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INHIBITION OF HERPESVIRUS REPLICATION, HERPESVIRUS-INDUCED DNA POLYMERASE, AND RETROVIRUS REVERSE TRANSCRIPTASE BY PHOSPHONOACETIC ACID AND PHOSPHONOFORMIC ACID

presented by

JOHN MARSHALL RENO

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Major professor

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AN ABSTRACT OF

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
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Department of Biochemistry

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Phosphonoformic acid was effective in treating herpesvirus infections in animal model systems. When mice or guinea pigs were inoculated intravaginally with herpes simplex type 2 and treated intravaginally with 10% trisodium phosphonoformate beginning at times up to 24 hours post infection, a significant reduction in virus titer was obtained. Phosphonoformic acid was not effective in mice inoculated intraperitoneally or intracerebrally with herpes simplex virus or intraperitoneally with murine cytomegalovirus.

The antiviral effects of phosphonoacetic acid and phosphonoformic acid are not entirely specific. Both compounds were shown to inhibit the DNA polymerase α from HeLa, Wi-38, and phytohemagglutin-stimulated lymphocytes, all human cells, and from Chinese hamster ovary cells and calf thymus. The apparent inhibition constants were about 30 μM. The mechanism of inhibition was analogous to that of the herpesvirus-induced DNA polymerase. The DNA polymerases β and γ were not sensitive to either phosphonoacetic acid or phosphonoformic acid.

Phosphonoformic acid, but not phosphonoacetic acid, was a potent inhibitor of DNA synthesis catalyzed by reverse transcriptase from avian myeloblastosis virus, Rous sarcoma virus, Moloney murine leukemia virus, and feline leukemia virus with poly(A)·oligo(dT) or activated DNA as substrate. With Mg^{2+} as the cofactor, 50% inhibition in the rate of DNA polymerization was observed with about 10 μM phosphonoformic acid. With Mn^{2+} as the cofactor, 50% inhibition was obtained with about 0.5 μM phosphonoformic acid. The endogenous reactions or reactions in which purified viral RNA was the substrate

were much less sensitive to phosphonoformate. The RNase H activity of AMV reverse transcriptase was not inhibited by either phosphonate in the presence of either Mg^{2+} or Mn^{2+}. Inhibition studies of both the DNA polymerization and the deoxyribonucleoside triphosphate-pyrophosphate exchange reaction were carried out. The results were different from those obtained with the herpesvirus-induced DNA polymerase and DNA polymerase α and are consistent with phosphonoformic acid interacting with the reverse transcriptase at the pyrophosphate binding site but functioning as a dead-end inhibitor.

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To

Kristine

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GENERAL INTRODUCTION

Herpesviruses, a large family of DNA containing viruses are the etiological agents for many human infectious diseases: herpes simplex type 1 causes skin and eye infections as well as fever blisters, herpes simplex type 2 causes genital herpes and is associated with a type of cervical carcinoma, varicella-zoster virus causes shingles in adults and chicken pox in children, human cytomegalovirus is associated with fetal damage and is a major cause of birth defects, Epstein-Barr virus causes infectious mononucleosis and is associated with Burkitt's lymphoma. There are also a number of herpesviruses that are pathogenic in animals: Marek's disease virus causes tumors in chickens and has been a serious cause of mortality in commercial poultry, pseudorabies virus is the causative agent of mad itch in swine and cattle, and host specific cytomegaloviruses infect many animals. Clearly the need exists for specific agents which have the potential for controlling and perhaps eliminating herpesvirus infections.

Along with their potential therapeutic value, inhibitors of replication are important for their ability to complement conditional mutants in the dissection of the complex events involved in nucleic acid synthesis. A much more efficient use of these inhibitors can be made if the target protein and the mechanism of

inhibition is known. Also the pharmacological factors and toxicity are more easily handled.

Following productive infection, herpesviruses induce the synthesis of a herpesvirus specific DNA polymerase. Because virus infected cells contain this unique enzyme and other proteins essential for virus reproduction, it should be possible to inhibit virus replication specifically. A compound, phosphonoacetic acid, is now known to specifically inhibit the herpesvirus-induced DNA polymerase. Although its efficacy as an antiviral has been demonstrated in many animal model systems, it has toxicity problems and its clinical usefulness is in doubt. Results contained in this thesis on investigations into the mechanism of inhibition showed that phosphonoacetic acid is acting as a pyrophosphate analog and thus provided enough information form the development of another effective antiherpesvirus compound, phosphoformic acid. Phosphonoformic acid may be of sufficiently different chemistry that it would be less toxic than phosphoacetic acid and therefore clinically useful.

Phosphoformic acid has also been discovered to be an inhibitor of the retrovirus reverse transcriptase. Retroviruses are RNA containing viruses but their intracellular state is as an integrated DNA. There are many different retroviruses and many cause cancer in animals. The retrovirus DNA polymerase, also called reverse transcriptase, is necessary for transcription of viral RNA into DNA from which progeny virus are made.

This thesis is organized in a series of articles that either have been published or will be submitted for publication.

Much of this work has been the result of several collaborations and will therefore be presented as appendices. Independent research will be presented in the body of the thesis as two articles, each with its own Abstract, Introduction, Methods, Results, Discussion, and References sections. It begins with a literature review in which much of the current information on phosphonoacetic acid is summarized. A complete review of the literature on phosphonoformic acid is also presented.

The antiherpesvirus effect of phosphonoacetic acid was initially discovered by Abbott Laboratories. Investigations into the mechanisms of inhibition of the herpesvirus-induced DNA polymerase showed that phosphonoacetic acid was interacting with the enzyme at its pyrophosphate binding site. This work is presented as Appendix A in the form of a reprint from the journal in which it was published. Another analog of pyrophosphate, phosphonoformic acid, was found to be a potent antiherpesvirus agent. Its synthesis, characterization, and mechanism of inhibition of the herpesvirus-induced DNA polymerase are contained in Article 1, also in the form of a reprint. After showing the effectiveness of phosphonoformic acid in cell culture infections with herpesviruses, its efficacy in animal model systems was tested. The results of this collaborative effort are presented in reprint form in Appendix B. The eukaryotic host cells contain three DNA polymerases designated α, β, and γ. Since phosphonoacetic acid and phosphonoformic acid could potentially interact with these enzymes in an analogous manner to the herpesvirus-induced DNA polymerase and be cytotoxic, a study of the effects

of these two drugs on the host cell DNA polymerases was carried out. The results are presented in reprint form in Appendix C.

Phosphosphonoformic acid and not phosphonoacetic acid was then discovered to be an inhibitor of reverse transcriptase. The results of a detailed study into the scope and mechanism of inhibition are presented in Article 2. This report will be submitted for publication. A preliminary report was submitted to the American Society of Biological Chemists for presentation at the 1980 meetings.

Dr. Lucy F. Lee was included as a coauthor on Article 1 in recognition of her assistance with some of the experiments. Dr. Hsing-Jien Kung, Assistant Professor, was included as a coauthor on Article 2 in appreciation for providing the AMV reverse transcriptase. Also, it is hoped that Dr. Kung will continue studying the biology of this problem.

LITERATURE REVIEW

Phosphonoacetic Acid

Since its efficacy as an antiviral agent was discovered in 1973, the literature on phosphonoacetic acid has burgeoned and has been reviewed several times (1-4). A comprehensive review by Boezi has recently been published (5). Therefore only a succinct review of the literature, for the purpose of introduction, will be presented here. This review also will include reports receiving little attention in past reviews as well as a survey of the newest literature. Phosphonoacetic acid will also be referred to as phosphonoacetate, its completely deprotonated salt.

Chemistry

Phosphonoacetic acid (Figure 1), an organophosphorus compound, was first synthesized by Nylen in 1924 (6). It is a white solid of molecular weight 140.03 and melting point of 142-143°C. The triacid has pKa values of 2.0 (P-OH), 5.11 (COOH), and 8.69 (P-OH) at zero ionic strength (7). Phosphonoacetic acid also forms stable complexes with a variety of divalent and trivalent cations. It is used in the extraction of rare earths (8) and its complexation with Am^{3+}, Cm^{3+}, and Pm^{3+} ions has been studied (9). Because of its biological importance, complexes of phosphonoacetic acid with Mg^{2+}, Ca^{2+}, Cu^{2+}, and Zn^{2+} have been investigated (10, P-H. Heubel and

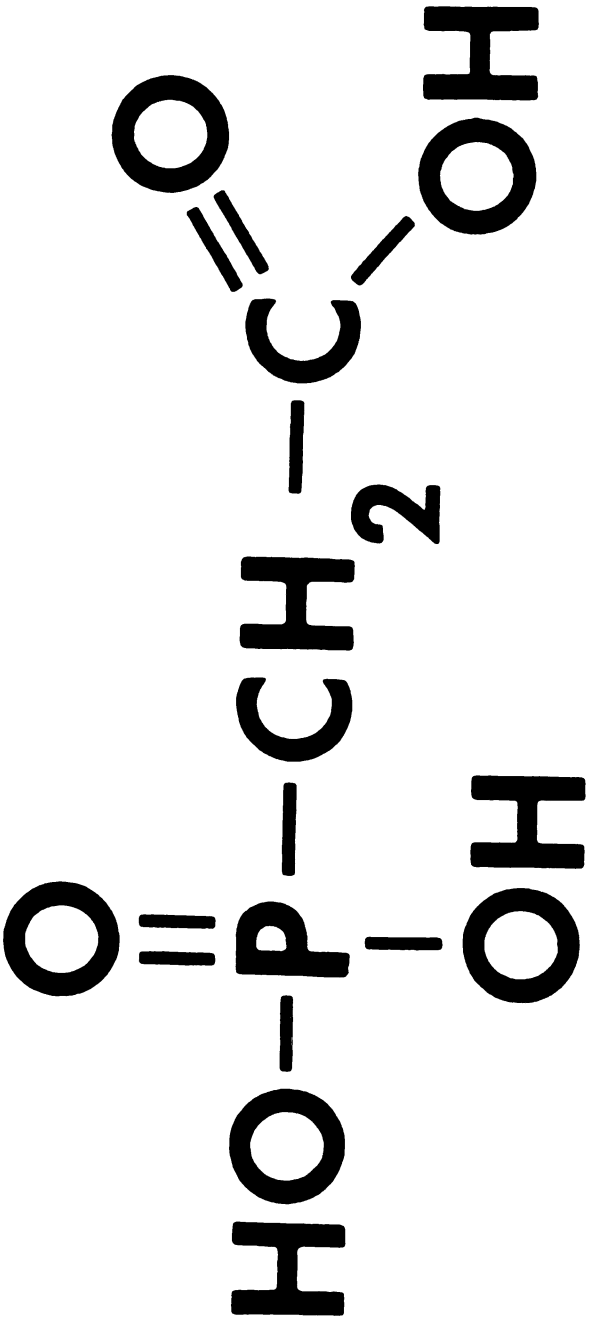

Figure 1.--Structure of Phosphonoacetic Acid.

A. Popov, personal communication). It forms 1:1 complexes with magnesium with log K_f values of 3 and 5.6 for the mono- and deprotonated forms, respectively.

Industrial Applications

The industrial uses of phosphonoacetate are numerous and it has been patented for widely different purposes. It has been used as a flame retardant in thermoplastics (11), as a corrosion inhibitor in boilers and water cooling systems (12), and to prevent the formation of dark spots on photographic plates due to iron and rust (13).

Its biological applications are also varied. In addition to its potential use as an anti-herpesvirus agent, phosphonoacetate has been proposed for use as an insecticide (14), as a herbicide, and as a plant growth regulator (15). It has also been proposed as a treatment for warts (16), and phosphonoacyl proline has been patented for use as a hypotensive (17).

Phosphonoacetate and Herpesvirus Infections

Tissue Culture Studies with Phosphonoacetate

Phosphonoacetate was first discovered to have antiviral properties by Abbott Laboratories in 1973 (18). Using a random testing of compounds with a tissue culture screen revealed that phosphonoacetate consistently inhibited herpes simplex virus at a concentration of 0.5 mM. It was effective in reducing the lesions and mortality of herpes simplex virus skin infections in mice and eye infections in rabbits. Herpes simplex virus infection of

human Wi-38 cells results in cell destruction; however in the presence of 0.5 mM phosphonoacetate the infected cells could not be distinguished from uninfected cells (19). Biochemical investigations revealed that phosphonoacetate had no effect on RNA or protein synthesis but was specific in inhibiting viral DNA synthesis. The herpes virus induced DNA polymerase was implicated as the target protein. Since these initial studies by Abbott Laboratories, reports from a large number of laboratories have confirmed and extended these results on the antiviral properties of phosphonoacetate.

In tissue culture studies it has been shown that phosphonoacetate is an effective inhibitor of the replication of all herpesviruses examined and that the inhibition is specific for viral DNA synthesis. A growing contention is that inhibition by phosphonoacetate is a new characteristic of the herpesvirus group. Those herpesviruses studied were: human, murine, and simian cytomegaloviruses (20, 21, 22), equine abortion herpesvirus (1), herpesvirus of turkeys (23), herpesvirus saimiri (24), Marek's disease herpesvirus, owl herpesvirus (23), varicella-zoster virus (25), and the Epstien-Barr virus (5). The replication of each of these herpesviruses was consistently and completely repressed by concentrations of phosphonoacetate of 0.5 mM or less.

The channel catfish herpesvirus is an exception in that to inhibit its replication in tissue culture some 10-20 times more phosphonoacetate is required (26). The virus only grows in cold blooded catfish cells which are also 10-20 times more resistant to phosphonoacetate than warm blooded cells. Further investigations are required

to determine if the virus itself is responsible for the decreased sensitivity.

Phosphonoacetate is clearly an antiviral agent selective for herpesviruses. It does not inhibit the replication of the DNA containing viruses simian virus -40 and human adenovirus -12. Insensitive RNA containing viruses are poliovirus, rhinovirus, and measles virus (5). Vaccinia virus is inhibited by phosphonoacetate but much higher concentrations are required (1).

Effect on Uninfected Cells in Tissue Culture

At concentrations of 0.2-0.5 mM at which it is an effective inhibitor of herpesvirus replication, phosphonoacetate induces no obvious morphological changes and there is no inhibition of cellular DNA, RNA, or protein synthesis (1, 5). At higher concentrations in the range 1-5 mM, phosphonoacetate is cytotoxic. Cell growth is arrested and cellular macromolecule synthesis is inhibited.

Effect of Phosphonoacetate on the Herpesvirus-Induced DNA Polymerase

Mao et al. (27) first observed that phosphonoacetate specifically inhibits the herpesvirus-induced DNA polymerase. When assayed in the presence of 1-2 μM phosphonoacetate, enzyme activity was reduced by 50%. All herpesvirus-induced DNA polymerases are sensitive to phosphonoacetate at this level (5).

Studies of phosphonoacetate resistant mutants of herpesvirus provided conclusive evidence that the inhibition of herpesvirus replication in cell culture is through a specific effect on the

herpesvirus-induced DNA polymerase (1, 5). The DNA polymerase induced by mutant viruses are much less sensitive to phosphonoacetate inhibition than is the polymerase induced by the wild type virus. Many of these mutants are temperature sensitive and the isolated DNA polymerases are also temperature sensitive. Revertants of the mutant viruses and their DNA polymerases are neither temperature sensitive nor phosphonoacetate resistant.

Effects on Other DNA Polymerases

The results of several studies on various eukaryotic cells have shown that DNA polymerase α is inhibited by phosphonoacetate (5). The apparent inhibition constants are about 15-30 times higher than the herpesvirus-induced DNA polymerase. DNA polymerase β and the γ-polymerase from eukaryotic cells are both insensitive to phosphonoacetate.

Various prokaryotes and viral DNA polymerases have been found to be insensitive to phosphonoacetate (5). They include *E. coli* DNA polymerase 1, *M. luteus* DNA polymerase, hepatitis B virus DNA polymerase and the reverse transcriptases of Rous sarcoma virus and avian myeloblastosis virus. The vaccinia virus-induced DNA polymerase is somewhat inhibited.

The non-polymerase enzymes are sensitive to phosphonoacetate. Phosphonoacetate is a competitive inhibitor for carbamyl phosphate in the aspartate transcarbamylase catalyzed reaction (28). The apparent inhibition constant is 0.32 mM. For pyruvate carboxylase, phosphonoacetate is noncompetitive with respect to ATP and the apparent inhibition constant is 0.5 mM (29).

Animal Studies with Phosphonoacetate

Phosphonoacetate has shown good efficicacy in the animal model systems for herpesvirus infections (5). Phosphonoacetate is efficacious for the herpes simplex diseases: herpes dermatitis, herpes keratitis, herpes iritis, herpes genitalis, and herpes encephalitis. Cytomegalovirus infection of mice and Marek's disease infection in chickens are also susceptible to phosphonoacetate. In general, topical administration of the drug shortly after infection gives the best results.

Phosphonoacetate is not effective for herpes infections which are latent (30). During latency, infectious virus cannot be detected, but can be recovered when re-activated. The state of the virus during latency is unknown.

Analogs of Phosphonoacetate

Studies of analogs of phosphonoacetate indicate that the structural requirements for antiviral actively are rather narrowly defined (5). The results indicate that to be an effective inhibitor of the herpesvirus-induced DNA polymerase, the inhibitor should have an unsubstituted phosphono group and an unsubstituted carboxyl group. Phosphonocarboxylic acids having a longer carbon chain length are not inhibitors. The methylene carbon should also be unsubstituted.

Some modification is allowed if the drug is used in tissue culture or animal systems. 1,3-Dipalmitoyl-2-phosphonoacetylglycerol is effective in treating herpes simplex skin infection in mice (31). Amides of phosphonoacetate of the type $R_2NCOCH_2P(O)(OH)_2$ are also

effective (32). Apparently the compounds are readily taken up by the cells in this form and metabolically hydrolized to give free phosphonoacetate which is then available to inhibit the herpesvirus-induced DNA polymerase.

Metabolism and Toxicity of Phosphonoacetate

Systematic studies on the toxicity of phosphonoacetate have not been carried out. In reports on the efficacy of phosphonoacetate in viral infections of animals there are some statements concerning this question. The LD_{50} dose for mice is 1500 mg/kg/day (33). At concentrations up to about one-half the LD_{50}, phosphonoacetate is well tolerated in mice. In rabbits, when applied to the eyes, the compound is also tolerated at concentrations at which it is effective (5).

In animals studies employing disodium [1-^{14}C] phosphonoacetate, no evidence was obtained for its conversion to other compounds (34). Most of the administered drug was excreted rapidly in the urine in an unchanged form. Whole-body autoradiography of treated animals demonstrated that the absorbed drug is accumulated in bone. After administration has ceased, phosphonoacetate is released from bone but very slowly. Toxicity problems associated with this deposition in bone have not been evaluated. In tissue culture studies monitoring calcification of tendon matrix it was found that phosphonoacetate inhibited the uptake of Ca^{2+} and inorganic phosphate by both uncalcified and previously calcified matrices (35). The inhibition is completely reversed when phosphonoacetate is washed off.

Severe toxic reactions have been observed in rabbits and patas monkeys. An intravenous concentration of 300 mg/kg phosphonoacetate produced severe tetanic muscular spasms in rabbits, often resulting in death; the same dose given to mice orally or intraperitoneally was well tolerated (5). In patas monkeys receiving a daily dose of 800 mg or higher there was liver degeneration, severe dermatitis, and a change in skin and hair color (36).

Phosphonoacetate was not mutagenic in the Salmonella typhimurium test (37). Animal carcinogenic studies have not been reported.

Abbott Laboratories has applied to the Food and Drug Administration for permission to classify phophonoacetate as an investigative drug for the purpose of initiating clinical studies. The FDA did not approve their request and has asked for further toxicity studies. Therefore, the prospect of phosphonoacetate being a clinically useful drug remains uncertain.

Phosphonoformic Acid

The synthesis, characterization, and assessment of the biological effects of phosphonoformic acid (Figure 2) are contained in Article 1 and Article 2 of this thesis. These results have been confirmed and extended by researchers at Astra Laboratories in Sodertalje, Sweden and at the University of Uppsala. Inhibition of herpesvirus replication by phosphonoformic acid has been included in reviews by Boezi (5) and Newton (39). A review of the Swedish effort has also appeared (40). A comprehensive review of the literature to date on phosphonoformic acid will be presented here.

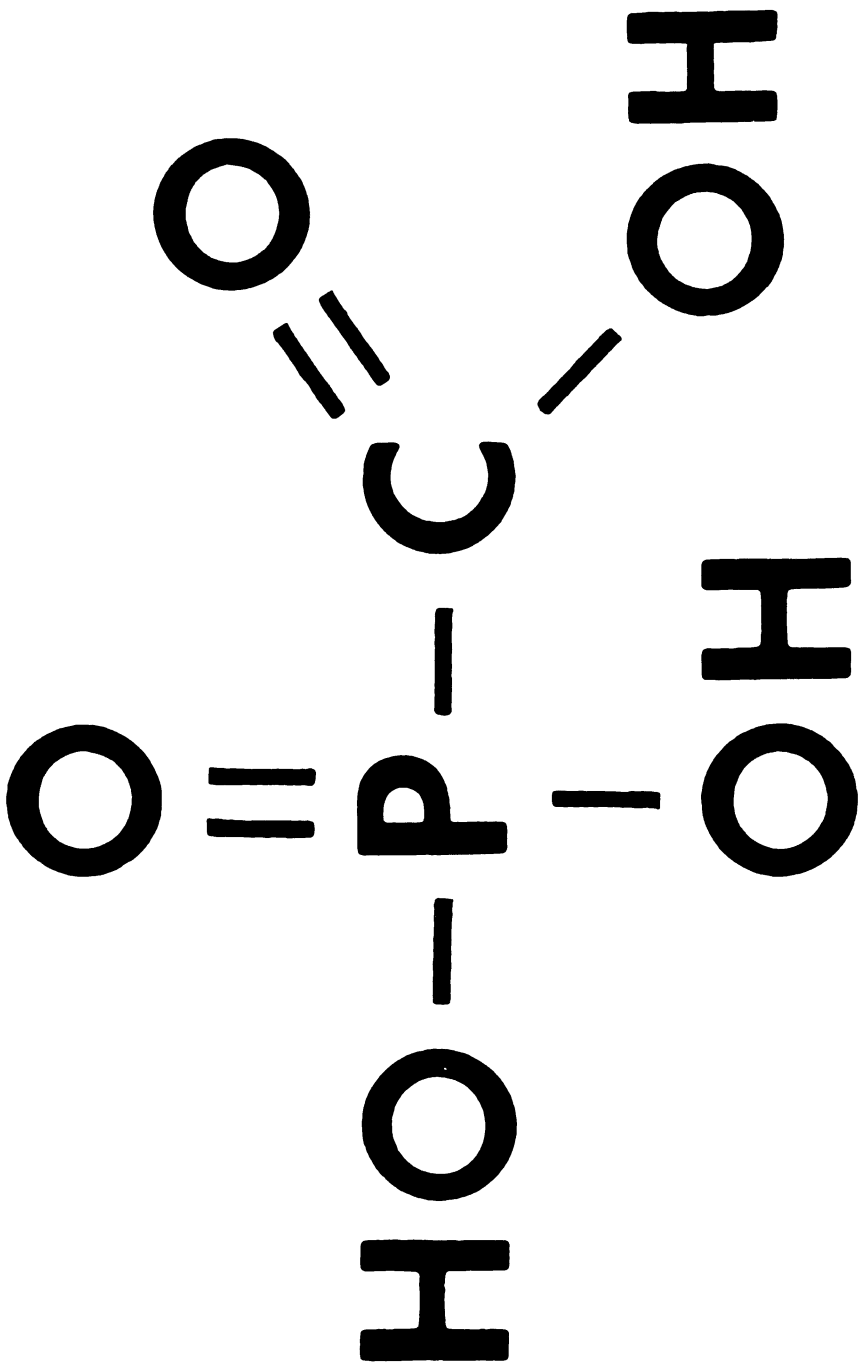

Figure 2. Structure of Phosphonoformic Acid.

Chemistry

Trisodiumphosphonoformate hexahydrate was first synthesized by Nylen in 1924 (6)--the same paper which describes the synthesis of phosphonoacetic acid. Zn, Mn, Cu, Pb, and Ag salts of phosphonoformate were also prepared. Upon converion to the completely protonated form, phosphoformic acid decomposes to form phosphorous acid and CO_2 (41, 42). The acidity constants at 25°C and ionic strength 0.05 are pK_1 of 1.7 (P-OH), pK_2 of 3.59 (COOH), and pK_3 of 7.56 (P-OH) (7). Phosphonoformate also forms stable complexes with magnesium with log K_f of 1.7 for the monoprotonated form and log K_f of 3.6 for the completely deprotonated form of phosphonoformate (P-H. Heubel and A. Popov, personal communication).

The crystal structure of trisodium phosphonoformate hexahydrate has been determined (43, 44). It consists of sodium ions surrounded octahedrally by six oxygen molecules, some from water molecules and from the phosphonoformate ions.

Esters of phosphonoformate are used in lubricating oils and hydraulic fluids because they have extreme pressure lubricating ability (45).

Effect on the Herpesvirus-Induced DNA Polymerase

The anti-herpesvirus effect of phosphonoformate was initially found by screening analogues of pyrophosphate as inhibitors of the herpesvirus-induced DNA polymerase (Article 1). The DNA polymerases induced by Marek's disease herpesvirus, the herpesvirus of turkeys, and herpes simplex virus are inhibited 50% by 1-3 μM

phosphonoformate (Article 1, 38). The molecular mechanisms by which phosphonoformate and phosphonoacetate inhibit the herpesvirus-induced DNA polymerase are analogous (Article 1).

Inhibition of Herpesvirus Replication in Cell Culture

Phosphonoformate is an effective inhibitor of herpesvirus replication in infected cells (Article 1). The addition of 60-70 μM phosphonoformate brought about a 50% reduction in the number of plaques observed in cell cultures infected with Marek's disease herpesvirus, the herpesvirus of turkeys and herpes simplex type 1. Herpes simplex type 2 and pseudorabies herpesvirus replication is inhibited by more than 90% with 100 μM phosphonoformate (38).

Vaccina virus, adenovirus type 2, and poliovirus are all insensitive to phosphonoformate (38). Influenza virus replication is inhibited by high concentrations of phosphonoformate. At 500 μM phosphonoformate influenza virus replication was inhibited by over 90% where the same concentration of phosphonoacetate had no effect. In each of the other viral systems the efficacy of phosphonoformate and phosphonoacetate were parallel (Article 1, 38).

Effect on Uninfected Cells in Culture

At the concentrations which are effective in blocking the replication of herpesviruses in cell culture, phosphonoformate has no obvious cytotoxic effects on duck embryo fibroblasts (Article 1), nor on African green monkey kidney cells, HeLa, and human lung cells (38, 46). Cell proliferation and cellular DNA synthesis is inhibited 50% by 1 mM phosphonoformate and RNA and protein synthesis could be

inhibited at concentrations exceeding 2.5 mM (46). HeLa cell division can be completely blocked with 10 mM phosphonoformate for 24 hours but is rapidly reversed after the drug is removed (46). Little cell death occurred during this time.

Effect on Other DNA Polymerases

The inhibition of herpesvirus-induced DNA polymerases by phosphonoformate is not entirely specific. DNA polymerase α from HeLa cells, Wi-38 cells, and phytohemaglutinin-stimulated lymphocytes are inhibited 50% by 30 μM phosphonoformate. DNA polymerase β from these sources is relatively insensitive (47). These results are analogous to those obtained with phosphonoacetate. Influenza RNA polymerase (38, 59) and the hepatitis B Dane particle DNA polymerase (48) are inhibited 50% by 20 μM phosphonoformate but not by phosphonoacetate. Replication of visna virus, an RNA virus, is also inhibited by phosphonoformate and this effect is ascribed to inhibition of the viral reverse transcriptase (49). Inhibition of reverse transcriptases from the RNA tumor viruses has been studied in depth (Article Ii, 50). A 50% reduction in the DNA polymerase activity is observed at about 10 μM. The mechanism of inhibition is different with reverse transcriptases than that for herpesvirus-induced DNA polymerases. The RNA dependent RNA polymerase of vesicular stomatitis virus is inhibited by high concentrations of phosphonoformate (51). A concentration of 5 mM is required to give 90% inhibition.

Herpesvirus Mutants Resistant to Phosphonoformate

Mutants of herpes simplex virus and Marek's disease herpesvirus resistant to phosphonoformate are easily obtained in cell culture (52, 53, 54). The induced DNA polymerases isolated from cells infected with these mutants are also resistant to phosphonoformate as well as phosphonoacetate. This provides evidence that the target protein for phosphonoformate is in fact the herpesvirus-induced DNA polymerase and that the merchanism of inhibition is the same for both phosphonates. However, the ease of which these mutants were obtained may indicate a limited clinical value for this compound.

Efficacy in Animal Model Herpesvirus Infections

Four reports on phosphonoformate efficacy in animal model studies are available and the results so far parallel those obtained with phosphonoacetate (55, 56, 57, 58). Topically applied phosphonoformate has a good therapeutic activity against established herpes simplex virus skin infections in guinea pigs (38, 55). Intravaginal treatment of herpes simplex virus genital infections in mice and guinea pigs with phosphonoformate reduces virus titer in the genital tract and the mortality rate but treatment must begin within 24 hours of infection (56, 57). Phosphonoformate has only minimal effectiveness in mice inoculated intra-cerebrally with herpes simplex virus or intraperitoneally with cytomegalovirus (56). Treatment of skin infections of herpes simplex virus in hairless mice

topically with phosphonoformate was very effective, even when delayed 24 hours (58). However, phosphonoformate could not prevent the establishment of latent infections in the nervous system of treated mice even when treatment was started at 3 hours post infection, whereas phosphonoacetate was effective.

Analogues of Phosphonoformate

Various esters of phosphonoformate have been synthesized and tested for their ability to inhibit the herpesvirus-induced DNA polymerase, herpesvirus replication in cell culture, and herpesvirus skin infection in guinea pigs (Helgstrand, E., N. G. Johansson, S. Stridh, and B. Oberg. Personal Communication, October 1979). None were effective in inhibiting the herpesvirus-induced DNA polymerase. Aryl esters of the phosphonate moiety were effective in cell culture at concentrations of 3-15 μM. It is suggested that these esters are more readily taken up into cells and then enzymatically hydrolyzed to yield free phosphonoformate. All were about as effective as free phosphonoformate in the animal model system.

Toxicity of Phosphonoformate

Systematic studies on the toxicology of phosphonoformate have not been reported. Summary statements of reports give some observations on the toxicity. Phosphonoformate does not cause the skin irritation seen with phosphonoacetate treatment (40, 56). Phosphonoformate is reported to be non-toxic to rats and dogs subcutaneously treated with the drug daily for 1 month at levels up to 195 mg/kg (40). Phosphonoformate is deposited in bone and cartilage tissues

from which it is only slowly released. No apparent toxicity is associated with the deposition in bone (40).

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ARTICLE 1

INHIBITION OF HERPESVIRUS-INDUCED DNA POLYMERASE AND HERPESVIRUS REPLICATION BY PHOSPHONOFORMATE

By

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Inhibition of Herpesvirus Replication and Herpesvirus-Induced Deoxyribonucleic Acid Polymerase by Phosphonoformate†

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Phosphonoformate was found to be an inhibitor of the deoxyribonucleic acid polymerase induced by the herpesvirus of turkeys. The apparent inhibition constants were 1 to 3 μM. Phosphonoformate was also able to block the replication in cell culture of Marek's disease herpesvirus, the herpesvirus of turkeys, and herpes simplex virus. It was as effective as phosphonoacetate. Phosphonoformate was not an effective inhibitor of a phosphonoacetate-resistant mutant of the herpesvirus of turkeys nor of its induced deoxyribonucleic acid polymerase.

Phosphonoacetate is an effective inhibitor of the replication of herpesviruses (11, 13, 23, 25). The inhibition of herpesvirus replication is through an effect on the viral-induced deoxyribonucleic acid (DNA) polymerase (9, 14-16, 18). In animal model studies, the efficacy of phosphonoacetate as an antiherpesvirus drug has been clearly demonstrated (7, 8, 12). Its clinical use, however, may be limited because it is somewhat toxic to test animals and because it is accumulated in bone (4).

Other phosphonate compounds are of interest as inhibitors of herpesvirus replication because they might exhibit an improved therapeutic ratio over phosphonoacetate either by being more effective inhibitors of virus replication or by being less toxic to animals. These compounds are also of interest at the enzymological level for the information that they might provide about the binding site on the herpesvirus-induced DNA polymerase. Consequently, using as an assay procedure the ability to inhibit the herpesvirus-induced DNA polymerase or the ability to block herpesvirus replication in cell culture or in animals, many other phosphonates have been looked at (10, 13, 14, 23). Only the low-molecular-weight carboxyl esters of phosphoacetate have proven to be effective inhibitors.

In this report, we demonstrate that phosphonoformate, a compound first synthesized in 1924 (17), is an effective inhibitor of the DNA polymerase induced by the herpesvirus of turkeys (HVT) and blocks the replication of Marek's disease herpesvirus, HVT, and herpes simplex virus (HSV). Further, we compare the effectiveness of phosphonoformate with phosphonoacetate. We also report the effect that phosphonoformate has on a phosphonoacetate-resistant mutant of HVT and point out that phosphonoformate inhibits herpesvirus replication through an effect on the DNA polymerase in an analogous manner to phosphonoacetate.

† Michigan Agricultural Station article no. 8188.

EXPERIMENTAL PROCEDURE

Reagents. Phosphonoacetate, disodium salt, was a gift from Abbott Laboratories. Triethyl phosphite and ethyl chloroformate were purchased from Aldrich Chemical Co. Triethyl phosphite was redistilled before use. Other reagents were from sources previously described (3) or were from the usual commercial sources.

Synthesis of triethyl phosphonoformate and trisodium phosphonoformate. Both triethyl phosphonoformate and trisodium phosphonoformate were prepared following the procedure of Nylen (17) as described by Warren and Williams (26). In brief, triethyl phosphonoformate was prepared by an Arbuzov reaction with ethyl chloroformate and triethyl phosphite and was purified by vacuum distillation. The infrared spectrum and proton nuclear magnetic resonance spectrum agreed with published spectra (21, 22). The ^{13}C-nuclear magnetic resonance proton-decoupled spectrum gave resonances at 14.6 ppm ($CO_2CH_2\underline{C}H_3$), 16.8 ppm (doublet, $J_{P\text{-}C}$ = 5.9 Hz, $POCH_2\underline{C}H_3$), 62.6 ppm (doublet, $J_{P\text{-}C}$ = 4.4 Hz, $CO_2\underline{C}H_2CH_3$), 64.9 ppm (doublet, $J_{P\text{-}C}$ = 6.6 Hz, $PO\underline{C}H_2CH_3$), and 168.2 ppm (doublet, $J_{P\text{-}C}$ = 265.4 Hz, $\underline{C}$ = O). The spectrum was obtained using a Bruker WP-60 spectrometer, and all chemical shifts are relative to tetramethylsilane. The triethyl ester was saponified

with NaOH, and the product was recrystallized several times from water to give trisodium phosphonoformate hexahydrate. Phosphorus analysis (1) for $CNa_3O_5P \cdot 6H_2O$ gave a molecular weight of 299 (in theory, 300). The ^{13}C-nuclear magnetic resonance proton-decoupled spectrum gave a resonance at 180 ppm (doublet, $J_{P\text{-}C}$ = 229.3 Hz). Trisodium phosphonoformate was further characterized by descending paper chromatography with the following solvent systems: (i) isopropyl alcohol-water-concentrated ammonia (7:2:1), R_f = 0.03; (ii) methanol-water-concentrated ammonia (6:1:3), R_f = 0.63; (iii) ethanol-1 M ammonium acetate, pH 7.5 (5:2), R_f = 0.06. The chromatograms were sprayed as described by Bandurski and Axelrod (2), and only a single blue spot was detected with each solvent system.

Virus strains. Marek's disease herpesvirus strain GA (MDHV) (19) was propagated in primary duck embryo fibroblasts as previously described (24). HVT strain FC-126 (HVT_{wt}) (28) and a phosphonoacetate resistant mutant of this strain (HVT_{pa}) (L. F. Lee, K. Nazerian, R. Witter, S. Leinbach, and J. Boezi, manuscript in preparation) were also propagated in primary duck embryo fibroblasts as previously described. The HSV type 1 used was the MP strain (20) adapted to duck embryo fibroblasts.

Assessment of phosphonate effect on herpesvirus replication. Triplicate duck embryo fibroblasts culture samples were inoculated with MDHV and incubated with various concentrations of phosphonoformate or phosphonoacetate according to the following regimen: with 0.035 mM phosphonate, cultures were inoculated with 50, 100, 500, and 1,000 plaque-forming units (PFU); with 0.07 mM phosphonate, cultures were inoculated with 100, 500, 1,000, and 5,000 PFU; at 0.14 mM phosphonate, with 5,000 and 10,000 PFU; and at 0.28 mM, with 50,000 and 100,000, and 500,000 PFU. Cultures were incubated, and plaques were counted 6 or 7 days postinfection. Relative numbers of plaques were determined by dividing the observed number of plaques formed by the input PFU.

Identical culture conditions were used for HVT_{wt} and HVT_{pa} except that triplicate cultures were all inoculated with 100 PFU in various concentrations of either phosphonate, and plaques were counted 5 days postinfection. The percentage of plaques surviving was calculated from cultures containing no phosphonate.

Identical culture conditions were also used for HSV except that it was inoculated at about 1,000 PFU/plate into cultures containing various concentrations of either phosphonate.

Preparation of HVT-induced DNA polymerase. Both HVT_{wt} and HVT_{pa} were treated in an identical manner. The preparation and growth of duck embryo fibroblasts and infection with virus was as previously described (3). The partial purification of the HVT-induced DNA polymerase was from the nuclear fraction of infected cells by phosphocellulose chromatography as described by Leinbach et al. (14). The specific enzymatic activity of the preparation used in the kinetic studies reported here was about 1,000 nmol of deoxynucleoside monophosphate incorporated into DNA/30 min per mg of protein. This enzyme fraction, when tested using the standard assay conditions for DNA polymerization, contained no detectable deoxyribonuclease activity, deoxyribonucleoside triphosphatase activity, or inorganic pyrophosphatase activity. The kinetic experiment with HVT_{wt}-induced DNA polymerase was also performed with a more highly purified preparation which had been purified by phosphocellulose and hydroxylapatite chromatography. No differences in the results were seen.

Inhibition patterns. Inhibition patterns and kinetic constants were defined according to the nomenclature of Cleland (5, 6). The data for the double reciprocal plots were evaluated using a computer program based on the method of Wilkinson (27). For evaluation of the apparent inhibition constants, replots of the intercepts and slopes of the double reciprocal plots were analyzed by the method of least squares.

RESULTS

Phosphonoformate inhibition of the DNA polymerization reaction catalyzed by HVT_{wt}-induced DNA polymerase. In the course of a study examining phosphonate compounds for their ability to inhibit the herpesvirus-induced DNA polymerase, it was discovered that phosphonoformate was an effective inhibitor of the HVT_{wt}-induced DNA polymerase. The addition of 2 to 3 μM phosphonoformate to the standard assay mixture resulted in a decrease in the rate of the DNA polymerization reaction by about 50%. The inhibition patterns produced by phosphonoformate were examined, and as shown in Fig. 1, phosphonoformate gave linear noncompetitive inhibition with the four deoxynucleoside triphosphates (dNTP's) as variable substrate and activated DNA at a saturating concentration of 200 μg/ml. The apparent inhibition constant determined from the replot of the vertical intercepts against phosphonoformate concentration (K_{ii}) was 1.1 μM. The apparent inhibition constant determined from the replot of the slopes against phosphonoformate concentration (K_{is}) was 0.9 μM. With activated DNA as the variable substrate and the four dNTP's at their apparent Michaelis constant

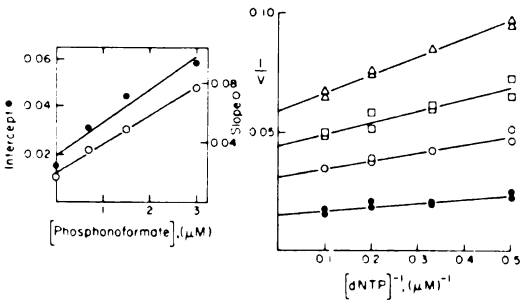

FIG. 1. *Double reciprocal plots of the HVT_{wt}-induced DNA polymerase-catalyzed reaction with the four dNTP's as the variable substrate and phosphonoformate as inhibitor. Activated DNA was at a concentration of 200 μg/ml. The initial velocities were expressed as picomoles of [^{3}H]thymidine 5'-monophosphate incorporated into DNA/30 min. Phosphonoformate concentrations were 0 (●), 0.7 μM (○), 1.5 μM (□), and 3.0 μM (△). Equimolar concentrations of each of the four dNTP's were present in the different reaction mixtures. The replots of the slopes (○) and intercepts (●) as a function of phosphonoformate concentration are shown in the left panel.*

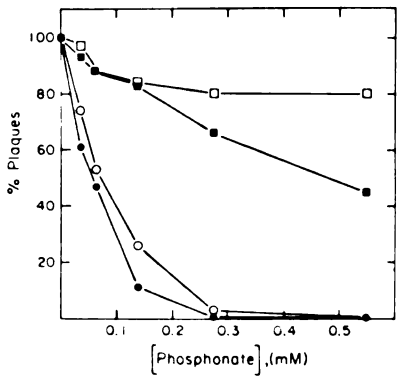

FIG. 2. *Effect of phosphonoformate and phosphonoacetate on the replication of HVT_{wt} and HVT_{pa}. A known titer of each virus preparation was used to infect secondary duck embryo fibroblast cultures in growth media without and with different concentrations of phosphonate. Plaques were enumerated 5 days postinfection. Each point represents the average of three experiments, and the number of plaques enumerated in cultures without phosphonate was considered as 100%. Viruses and inhibitors were HVT_{wt} with phosphonoformate (●), HVT_{wt} with phosphonoacetate (○), HVT_{pa} with phosphonoformate (■), and HVT_{pa} with phosphonoacetate (□).*

concentration of 2.5 μM each, phosphonoformate gave linear noncompetitive inhibition (data not shown). A replot of the vertical intercepts yielded a K_{ii} of 2.6 μM, and a replot of the slopes yielded a K_{is} of 2.3 μM. The inhibition patterns and the apparent inhibition constants are similar to those obtained with phosphonoacetate (14).

Effect of phosphonoformate on the replication of herpesviruses in cell culture. After this discovery, phosphonoformate was tested for its ability to block the replication of HVT_{wt}, MDHV, and HSV in cell culture. Again, phosphonoformate was an effective inhibitor. With HVT_{wt}, phosphonoformate was as effective an inhibitor as phosphonoacetate (Fig. 2). The addition of 0.06 to 0.07 mM of either phosphonate to the culture medium brought about a 50% reduction in the number of plaques observed. Essentially no plaques were observed at concentrations above 0.3 mM.

Phosphonoformate and phosphonoacetate also exhibited a parallel ability to block the replication of MDHV. The addition of either phosphonate to a final concentration of about 0.02 mM brought about a 50% reduction in the number of plaques produced. Either phosphonate at 0.14 mM reduced the number of plaques by more than three orders of magnitude (Fig. 3). At 0.28 mM, no plaques at all were observed (data not shown).

Phosphonoformate also inhibited the replication of HSV type 1 in cell culture (data not shown). Again, phosphonoformate was about as effective an inhibitor as phosphonoacetate.

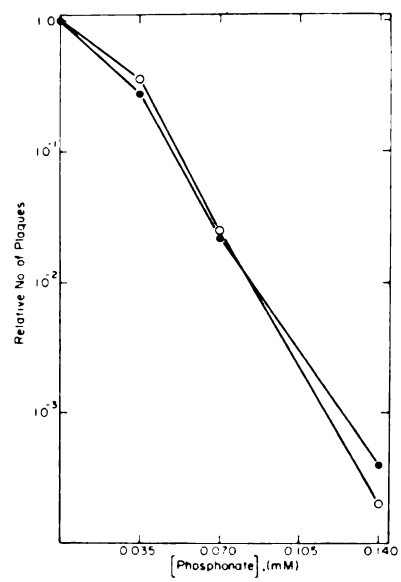

FIG. 3. *Semilog plot of the effect of phosphonoformate (●) and phosphonoacetate (○) on the replication of MDHV. A known titer of virus was used to infect secondary duck embryo fibroblast cultures in growth media without and with different concentrations of phosphonate. Plaques were enumerated 6 to 7 days postinfection. Each point represents the average of three experiments. The number of plaques observed in each experiment was normalized to the input number of PFU.*

At concentrations up to 0.55 mM, phosphonoformate had no obvious cytotoxic effect on the growth of normal duck embryo fibroblasts. The treated cells formed a confluent monolayer in the usual time, and maintenance of the monolayer was normal.

In the above studies, phosphonoformate was an effective inhibitor of the replication of MDHV, HVT_{wt}, and HSV in cell culture. Phosphonoformate, however, was not an effective inhibitor of the replication of HVT_{pa} in cell culture. As seen in Fig. 2, the replication of HVT_{pa} is less sensitive to phosphonoformate than is the replication of HVT_{wt}. This indicates that the mutation to phosphonoacetate resistance also leads to phosphonoformate resistance and suggests that the DNA polymerase of HVT_{pa} is altered so as to be less sensitive to phosphonoformate as well as phosphonoacetate.

Phosphonoformate inhibition of the DNA polymerization reaction catalyzed by the HVT_{pa}-induced DNA polymerase. Indeed, phosphonoformate, like phosphonoacetate, was not an effective inhibitor of the DNA polymerase of this phosphonoacetate-resistant mutant. With the four dNTP's as the variable substrate, phosphonoformate again gave linear noncompetitive inhibition (Fig. 4). The K_{ii} was 8 μM and K_{is} was 18 μM. This represents an increase of 10 to 20 times over the values obtained for the HVT_{wt}-induced DNA polymerase and is similar to the increase seen with phosphonoacetate (L. F. Lee et al., manuscript in preparation). With activated DNA as the variable substrate and the four dNTP's at their apparent Michaelis concentration of 5 μM, phosphonoformate gave linear noncompetitive inhibition, a K_{ii} of 13 μM, and a K_{is} of 43 μM. Again, this represents an increase of about 10 to 20 times over the same values obtained for the HVT_{wt}-induced DNA polymerase, as was also seen with phosphonoacetate.

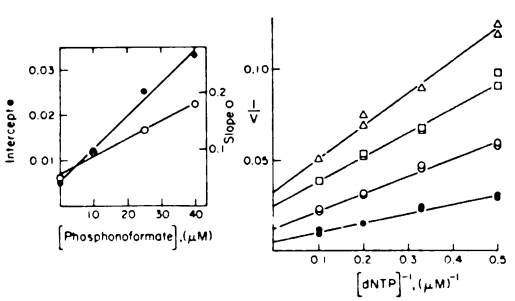

FIG. 4. *Double reciprocal plots of the HVT_{pa}-induced DNA polymerase-catalyzed reaction with the four dNTP's as the variable substrate and phosphonoformate as inhibitor. Activated DNA was at a concentration of 200 μg/ml. The initial velocities were expressed as picomoles of [^{3}H]thymidine 5'-monophosphate incorporated into DNA/30 min. Phosphonoformate concentrations were 0 (●), 10 μM (○), 25 μM (□), and 40 μM (△). Equimolar concentrations of each of the four dNTP's were present in the reaction mixtures. The replots of the slopes (○) and intercepts (●) as a function of phosphonoformate concentration are shown in the left panel.*

DISCUSSION

The replication of MDHV, HVT_{wt}, and HSV in cell culture was effectively inhibited by phosphonoformate. This inhibition was as effective as the inhibition by phosphonoacetate. The inhibition patterns seen in the steady-state enzyme kinetic analysis of the HVT_{wt}-induced DNA polymerase with phosphonoformate as inhibitor were identical to those reported for phosphonoacetate (14). Both phosphonates showed noncompetitive inhibition with the four dNTP's as variable substrate. With activated DNA as the variable substrate and the four dNTP's at their Michaelis concentration, noncompetitive inhibition was also observed. The apparent inhibition constant values were similar. These observations, taken together with the resistance of HVT_{pa} replication to the effect of phosphonoformate and the much higher inhibition constant values obtained for the HVT_{pa}-induced DNA polymerase, indicate that the inhibition of herpesvirus replication by phosphonoformate is through an effect on the herpesvirus-induced DNA polymerase in a manner analogous to the inhibition by phosphonoacetate (9, 14–16).

Although phosphonoformate is a potent inhibitor of the herpesvirus-induced DNA polymerase, it is not entirely specific. Recent experiments in this laboratory have shown that the α-polymerase of Hela, KB, and Wi-38 cells was inhibited by phosphonoformate (C. L. K. Sabourin, J. Reno, and J. Boezi, manuscript in preparation). Phosphonoacetate also inhibits these enzymes. The apparent inhibition constant values for either phosphonate were about 30 μM. The β and γ polymerases are not effectively inhibited by phosphonoformate or phosphonoacetate.

The results of previous studies on analogs of phosphonoacetate demonstrated that the structural requirements for inhibition were rather narrowly defined (10, 13, 14, 23). For example, analogs containing a mono- or diester on the phosphono group or containing a carboxyl or sulfo substitution for the phosphono group were not inhibitors. Analogs that contained a methylamino- or phenyl-substituted methylene carbon also were not inhibitors. Apparently some modification at the carboxyl end of phosphonoacetate is permissible. Low-molecular-weight carboxyl esters are reported to be effective inhibi-

tors (10). Aldehyde, amide, and acetonyl substitutions for the carboxyl group of phosphonoacetate, however, did not yield effective inhibitors (J. A. Boezi, unpublished results). Phosphonates having longer carbon chain length than phosphonoacetate (for example, phosphonopropionate or phosphonobutyrate) were not inhibitors. This report demonstrates that the shorter chain length of phosphonoformate yielded an effective inhibitor.

Now that phosphonoformate has been shown to be an effective inhibitor of herpesvirus replication, its efficacy as an antiherpesvirus drug in animals must be determined. Recent results have shown that phosphonoformate is as effective as phosphonoacetate against HSV types 1 and 2 in mice and guinea pigs (E. R. Kern, J. Overall, L. Glasgow, J. Reno, and J. Boezi, manuscript in preparation). Additional animal model systems will be tested, and toxicity studies will follow. Phosphonoformate may be of sufficiently different chemistry that it would be less toxic, would not be accumulated in bone, and might become a useful drug in the treatment of herpesvirus infections.

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ARTICLE 2

MECHANISM OF PHOSPHONOFORMATE INHIBITION OF REVERSE TRANSCRIPTASE

By

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ABSTRACT

Phosphonoformate, and not phosphonoacetate, was a potent inhibitor of DNA synthesis catalyzed by reverse transcriptase from avian myeloblastosis virus, Rous sarcoma virus, Moloney murine leukemia virus, and feline leukemia virus with poly(a)·oligo(dT), activated DNA or poly(A)·containing RNA·oligo (dT), as substrate. With Mg^{2+} as the cofactor, 50% inhibition in the rate of polymerization was observed with about 10 μM phosphonoformate. With Mn^{2+} as the cofactor, 50% inhibition was obtained with about 0.5 μM phosphonoformate. The endogenous reactions or reactions in which purified viral RNA was the substrate were much less sensitive to phosphonoformate. The RNase H activity of AMV reverse transcriptase was not inhibited by either phosphonoate in the presence of either Mg^{2+} or Mn^{2+}. A steady-state enzyme inhibition kinetic analysis of both the DNA polymerization and the deoxyribonucleoside triphosphate-pyrophosphate exchange reaction was carried out. The results are consistent with phosphonoformate interacting with the reverse transcriptase at the pyrophosphate binding site and functioning as a dead-end inhibitor.

INTRODUCTION

Inhibitors of nucleic acid synthesis that interact directly with a target enzyme are few in number (1). Phosphonoacetate (2, 3, 4), one such compound, has been the subject of several reviews (5, 6, 7). It is an effective inhibitor of herpesvirus replication in infected cell cultures at concentrations at which cell proliferation is unaffected and is efficacious as an antiherpesvirus agent in animal model systems. Its target protein is the herpesvirus-induced DNA polymerase and it inhibits by interacting with the enzyme at its pyrophosphate binding site with an apparent inhibition constant of 1 μM (8, 9). Phosphonoacetate also inhibits eukaryotic DNA polymerase α in an analogous manner but with an apparent inhibiton constant of about 30 μM (9, 10). Eukaryotic β- and γ-polymerases are not inhibited.

Of the many phosphonoacetate analogs tested, only phosphonoformate was found to be an effective inhibitor of the herpesvirus-induced DNA polymerase (11, 12). Its mechanism of inhibition and effectiveness are similar to that of phosphonoacetate. Like phosphonoacetate, phosphonoformate blocks herpesvirus replication in infected cell cultures and in animal model systems (13, 14, 15, 16).

Sundquist and Larner (17) reported that phosphonoformate, but not phosphonoacetate, inhibited the replication of visna virus in cultures of sheep choroid plexus cells. Visna virus is a retrovirus that causes a slow degenerative disease of the central nervous system of sheep (18). In contrast to the oncornaviruses which transform cells in culture, visna virus causes a productive lytic infection in cultures of sheep cells (19). At 100 μM, the lowest concentration tested, phosphonoformate but not phosphonoacetate inhibited polymerization by the reverse transcriptase of this virus (17).

In this report, we examine the phosphonoformate inhibition of reverse transcriptase of some oncogenic retroviruses from avian and mammalian sources. With purified avian myeloblastosis virus DNA polymerase, we report the results of our enzyme kinetic inhibition studies and propose a mechanism by which phosphonoformate inhibits polymerization. We also look into the effect of phosphonoformate on the RNase H activity of reverse transcriptase.

METHODS

Avian myeloblastosis virus and purified AMV DNA polymerase (specific enzymatic activity of 65.3 μmoles of dTMP incorporated per 10 min per mg of protein) were obtained from Dr. J. Beard, Life Science Research Laboratories. Rous sarcoma virus and RSV RNA were purified as previously described (20), Feline leukemia virus and Moloney murine leukemia virus clone 1 were gifts from Dr. L. Velicer, Department of Microbiology, Michigan State University. E. coli RNA polymerase and poly(A)-containing RNA from bovine pituitary glands were gifts from Dr. A. Revzin and Dr. J. Nilson, respectively, of the Department of Biochemistry. Phosphonoacetic acid was from Richmond Organics. Trisodium phosphonoformate hexahydrate was prepared following the procedure of Nylen (21). Aphidicolin was obtained from the Developmental Therapeutics Program, Chemotherapy, National Cancer Institute. All other reagents were from sources previously described (9).

Assay of the DNA Polymerization Reaction Catalyzed by Reverse Transcriptase. The standard reaction mixture contained in 200 μl: 50 mM Tris-HCl, pH 8, 1 mM dithiothreitol, 60 mM KCl, 100 μg per ml bovine serum albumin, either 10 mM $MgCl_2$ or 0.5 mM $MnCl_2$, 18 μg per ml poly(A)·oligo$(dT)_{12-18}$, 100 μM [^{3}H]dTTP (specific radioactivity 30-250 cpm per pmol), and DNA polymerase. With 18 μg

per ml poly(C)·oligo$(dG)_{12-18}$ as template-primer, 100 μM [^{3}H]dGTP (specific radioactivity 40-160 cpm per pmol) was used. When 200 μg per ml activated calf thymus DNA was the substrate, the three unlabeled dNTPs were at 100 μM and [^{3}H]dTTP (specific radioactivity 100-400 cpm per pmol) was at 20 μM. With 4.2 μg per ml poly(A)-containing RNA as substrate, oligo(dT) was added at 5 μg per ml, the three unlabeled dNTPs were at 100 μM and [^{3}H]dTTP (specific radioactivity 900-1600 cpm per pmol) was at 20 μM. For experiments with 55 μg per ml RSV RNA as substrate, the three unlabeled dNTPs were at 100 μM and [α-^{32}P]dCTP (specific radioactivity 2100 cpm per pmol) was at 20 μM.

For reactions in which purified virus was used as the source of DNA polymerase and substrate RNA, 0.05% NP-40 was used, the three unlabeled dNTPs were at 100 μM and [^{3}H]dTTP (specific radioactivity 300-650 cpm per pmol) was at 20 μM.

For experiments when aphidicolin was evaluated as an inhibitor, it was dissolved in dimethylsulfoxide. The concentration of dimethylsulfoxide in the reaction mixtures, including controls to which no aphidicolin was added, was kept constant at 6%.

The amounts of DNA polymerase or purified virions added to the reaction mixtures were within the activity range which gave direct proportionality with the rate of DNA synthesis and with time. The stock enzyme solution was diluted with buffer containing 50 mM Tris-HCl, pH 8, 1 mM dithiothreitol, and 1 mg per ml bovine serum albumin. For the kinetic studies, changes in concentration of assay components were as noted in the Legends to the Figures. Incubation

was for 30 min at 37°C. The reactions were terminated by addition of 2 ml of a cold solution of 10% trichloroacetic acid-1% socium pyrophosphate. After 5 min at 0°C, the reaction mixtures were filtered onto glass fiber filters (GF/C). The filters were washed, dried, and monitored for radioactivity by liquid scintillation spectrometry.

Assay of the RNase H Reaction Catalyzed by AMV DNA Polymerase. The substrate [^{3}H]poly(A)·poly(dT) was synthesized using E. coli RNA polymerase with poly(dT) as template and [^{3}H]ATP (specific radioactivity 400 cpm per pmol) using the conditions described by Watson et al. (22). After 2.5 hr at 37°C the reaction mixture was concentrated with n-butanol and the product purified on a Sephadex G-50 column.

The RNase H assay mixture contained in 200 µl: 50 mM Tris-HCl, pH 8, either 10 mM $MgCl_2$ or 0.5 mM $MnCl_2$, 1 mM dithiothreitol, 100 µg per ml bovine serum albumin, 2 nmol [^{3}H]poly(A)·poly(dT) as AMP residues, and 9.6 ng AMV DNA polymerase. The reaction was stopped on ice with the addition of 250 µl of cold 10% $HClO_4$ and 50 µl of 5 mg per ml bovine serum albumin. After standing 5 min at 0°C, the reaction mixtures were centrifuged at 9000 rpm for 5 min and an aliquot of the supernatant was counted in Triton X-100 scintillation cocktail. The assay was linear with the amount of DNA polymerase added over the time course of the reaction.

Assay of the dNTP-Pyrophosphate Exchange Reaction Catalyzed by AMV DNA Polymerase. The exchange reaction was routinely assayed in 100 µl with 50 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 60 mM KCl, 1 mM

dithiothreitol, 100 μg per ml bovine serum albumin, 100 μM dTTP, 18 μg per ml poly(A)·oligo(dT), 2 mM sodium [^{32}P]pyrophosphate (specific radioactivity 200 cpm per pmol), and AMV DNA polymerase. The kinetic experiment (Figure 5) was performed in 200 μl with 50 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 60 mM KCl, 1 mM dithiothreitol, 100 μg per ml bovine serum albumin, 1 mM each of the four dNTPs, 200 μg per ml of activated calf thymus DNA, 106 ng AMV DNA polymerase, and various concentrations (0.82-2.0 mM) of sodium [^{32}P]pyrophosphate (specific radioactivity 100 cpm per pmol). The stock solution of unlabeled sodium pyrophosphate was treated with Chelex 100 before use. In addition to the 10 mM $MgCl_2$ in the reaction mixture, supplementary $MgCl_2$, in an amount equimolar to the sodium pyrophosphate in the reaction, was used.

Incubation was for 30 min at 37°C. Reactions were stopped on ice with 1 ml of 2 N $HClO_4$-0.4 M sodium pyrophosphate. After addition of 0.2 ml of 25% acid-washed Norit, the reaction mixtures were centrifuged, the supernatant removed, and the Norit collected on glass fiber filters (GF/C). The filters were washed with 50 ml of 10 mM sodium pyrophosphate, pH 6, dried, and counted in a Nuclear Chicago low background flow counter.

The pyrophosphate exchange reaction was shown to be dependent on added enzyme, template-primer, $MgCl_2$, and dNTPs. The reaction was linear with time through the course of the reaction and was directly proportional to the amount of AMV DNA polymerase added to the reaction mixture.

Assay of the DNA Polymerization Reaction Catalyzed by the α- and β-Polymerases from Chinese Hamster Ovary Cells (CHO). The CHO α- and β-polymerases were purified and assayed as described previously (10).

Inhibition Patterns. Inhibition patterns and kinetic constants were defined according to the nomenclature of Cleland (23, 24). Analysis of each reaction mixture was done in duplicate. The data for the double reciprocal plots were evaluated using a computer program based on the method of Wilkinson (25). For evaluatin of the apparent inhibition constants, replots of the intercepts and slopes of the double reciprocal plots were analyzed by the method of least squares.

RESULTS

Inhibition of DNA Synthesis Catalyzed by Reverse Transcriptase. Phosphonoformate is a potent inhibitor of DNA synthesis catalyzed by AMV DNA polymerase (Figure 1, upper and lower panels on left). With poly(A)·oligo(dT) as the template-primer and Mn^{2+} as the cofactor, the rate of dTTP polymerization was decreased 50% by 0.25 μM phosphonoformate. With Mg^{2+} as the cofactor, the polymerization rate was decreased 50% by 7 μM phosphonoformate. With phosphonoacetate, a 50% inhibition in the rate of dTTP polymerization was observed at 180 μM with Mn^{2+} as the cofactor and no inhibition was observed with Mg^{2+} at concentrations up to 500 μM (Figure 1, upper and lower panels on right).

The concentration of phosphonoformate required to inhibit DNA polmerization by 50% was found to be dependent on the template-primer (Table 1). Phosphonoformate was essentially equally effective in inhibiting DNA polymerization with poly(A)·oligo(dT), activated DNA, and poly(A)-containing RNA·oligo(dT) as template-primers. With poly(C)·oligo(dG) and with RSV RNA, however, higher concentrations of phosphonoformate were required to yield 50% inhibition.

The phosphonoformate sensitivity of reverse transcriptase from some different viral sources was examined (Table 2). Using poly(A)·oligo(dT) as the template-primer and detergent-treated virions, no significant differences in phosphonoformate sensitivity

FIGURE 1. Inhibition of AMV DNA polymerase by phosphonoformate and phosphonoacetate. V_o represents pmol [^{3}H]dTMP incorporated into poly(A)·oligo(dT) per 30 min in the absence of phosphonate. V represents dTMP incorporation in the presence of phosphonate. The phosphonate concentration which inhibits the enzyme activity by 50% is that concentration for which V_o/V is 2.

Upper and lower panels on left: Inhibition by phosphonoformate in the presence of Mg^{2+} (o) for which V_o was 430 pmol per 30 min and in the presence of Mn^{2+} (●) for which V_o was 200 pmol per 30 min.

Upper and lower panels on right: Inhibition by phosphonoacetate in the presence of Mg^{2+} (o) for which V_o was 360 pmol per 30 min and in the presence of Mn^{2+} (●) for which V_o was 180 pmol per 30 min.

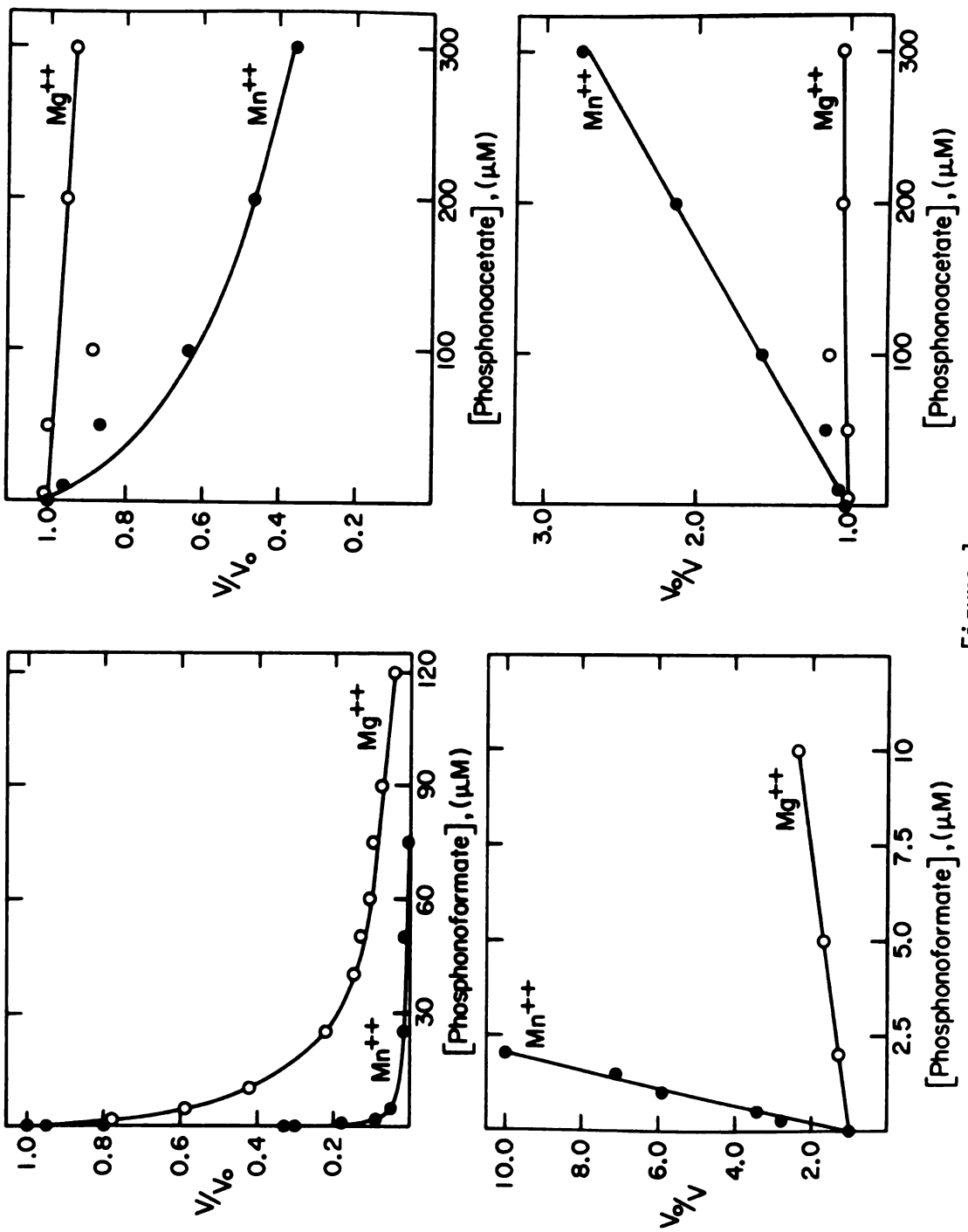

Figure 1

TABLE 1.--Effect of template-primer on phosphonoformate inhibition of AMV DNA polymerase.

Template-primer	Deoxyribonucleoside Triphosphates	Concentration of phosphonoformate that produced 50% inhibition in the rate of polymerization	
		Mn^{2+}	Mg^{2+}
		μM	
Poly(A)·oligo(dT)	[^{3}H]dTTP	0.25	7.2
Poly(C)·oligo(dG)	[^{3}H]dGTP	8	100
Activated DNA	[^{3}H]dTTP, dATP dCTP, dGTP	0.5	6
Activated DNA	[^{3}H]dGTP, dTTP dATP, dCTP	-	5.6
Poly(A)-containing RNA·oligo(dT)	[^{3}H]dTTP, dATP dCTP, dGTP	0.7	15
RSV RNA	[^{32}P]dCTP, dATP dGTP, dTTP	2.5	50

The standard reaction mixture with the appropriate template-primer, deoxyribonucleoside triphosphates, and metal ion cofactor was used as described under Methods. The concentrations of phosphonoformate that produced 50% inhibition were determined as in Figure 1.

TABLE 2.--Phosphonoformate inhibition of reverse transcriptase.

DNA Polymerase	Template-primer	Concentration of phosphonoformate that produced 50% inhibition in the rate of polymerization	
		Mn^{2+}	Mg^{2+}
		μM	
AMV	Poly(A)·oligo(dT)	0.2	8
M-MuLV	Poly(A)·oligo(dT)	0.7	35
FeLV	Poly(A)·oligo(dT)	0.6	13
RSV	Poly(A)·oligo(dT)	0.3	13
RSV	Endogenous RNA	7.2	150

Assays were performed using detergent treated virions as described under Methods. The concentrations of phosphonoformate that produced 50% inhibition were determined as in Figure 1.

were observed for the reverse transcriptases of AMV, M-MuLV, FeLV, and RSV. For RSV, a 10 to 20-fold higher concentration of phosphonoformate was required to give 50% inhibition with endogenous RNA as the template-primer as with poly(A)·oligo(dT).

Effect of Phosphonates on the RNase H Activity of AMV DNA Polymerase. The RNase H activity of AMV DNA polymerase, as assayed by the hydrolysis of [^{3}H]poly(A)·poly(dT), was not inhibited by either phosphonoformate or phosphonoacetate in the presence of either Mn^{2+} or Mg^{2+} (Figure 2). When dTTP was added to the reaction mixture, again, no inhibition of RNase H activity was observed.

Phosphonoformate Inhibition Patterns for the DNA Polymerization Reaction Catalyzed by AMV DNA Polymerase. With poly(A)·oligo(dT) at a saturating concentration of 18 μg per ml and Mn^{2+} as the cofactor, phosphonoformate gave linear noncompetitive inhibition with dTTP as the variable substrate (Figure 3). The apparent Km for dTTP was 14 μM. The apparent inhibition constants were determined from the replots of intercept and slope against phosphonoformate concentration. K_{ii} was 0.19 μM and K_{is} was 0.11 μM. Linear noncompetitive inhibition was also observed with Mg^{2+} as cofactor. The apparent K_m for dTTP was 81 μM, K_{ii} was 14 μM, and K_{is} was 4.9 μM.

With activated DNA at a saturating concentration of 200 μg per ml and Mg^{2+} as cofactor, phosphonoformate again gave linear noncompetitive inhibition with the four dNTPs as the variable substrate. The apparent K_m for dNTP was 24 μM, K_{ii} was 11 μM, and K_{is} was 4.8 μM.

FIGURE 2. Effect of phosphonoformate (●) and phosphonoacetate (o) on AMV RNase H activity. V_o represents cpm [^{3}H]AMP rendered acid soluble in 30 min in the absence of phosphonate. V represents acid soluble cpm in the presence of phosphonate.

Top: Mg^{2+} as cofactor for which V_o was 9540 cpm.

Bottom: Mn^{2+} as cofactor for which V_o was 9350 cpm.

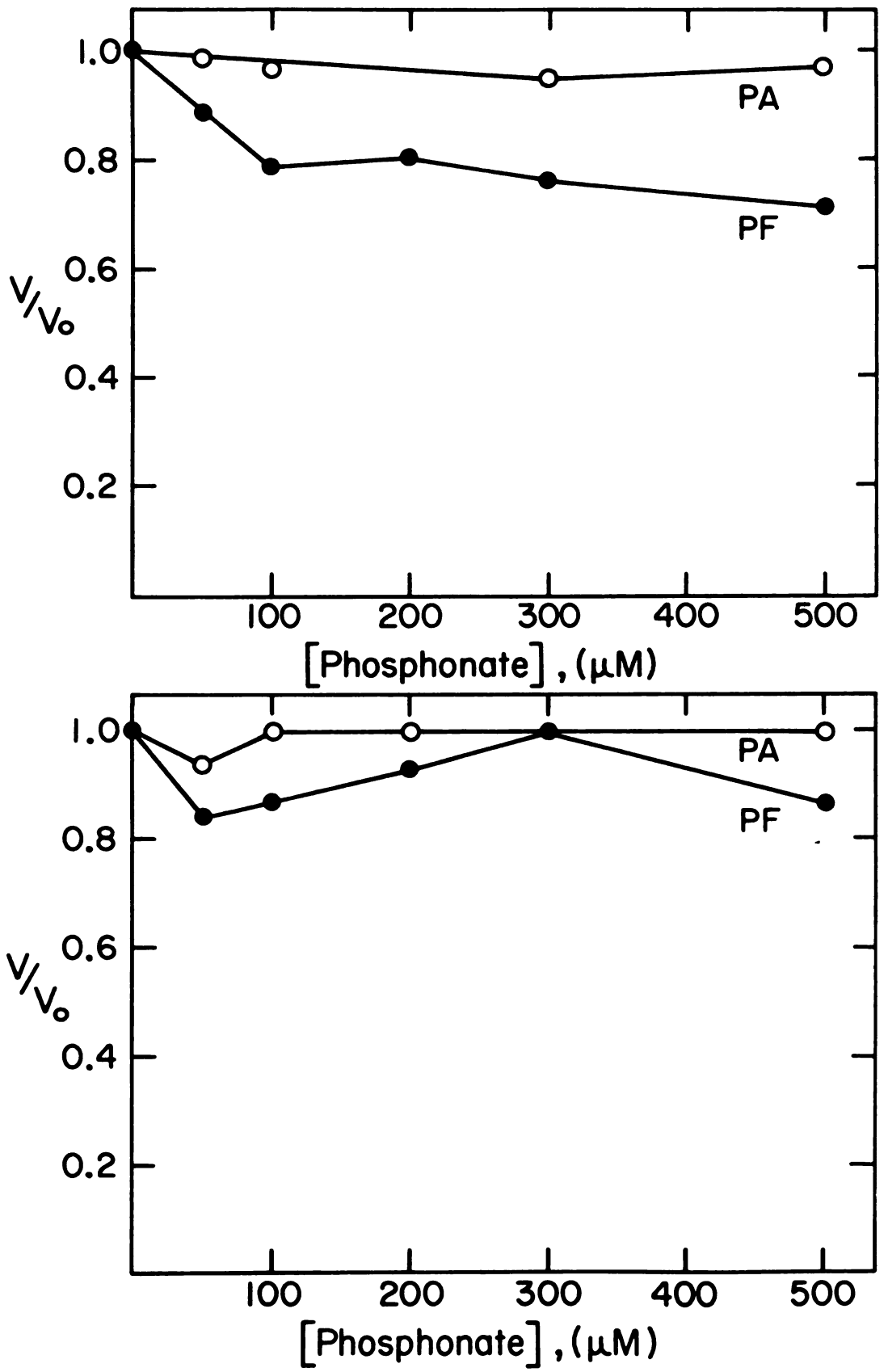
1.0
0.8
0.6
0.4
0.2
V/Vo
PA
PF
100
200
300
400
500
[Phosphonate], (μM)
1.0
0.8
0.6
0.4
0.2
V/Vo
PA
PF
100
200
300
400
500
[Phosphonate], (μM)

Figure 2

FIGURE 3. Double reciprocal plots of the AMV DNA polymerase catalyzed reaction with dTTP as variable substrate and Mn^{2+} as cofactor. Poly(A)·oligo(dT) was at 18 μg per ml. The initial velocities were expressed as pmol of [^{3}H]dTTP polymerized in 30 min. Phosphonoformate concentrations were 0 (●), 0.2 μM (□), 0.4 μM (○), and 0.7 μM (■). The replots of the slopes (○) and intercepts (●) as a function of phosphonoformate are shown in the left panel.

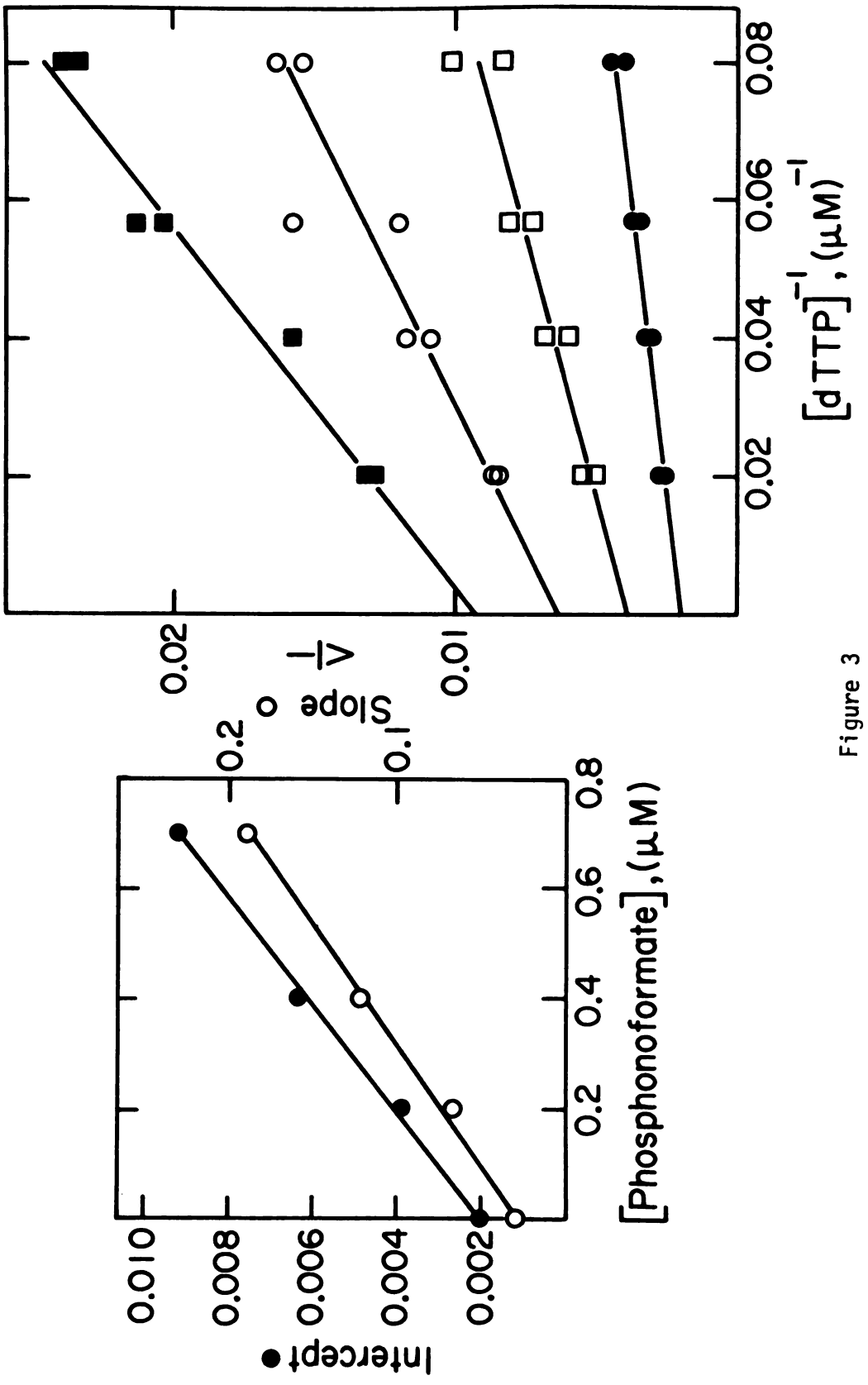
0.02
0.01
Slope ○
1/V
0.2
0.1
0.02
0.04
0.06
0.08
[dTTP]⁻¹, (μM)⁻¹
0.010
0.008
0.006
0.004
0.002
Intercept ●
0.2
0.4
0.6
0.8
[Phosphonoformate], (μM)

Figure 3

With poly(A)·oligo(dT) as the variable substrate, Mn^{2+} as the cofactor and dTTP at 20 μM, phosphonoformate gave linear uncompetitive inhibition (Figure 4). A replot of the vertical intercepts yielded a K_{ii} of 0.10 μM. The apparent K_m for poly(A)·oligo(dT) was 3.5 μg per ml. Phosphonoformate also gave linear uncompetitive inhibition with dTTP at concentrations of 2 μM and 100 μM. With Mg^{2+} as the cofactor and with dTTP at 100 μM, again linear uncompetitive inhibition was observed.

Phosphonoformate Inhibition of the dNTP-Pyrophosphate Exchange Reaction Catalyzed by AMV DNA Polymerase. The effect of the phosphonoates on the AMV DNA polymerase catalyzed pyrophosphate exchange reaction was studied. With poly(A)·oligo(dT) as the template-primer and Mg^{2+} as the cofactor, phosphonoformate gave 50% inhibition in the rate of dTTP-pyrophosphate exchange reaction at a concentration of 9 μM. Under the same conditions, phosphonoacetate did not inhibit the exchange reaction at concentrations up to 300 μM.

Because AMV DNA polymerase is more active in catalyzing the pyrophosphate exchange reaction with activated DNA and the four dNTPs than with poly(A)·oligo(dT) and dTTP (26), activated DNA and the four dNTPs with Mg^{2+} as the cofactor was used to determine the phosphonoformate inhibition pattern for the pyrophosphate exchange reaction. Phosphonoformate was a competitive inhibitor of pyrophosphate (Figure 5). The apparent K_{is} was 12 μM and the apparent K_m for pyrophosphate was 2.7 mM.

FIGURE 4. Double reciprocal plots of the AMV DNA polymerase catalyzed reaction with poly(A)·oligo(dT) as the variable substrate and Mn^{2+} as cofactor. dTTP was at 20 μM. Phosphonoformate concentrations were 0 (●), 0.3 μM (O), 0.6 μM (□), and 0.7 μM (Δ).

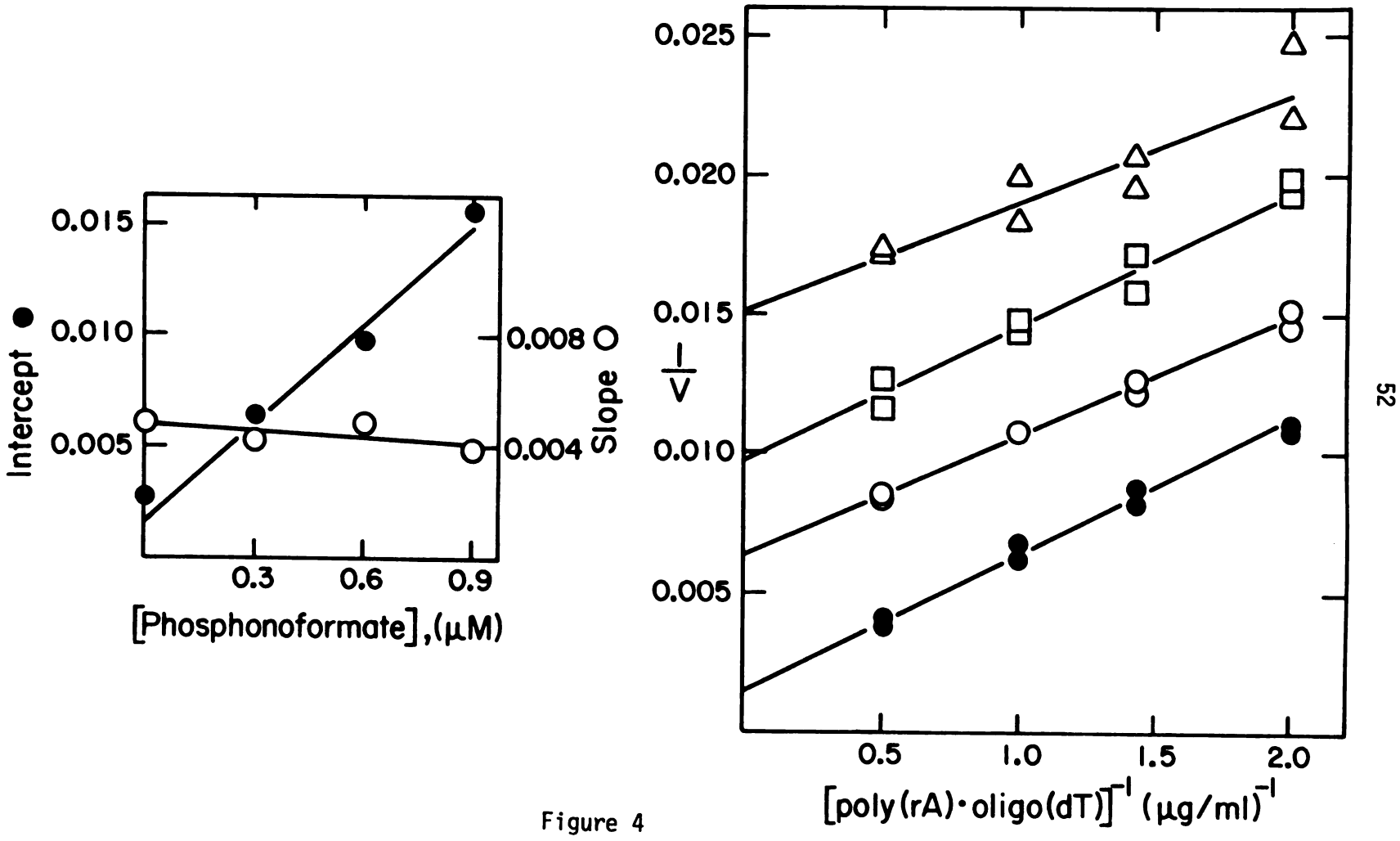
0.025
0.020
0.015
0.010
0.005
1/V
0.5
1.0
1.5
2.0
[poly(rA)·oligo(dT)]⁻¹ (μg/ml)⁻¹
Intercept ●
0.015
0.010
0.005
0.008 ○
0.004
Slope
0.3
0.6
0.9
[Phosphonoformate],(μM)

Figure 4

FIGURE 5. Double reciprocal plots of the dNTP-pyrophosphate exchange reaction catalyzed by AMV DNA polymerase with pyrophosphate as the variable substrate. The initial velocities were expressed as pmol [^{32}P] pyrophosphate converted to a Norit adsorbable form per 30 min. Phosphonoformate concentrations were 0 (●), 8 μM (0), and 20 μM (□).

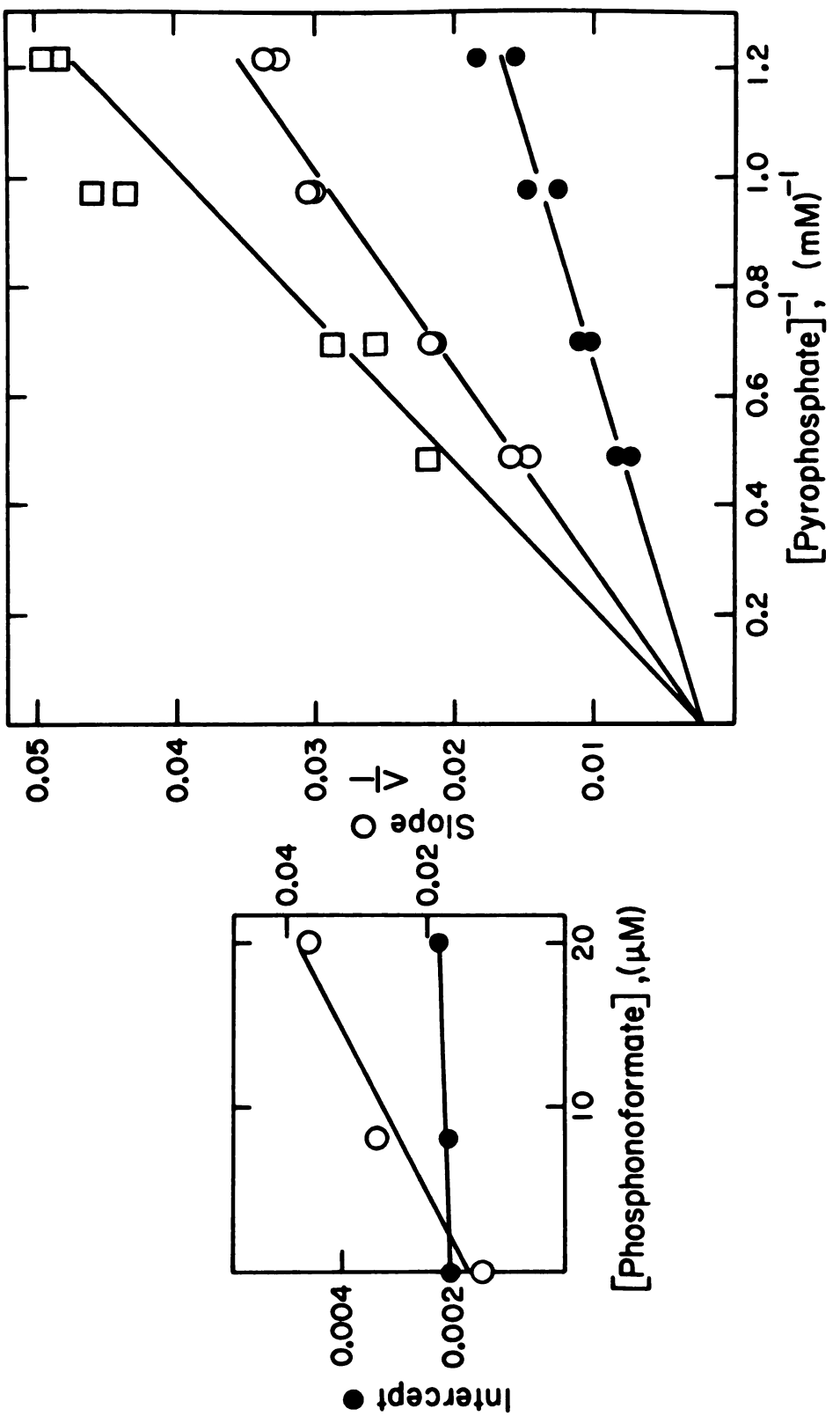
0.05
0.04
0.03
0.02
0.01
Slope ○
ΔA/
0.2
0.4
0.6
0.8
1.0
1.2
[Pyrophosphate]⁻¹, (mM)⁻¹
0.04
0.02
Intercept ●
0.004
0.002
10
20
[Phosphonoformate], (μM)

Figure 5

Effect of Mn^{2+} on Phosphonate Inhibition of Eukaryotic DNA Polymerases α and β. In studies utilizing Mg^{2+} as the cofactor, DNA polymerase α, but not β, was found to be inhibited by phosphonoformate and phosphonoacetate (9, 10). With Mg^{2+} as the cofactor and activated DNA as the template-primer, a 50% decrease in the rate of DNA synthesis by α-polymerase was observed at a concentration of approximately 30 μM with either phosphonate. No inhibition by phosphonoformate or phosphonoacetate for the β-polymerase was observed at concentrations up to 300 μM. Since in the presence of Mn^{2+}, a 15 to 30-fold greater sensitivity to inhibition by the phosphonates was observed for AMV DNA polymerase, the sensitivity of the α- and β-polymerases with Mn^{2+} as the cofactor was examined. For CHO α-polymerase with activated DNA as the template-primer, a 50% inhibition in the rate of DNA synthesis was observed in the presence of 0.4 μM phosphonoformate and 0.9 μM phosphonoacetate. These values are some 30-times lower than those observed with Mg^{2+} as the cofactor. For CHO β-polymerase with Mn^{2+} as the cofactor, neither phosphonate inhibited DNA synthesis at concentrations up to 300 μM.

Effect of Aphidicolin on DNA Synthesis Catalyzed by AMV DNA Polymerase. Aphidicolin, an inhibitor of eukaryotic DNA polymerase α, did not inhibit AMV DNA polymerase with poly(Cm)·oligo(dG) as template-primer (27). However, aphidicolin was subsequently shown to be a competitive inhibitor of dCTP, a substrate not required for poly(Cm)·oligo(dG) directed polymerization (28). In the course of

our experiments with phosphonoformate, aphidicolin was tested as an inhibitor of AMV DNA polymerase using activated DNA as the template-primer and all four dNTPs as substrates. Aphidicolin did not inhibit at concentrations up to 600 μM. Under identical conditions, a 50% decrease in the rate of DNA synthesis catalyzed by CHO DNA polymerase α was observed at 5 μM aphidicolin.

DISCUSSION

The results of our study are consistent with the mechanism of phosphonoformate inhibition of reverse transcriptase presented in Figure 6. We propose that phosphonoformate binds to the enzyme at the pyrophosphate binding site and functions as a dead-end inhibitor. For such a reaction scheme, the rate equation describing DNA synthesis in the presence of template-primer, dNTP, and phosphonoformate is given by Equation 1,* where K_{T-P}, K_{iT-P} and $K_{T-P'}$, $K_{iT-P'}$ refer to template-primer binding as substrate and product, respectively.

Under the experimental conditions that we have used, all terms containing the product, inorganic pyrophosphate, can be eliminated. The reciprocal of this equation arranged with dNTP as the variable substrate is given by Equation 2; the reciprocal arranged with the template-primer as the variable substrate is given by Equation 3. With dNTP as the variable substrate, both the slope and intercept terms are a function of phosphonoformate concentration and the inhibition pattern is noncompetitive. With the template-primer as the variable substrate and dNTP as the fixed substrate only the intercept is a function of phosphonoformate concentration;

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*The rate equation was first written in terms of individual rate constants by the method of King and Altman (38). It was then transformed into a rate equation in terms of kinetic constants as described by Cleland (23).

Figure 6. Proposed mechanism of phosphonoformate inhibition of reverse transcriptase. The postulated basic reaction mechanism in the absence of phosphonoformate is a modified ordered bi bi mechanism (9, 12, 36, 37). E denotes reverse transcriptase, T-P is template-primer, and PF is phosphonoformate.

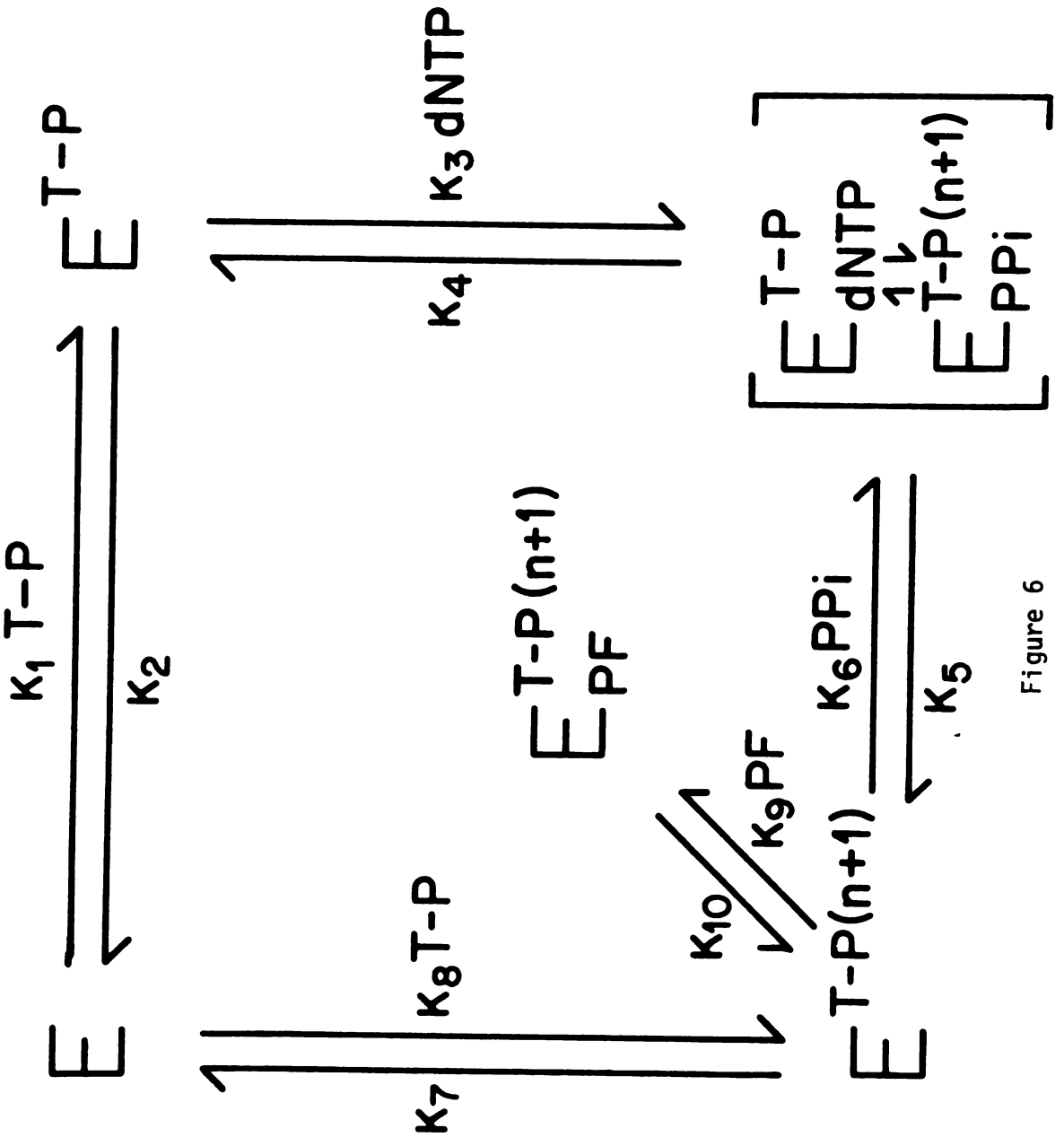
E
T-P
K1 T-P
K2
K3 dNTP
K4
T-P
dNTP
T-P(n+1)
PPi
T-P(n+1)
PF
K8 T-P
K7
K10
K9 PF
T-P(n+1)
K6 PPi
K5

Figure 6

$$\frac{v}{E_t} = \frac{V_1(T\text{-}P)(dNTP) - V_2\left(\frac{K_{dNTP}K_{iT\text{-}P}}{K_{iT\text{-}P'}K_{PPi}}\right)(T\text{-}P)(PPi)}{K_{iT\text{-}P}K_{dNTP} + K_{T\text{-}P}(dNTP) + K_{dNTP}\left(1 + \frac{K_{iT\text{-}P}}{K_{iT\text{-}P'}}\right)(T\text{-}P) + \left(1 + \frac{K_{T\text{-}P}}{K_{iT\text{-}P'}}\right)(T\text{-}P)(dNTP)} \quad (1)$$

$$+ \frac{K_{dNTP}K_{iT\text{-}P}}{K_{iT\text{-}P'}K_{PPi}}\left[K_{T\text{-}P'} + \left(1 + \frac{K_{T\text{-}P'}}{K_{iT\text{-}P}}\right)(T\text{-}P)\right](PPi)$$

$$+ \left(\frac{K_{dNTP}K_{iT\text{-}P}}{K_{iT\text{-}P'}K_{idNTP}K_{PPi}} + \frac{1}{K_{iPPi}}\right)(T\text{-}P)(dNTP)(PPi)$$

$$+ \left[\frac{K_{dNTP}K_{iT\text{-}P}}{K_{iT\text{-}P'}K_{PF}} + \left(\frac{K_{iT\text{-}P'}K_{PF} + K_{T\text{-}P}K_{iPF}}{K_{iPF}K_{iT\text{-}P'}K_{PF}}\right)(dNTP)\right](T\text{-}P)(PF)$$

$$\frac{E_t}{v} = \frac{1}{V_1}\left[\left[K_{dNTP}\left(1 + \frac{K_{iT\text{-}P}}{K_{iT\text{-}P'}}\right) + \frac{K_{iT\text{-}P}K_{dNTP}}{(T\text{-}P)} + \frac{K_{dNTP}K_{iT\text{-}P}}{K_{iT\text{-}P'}K_{PF}}(PF)\right]\frac{1}{(dNTP)} + \left(1 + \frac{K_{T\text{-}P}}{K_{iT\text{-}P'}}\right) + \frac{K_{T\text{-}P}}{(T\text{-}P)}\right.$$

$$\left. + \left(\frac{1}{K_{iPF}} + \frac{K_{T\text{-}P}}{K_{iT\text{-}P'}K_{PF}}\right)(PF)\right] \quad (2)$$

$$\frac{E_t}{v} = \frac{1}{V_1}\left[\left[K_{T\text{-}P} + \frac{K_{iT\text{-}P}K_{dNTP}}{(dNTP)}\right]\frac{1}{(T\text{-}P)} + \left(1 + \frac{K_{T\text{-}P}}{K_{iT\text{-}P'}}\right) + \frac{K_{dNTP}\left(1 + \frac{K_{iT\text{-}P}}{K_{iT\text{-}P'}}\right)}{(dNTP)} + \left[\frac{K_{dNTP}K_{iT\text{-}P}}{K_{iT\text{-}P'}K_{PF}} + \frac{1}{K_{iPF}}\right.\right. \quad (3)$$

$$\left.\left. + \frac{K_{T\text{-}P}}{K_{iT\text{-}P'}K_{PF}}\right](PF)\right]$$

the slope is independent of phosphonoformate concentration and the pattern is uncompetitive. Experimentally, these inhibition patterns were observed (Figures 3 and 4). Furthermore, the competitive inhibition pattern observed for phosphonoformate with pyrophosphate as the variable substrate in the exchange reaction (Figure 5) is consistent with the two compounds binding to the same site on the enzyme.

Phosphonoacetate and phosphonoformate inhibit the herpesvirus-induced DNA polymerase by binding to the enzyme at its pyrophosphate binding site (9, 12). In this case, however, the phosphonates may be acting as alternate product inhibitors because, as predicted by the rate equation for an alternate product inhibitor, noncompetitive inhibition is observed with activated DNA as the variable substrate.

Phosphonoformate, although an effective inhibitor of the polymerization reaction catalyzed by AMV DNA polymerase, does not inhibit the RNase H reaction. For AMV DNA polymerase, polymerization activity and RNase H activity are present on the same polypeptide chain (29) and are mechanistically independent (30) although the same template-primer binding site may be used for both activities (31). The phosphonoformate results may be interpreted to mean that $E_{PF}^{T\text{-}P(n+1)}$, a form of the enzyme that is inactive in polymerization, still has an active RNase H function. Like phosphonoformate, inorganic pyrophosphate inhibits polymerization but not the RNase H

activity of AMV DNA polymerase (32). Pyridoxal 5' phosphate is another compound that inhibits polymerization but not RNase H activity (33, 34). Since pyridoxal 5'-phosphate is a competitive inhibitor of the dNTPs, the $E^{T-P}_{B_6P}$ form of the enzyme, where B_6P is pyridoxal 5'-phosphate, must also have an active RNase H function. Knopf (35) recently has reported that herpes simplex virus DNA polymerase has an associated 3' to 5' exonuclease activity which is inhibited by phosphonoacetate. For this enzyme, both the DNA polymerase and exonuclease activities are inhibited by phosphonoacetate.

In the Mn^{2+}-containing DNA synthesis reactions catalyzed by AMV DNA polymerase, phosphonoformate was a much more potent inhibitor than in the Mg^{2+}-containing reactions. The apparent inhibition constants were lower by 50 to 100 times. The apparent K_ms for poly(A)·oligo(dT) and dTTP were also lower, but only by 5 to 10 times. This greater sensitivity to inhibition by phosphonoformate with Mn^{2+} than with Mg^{2+} was seen with all the template-primers tested with AMV DNA polymerase (Table 1), with four different reverse transcriptases (Table 2), and with DNA polymerase α.

Eukaryotic DNA polymerase α, but not β or γ, is inhibited by phosphonoformate (10). The phosphonoformate apparent inhibition constants for the α-polymerase were about 30 μM with activated DNA as the variable substrate and Mg^{2+} as the cofactor. When tested under identical conditions the phosphonoformate apparent inhibition constants for AMV DNA polymerase were 5 to 10 μM. For the herpesvirus of turkeys induced DNA polymerase, the apparent inhibition

constants were 1 to 3 μM (12). AMV DNA polymerase, however, was considerably less sensitive to phosphonoformate inhibition with purified RSV RNA as the template-primer or when the endogenous reaction was monitored with detergent-treated virions (Tables 1 and 2).

Since the reaction catalyzed by reverse transcriptase in vivo might not be as sensitive to phosphonoformate inhibition as that catalyzed by the α-polymerase, the selective inhbition of retrovirus replication by phosphonoformate at concentrations where cell proliferation would not be affected might not be realized. Sundquist and Larner (17) however, reported that phosphonoformate inhibited replication of visna virus at concentrations of 20 to 80 μM where cell growth was not affected. In contrast, RSV replication is not inhibited by phosphonoformate when tested at 500 μM (H-J. Kung, unpublished results).

Through the use of selective inhibitors, we would like to study the in vivo roles that reverse transcriptase and cellular DNA polymerases play in the synthesis of viral DNA and its integration into cellular DNA. Perhaps phosphonoformate will not be selective enough in its inhibition to enable us to differentiate the role of reverse transcriptase from that of the α-polymerase. Experiments are underway to decide. Aphidicolin, however, should be useful in defining the role of the α-polymerase in retrovirus replication. Aphidicolin is an effective inhibitor of the α-polymerase but not the β- or γ-polymerases (28), and as described in the Results, aphidicolin does not inhibit reverse transcriptase.

While this manuscript was in preparation, but after an abstract of our studies had been submitted to the American Society of Biological Chemists for presentation at the 1980 meetings, a report by Sundquist and Oberg (39) appeared describing their studies on the phosphonoformate inhibition of reverse transcriptase of avian and mammalian retroviruses. While their study emphasized the general aspects of phosphonoformate inhibitinn, our study emphasized mechanism.

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APPENDICES

APPENDIX A

MECHANISM OF PHOSPHONOACETATE INHIBITION OF HERPESVIRUS-INDUCED DNA POLYMERASE

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Mechanism of Phosphonoacetate Inhibition of Herpesvirus-Induced DNA Polymerase†

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ABSTRACT: Phosphonoacetate was an effective inhibitor of both the Marek's disease herpesvirus- and the herpesvirus of turkey-induced DNA polymerase. Using the herpesvirus of turkey-induced DNA polymerase, phosphonoacetate inhibition studies for the DNA polymerization reaction and for the deoxyribonucleoside triphosphate-pyrophosphate exchange reaction were carried out. The results demonstrated that phosphonoacetate inhibited the polymerase by interacting with it at the pyrophosphate binding site to create an alternate reaction pathway. A detailed mechanism and rate equation for the inhibition were developed. For comparison to phosphonoacetate, pyrophosphate inhibition patterns and apparent inhibition constants were determined. Twelve analogues of phosphonoacetate were tested as inhibitors of the herpesvirus of turkey-induced DNA polymerase. At the concentrations tested, only one, 2-phosphonopropionate, was an inhibitor. The apparent inhibition constant for it was about 50 times greater than the corresponding apparent inhibition constant for phosphonoacetate. DNA polymerase α of duck embryo fibroblasts, the host cell for the herpesviruses, was inhibited by phosphonoacetate. The apparent inhibition constants for the α polymerase were about 10–20 times greater than the corresponding inhibition constants for the herpesvirus-induced DNA polymerase. Duck DNA polymerase β, *Escherichia coli* DNA polymerase I, and avian myeloblastosis virus reverse transcriptase were not inhibited by phosphonoacetate.

Using a random testing of compounds with a cell culture screen, workers at Abbott Laboratories discovered that phosphonoacetate was an effective inhibitor of the replication of herpes simplex virus types 1 and 2. Three reports, all from Abbott Laboratories, have been published on studies with phosphonoacetate. Shipkowitz et al. (1973) reported that phosphonoacetate, when administered orally or topically to mice experimentally infected with herpes simplex virus, was able to significantly reduce the mortality associated with the viral infection. Overby et al. (1974) reported that the mode of inhibition of the replication of herpes simplex virus by phosphonoacetate appeared to be a result of the inhibition of herpes simplex viral DNA synthesis. Mao et al. (1975) reported that phosphonoacetate was an effective inhibitor of the herpes simplex-induced DNA polymerase. The major and minor DNA polymerase activities, presumably α and β, from the uninfected cells (Wi-38) were not inhibited by phosphonoacetate. Thus, phosphonoacetate probably inhibits herpes simplex DNA synthesis by specific inhibition of the viral induced DNA polymerase.

Phosphonoacetate also inhibits the replication of Marek's disease herpesvirus (MDHV)[1] and the herpesvirus of turkeys (HVT) (L. F. Lee et al., manuscript in preparation). MDHV is an oncogenic herpesvirus that causes a highly contagious malignant lymphoma of chickens (Marek, 1907; Churchill and Biggs, 1967; Nazerian et al., 1968; Solomon et al., 1968). HVT is used as a vaccine against Marek's disease (Purchase et al., 1971).

In this report, we demonstrate that phosphonoacetate is an effective inhibitor of the DNA polymerase induced by these avian herpesviruses. We report the results of a study of the inhibition patterns produced by phosphonoacetate on in vitro DNA synthesis by the partially purified HVT-induced DNA polymerase and propose a mechanism by which the inhibitor works. Finally, we report the results of a study of the effect of some structural analogues of phosphonoacetate on in vitro DNA synthesis by HVT-induced DNA polymerase and examine the effect of phosphonoacetate on four other DNA polymerases.

Materials and Methods

Reagents. Phosphonoacetate, disodium salt, was a gift from Abbott Laboratories. ^{32}P-labeled sodium pyrophosphate was purchased from New England Nuclear. Phosphonopropionate, the trimethyl ester of phosphonoacetate, α-phenylphosphonoacetate, 2-aminophosphonoacetate, 2-methyl-2-phosphonopropionate, and 2-phosphonopropionate were synthesized using published procedures (Chambers and Isbell, 1964; Berry et al., 1972; Isbell et al., 1972). Other reagents were from sources previously described (Boezi et al., 1974), or were from the usual commercial sources.

Purification of HVT-Induced DNA Polymerase. The preparation and growth of duck embryo fibroblasts and infection with HVT was as previously described (Boezi et al., 1974). The purification of the HVT-induced DNA polymerase and description of its catalytic and structural properties will be presented in detail elsewhere (manuscript in preparation). In short, however, HVT-induced DNA polymerase was purified from the nuclear fraction of infected cells by chromatography on phosphocellulose as described by Boezi et al. (1974). The peak fractions of HVT-induced DNA polymerase activity which eluted from the phospho-

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[1] Abbreviations used are: MDHV, Marek's disease herpesvirus; HVT, herpesvirus of turkeys.

cellulose column at about 0.30 *M* KCl were pooled, made 50% (v/v) in glycerol, and stored at −20°C. Only small amounts of HVT-infected duck embryo fibroblasts (about 1 g wet weight of cells) were used in a single purification procedure and throughout the procedure, bovine serum albumin was used to stabilize polymerase activity (Boezi et al., 1974). For these reasons, the specific enzymatic activity of HVT-induced DNA polymerase purified through phosphocellulose chromatography can only be estimated to be about 200 nmol of dNMP incorporated per 30 min per mg of protein and to amount to about a 25-fold purification over the crude nuclear fraction. Further purification was achieved by chromatography on DEAE-cellulose as described by Weissbach et al. (1971). For the experiments reported here, HVT-induced DNA polymerase purified through phosphocellulose was used. This enzyme fraction, when tested using the standard assay conditions for DNA polymerization contained no detectable DNase activity, deoxyribonucleoside triphosphatase activity, or inorganic pyrophosphatase activity. Many of the experiments reported here were also performed using the more highly purified HVT-induced DNA polymerase purified through DEAE-cellulose. No differences in the results were seen.

Other Polymerases. Escherichia coli DNA polymerase I was purchased from Boehringer Mannheim. AMV reverse transcriptase was a gift of Dr. J. Beard, Life Science Research Laboratories. Duck embryo fibroblast DNA polymerase α from the cytoplasmic fraction and DNA polymerase β from the nuclear fraction were purified through DEAE-cellulose as described by Weissbach et al. (1971). MDHV-induced DNA polymerase was purified from the nuclear fraction of infected duck embryo fibroblasts by phosphocellulose chromatography.

Assay of the DNA Polymerization Reaction. The standard reaction mixture employed for the HVT- or the MDHV-induced DNA polymerase contained in 200 μl: 50 m*M* Tris-HCl (pH 8), 1 m*M* dithiothreitol, 200 m*M* KCl, 2 m*M* $MgCl_2$, 500 μg/ml of bovine serum albumin, 200 μg/ml of activated calf thymus DNA (DNase I treated, Boezi et al., 1974), 20 μM ^{3}H-labeled deoxyribonucleoside triphosphate (specific radioactivity of 200–1000 cpm/pmol), 100 μM each of the other three deoxyribonucleoside triphosphates, and DNA polymerase. Incubation was at 37°C for 30 min. Assay of the conversion of ^{3}H-labeled deoxyribonucleoside triphosphate into a trichloroacetic acid insoluble form was as previously described (Boezi et al., 1974). Assay conditions were used so that the rate of DNA polymerization was linear with time and with the amount of DNA polymerase. For the kinetic studies, changes in concentrations of assay components are as noted in the legends to the figures.

In the experiments in which pyrophosphate was added to the reaction mixture as a product inhibitor, supplementary $MgCl_2$, in an amount equimolar to the sodium pyrophosphate added, was used. This supplementary $MgCl_2$ which was in addition to the 2 m*M* $MgCl_2$ routinely added to the standard reaction mixture was used to compensate for the chelation of Mg^{2+} ions by pyrophosphate. The amount of supplementary $MgCl_2$ to be added to the reaction mixtures was determined using the equations described by Moe and Butler (1972).

Assay of the dNTP-Pyrophosphate Exchange Reaction. The reaction mixture contained in 200 μl: 50 m*M* Tris-HCl (pH 8), 1 m*M* dithiothreitol, 200 m*M* KCl, 1 m*M* $MgCl_2$, 500 μg/ml of bovine serum albumin, 200 μg/ml of activated calf thymus DNA, 0.1 m*M* each of the four deoxyribonucleoside triphosphates, HVT-induced DNA polymerase, and various concentrations (0.14–1.1 m*M*) of ^{32}P-labeled sodium pyrophosphate (specific radioactivity of approximately 100 cpm/pmol). In addition to the 1 m*M* $MgCl_2$ 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°C for 30 min. The assay measuring the conversion of ^{32}P-labeled pyrophosphate to a Norit-adsorbable form was performed as described by Deutscher and Kornberg (1969). 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 m*M* ^{32}P-labeled sodium pyrophosphate, the reaction was found to be linear with time for at least 120 min 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.

Inhibition Patterns. Inhibition patterns and kinetic constants were defined according to the nomenclature of Cleland (1963a,b). Analysis of each reaction mixture was done in duplicate. The data for the double reciprocal plots were evaluated using a computer program based on the method of Wilkinson (1961). For evaluation of the apparent inhibition constants, replots of the intercepts and slopes of the double reciprocal plots were analyzed using a computer program for least-squares analysis.

Results

Phosphonoacetate Inhibition of the DNA Polymerization Reaction Catalyzed by Herpesvirus-Induced DNA Polymerase. Phosphonoacetate was an effective inhibitor of the DNA polymerization reaction catalyzed by MDHV- and by HVT-induced DNA polymerase. The addition of 2–3 μM phosphonoacetate to the standard reaction mixture resulted in a decrease in the rate of the DNA polymerization reaction by about 50%. Either in the presence or absence of phosphonoacetate, the rate of the reaction was linear for at least 1 hr.

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 the activated DNA at a saturating concentration of 200 μg/ml (Figure 1). The apparent inhibition constant (K_{ii}) determined from the replot of the vertical intercepts against phosphonoacetate concentration was 1.5 μM. The apparent inhibition constant (K_{is}) determined from the replot of the slopes against phosphonoacetate concentration was 1 μM.

With activated DNA as the variable substrate, and the four dNTPs at their apparent Michaelis constant concentrations of 2.5 μM each, phosphonoacetate gave linear noncompetitive inhibition (Figure 2). A replot of the vertical intercepts yielded a K_{ii} of 1.5 μM and a replot of the slopes yielded a K_{is} of 2.5 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 μM and K_{is} was about 20 μM. The higher K_{is} value seen at 100 μM dNTP compared to that seen at 2.5 μM dNTP indicated that the phosphonoacetate inhibition pattern was more nearly uncompetitive at

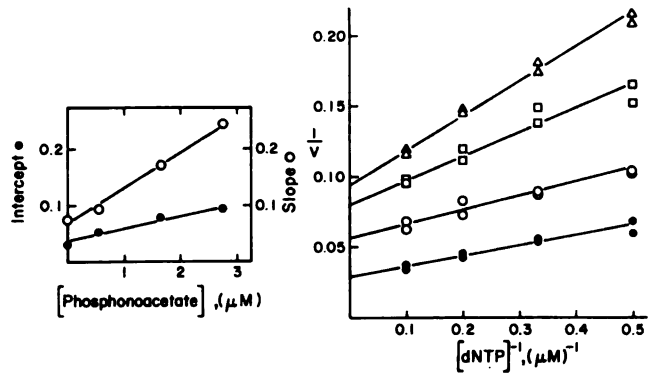

FIGURE 1: Double reciprocal plots with the four dNTPs as the variable substrate and phosphonoacetate as inhibitor. Activated DNA was at 200 μg/ml. The initial velocities were expressed as pmol of ^{3}H-labeled dCMP incorporated into DNA per 30 min. Phosphonoacetate concentrations were 0 (●), 0.55 μ*M* (○), 1.65 μ*M* (□), and 2.75 μ*M* (△). Equimolar concentrations of each of the four dNTPs were present in the different reaction mixtures. The replots of the slopes (○) and intercepts (●) as a function of phosphonoacetate concentration are shown in the left panel.

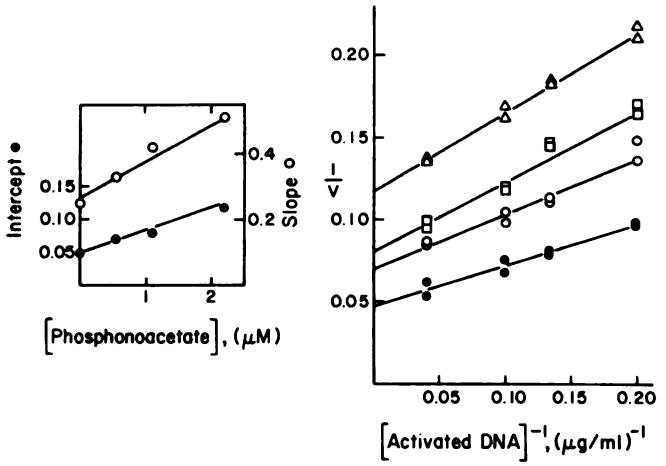

FIGURE 2: Double reciprocal plots with activated DNA as the variable substrate and phosphonoacetate as inhibitor. The four dNTPs were at 2.5 μ*M* each. Phosphonoacetate concentrations were 0 (●), 0.55 μ*M* (○), 1.1 μ*M* (□), and 2.2 μ*M* (△).

the higher concentration of dNTP than it was at the lower concentration.

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 3). The apparent K_{is} value for phosphonoacetate was 1.3 μ*M*. The apparent K_m value for pyrophosphate was 0.24 m*M*.

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 were performed. With the four dNTPs as the variable substrate, pyrophosphate gave linear noncompetitive inhibition. K_{ii} was 1.3 m*M* and K_{is} was 0.7 m*M*. With activated DNA as the variable substrate and with the four dNTPs at 2.5 μ*M* each, pyrophosphate gave

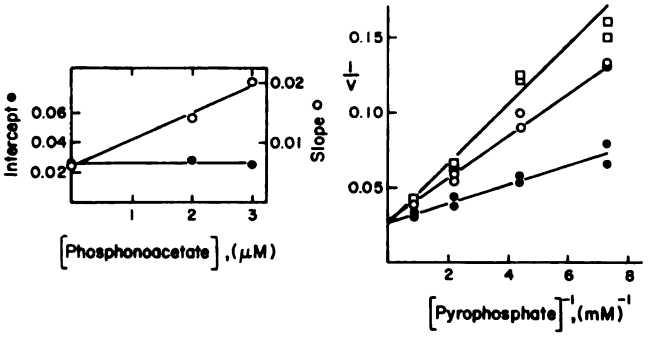

FIGURE 3: Double reciprocal plots of the dNTP-pyrophosphate exchange reaction with pyrophosphate as the variable substrate and phosphonoacetate as inhibitor. The initial velocities were expressed as picomoles of ^{32}P-labeled pyrophosphate converted to a Norit-adsorbable form per 30 min. Phosphonoacetate concentrations were 0 (●), 2 μ*M* (○), and 3 μ*M* (□).

linear noncompetitive inhibition. K_{ii} was 0.95 m*M* and K_{is} was 1.7 m*M*. Pyrophosphate gave linear uncompetitive inhibition with activated DNA as the variable substrate and with the four dNTPs at 20 μ*M* each. K_{ii} was about 0.9 m*M*.

Inhibition by Structural Analogues of Phosphonoacetate. When tested at a concentration of 200 μ*M*, 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, phosphonopropionate, amino methyl phosphonate, α-amino ethyl phosphonate, trimethyl ester of phosphonoacetate, α-phenylphosphonoacetate, 2-aminophosphonoacetate, 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 μ*M*.

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 10–20 times greater. DNA polymerase β, when tested to a phosphonoacetate concentration of 200 μ*M*, was not significantly inhibited. Likewise, neither *E. coli* DNA polymerase I nor AMV reverse transcriptase was significantly inhibited.

Discussion

The results of our study are consistent with the mechanism of phosphonoacetate inhibition presented in Figure 4. We propose that in the presence of phosphonoacetate an alternate pathway exists in addition to the basic polymerization pathway. Phosphonoacetate binds to the polymerase at the pyrophosphate binding site and is, thus, a competitive inhibitor of pyrophosphate in the exchange reaction. Phosphonoacetate may simply dissociate from the $E_{PA}^{DNA(n+1)}$ complex or may undergo reaction with the nucleotide at the 3′-end of the DNA primer chain to yield the postulated nucleotide, dNMP-PA, and E^{DNA}. Thus, phosphonoacetate inhibition occurs because the $E^{DNA(n+1)}$ complex is diverted by phosphonoacetate from the main polymerization pathway into an alternate pathway.

For such a reaction scheme, the rate equation describing DNA synthesis in the presence of DNA, dNTP, and phosphonoacetate is given by eq 1, where K_{DNA}, K_{iDNA} and $K_{DNA'}$, $K_{iDNA'}$ refer to DNA binding as substrate and product, respectively.[2]

$$v/E_t = [V_{1(DNA,dNTP)}(DNA)(dNTP) - V_{2(DNA,PA)}(K_{dNTP}K_{iDNA}/K_{iDNA'}K_{PA}) \times (DNA)(PA)]/[K_{iDNA}K_{dNTP} + K_{DNA}(dNTP) + K_{dNTP} \times (1 + K_{iDNA}/K_{iDNA'})(DNA) + (1 + K_{DNA}/K_{iDNA'}) \times (DNA)(dNTP)] + (K_{dNTP}K_{iDNA}/K_{PA}K_{iDNA'})[K_{DNA'} + (1 + K_{DNA'}/K_{iDNA})(DNA)](PA) + [(1/K_{iPA}) + (K_{dNTP}K_{iDNA}/K_{idNTP}K_{iDNA'}K_{PA})] \times (DNA)(dNTP)(PA)] \quad (1)$$

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

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

Our results are consistent with the predictions of eq 2. With activated DNA as the variable substrate and with the dNTP concentration at K_m levels, noncompetitive inhibition was observed (Figure 2). With the dNTP concentration at $40K_m$, the inhibition pattern was more nearly uncompetitive.

If eq 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 activated DNA concentration at about $20K_m$, the inhibition pattern was noncompetitive (Figure 1). Again, our results are consistent with the prediction from the rate equation.

Our results are not consistent with phosphonoacetate being a simple dead-end inhibitor in which it could only bind and dissociate from the $E^{DNA(n+1)}$ complex. For the case of a dead-end inhibitor, the rate equation[3] predicts that with DNA as the variable substrate the phosphonoacetate inhibition pattern will be uncompetitive regardless of the concentration of dNTP. However, as shown in Figure 2, at K_m levels of dNTP, the inhibition pattern which we observed was noncompetitive.

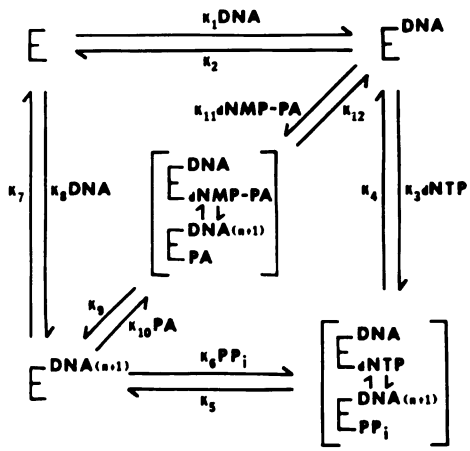

FIGURE 4: Proposed mechanism of phosphonoacetate inhibition of herpesvirus-induced DNA polymerase. The basic reaction mechanism in the absence of phosphonoacetate (PA) is a modified ordered bi-bi mechanism (Kornberg, 1969; McClure and Jovin, 1975). Initial velocity studies (S. S. Leinbach et al., unpublished data) and the pyrophosphate product inhibition studies presented here are consistent with this mechanism for the HVT-induced DNA polymerase. The postulated compound, dNMP-PA, is a deoxyribonucleoside 5'-monophosphate covalently linked to phosphonoacetate by a phosphodiester bond.

To verify our proposed scheme, the postulated nucleotide, dNMP-PA, must be identified in the reaction mixtures. Our first attempts to identify the nucleotide have not proved successful. When unlabeled phosphonoacetate and labeled DNA were used as substrates for the reverse reaction to polymerization, no labeled nucleotide was detected. The reverse reaction to polymerization, however, apparently goes very poorly since labeled nucleotide (dNTP) was not detected in reaction mixtures which contained either unlabeled pyrophosphate and labeled DNA or labeled pyrophosphate and unlabeled DNA as the substrates. Success in identifying the nucleotide is more likely to come from studies using radioactive phosphonoacetate of high specific activity as a substrate in an exchange-type reaction with unlabeled dNTP. Such studies are now being pursued.

In our proposed scheme, both phosphonoacetate and pyrophosphate inhibit DNA synthesis in an analogous manner. Indeed, the inhibition patterns for these two compounds are similar. The apparent inhibition constants for pyrophosphate, however, are two to three orders of magnitude greater than those for phosphonoacetate.

The studies with the analogues of phosphonoacetate give us 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 methyl amino, or a phosphono group cannot substitute for the carboxyl group.

Since phosphonoacetate seems to be a general inhibitor of the DNA polymerases induced by the herpesviruses, the pyrophosphate binding site for this group of DNA polymerases must be quite similar. The pyrophosphate binding site of DNA polymerase α of ducks appears to be somewhat

[2] The steady-state rate equation was first written in terms of individual rate constants by the method of King and Altman (1956). It was then transformed into a rate equation in terms of kinetic constants as described by Cleland (1963a). Using these procedures, we have also derived and verified the rate equation presented by McClure and Jovin (1975) for the modified ordered bi-bi mechanism.

[3] The rate equation for the dead-end inhibitor was derived by the method described by Cleland (1963b).

similar to this site. The pyrophosphate binding sites of DNA polymerase β of ducks, *E. coli* DNA polymerase I, AMV reverse transcriptase, and the α and β polymerases of human Wi-38 cells (Mao et al., 1975), however, appear to be different from this site on the herpesvirus-induced DNA polymerase.

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APPENDIX B

TREATMENT OF EXPERIMENTAL HERPESVIRUS INFECTIONS WITH PHOSPHONOFORMATE AND SOME COMPARISONS WITH PHOSPHONOACETATE

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Treatment of Experimental Herpesvirus Infections with Phosphonoformate and Some Comparisons with Phosphonoacetate†

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Phosphonoformate (PF) at a concentration of 5 to 10 μg/ml inhibited the growth of type 1 strains of herpes simplex virus (HSV) in tissue culture, whereas 20 to 30 μg/ml was required for inhibition of type 2 strains and about 50 μg/ml was required for murine cytomegalovirus. In mice inoculated intraperitoneally or intracerebrally with HSV or intraperitoneally with murine cytomegalovirus, treatment with 250 to 400 mg of PF per kg twice daily for 5 days had only minimal effectiveness. When mice were inoculated intravaginally (i.vg.) with HSV type 2 and treated i.vg. with 10% PF beginning 3 h after viral inoculation, treatment was effective in completely inhibiting viral replication in the genital tract. If i.vg. therapy was initiated 24 h after infection, when the mice had a mean virus titer of 10^5 plaque-forming units in vaginal secretions, a significant reduction in the mean virus titer was observed on days 3, 5, and 7 after infection as compared with control animals. In guinea pigs treated i.vg. with 10% PF beginning 6 h after i.vg. inoculation with HSV type 2 there was also complete inhibition of viral replication in the genital tract, and no extenal lesions developed. When therapy was initiated 24 h after infection there was a 4 to 5-log decrease in viral titers on days 3, 5, and 7 of the infection and a slight delay in the development of external lesions.

Phosphonoacetate (PA) has been reported to be active against herpes simplex virus (HSV) types 1 and 2 and both human and murine cytomegalovirus (MCMV) replication in tissue culture (7, 11, 13) and to be effective when applied topically to treat skin or mucous membrane HSV infections of animals (2, 3, 7, 10, 17). The compound has been less effective, however, when administered systemically to HSV-or MCMV-infected animals (1, 2, 7-9, 11). Because PA accumulates in the bone of a variety of experimental animals it has not been considered for use in humans (B. A. Bopp, C. B. Estep, and D. J. Anderson, Fed. Proc. **36**: 939, 1977). The efficacy of this drug in herpesvirus infections of animals has generated sufficient interest that a number of analogs have been synthesized in the hope that modification of the parent compound might result in maintenance of antiviral activity with a reduction in toxicity. One of these analogs, phosphonoformate (PF), has been reported to be as active as PA in inhibiting the replication of HSV, Marek's disease herpesvirus, and herpesvirus of turkeys (16). The mechanism of action also appears to be similar to PA, that is, inhibition of viral DNA polymerase.

The purpose of our experiments was to determine the antiviral activity of PF in tissue culture and in experimental herpesvirus infections of animals that are models of human disease and to compare its activity with that of PA. The experimental viral infections utilized were: (i) intraperitoneal (i.p.) or intracerebral (i.c.) inoculation of mice with HSV type 2, models of herpes encephalitis; (ii) i.p. inoculation of mice with MCMV, a model for disseminated cytomegalovirus infection; and (iii) intravaginal (i.vg.) inoculation of mice or guinea pigs with HSV type 2, models for genital herpes. With the exception of the guinea pig infection, the pathogenesis of these experimental infections has been reported in detail in previous publications (8, 11, 12). Due to the lack of availability of PA, the antiviral activities of PA and PF were compared directly only in tissue culture and in the genital infections of mice and guinea pigs.

MATERIALS AND METHODS

Animals and virus inoculation. Three-week-old female Swiss Webster mice (Simonsen Laboratories,

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Gilroy, Calif.) were inoculated by the i.p. route with 2 × 10^4 plaque forming units (PFU) of HSV or 10^6 PFU of MCMV, which usually resulted in 80 to 100% mortality. Two-week-old mice were inoculated i.c. with 2 to 5 PFU of HSV, which resulted in 95 to 100% mortality. Seven-week-old female mice or 200-g female guinea pigs (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were inoculated i.vg. with 10^6 or 10^4 PFU of HSV type 2, respectively, using a plastic catheter attached to a syringe. These concentrations of virus usually resulted in a 70 to 90% genital infection rate.

Viruses, media, cell cultures, and virus assay. The McIntyre, E-377, MS, E-326, and X-79 strains of HSV were obtained from Andre Nahmias, Emory University, Atlanta, Ga. Strain E-196 was from Harold Haines, University of Miami, Miami, Fla. The strains designated HL-3, HL-34, Wilson, and Heeter were isolated in our laboratory from patients with oral or genital lesions. These strains were typed using a modification of the chick embryo microtest as described by Yang et al. (19). The origin and preparation of pools of the Smith strain of MCMV and vaccinia virus have been previously described (4, 5). The media utilized, preparation of cell cultures, and assay for HSV have been described in a previous publication (6).

Antiviral agents. The disodium salt of PA (molecular weight 184) was provided by Abbott Laboratories, North Chicago, Ill. The trisodium PF hexahydrate (molecular weight 300) was synthesized in the Department of Biochemistry laboratories, Michigan State University, East Lansing, Mich. (16). Both PA and PF were dissolved in phosphate-buffered saline. In addition, a 5% PA cream and a 10% suspension of PF in 0.4% agarose were utilized. All drug solutions were prepared just before use and administered i.p. or i.vg. in a volume of 0.1 ml.

Susceptibility of viral strains to PF and PA. The susceptibility of a number of HSV strains and of MCMV and vaccinia virus to the two drugs was determined by a 50% plaque-reduction assay as described previously (6). Serial twofold dilutions of each drug in twice-concentrated minimal essential medium were mixed with an equal volume of 1.0% agarose. The overlay mixture containing drug was added to monolayer cultures of mouse embryo fibroblast (MEF) cells 1 h after inoculation with about 50 PFU of each of the virus strains.

Assay for virus in vaginal secretions. Vaginal swabs for isolation of HSV were obtained on days 1, 3, 5, 7, and 10 after inoculation. The swabs were placed in 1.0 ml of tissue culture medium, and frozen at −70°C until assayed for virus on fetal lamb kidney cells. Viral titers are expressed as PFU per milliliter of tissue culture medium. To rule out carry-over of drug from the genital tract to the viral assay system, all swabs were collected 12 h after treatment. Samples from drug-treated animals were also tested in vitro for antiviral activity. No evidence of drug in sufficient concentration to inhibit HSV replication was detected in the vaginal swabs tested.

Statistical evaluation. For comparison of final mortality rates in untreated and drug-treated mice, the data were evaluated by the Fisher exact test. The Mann-Whitney U test was used for comparing the mean day of death between untreated and drug-treated animals. The virus titer-day area under the curve and the lesion score-day area under the curve were generated through the use of a computer program. These data from untreated and drug-treated animals were compared also by the Mann-Whitney U test. For all statistical analyses, a P value of <0.05 was considered to be significant.

RESULTS

Susceptibility of viral strains to PA and PF in vitro. The susceptibility of five strains of HSV type 1, five strains of HSV type 2, MCMV, and vaccinia virus to the two drugs was determined in MEF cells. The mean values from two separate experiments are listed in Table 1. Since the two compounds have different molecular weights, the 50% inhibitory levels are expressed both in micrograms per milliliter and millimolar concentration. Most of the type 1 strains of HSV were inhibited by 4 to 10 μg of PA per ml and 5 to 15 μg of PF per ml. The type 2 strains required 10 to 20 μg of PA per ml and 20 to 30 μg of PF per ml for 50% inhibition. When the inhibitory levels of the two drugs are compared on an equimolar basis, however, there is little difference between the susceptibility of either virus type to PA or PF. The type 1 strains were about twofold more sensitive than type 2 strains to both compounds. Another member of the herpesvirus group, MCMV, was inhibited by about 15 μg of PA per ml and about 50 μg of PF per ml. Vaccinia virus, another DNA virus, was not inhibited by concentrations of 50 to 100 μg of either drug per ml.

Effect of treatment with PF on mortality of mice inoculated i.c. or i.p. with HSV type 2. After i.c. inoculation, untreated mice became paralyzed on days 5 to 7 and died on days 6 to 8. The effect of treatment with 400 mg of PF per kg given i.p. twice daily for 5 days is summarized in Table 2. In experiment 1 the virus control group treated with phosphate-buffered saline had a final mortality of 91% and a mean day of death of 6.6 days. Treatment with PF initiated 2, 24, or 48 h after virus inoculation had no effect on final mortality, but did significantly increase the mean day of death. Similar data were obtained in experiment 2.

After i.p. inoculation, untreated mice developed ruffled fur and hunching on days 5 to 7, and most of the animals died between 7 and 12 days after viral challenge. The results of treatment in this model infection with 250 to 300 mg of PF per kg i.p. twice daily for 5 days are summarized in Table 3. In experiment 1 treatment initiated 2 or 24 h after infection was effective in reducing final mortality. In the sec-

TABLE 1. *Susceptibility of type 1 and 2 strains of HSV, vaccinia virus and MCMV to PA and PF in MEF cells*

Virus strains	50% inhibitory levels[a]			
	μg/ml ± SD		mM	
	PA	PF	PA	PF
HSV type 1				
McIntyre	30.8 ± 8.2	78.1 ± 31.0	0.167	0.260
E-377	4.6 ± 2.2	8.4 ± 2.1	0.025	0.028
HL-3	3.6 ± 0.9	4.9 ± 2.7	0.020	0.016
HL-34	6.1 ± 4.4	13.6 ± 5.1	0.033	0.045
Wilson	4.1 ± 1.6	8.0 ± 0.1	0.022	0.027
HSV type 2				
MS	15.8 ± 10.5	32.8 ± 7.5	0.086	0.110
E-326	12.9 ± 4.2	26.9 ± 4.4	0.070	0.090
X-79	15.6 ± 8.3	31.0 ± 7.0	0.085	0.103
E-196	13.1 ± 6.3	20.5 ± 0.7	0.071	0.068
Heeter	13.5 ± 5.0	22.5 ± 0.07	0.073	0.075
MCMV	15.1	57.0	0.082	0.190
Vaccinia	>50.0	>50.0		

[a] Determined by plaque reduction assay. SD, Standard deviation.

TABLE 2. *Effect of treatment with PF on the mortality of 2-week-old mice inoculated i.c. with HSV type 2*

Treatment[a]	Mortality		MDD[b]
	No.[c]	%	
Experiment 1			
PBS	20/22	91	6.6
PF +2 h	17/20	85	8.9[d]
+24 h	16/19	84	7.4[e]
+48 h	18/19	95	7.6[e]
Experiment 2			
PBS	20/20	100	5.4
PF +2 h	18/18	100	9.3[d]
+24 h	20/20	100	6.8[e]
+48 h	19/20	95	7.1[d]
Drug control	1/10	10	

[a] Treatment was 400 mg of PF per kg i.p. twice daily for 5 days.
[b] MDD, Mean day of death.
[c] Number of mice dead/number treated.
[d] $P < 0.001$.
[e] $P < 0.01$.

ond experiment, where the dosage of drug was slightly increased, only therapy begun 2 h after viral inoculation reduced mortality.

Effect of treatment with PF on mortality of mice inoculated i.p. with MCMV. To determine the effectiveness of treatment with PF in another disseminated viral infection, mice were inoculated i.p. with MCMV. Control animals exhibited diminished activity, ruffled fur, and hunching by day 3, and most animals died between days 5 and 7. The results of treatment with 250 mg of PF per kg i.p. twice daily for 5 days are listed in Table 4. In experiment 1 only treatment begun 2 h after infection effectively reduced mortality; no reduction at any of the time periods was observed in the second experiment.

Effect of i.vg. treatment with PF on a genital HSV type 2 infection of mice. Genital

TABLE 3. *Effect of treatment with PF on the mortality of weanling mice inoculated i.p. with HSV type 2*

Treatment	Mortality		MDD[a]
	No.[b]	%	
Experiment 1[c]			
PBS	13/15	87	11.0
PF +2 h	1/13	8[d]	12.0
+24 h	6/15	40[e]	10.8
+48 h	8/15	53	11.5
Experiment 2[f]			
PBS	14/14	100	8.0
PF +2 h	1/12	8[d]	5.0
+24 h	12/15	80	7.9
+48 h	9/12	75	10.0[g]
Drug control	1/13	8	

[a] MDD, Mean day of death.
[b] Number of mice dead/number treated.
[c] 250 mg of PF per kg i.p. twice daily for 5 days.
[d] $P < 0.001$.
[e] $P < 0.05$.
[f] 300 mg of PF per kg i.p. twice daily for 5 days.
[g] $P < 0.01$.

TABLE 4. *Effect of treatment with PF on the mortality of weanling mice inoculated i.p. with MCMV*

Treatment[a]	Mortality		MDD[b]
	No.[c]	%	
Experiment 1			
PBS	10/15	67	5.9
PF +2 h	3/15	20[d]	5.0
+24 h	6/15	40	7.0
+48 h	6/15	40	6.5
Experiment 2			
PBS	12/15	80	5.5
PF +2 h	9/15	60	5.6
+24 h	9/15	60	6.9
+48 h	7/15	47	7.4[d]
Drug control	1/15	7	

[a] 250 mg of PF per kg i.p. twice daily for 5 days.
[b] MDD, Mean day of death.
[c] Number of mice dead/number treated.
[d] $P < 0.05$.

HSV infections of humans are one of the major areas of emphasis for viral chemotherapy. Since PA has been one of the most effective compounds we have tested in our mouse model of genital HSV infection, we next evaluated PF in this infection. The effect of treatment with 10% PF suspended in 0.4% agarose administered i.vg. twice daily for 7 days on viral replication in the genital tract is illustrated in Fig. 1. Treatment was begun either 3 or 24 h after viral inoculation. A group of mice that received a 5% PA cream preparation beginning 3 h after infection was also included. In the untreated control group the geometric mean titer of virus in vaginal secretions reached levels of about 10^4 PFU/ml on days 1, 3, and 5 and dropped to about 10^3 PFU/ml on day 7. Similar titers of virus were detected in animals treated with the agarose placebo. Treatment i.vg. with PA or PF initiated 3 h after viral inoculation was highly successful in preventing viral replication in that virus was

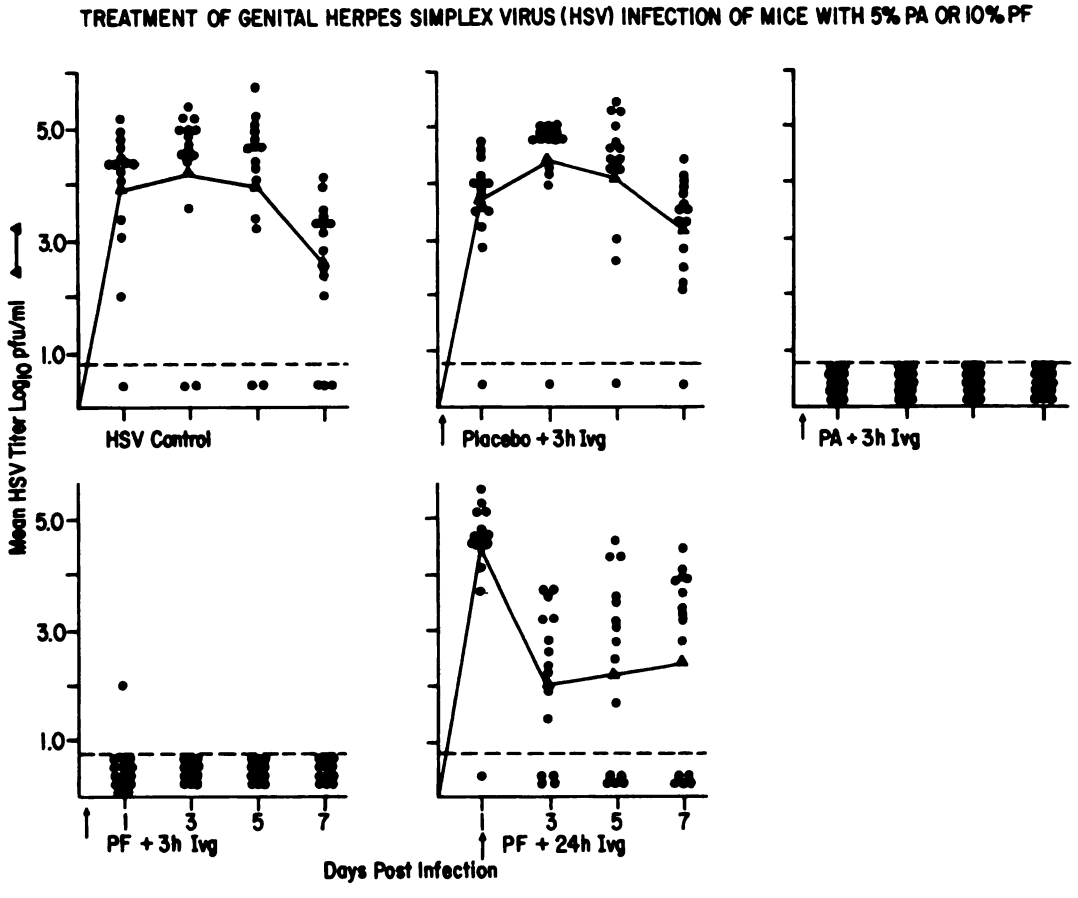

FIG. 1. *Treatment of genital HSV type 2 infection of mice with PA or PF. Treatment by the i.vg. route with 5% PA or 10% PF, twice daily for 5 days, was initiated at 3 or 24 h after infection. Symbols: (●) virus titer for each animal; (▲——▲)geometric mean HSV titer of all animals for each day sampled.*

isolated from none of the PA-treated mice and from only one of those that received PF. Treatment with PF initiated 24 h after infection, when 14 of 15 animals were infected with a mean titer of virus of $10^{4.5}$ PFU/ml, reduced the number of infected mice to 10 of 15 with a mean viral titer of 10^2 PFU/ml.

To compare statistically the differences in mean viral titers between untreated and drug-treated animals, we utilized a computer program to calculate the mean virus titer-day area under the curve (18). The mean areas for each group were then compared by the Mann-Whitney U test (Table 5). Treatment either with PA at 3 h or with PF at 3 or 24 h after viral inoculation significantly reduced viral replication in the genital tract. The placebo had no effect.

The final mortality rate of the infected animals in the previous experiment was reduced from 64 and 93% in the control and placebo-treated groups, respectively, to 0% in the groups treated with PA or PF at 3 h after viral inoculation. Therapy with PF was not effective in reducing mortality (64%) when initiated 24 h after infection.

Development of a genital HSV type 2 infection of guinea pigs and treatment with PF. The use of a genital infection of mice as a model for human herpes genitalis has two major drawbacks: (i) most infected mice develop an acute encephalitis and die within 10 days, so long-term follow-up is not possible, and (ii) with the virus strains we have utilized no external lesions develop, so we are unable to assess the efficacy of therapy on the course of lesion development. To develop a model which more closely resembles HSV genitalis in humans, we inoculated 200-g guinea pigs i.vg. with the MS strain of HSV type 2. After inoculation with this strain, there is a pattern of viral replication in the genital tract similar to that observed in mice and, more importantly, lesions appear on the external genitalia beginning 3 to 4 days after i.vg. inoculation. Lesions were scored by a single investigator throughout the experiment according to the scale: 1+, redness or swelling; 2+, a few small vesicles; 3+, several large vesicles; 4+, several large ulcers and maceration. A declining score was used during the healing stage. The mean lesion score for all the animals was calculated for each day of observation.

The effect of i.vg. treatment with 10% PF suspended in 0.4% agarose is shown in Fig. 2. Treatment was initiated 6 or 24 h after viral inoculation and continued twice daily for 7 days. In the first experiment (Fig. 2, upper row) the untreated HSV infected animals had peak mean vaginal viral titers of 10^5 PFU/ml on day 1; these gradually declined through day 10. External lesions first appeared on day 4, and peak mean lesion scores were observed on days 8 to 10. Similar results were obtained in the agarose placebo-treated group. When treatment with PF was begun 6 h after viral inoculation, there was complete inhibition of viral replication in the genital tract of most animals, and no external lesions developed. In the second experiment (Fig. 2, bottom row), the untreated virus control and the placebo-treated animals followed the same course as described above. If i.vg. treat-

TABLE 5. *Area under the curve analysis*[a]

Experimental groups			Mean area under the curve	
Animals	Group		Vaginal virus titers	Lesion scores
Mice				
	Control		26.0	
	Placebo	+3 h	27.5[b]	
	PA	+3 h	0[c]	
	PF	+3 h	0.1[c]	
	PF	+24 h	18.9[c]	
Guinea pigs				
	Experiment 1			
	Control		34.0	25.3
	Placebo	+6 h	33.3[b]	21.9[b]
	PF	+6 h	3.6[c]	0[c]
	Experiment 2			
	Control		30.0	25.3
	Placebo	+24 h	29.3[b]	20.3[b]
	PF	+24 h	13 1[c]	18.2[d]

[a] Area under the curve analysis for comparison of mean vaginal virus titers and mean external lesion scores between untreated and drug-treated mice and guinea pigs inoculated intravaginally with HSV type 2.

[b] Placebo not significantly different from untreated control.

[c] $P < 0.001$ when compared with placebo.

[d] Not significantly different from placebo; $P = 0.05$ when compared with untreated control.

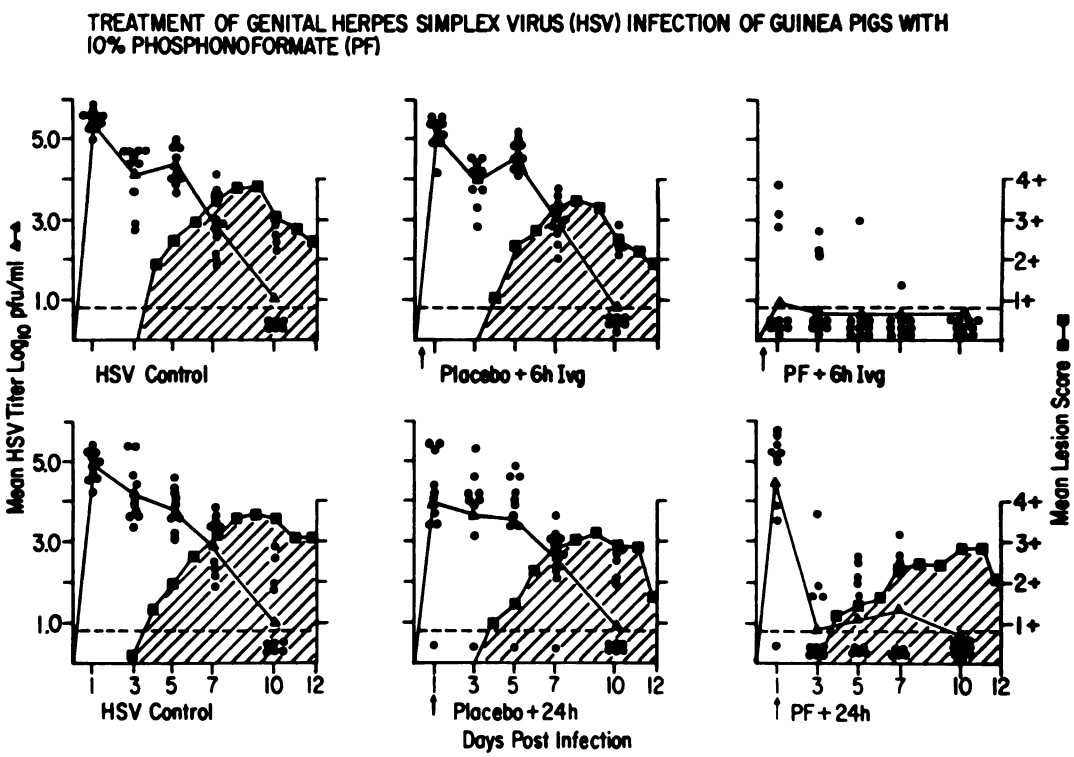

FIG. 2. *Treatment of genital HSV type 2 infection of guinea pigs with PF. Treatment by the i.vg. route was initiated at 6 or 24 h after infection with 10% PF twice daily for 7 days. Symbols: (●) virus titer for each animal; (▲——▲) geometric mean HSV titer of all animals for each day sampled; (■——■) mean score of external lesions.*

ment with PF was initiated at 24 h after infection, when 9 of 10 animals were infected with a mean viral titer of $10^{4.5}$, there was a reduction in the number of infected animals to four, greater than a 4-log decrease in mean titers of virus on day 3, and a slight delay in the development of lesions. In other experiments, treatment with PA produced similar results (data not presented). Values for the mean virus titer-day area under the curve and mean lesion score-day area under the curve for each group were calculated and analyzed for statistical significance (Table 5). Treatment with PF, initiated either 6 or 24 h after infection, significantly altered viral replication in the genital tract, but only early treatment had an effect on lesion development.

DISCUSSION

The minimal inhibitory levels of PF determined in our plaque reduction assay for several type 1 and type 2 strains of HSV and for MCMV are similar to those reported for PA by us and other investigators (7, 11, 13). When compared on a micrograms-per-milliliter basis, the viruses tested were generally about twofold more susceptible to PA than PF; however, when compared on a millimolar basis, little difference was noted.

In mice inoculated i.c. or i.p. with HSV or i.p. with MCMV, treatment with PF was successful only if begun 2 to 24 h after viral inoculation. In these same animal model infections, treatment with PA has also been effective only with early treatment (1, 2, 8, 11). Therefore, with systemic administration for HSV and MCMV infections of mice, PF appeared to be about as effective as PA. We have reported previously that in mice inoculated with HSV by the i.c. or i.p. route treatment with either adenine arabinoside or adenine arabinoside 5'-monophosphate was effective in preventing mortality when therapy was initiated 48 to 96 h after viral inoculation (8). From these data it appears that both PA and PF were less effective than adenine arabinoside or adenine arabinoside 5'-monophosphate in these experimental infections. In a genital HSV infection of mice, i.vg. treatment with PA initiated 3 or 24 h after viral inoculation was effective in preventing or reducing HSV replication in the genital tract (7). Treatment i.vg. with PF was also highly effective in altering HSV replication in the genital tract of both mice

and guinea pigs when administered as late as 24 h after viral inoculation. Additionally, early treatment (6 h after challenge) was effective in preventing the development of external lesions in guinea pigs. In contrast, treatment initiated later in the course of infection in guinea pigs, i.e., 24 h after infection, failed to retard the development of the lesions. In both genital HSV model infections PF appeared to be as effective as PA. PA has also been reported to be efficacious against a genital HSV infection of hamsters (15), skin lesions induced by HSV and vaccinia virus in mice and rabbits (2, 17), and herpes keratitis in rabbits (3, 17). The data reported in this paper suggest that PF would also be effective in these other model infections.

Although PA is a very effective antiviral agent when applied topically against a number of experimental HSV infections, it has not been tested in humans because of potential toxicity. Several investigators have noted skin irritation associated with the drug (14), and there is one report in which the drug accumulated in bone of a number of animal species (Bopp et al., Fed. Proc. **36**:939, 1977). In our studies using topical treatment with PA or PF on mouse and guinea pig genitalia, no adverse effects were noted. Further toxicology studies are needed, however, to determine if alteration of the molecule has eliminated the toxicity associated with PA. The effectiveness of PF in the treatment of genital HSV infections of mice and guinea pigs indicates that additional studies are needed to further define the potential for this drug in the treatment of mucocutaneous HSV infections in humans.

ACKNOWLEDGMENTS

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APPENDIX C

INHIBITION OF EUKARYOTIC DNA POLYMERASES BY PHOSPHONOACETATE AND PHOSPHONOFORMATE

By

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Inhibition of Eucaryotic DNA Polymerases by Phosphonoacetate and Phosphonoformate[1]

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Phosphonoacetate was found to be an inhibitor of the DNA polymerase α from three human cells, HeLa, Wi-38, and phytohemagglutinin-stimulated lymphocytes. The inhibition patterns were determined. The apparent inhibition constants (K_{ii}) were about 30 μM. Thus the DNA polymerase α is 15 to 30 times less sensitive to phosphonoacetate than the herpesvirus-induced DNA polymerase. The DNA polymerase α from Chinese hamster ovary cells and calf thymus was also inhibited. The DNA polymerases β and γ from the eucaryotic cells were relatively insensitive to phosphonoacetate. The sensitivity of the DNA polymerase α and the relative insensitivity of the DNA polymerase β and γ appeared to be general characteristics of the vertebrate polymerases. DNA polymerases from two other eucaryotic cells, yeast DNA polymerase A and B and tobacco cell DNA polymerase, were inhibited by phosphonoacetate, and to about the same extent as the α-polymerases. Fourteen phosphonate analogs were examined for inhibition of the HeLa DNA polymerase α. Only one, phosphonoformate, was an inhibitor. The mechanism of inhibition for phosphonoformate was analogous to that for phosphonoacetate.

Phosphonoacetate is an effective inhibitor of the replication of herpesviruses (1-4). The inhibition of viral replication is through an effect on the viral-induced DNA polymerase (5-9). At the concentrations at which phosphonoacetate is an effective antiherpesvirus agent, it has no obvious cytotoxic effects. At higher concentrations, however, phosphonoacetate is cytotoxic (2, 5, 10) and cellular DNA synthesis is inhibited. Cell growth is impaired and the cells are apparently arrested at interphase.

Mao *et al.* (6) and Mao and Robishaw (7) reported that the inhibitory effect of phosphonoacetate on the herpesvirus-induced DNA polymerase was specific. They reported that the herpesvirus-induced DNA polymerase was highly sensitive to phosphonoacetate, but that the cellular DNA polymerases α and β of human Wi-38 cells, the host cells for their herpesvirus infections, were not inhibited by phosphonoacetate. Huang (2) and Hirai and Watanabe (11) also reported that the DNA polymerases α and β of Wi-38 cells were not inhibited. In contrast, Bolden *et al.* (12) showed that, although the β-polymerase of human HeLa cells was not inhibited by phosphonoacetate, the α-polymerase was inhibited. Moreover, Bolden *et al.* (12) reported that the HeLa α-polymerase was as sensitive to phosphonoacetate as the herpesvirus-induced DNA polymerase. For duck embryo fibroblasts, Leinbach *et al.* (9) demonstrated that the α-polymerase was sensitive to phosphonoacetate, but that it was 15 to 30 times less sensitive to the inhibitor than the herpesvirus-induced DNA polymerase.

With regard to the human cells, Overby *et al.* (13) suggested that the apparent differences in the sensitivities of the α-polymerases from Wi-38 and HeLa cells might be explained by the fact that the two cell types arose from different sources.

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Wi-38 cells originated from normal embryonic lung tissue (14) and HeLa cells arose from cervical cancer tissue (15). They argued that normal and transformed cells might have different α-polymerases.

In this report, we have reexamined the effect of phosphonoacetate on the α-polymerases of Wi-38 and HeLa cells. We have also examined the α-polymerase of phytohemagglutinin-stimulated human lymphocytes. In addition, we have looked at the α-polymerases of Chinese hamster ovary cells and calf thymus as well as the DNA polymerases from two nonvertebrate eucaryotic cells, yeast and tobacco cells. Finally we have investigated the effect of several phosphonate analogs on the HeLa α-polymerase.

EXPERIMENTAL PROCEDURES

Reagents. Phosphonoacetic acid was from Richmond Organics. Trisodium phosphonoformate and triethyl phosphonoformate were prepared following the procedure of Nylen (16). Deoxythymidine 5'-phosphophosphonoacetate was a gift from Dr. Susan S. Leinbach of this laboratory. Phosphonoacetamide and *N*-methylphosphonoacetamide were gifts from Dr. George Stark, Stanford University. Acetonyl phosphonate was a gift from Dr. Ronald Kluger, University of Toronto. The α-polymerase from calf thymus and the γ-polymerase from fetal calf liver were obtained from Worthington. Other reagents were from sources previously described (9) or from the usual commercial sources.

Cell cultures. HeLa (CCL 2) and Wi-38 (CCL 75) cells were purchased from the American Type Culture Collection. Chinese hamster ovary (CHO)[2] cells, proline requiring, were obtained from Dr. Louis Siminovitch, University of Toronto. HeLa, Wi-38, and CHO cell cultures were tested at 37°C aerobically and anaerobically for mycoplasma contamination, as described by Hayflick (17), and were not found to be contaminated. HeLa and CHO cells were grown in suspension culture in F-14 and F-19 media (Gibco), respectively, supplemented with 10% fetal calf serum. Wi-38 cells (passages 16–29) were grown in monolayer culture in G-13 medium (Gibco) supplemented with 10% fetal calf serum. *Saccharomyces cerevisiae*, X2180 diploid strain, was obtained from Dr. William L. Smith of this department and was grown in glucose-yeast extract-casamino acids medium. Tobacco XD cells (*Nicotiana tabacum* L. var. Xanthi) were a gift from Dr. Philip Filner of this department and were grown according to the procedure of Filner (18). All cells were harvested in midlog phase and frozen at −70°C until use. Lymphocytes were purified from human blood by centrifuging over Ficoll-Paque (Pharmacia) (19). The lymphocytes were diluted in G-13 medium containing 10% fetal calf serum and 0.8% (v/v) phytohemagglutinin-*P* (Difco) and cultured for 4.5 days.

Assay of the DNA polymerization reaction. The standard reaction mixture for the α-polymerase contained in 200 μl: 50 mM Tris-hydrochloride (pH 8.0), 1 mM dithiothreitol, 500 μg/ml of bovine serum albumin, 10 mM $MgCl_2$, 20 mM KCl, 200 μg/ml of activated calf thymus DNA (DNase I treated), 20 μM ^{3}H-labeled deoxyribonucleoside triphosphate, 100 μM each of the other three deoxyribonucleoside triphosphates, and α-polymerase. For the β-polymerase and the yeast DNA polymerases, KCl was omitted from the standard reaction mixture. For the tobacco cell DNA polymerase, KCl was omitted, dithiothreitol was 20 mM, and $MgCl_2$ was 6 mM. The standard reaction mixture for the fetal calf liver γ-polymerase contained in 200 μl: 50 mM Tris-hydrochloride (pH 8.0), 2.5 mM dithiothreitol, 500 μg/ml of bovine serum albumin, 4 mM $MgCl_2$, 100 mM KCl, 50 μg/ml of poly(rA)·oligo$(dT)_{12-18}$, 100 μM ^{3}H-labeled dTTP, and γ-polymerase. Incubation was at 37°C for 30 min. Assay of the conversion of ^{3}H-labeled deoxyribonucleoside triphosphate into a trichloroacetic acid-insoluble form was as previously described (20). Assay conditions were used so that the rate of DNA polymerization was linear with time and with the amount of DNA polymerase. For the kinetic studies, changes in the concentrations of assay components are as noted in the legends to the figures.

Purification of the DNA polymerases. HeLa and CHO cells were fractionated into a nuclear and a cytoplasmic fraction according to the procedure of Chang and Bollum (21). The purification of the α-polymerase from the cytoplasmic fraction and the β-polymerase from the nuclear fraction was as described in the procedure of Weissbach *et al.* (22). This procedure involved purification by DEAE-cellulose and phosphocellulose column chromatography. The peak fractions of DNA polymerase activity from the phosphocellulose column were pooled, made 50% (v/v) in glycerol, and stored at −20°C. The specific enzymatic activity (nanomoles of deoxyribonucleoside triphosphate incorporated into an acid-insoluble form in 30 min at 37°C per milligram of protein) was 2400 for the HeLa α-polymerase and 1300 for the CHO α-polymerase. The purified α-polymerases showed a single peak of enzymatic activity at about 7.5 S in a linear 20 to 40% glycerol gradient containing 0.45 M KCl. The Wi-38 α-polymerase was purified as above except that the phosphocellulose chromatography step was omitted. Glycerol gradient centrifugation was used to purify the

[2] Abbreviations used: CHO, chinese hamster ovary; DEAE, diethylaminoethyl; dTTP, deoxythymidine 5'-triphosphate; dNTP, deoxyribonucleoside triphosphate.

α-polymerase from phytohemagglutinin-stimulated lymphocytes, as described by Bertazzoni *et al.* (23). DNA polymerases A and B from *S. cerevisiae* were isolated through DEAE-cellulose chromatography according to Wintersberger and Wintersberger (24). The DNA polymerase from tobacco cells was prepared as described by Srivastava (25) and was further purified by DEAE-cellulose chromatography.

Inhibition patterns. Inhibition patterns and kinetic constants were defined according to the nomenclature of Cleland (26, 27). Analysis of each reaction mixture was done in duplicate. The data for the double-reciprocal plots were evaluated using a computer program based on the method of Wilkinson (28). For evaluation of the apparent inhibition constants, replots of the intercepts and slopes of the double-reciprocal plots were analyzed using a computer program for least-squares analysis.

RESULTS

Phosphonoacetate inhibition of the DNA polymerization reaction catalyzed by HeLa DNA polymerase α. Phosphonoacetate was an inhibitor of the DNA polymerization reaction catalyzed by the HeLa α-polymerase. The addition of 25 to 30 μM phosphonoacetate to the standard reaction mixture resulted in a decrease in the rate of the DNA polymerization reaction by about 50%. In either the presence or the absence of phosphonoacetate, the rate of the reaction was linear for at least 1 h. Phosphonoacetate gave linear noncompetitive inhibition with the four dNTPs as the variable substrate and activated DNA at a saturating concentration of 200 μg/ml (Fig. 1). The apparent inhibition constant (K_{ii}) determined from the replot of the vertical intercepts against the phosphonoacetate concentration was 29 μM. The apparent inhibition constant (K_{is}) determined from the replot of the slopes against phosphonoacetate concentration was 53 μM. Phosphonoacetate also gave linear noncompetitive inhibition with activated DNA as the variable substrate, the three dNTPs at 100 μM each, and the ^{3}H-labeled dTTP at 20 μM. The K_{ii} for phosphonoacetate was determined to be 29 μM and the K_{is} 200 μM.

The above results therefore demonstrate that the HeLa α-polymerase is indeed sensitive to phosphonoacetate. It is, however, less sensitive than the herpesvirus-induced DNA polymerase. The apparent inhibition constants for the herpesvirus-induced DNA polymerase were in the 1 to 2 μM range (7, 9).

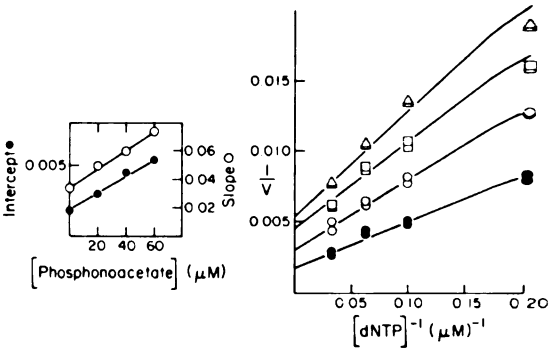

FIG. 1. Double-reciprocal plots with the four dNTPs as the variable substrate and phosphonoacetate as inhibitor of the purified HeLa α-polymerase. Activated DNA was at 200 μg/ml. The initial velocities were expressed as picomoles of ^{3}H-labeled dTMP incorporated into DNA per 30 min. Phosphonoacetate concentrations were 0 (●), 20 (○), 40 (□), and 60 μM (△). Equimolar concentrations of each of the four dNTPs were present in the different reaction mixtures. The replots of the slopes (○) and intercepts (●) as a function of phosphonoacetate concentration are shown in the left panel.

Phosphonoacetate inhibition of the DNA polymerization reaction catalyzed by Wi-38 DNA polymerase α. Contrary to the results published by Mao *et al.* (6), Mao and Robishaw (7), Huang (2), and Hirai and Watanabe (11), phosphonoacetate was an inhibitor of the Wi-38 α-polymerase. The inhibition constants and the inhibition patterns were similar to those of the HeLa α-polymerase. Phosphonoacetate gave linear noncompetitive inhibition with the four dNTPs as the variable substrate and activated DNA at a saturating concentration of 200 μg/ml. The K_{ii} was 15 μM and the K_{is} was 25 μM. With activated DNA as the variable substrate, the three dNTPs at 100 μM each, and the ^{3}H-labeled dTTP at 20 μM, phosphonoacetate gave linear noncompetitive inhibition. The K_{ii} was 33 μM and the K_{is} was 150 μM.

The effect of phosphonoacetate on the DNA polymerization reaction catalyzed by other α-polymerases. The α-polymerase from a third human cell source was examined for its sensitivity to phosphonoacetate. The α-polymerase from phytohemaggluti-

nin-stimulated lymphocytes was as sensitive as the α-polymerase from HeLa and Wi-38 cells. Indeed, the inhibition by phosphonoacetate seems to be a general characteristic of α-polymerases. For example, the CHO α-polymerase and the one from calf thymus were inhibited. For the CHO α-polymerase the inhibition patterns and apparent inhibition constants were examined and found to be the same as those reported above for the HeLa α-polymerase.

The effect of phosphonoacetate on the DNA polymerization reaction catalyzed by other DNA polymerases. Initial results by Mao *et al.* (6), Bolden *et al.* (12), and Leinbach *et al.* (9) showed that the β-polymerase of eucaryotic cells was not significantly inhibited by phosphonoacetate. Our results agree. The addition of 200 μM phosphonoacetate to the standard reaction mixture produced no significant inhibition in the rate of the DNA polymerization reaction catalyzed by the HeLa or CHO β-polymerase. The β-polymerases of Wi-38 cells and human lymphocytes were also insensitive to phosphonoacetate. Likewise, the γ-polymerase of fetal calf liver was not significantly inhibited. The HeLa γ-polymerase had previously been shown by Knopf *et al.* (29) not to be significantly sensitive to phosphonoacetate.

The DNA polymerases from two nonvertebrate eucaryotic cells were examined for their susceptibility to phosphonoacetate. The DNA polymerases A and B from *S. cerevisiae* were inhibited. The rate of DNA synthesis for both DNA polymerases A and B was decreased 50% by the addition of 15–20 μM phosphonoacetate to the standard reaction mixture. Similarly, the DNA polymerase from tobacco cells was sensitive to phosphonoacetate at about the same concentration.

The effect of other phosphonate compounds on the DNA polymerization reaction catalyzed by HeLa DNA polymerase α. Recently it was discovered by Reno *et al.* (submitted for publication) that phosphonoformate is an effective inhibitor of the herpesvirus-induced DNA polymerase. Phosphonoformate is also an inhibitor of the HeLa α-polymerase. With the four dNTPs as the variable substrate and activated DNA at a saturating concentration of 200 μg/ml, phosphonoformate, like phosphonoacetate, gave linear noncompetitive inhibition (Fig. 2). A replot of the vertical intercepts yielded a K_{ii} of 24 μM and a replot of the slopes gave a K_{is} of 59 μM. Phosphonoformate also gave linear noncompetitive inhibition with activated DNA as the variable substrate. A replot of the vertical intercepts gave a K_{ii} of 32 μM and a replot of the slopes yielded a K_{is} of 176 μM. Phosphonoformate, therefore, appears to be equally effective as phosphonoacetate in its inhibition of the HeLa α-polymerase.

Reno *et al.* (submitted for publication) have shown that phosphonoformate inhibits the herpesvirus-induced DNA polymerase in a manner analogous to that of phosphonoacetate. This also seems to be the case for the HeLa α-polymerase. A multiple inhibition analysis (30, 31) of the DNA polymerization reaction indicated that phosphonoformate and phosphonoacetate are mutually exclusive inhibitors and, therefore, bind at the same site on the α-polymerase. For the multiple inhibition analysis the concentration of phosphonoformate was varied in the presence of fixed concentrations of phosphonoacetate. As shown in Fig. 3, a plot of 1/V against phosphonoformate concentration resulted in a series of parallel lines, indicating that the two phosphonate com-

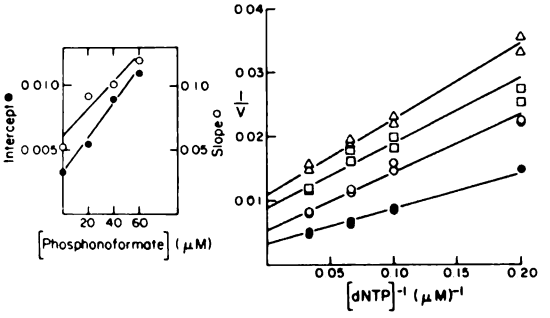

FIG. 2. Double-reciprocal plots with the four dNTPs as the variable substrate and phosphonoformate as inhibitor of the purified HeLa α-polymerase. Activated DNA was at 200 μg/ml. Phosphonoformate concentrations were 0 (●), 20 (○), 40 (□), and 60 μM (△).

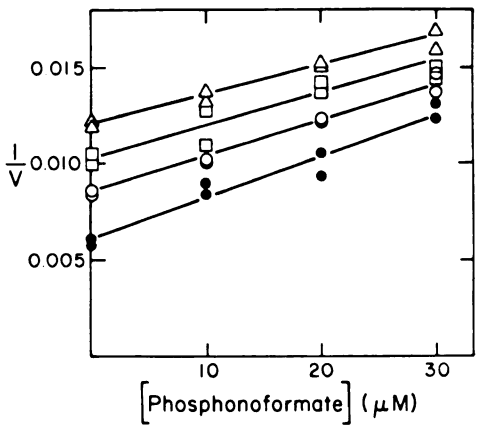

FIG. 3. Multiple inhibition of the HeLa α-polymerase by phosphonoacetate and phosphonoformate. Activated DNA was at 200 μg/ml and the four dNTPs were at 7 μM each. Phosphonoacetate concentrations were 0 (●), 10 (○), 20 (□), and 30 μM (△).

pounds are mutually exclusive inhibitors.

Other phosphonate compounds produced no significant inhibition of the rate of the polymerization reaction catalyzed by the HeLa α-polymerase when tested to a concentration of 200 μM. The compounds which were tested were imidodiphosphate, methylene diphosphate, sulfoacetate, 2-phosphonopropionate, 3-phosphonopropionate, 2-phenylphosphonoacetate, deoxythymidine 5'-phosphophosphonoacetate, trimethyl phosphonoacetate, triethyl phosphonoacetate, triethyl phosphonoformate, phosphonoacetamide, *N*-methylphosphonoacetamide, and acetonyl phosphonate.

DISCUSSION

This report demonstrates that the α-polymerases from three human cells, HeLa, Wi-38, and phytohemagglutinin-stimulated lymphocytes, and the enzymes from calf thymus and CHO cells are inhibited by phosphonoacetate. The apparent inhibition constants (K_{ii}) are about 30 μM. The HeLa α-polymerase has previously been shown by Bolden *et al.* (12) to be inhibited by phosphonoacetate. This report confirms their result. In addition, we report the apparent inhibition constants and inhibition patterns for the α-polymerase. In contrast to the results of Bolden *et al.* (12), who reported that the α-polymerase and the herpesvirus-induced DNA polymerase were equally sensitive to phosphonoacetate, our results demonstrate that the α-polymerase is, in fact, 15 to 30 times less sensitive. Our results showing that the Wi-38 α-polymerase is sensitive to phosphonoacetate contradict the results reported by other authors (2, 6, 7, 11).

We found that the β-polymerases of three human cells, HeLa, Wi-38, and lymphocytes, are relatively insensitive to phosphonoacetate. Our result for HeLa is in agreement with that of Bolden *et al.* (12) and our result for Wi-38 agrees with the results reported by others (2, 6, 7, 11). Moreover, the β-polymerase of CHO cells, as reported here, and the β-polymerase of duck embryo fibroblasts, as previously reported by Leinbach *et al.* (9), are not inhibited by phosphonoacetate. As observed by Knopf *et al.* (29) with HeLa γ-polymerase, the γ-polymerase of fetal calf liver was also relatively insensitive to phosphonoacetate. Therefore, the sensitivity of the α-polymerase and the relative insensitivity of the β- and γ-polymerases appear to be general characteristics of vertebrate polymerases.

Of the many phosphonate compounds which were examined for inhibition of the HeLa α-polymerase, only one, phosphonoformate, was shown to be an inhibitor. Phosphonoformate was also an inhibitor of the yeast DNA polymerases A and B and the tobacco cell DNA polymerase. Recently, in this laboratory, Reno *et al.* (submitted for publication) discovered that phosphonoformate is an effective inhibitor of herpesvirus-induced DNA polymerase and that the mechanism of inhibition by phosphonoformate is analogous to that of phosphonoacetate. Leinbach *et al.* (9) reported that phosphonoacetate inhibits the herpesvirus-induced DNA polymerase by interacting at the pyrophosphate-binding site. For the experiments reported here with HeLa α-polymerase, phosphonoformate and phosphonoacetate were shown to be mutually exclusive inhibitors which bind at the same site on the α-polymerase. Presumably, this site is the pyrophosphate-binding site.

At high concentrations, about 10 to 20 times greater than the concentration which effectively blocks herpesvirus replication, phosphonoacetate is cytotoxic (2, 5, 10) and cellular DNA synthesis is inhibited. Although not proven, it is not unreasonable to assume that at high concentrations, phosphonoacetate inhibits cell growth as a result of the inhibition of cellular DNA synthesis through a specific inhibition of the α-polymerase. This phosphonoacetate inhibition of cellular DNA synthesis by inhibition of the α-polymerase would be analogous to the inhibition of herpesvirus DNA synthesis by inhibition of the herpesvirus-induced DNA polymerase (7). The difference would be in the concentrations of phosphonoacetate required to inhibit the two processes. If this is so, then phosphonoacetate-resistant cell mutants could contain an altered α-polymerase. These mutants might prove useful in evaluating the role that α-polymerase plays in cellular DNA replication.

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PUBLICATIONS AND ABSTRACTS

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1. Leinbach, S. S., J. M. Reno, L. F. Lee, A. F. Isbell, and J. A. Boezi. Mechanism of Phosphonoacetate Inhibition of Herpesvirus-Induced DNA Polymerase. 1976. Biochemistry 15: 426-430.

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