mM $MgCl_2$ and 200 mM KCl, a DNA polymerase with properties similar to those of the HVT- and MDHV, strain BC-1-, induced DNA polymerases was found.

When HVT was grown in DEF in the presence of increasing concentrationsof phosphonoacetate, a phosphonoacetate-resistant mutant of HVT resulted. This mutant replicated as well as the wild type virus at 37° but not as well as the virus type virus at 41°. The DNA polymerase induced by the PA-resistant mutant had an apparent inhibition constant for phosphonoacetate, an apparent inhibition constant for pyrophosphate and an apparent Michaelis constant for dNTP of about 10-, 2-, and 2.5-fold, respectively, greater than the wild-type enzyme. *In vitro* thermal inactivation studies with the HVT_{PA}- and HVT_{WT}-induced DNA polymerases demonstrated that the HVT_{PA}-induced DNA polymerase was inactivated 1.7 times faster than the HVT_{WT}-induced DNA polymerase at 52°. Thus, a mutation in HVT resulted in an alteration in the structure of the viral-induced DNA polymerase. These studies suggest that the HVT-induced DNA polymerase is viral coded and that inhibition by phosphonoacetate occurs by direct interaction with the herpesvirus-induced DNA polymerase. Hay and Subak-Sharpe and Klein and Friedman-Kien have also reported PA-resistant mutants of HSV (79,111).

The properties of the MDHV- and HVT-induced DNA polymerase were similar to those of the HSV-induced DNA polymerase (64,75). All exhibited maximum activity in the presence of 1-2 mM $MgCl_2$. All were stimulated to similar extents in the presence of salt, although maximum stimulation was observed with different salts. The HSV-induced DNA polymerase was stimulated 25- to 50-fold in the presence of 150 mM K_2SO_4 and only 0.8 as well in the presence of 250 mM KCl.

The MDHV-induced DNA polymerase was stimulated 10- to 60-fold in the presence of 200 mM KCl and only 0.6 as well in the presence of 110 mM K_2SO_4. All were inhibited about 50% in the presence of 1-3 μM phosphonoacetate. All were high molecular weight DNA-dependent DNA polymerases. The molecular weight of the HSV-induced DNA polymerase was about 180,000-200,000 daltons. That of the MDHV- and HVT-induced DNA polymerases was about 130,000 daltons. Both the HSV-induced DNA polymerase and the HVT-induced DNA polymerase eluted from DEAE-cellulose columns in the same region of the gradient as two peaks of activity. There is evidence that both enzymes are viral coded (65,111). Therefore, it appears that general structural and catalytic properties exist for all herpesvirus-induced DNA polymerases. Nevertheless, the HVT-induced DNA polymerase was not inhibited by antiserum prepared against HSV-infected cells (data not shown) indicating that these enzymes are antigenically distinct.

Infection of DEF with MDHV results in a productive infection. Cellular necrosis occurs and viral antigens and virus particles are produced, although no cell-free infectious virus is produced. Such a condition also exists in a small percentage of the cell population of the MSB-1 lymphoblastoid cell line. In both cases MDHV DNA polymerase is induced. In MSB-1 cultures this induction is more readily apparent in cells which have been cultured for six days than in cells which had been cultured for two days. This is because the levels of host DNA polymerase activities are lower in the 6-day-old cells since fewer cells are viable. These results suggest that in order to determine if the MDHV DNA polymerase is required to replicate the resident MDHV genomes in tumor cells, a more specific, sensitive technique for the detection of the MDHV DNA polymerase is necessary.

Immunofluorescence using antibody prepared against the MDHV DNA polymerase would be such a technique.

The studies with Raji cells grown in the presence of phosphonoacetate suggest that a herpesvirus-induced DNA polymerase does not replicate the resident EBV genomes in these transformed cells (81,82). However, the existence of an EBV-induced DNA polymerase has not yet been demonstrated directly and direct evidence for or against the existence of a herpesvirus-induced DNA polymerase in tumor cells is most important.

Mechanism of Phosphonoacetate Inhibition of Herpesvirus-Induced DNA Polymerase

The purpose of this study was to elucidate the mechanism by which phosphonoacetate inhibits the HVT-induced DNA polymerase. This problem was approached using analysis by steady state kinetics. Phosphonoacetate was a competitive inhibitor of pyrophosphate in the dNTP-pyrophosphate exchange reaction. Multiple inhibition studies with phosphonoacetate and pyrophosphate showed that the two were mutually exclusive inhibitors. Therefore, phosphonoacetate inhibits by binding to the pyrophosphate binding site of the HVT-induced DNA polymerase. At this site phosphonoacetate could inhibit as a dead end inhibitor or as an alternate product inhibitor. As a dead end inhibitor phosphonoacetate could simply bind to and dissociate from the pyrophosphate binding site. As an alternate product inhibitor phosphonoacetate could reverse the DNA polymerization reaction. An attempt was made to determine which of these two mechanisms best described the mechanism of inhibition by phosphonoacetate. Rate equations were derived for each. The predictions which emerged were then tested with the data obtained from the phosphonoacetate inhibition studies.

The alternate product inhibition model is presented in Figure 20. In the presence of phosphonoacetate an alternate reaction pathway exists in addition to the basic polymerization pathway. Thus, as shown, phosphonoacetate binds to the $E^{DNA}(n+1)$ complex. It could then simply dissociate from the complex or it could remain associated with the complex and undergo reaction with the nucleotide at the 3'-end of the DNA primer chain to form the $E^{DNA}_{dNMP-PA}$ complex. This complex would in turn yield E^{DNA} and dNMP-PA, a nucleotide analogue of phosphonoacetate and an alternate substrate for the polymerization reaction. Thus, phosphonoacetate inhibition occurs because the $E^{DNA}(n+1)$ complex is diverted by phosphonoacetate from the main polymerization pathway into an alternate pathway.

The steady state rate equation for this mechanism is given by equation 1, where K_{DNA} and $K_{DNA'}$ are Michaelis constants for DNA as substrate and product, respectively; K_{iDNA} and $K_{iDNA'}$ are inhibition constants for DNA as substrate and product, respectively (see Appendix B for method of derivation).

Under the experimental conditions that were used for the phosphonoacetate inhibition experiments, the numerator of equation 1 is approximated by the $V_{1(DNA,DNTP)}(DNA)(dNTP)$ term. The reciprocal of equation 1 arranged with DNA as the variable substrate takes the form of equation 2. At low dNTP concentrations both the slope and intercept terms are a function of phosphonoacetate concentration and the inhibition pattern is noncompetitive. At high dNTP concentrations, however, the slope term is independent of phosphonoacetate concentration and the inhibition pattern is uncompetitive.

The experimental results are consistent with the predictions of equation 2. With activated DNA as the variable substrate and with

Figure 20. Alternate product inhibition model of phosphonoacetate inhibition of herpesvirus-induced DNA polymerase. The basic reaction mechanism in the absence of phosphonoacetate is a modified ordered bi bi mechanism (Appendices A and C).

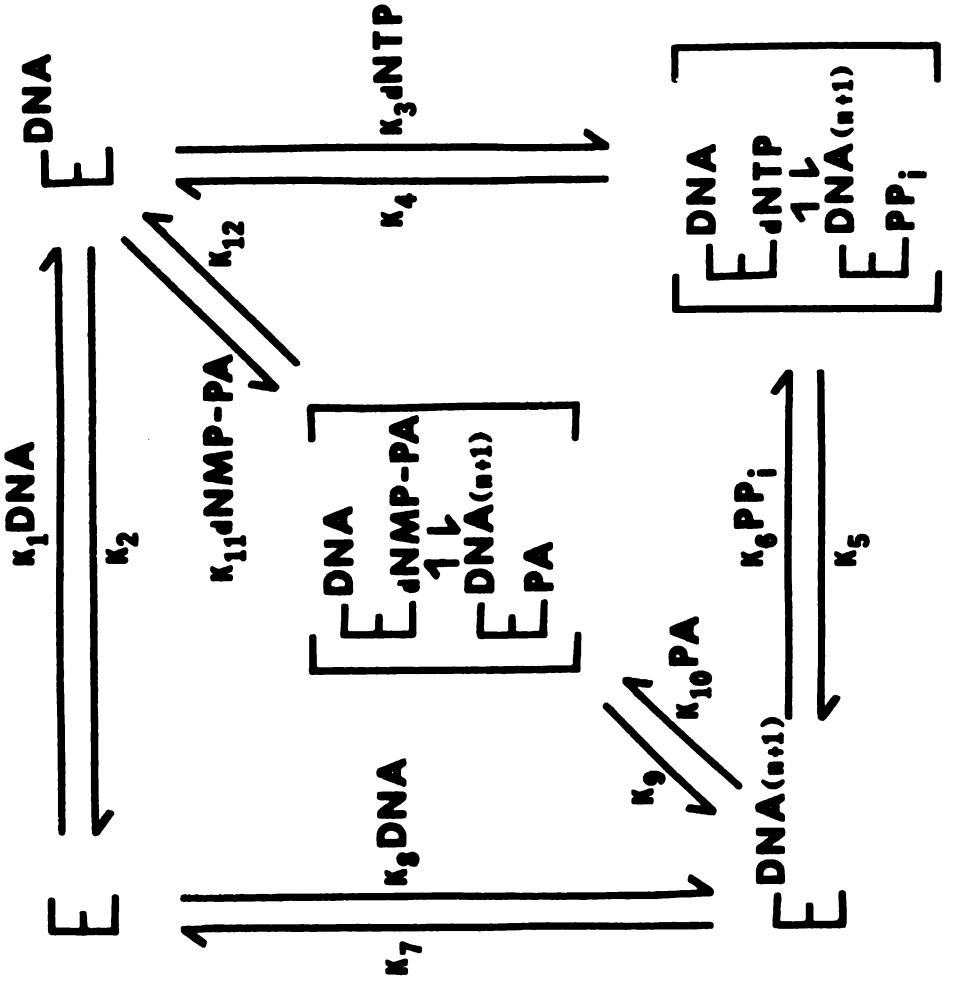
DNA
k_3dNTP
k_4
k_{12}
k_{11}dNMP-PA
DNA
dNMP-PA
1
DNA(n+1)
PA
DNA
dNTP
1
DNA(n+1)
PP_i
k_1DNA
k_2
k_6PP_i
k_5
k_9
k_{10}PA
k_8DNA
k_7
DNA(n+1)

Figure 20

$$\frac{v}{E_t} = \frac{V_{1(DNA,dNTP)}(DNA)(dNTP) - V_{2(DNA,PA)}\left(\frac{K_{dNTP}\,K_{iDNA}}{K_{iDNA'}\,K_{PA}}\right)(DNA)(PA)}{K_{iDNA}K_{dNTP} + K_{DNA}(dNTP) + K_{dNTP}\left(1+\frac{K_{iDNA}}{K_{iDNA'}}\right)(DNA) + \left(1+\frac{K_{DNA}}{K_{iDNA'}}\right)(DNA)(dNTP)} \quad (1)$$

$$+ \frac{K_{dNTP}\,K_{iDNA}}{K_{PA}\,K_{iDNA'}}\left[K_{DNA'} + \left(1+\frac{K_{DNA'}}{K_{iDNA}}\right)(DNA)\right](PA)$$

$$+ \left(\frac{1}{K_{iPA}} + \frac{K_{dNTP}\,K_{iDNA}}{K_{idNTP}\,K_{iDNA'}\,K_{PA}}\right)(DNA)(dNTP)(PA)$$

$$\frac{E_t}{v} = \frac{1}{V_{1(DNA,dNTP)}} \left[\left[K_{DNA} + \frac{K_{iDNA}\, K_{dNTP}}{(dNTP)} + \frac{K_{iDNA}\, K_{DNA'}\, K_{dNTP}\, (PA)}{K_{iDNA'}\, K_{PA}(dNTP)} \right] \frac{1}{(DNA)} \right. \tag{2}$$

$$+ \left(1 + \frac{K_{DNA}}{K_{iDNA'}}\right) + \frac{K_{dNTP}}{(dNTP)} \left(1 + \frac{K_{iDNA}}{K_{iDNA'}}\right) + \left[\frac{1}{K_{iPA}} + \frac{K_{iDNA}\, K_{dNTP}}{K_{iDNA'}\, K_{idNTP}\, K_{PA}} \right.$$

$$\left. \left. + \frac{K_{iDNA}\, K_{dNTP}}{K_{iDNA'}\, K_{PA}(dNTP)} \left(1 + \frac{K_{DNA'}}{K_{iDNA}}\right) \right] (PA) \right]$$

a dNTP concentration of 2.5 μM, noncompetitive inhibition was observed. With a dNTP concentration of 100 μM, the inhibition pattern was nearly uncompetitive.

If equation 2 is rearranged with dNTP as the variable substrate, the prediction emerges that the phosphonoacetate inhibition pattern will be noncompetitive regardless of the concentration of DNA. With dNTP as the variable substrate and with the activated DNA concentration at 200 μg per ml, the inhibition pattern was noncompetitive. Again these results are consistent with equation 2.

The kinetic data obtained from the phosphonoacetate inhibition studies of the HVT-induced DNA polymerization reaction are not consistent with phosphonoacetate as a dead end inhibitor. If phosphonoacetate complexes with the $E^{DNA}(n+1)$ enzyme form as a dead end inhibitor, the rate equation for this model predicts that with DNA as variable substrate inhibition by phosphonoacetate would be uncompetitive regardless of the concentration of dNTP (see Appendix B for method of derivation). This same prediction emerges from the rate equation which describes phosphonoacetate acting as a dead end inhibitor with both the $E^{DNA}(n+1)$ and E^{DNA} enzyme forms. If phosphonoacetate complexes with both the $E^{DNA}(n+1)$ and E enzyme forms, the rate equation predicts that with DNA as variable substrate noncompetitive inhibition would be observed regardless of dNTP concentration. Experimentally, when DNA was variable substrate, inhibition by phosphonoacetate was noncompetitive at 2.5 μM dNTP and nearly uncompetitive at 100 μM dNTP.

Thus, the phosphonoacetate inhibition studies of the HVT-induced DNA polymerization reaction favor the alternate product inhibition model. An attempt was made to provide direct evidence

for this model by demonstrating the existence of the alternate reaction pathway predicted by the model. Deoxythymidine 5'phosphophosphonoacetate was synthesized and assayed as an alternate substrate in the polymerization reaction.

As an alternate substrate, dTMP-PA should replace dTTP in HVT-induced DNA polymerization reaction mixtures. In an activated DNA-directed reaction incorporation of [^{3}H]dCMP in the presence of dATP and dGTP was not enhanced by dTMP-PA. As an alternate substrate, dTMP-PA should be a competitive inhibitor of dTTP. When [^{3}H]dTMP incorporation was measured with dNTP as variable substrate and activated DNA at a concentration of 200 µg per ml in the presence of changing fixed concentrations of dTMP-PA, a noncompetitive inhibition pattern resulted. Thus, dTMP-PA was not demonstrated to be a substrate for the HVT-induced DNA polymerase or to bind to the dNTP binding site of the enzyme.

As an alternate substrate, dTMP-PA should be generated in an exchange reaction during phosphonoacetate inhibition. When 2.7 µM [^{3}H]phosphonoacetate was present in standard reaction mixtures, no exchange of [^{3}H]phosphonoacetate into dTMP-PA was demonstrated by Dowex-1 (Cl^-) chromatography of reaction mixtures. Moreover, since phosphonoacetate was identified as the only radioactive component in the HVT-induced DNA polymerization reaction, it is unlikely that the phosphonoacetate analogue with an acyl phosphate linkage was generated in measurable quantity either. Such an acyl phosphate linkage might be unstable. However, no [^{3}H]dTMP was generated in standard reaction mixtures containing 2.5 µM phosphonoacetate during [^{3}H]dTMP incorporation into activated DNA.

Therefore, no direct evidence has been obtained in support of the alternate product inhibition model as the mechanism of phosphonoacetate inhibition. Nevertheless, the conclusion from the kinetic inhibition studies remains that phosphonoacetate inhibits the herpesvirus-induced DNA polymerase by binding to the pyrophosphate binding site of the enzyme. This binding probably involves a magnesium-phosphonoacetate species.

Phosphonoacetate may be considered as an analogue of pyrophosphate. The two exhibited similar inhibition patterns. However, quantitatively they appear to interact differently with the HVT-induced DNA polymerase. The apparent inhibition constants for phosphonoacetate were μM. The apparent inhibition constants for pyrophosphate were mM. The apparent affinity of the HVT-induced DNA polymerase for phosphonoacetate is, therefore, three orders of magnitude greater than the apparent affinity of the enzyme for pyrophosphate. In addition, pyrophosphate reverses the polymerization reaction in an exchange reaction whose rate is about 25% of the rate of the polymerization reaction. Phosphonoacetate may reverse the polymerization reaction in an exchange reaction whose rate is less than 3% of the rate of the polymerization reaction.

Therefore, while the kinetic studies on phosphonoacetate inhibition suggest that phosphonoacetate acts as an alternate product inhibitor by binding to the pyrophosphate binding site of the HVT-induced DNA polymerase, the alternate substrate, dNMP-PA, is released from the enzyme too slowly to be detected. It appears, then, that phosphonoacetate is an effective inhibitor of the HVT-induced DNA polymerase because of its tight binding to the enzyme.

Mao and Robishaw (77) published a partial kinetic analysis of the inhibition of HSV-induced DNA polymerase by phosphonoacetate at the same time this study was published (112). They obtained results similar to those reported here. With [^{3}H]dTTP as variable substrate and with saturating concentrations of dATP, dGTP, dCTP and activated DNA, inhibition by phosphonoacetate was noncompetitive. With activated DNA as variable substrate and saturating concentrations of the four dNTPs, inhibition by phosphonoacetate was uncompetitive. A study of phosphonoacetate inhibition with activated DNA as variable substrate and low dNTP concentrations was not reported. The similarity in the results of kinetic inhibition studies with the HVT- and HSV-induced DNA polymerases suggests that the mechanism of inhibition by phosphonoacetate is the same for all herpesvirus-induced DNA polymerases.

Phosphonoacetate appears to be a universal inhibitor of the DNA polymerases induced by the herpesviruses. Therefore, the pyrophosphate binding site for this group of DNA polymerases must be quite similar. The pyrophosphate binding sites of DNA polymerase α of ducks and HeLa cells and of the vaccinia virus-induced DNA polymerase (76) appear to be slightly related to this site. The pyrophosphate binding sites of DNA polymerase β of ducks and HeLa cells, of DNA polymerase γ of HeLa cells (76), of DNA polymerases of human WI-38 cells (73,75), of AMV and RSV reverse transcriptases (77), and of *E. coli* DNA polymerase I and *M. luteus* DNA polymerase (77), however, appear to be unrelated to this site on the herpesvirus-induced DNA polymerase.

The studies with the analogues of phosphonoacetate give some information about the structural requirements for binding at the

HVT-induced DNA polymerase pyrophosphate binding site. The results demonstrate that the carbon chain of the phosphono-compound must be of specific chain length, that a carboxyl or sulfo-group cannot substitute for the phosphono-group, that the methylene carbon cannot have bulky or charged substituents, and that an amino, a methylamino, an acetaldehyde, an acetonyl, an N-methylacetamide or a phosphono-group cannot substitute for the carboxyl group.

APPENDICES

APPENDIX A

NOMENCLATURE USED IN STEADY STATE KINETIC ANALYSES OF THE HVT-INDUCED DNA POLYMERIZATION REACTION

The following nomenclature as defined by Cleland (99,100) was used in determining the kinetic mechanism of the HVT-induced DNA polymerase and the kinetic mechanism for phosphonoacetate inhibition.

Steady State Kinetics - The study of enzyme catalyzed reaction rates in which reactant concentrations and their Michaelis constants greatly exceed enzyme concentration. Under these conditions all enzyme intermediates are at constant steady state levels.

Initial Velocity Study - Steady state kinetic experiment used to determine if a kinetic mechanism is sequential or ping pong. The concentration of one substrate, the variable substrate, is varied in the presence of fixed concentrations of the second substrate, the changing fixed substrate, in the absence of products. When a double reciprocal plot of variable substrate against velocity at different changing fixed substrate concentrations is made, a family of lines, an initial velocity pattern, results. Values of the vertical intercepts and of the slopes from the initial velocity pattern are then replotted against reciprocal changing fixed substrate concentration in order to identify a kinetic mechanism as sequential or ping pong. For a sequential mechanism values of both vertical intercepts and slopes increase as a linear function of the reciprocal changing fixed

substrate concentration. For a ping pong mechanism slope values from the initial velocity pattern are independent of reciprocal changing fixed substrate concentration while vertical intercept values increase as a linear function of reciprocal changing fixed substrate concentration. Kinetic constants for the substrates of the enzyme reaction and V_{max} are also determined from the replots.

Sequential Mechanism - Kinetic mechanism in which all substrates must add to the enzyme before any products are released.

Ping Pong Mechanism - Kinetic mechanism in which one or more products are released from the enzyme before all the substrates have added.

Ordered bi bi Mechanism - Sequential kinetic mechanism in which the two substrates add to the enzyme in an obligatory order before the two products are released from the enzyme in an obligatory order.

Modified Ordered bi bi Mechanism - Ordered bi bi mechanism in which a polymer is both a substrate and product in an enzyme reaction. The polymer product cannot be distinguished from the polymer substrate. Consequently, a reaction product is always present during enzyme reaction (113).

Inhibitor - A compound which slows down the rate of an enzyme catalyzed reaction by forming complexes with particular forms of the enzyme. This results in a decrease in the amount of enzyme available for participation in the normal reaction sequence.

Three types of inhibitors are defined by Cleland (100) and were considered in this study:

Product Inhibitor - A compound which is a product of a particular enzyme reaction which, when added in sufficient quantity to reaction mixtures, inhibits by forming reaction complexes for the reverse enzyme reaction.

Alternate Product Inhibitor - A compound which is a product of a particular enzyme reaction when an alternate substrate is used. It inhibits in the same way as a product inhibitor. The enzyme complexes formed, however, are those of an alternate reaction pathway.

Dead End Inhibitor - A compound which forms reversible enzyme complexes which cannot participate in the reaction pathway.

Three basic types of inhibition were observed in these studies. Each type is defined on the basis of the effect the inhibitor has on the slope and intercept of double reciprocal plots where variable substrate is plotted against velocity at different fixed inhibitor concentrations.

Linear Competitive Inhibition - Observed if the slopes of double reciprocal plots are a linear function of inhibitor concentration but the intercepts are independent of inhibitor concentration.

Linear Uncompetitive Inhibition - Observed if the intercepts of double reciprocal plots are a linear function of inhibitor concentration but the slopes are independent of inhibitor concentration.

Linear Noncompetitive Inhibition - Observed if both the intercepts and slopes of double reciprocal plots increase as a linear function of inhibitor concentration.

Inhibition constants may be determined from slope and intercept replots. The inhibition constant from the slope replot, K_{is}, is obtained from the X-intercept of the replot. The inhibition constant from the vertical intercept replot, K_{ii}, is obtained from the X-intercept of the replot. For complex kinetic mechanisms these inhibition constants are apparent inhibition constants. They are a combination of kinetic constants including the inhibition constant.

APPENDIX B

PROCEDURE FOR THE DERIVATION OF STEADY STATE RATE EQUATIONS

A steady state rate equation for a particular kinetic mechanism is derived by first diagramming a kinetic mechanism which illustrates the order of addition of substrates to and release of products from different enzyme forms and the rate constants involved. This mechanism is then translated into a rate equation composed of individual rate constants and reactant concentrations by the method of King and Altman (107,114). Finally, this rate equation is transformed into a rate equation expressed in terms of kinetic constants as described by Cleland (99). The complete steady state equation is then expressed in terms of one Michaelis constant and one inhibition constant for each reactant and two maximum velocity terms.

Steady state rate equations for dead end inhibitors are derived by multiplying certain terms in the denominator of the full rate equation by the factor $(1 + I/K_i)$ where K_i is the dissociation constant of the enzyme-inhibitor complex. The terms multiplied by this factor are those which represent the enzyme form with which the inhibitor combines. In certain cases the rate equation for dead end inhibition cannot be expressed entirely in kinetic constants because a kinetic constant is often a combination of rate constants representing different enzyme forms.

APPENDIX C

DETERMINATION OF THE KINETIC MECHANISM FOR THE HVT-INDUCED DNA POLYMERIZATION REACTION

A modified ordered bi bi mechanism was postulated for the HVT-induced DNA polymerization reaction. In this mechanism DNA would add to the enzyme as the first substrate, followed by dNTP as the second substrate. After addition of dNMP to the 3'-end of the DNA primer chain by phosphodiester bond formation, PP_i would be released from the enzyme as the first product, followed by DNA as the second product (Figure 20, outside square). The steady state rate equation for this mechanism is given by equation 4, where K_{DNA} and $K_{DNA'}$ are Michaelis constants for DNA as substrate and product, respectively, and K_{iDNA} and $K_{iDNA'}$ are inhibition constants for DNA as substrate and product, respectively. The validity of this equation for the HVT-induced DNA polymerization reaction was tested by initial velocity studies and pyrophosphate product inhibition studies.

In an initial velocity study, initial velocities in the absence of pyrophosphate are measured. Therefore, the negative velocity term in the numerator of equation 4 and all terms containing (PP_i) in the denominator of equation 4 can be eliminated. The reciprocal of this simplified equation with dNTP as variable substrate and activated DNA as changing fixed substrate takes the form of equation 5. This equation predicts that the slope values and the intercept values of this initial velocity pattern should be linear functions of reciprocal

$$\frac{V}{E_t} = \frac{V_{1(DNA,dNTP)}(DNA)(dNTP) - V_{2(DNA,PP_i)}\left(\frac{K_{dNTP}\,K_{iDNA}}{K_{iDNA'}\,K_{PP_i}}\right)(DNA)\,(PP_i)}{K_{iDNA}K_{dNTP} + K_{DNA}(dNTP) + K_{dNTP}\left(1 + \frac{K_{iDNA}}{K_{iDNA'}}\right)(DNA) + \left(1 + \frac{K_{DNA}}{K_{iDNA'}}\right)(DNA)}$$

$$(dNTP) + \frac{K_{dNTP}\,K_{iDNA}}{K_{PP_i}\,K_{iDNA'}}\left[K_{DNA'} + \left(1 + \frac{K_{DNA'}}{K_{iDNA}}\right)(DNA)\right](PP_i) \qquad (4)$$

$$+ \left(\frac{1}{K_{iPP_i}} + \frac{K_{dNTP}\,K_{iDNA}}{K_{idNTP}\,K_{iDNA'}\,K_{PP_i}}\right)(DNA)(dNTP)(PP_i)$$

$$\frac{E_t}{V} = \frac{K_{iDNA}K_{dNTP}}{V_{1(DNA,dNTP)}(DNA)} + \frac{K_{dNTP}}{V_{1(DNA,dNTP)}}\left(1 + \frac{K_{iDNA}}{K_{iDNA'}}\right)\frac{1}{(dNTP)} + \frac{1}{V_{1(DNA,dNTP)}}\left(\frac{K_{DNA}}{(DNA)} + \left(1 + \frac{K_{DNA}}{K_{iDNA'}}\right)\right) \quad (5)$$

DNA concentration. These results were observed (Figure 6). Therefore, the HVT-induced DNA polymerization reaction is consistent with a sequential mechanism as would be expected for a modified ordered bi bi reaction.

Kinetic constants for dNTP and activated DNA were evaluated using equation 5 and the slope and intercept replots of Figure 6. For these evaluations it was necessary to assume that $K_{iDNA'}$ was very large; $K_{iDNA'}$ is defined as K_7/K_8 (Figure 20) so that in this initial velocity study this assumption would be valid. The values of these kinetic constants are given in Table 7. The kinetic constants calculated when dNTP was variable substrate and activated DNA was changing

Table 7. Kinetic constants for HVT-induced DNA polymerase

	Variable Substrate	dNTP	Activated DNA
Kinetic constant	Changing Fixed Substrate	Activated DNA	dNTP
$V_{1(DNA,dNTP)}$		36.5 pmol per 30 min	37.7 pmol per 30 min
K_{dNTP}		2.1 μM	2.1 μM
K_{DNA}		6.9 μg per ml	7.2 μg per ml
K_{iDNA}		2.1 μg per ml	2.6 μg per ml

Kinetic constants were evaluated using equation 5 and slope and intercept replots of initial velocity patterns with variable substrate and changing fixed substrate as indicated.

fixed substrate (Figure 6) are in good agreement with those calculated when activated DNA was variable substrate and dNTP was changing fixed substrate (data not shown).

Pyrophosphate product inhibition experiments were used to determine if the HVT-induced DNA polymerization reaction did fit the full steady rate equation for the modified ordered bi bi mechanism (equation 4). Under the experimental conditions that were used, the numerator of equation 4 is approximated by the term $V_{1(DNA,dNTP)}(DNA)(dNTP)$. The reciprocal of equation 4 arranged with DNA as the variable substrate takes the form of equation 6. At low concentrations of dNTP, both the slope and the intercept terms are a function of pyrophosphate concentrations and the inhibition pattern is noncompetitive. At high concentrations of dNTP the slope term is independent of pyrophosphate concentration and the inhibition pattern is uncompetitive.

These predictions were satisfied experimentally. When dNTP levels were 2.5 μM a noncompetitive inhibition pattern was observed. When dNTP levels were 20 μM an uncompetitive inhibition pattern was observed (p. 72).

If equation 6 is rearranged with dNTP as variable substrate, the prediction emerges that the pyrophosphate inhibition pattern would be noncompetitive regardless of DNA concentration. With dNTP as the variable substrate and activated DNA at a concentration of 200 μg per ml, the inhibition pattern was noncompetitive as predicted (p. 72).

Therefore, the results from initial velocity experiments and pyrophosphate inhibition experiments are consistent with a modified ordered bi bi kinetic mechanism for the HVT-induced DNA polymerization reaction. Evidence for this same kinetic mechanism has also been presented for *E. coli* DNA polymerase I (108).

$$\frac{E_t}{V} = \frac{1}{V_{1(DNA,dNTP)}} \left\{ \left[K_{DNA} + \frac{K_{iDNA}\, K_{dNTP}}{(dNTP)} + \frac{K_{iDNA}\, K_{DNA'}\, K_{dNTP}\, (PP_i)}{K_{iDNA'}\, K_{PP_i}\, (dNTP)} \right] \frac{1}{(DNA)} \right.$$

$$+ \left(1 + \frac{K_{DNA}}{K_{iDNA'}}\right) + \frac{K_{dNTP}}{(dNTP)} \left(1 + \frac{K_{iDNA}}{K_{iDNA'}}\right) + \left[\frac{1}{K_{iPP_i}} + \frac{K_{iDNA}\, K_{dNTP}}{K_{iDNA'}\, K_{idNTP}\, K_{PP_i}} \right. \tag{6}$$

$$\left. \left. + \frac{K_{iDNA}\, K_{dNTP}}{K_{iDNA'}\, K_{PP_i}\, (dNTP)} \left(1 + \frac{K_{DNA'}}{K_{iDNA}}\right) \right] (PP_i) \right\}$$

APPENDIX D

MULTIPLE INHIBITION ANALYSIS TO DETERMINE THAT TWO INHIBITORS ARE MUTUALLY EXCLUSIVE

Two inhibitors are mutually exclusive if, when the concentration of inhibitor$_1$ is varied in the presence of changing fixed concentrations of inhibitor$_2$, a plot of 1/v versus inhibitor$_1$ concentration yields a series of parallel lines. In order to understand the validity of this statement, the circumstances under which a series of parallel lines arises from plots of a rate equation must be defined. Then a rate equation which describes phosphonoacetate and pyrophosphate as both acting at the pyrophosphate binding site of the DNA polymerase will be inspected.

In general, a steady state kinetic experiment involves independent components (variable substrate, variable inhibitor) and changing fixed components (changing fixed substrate in initial velocity studies or changing fixed inhibitor in inhibition studies). When values of 1/v are plotted against values of reciprocal variable substrate concentrations or variable inhibitor concentrations, a series of lines is obtained as a result of the different changing fixed component concentrations used in the experiment. These lines are parallel if the slope term of the equation describing the relationship between 1/v and the independent component does not contain changing fixed component concentration terms.

For the HVT-induced DNA polymerization reaction, evidence exists that phosphonoacetate and pyrophosphate are mutually exclusive inhibitors (Figure 10). The kinetic model which best describes this fact is the alternate product inhibition model (Figure 20). The rate equation which describes the alternate product inhibition model when both phosphonoacetate and pyrophosphate are present will be inspected. Because terms for both phosphonoacetate and pyrophosphate must be considered in the rate equation, it will be very complex. Therefore, the rate equation will be written in "coefficient" form: for each term in the rate equation, the constants are lumped together and are identified as the coef of the reactant concentration factor in that term in the denominator and as num_1 or num_2 in the numerator (99).

Using this simplification, the rate equation appropriate for a 1/v against phosphonoacetate concentration plot (Figure 10) is given by equation 7. The slope term of this equation contains no (PP_i) terms, where pyrophosphate was the changing fixed inhibitor in this experiment. Therefore, equation 7, which describes the fact that phosphonoacetate and pyrophosphate both act at the pyrophosphate binding site of the DNA polymerase, predicts that a 1/v against phosphonoacetate concentration plot will be a series of parallel lines as was observed.

$$\frac{E_t}{V} = \frac{1}{num_1}\left[\text{coef DNA,dNTP,PA} + \text{coef DNA',dNTP,PA} + \frac{\text{coef DNA,PA}}{(\text{dNTP})} + \frac{\text{coef PA}}{(\text{dNTP})(\text{DNA})} + \frac{\text{coef DNA',PA}}{(\text{dNTP})}\right)(\text{PA})$$

$$+ \left(\text{coef DNA,dNTP,PP}_i + \text{coef DNA',dNTP,PP}_i + \frac{\text{coef DNA,PP}_i}{(\text{dNTP})} + \frac{\text{coef DNA',PP}_i}{(\text{dNTP})}\right.$$

(7)

$$+ \frac{\text{coef PP}_i}{(\text{dNTP})(\text{DNA})}(\text{PP}_i) + \text{coef DNA,dNTP} + \text{coef DNA',dNTP} + \frac{\text{coef DNA}}{(\text{dNTP})} + \frac{\text{coef DNA'}}{(\text{dNTP})}$$

$$\left. + \frac{\text{coef dNTP}}{(\text{DNA})} + \frac{\text{const}}{(\text{DNA})(\text{dNTP})}\right]$$

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