This is to certify that the

thesis entitled

INHIBITION OF EUKARYOTIC DNA POLYMERASES BY

PHOSPHONOACETATE AND PHOSPHONOFORMATE

presented by

Carol Lee Kempen Sabourin

has been accepted towards fulfillment
of the requirements for

M.S. degree in Biochemistry

John A. Boezi
Major professor

Date 10/27/77

O-7639

INHIBITION OF EUKARYOTIC DNA POLYMERASES BY PHOSPHONOACETATE AND PHOSPHONOFORMATE

By

Carol Lee Kempen Sabourin

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Biochemistry

1977

ABSTRACT

INHIBITION OF EUKARYOTIC DNA POLYMERASES BY
PHOSPHONOACETATE AND PHOSPHONOFORMATE

By

Carol Lee Kempen Sabourin

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 eukaryotic cells were relatively insensitive to phosphonoacetate. The sensitivity of the DNA polymerase α and the relative insensitivity of the DNA polymerases β and γ appeared to be a general characteristic of the vertebrate polymerases. DNA polymerases from two other eukaryotic cells, yeast DNA polymerase A and B and tobacco cell DNA polymerase, were inhibited by phosphonoacetate. Fourteen phosphonate analogues 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.

DEDICATION

To my parents and my husband, Pat

ACKNOWLEDGEMENTS

The author wishes to express her deepest appreciation to Dr. J. A. Boezi for his guidance, criticism, and encouragement which made possible this study. Sincere appreciation also goes to the members of my guidance committee, Dr. A. Revzin and Dr. L. F. Velicer. I would also like to thank John Reno for his helpfulness and Betty Baltzer for her technical assistance. Finally, I am indebted to the Department of Biochemistry at Michigan State University and the National Cancer Institute for financial support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

HSV	herpes simplex virus
EBV	Epstein-Barr virus
IUdR	5-iodo-2'-deoxyuridine
dNTP	deoxynucleoside triphosphate
Tris	tris-(hydroxymethyl)-methylamine
DEAE	diethylaminoethyl

INTRODUCTION

Phosphonoacetate is an effective inhibitor of the replication of herpesviruses (1,2,3,4). The inhibition of viral replication is through an effect on the viral-induced DNA polymerase (5,6,7,8,9). At the concentrations at which phosphonoacetate is an effective anti-herpesvirus 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. 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 the effect of phosphonoacetate on the α-polymerase of Wi-38 and HeLa cells was reexamined. The α-polymerase of phytohemagglutinin-stimulated human lymphocytes was also examined. In addition, the α-polymerases of Chinese hamster ovary cells and calf thymus as well as the DNA polymerases from two non-vertebrate eukaryotic cells, yeast and tobacco cells, were looked at. Finally the effect of several phosphonate analogues on the HeLa α-polymerase was investigated.

LITERATURE REVIEW

Vertebrate DNA Polymerases

Three general classes of DNA polymerases are found in cells of all vertebrates: DNA polymerase α, DNA polymerase β, and DNA polymerase γ. The Greek letters indicate the historical order of discovery of the DNA polymerases. These DNA polymerases can be distinguished from one another by their chromatographic properties, molecular weight, sensitivity to N-ethylmaleimide, salts, and heat, and ability to copy various templates. Each of these enzymes has been reported from numerous sources, as has been reviewed by Weissbach (38,39) and Wintersberger (40). In this discussion the general properties of the enzymes will be noted.

DNA Polymerase α

This class of DNA polymerase is exemplified by the calf thymus polymerase which was purified and extensively studied by Bollum and his co-workers (41,42). These studies provided a framework for future studies from other origins. The α-polymerase is referred to as the high molecular weight vertebrate polymerase which when dis-aggregated exhibits a sedimentation coefficient of 6 to 8S. The purified enzyme from human KB cells shows a native molecular weight of 175,000 daltons and contains an 87,000 dalton peptide subunit (43). The α-polymerase of calf thymus is a single polypeptide of molecular weight 155,000 to 170,000 and seems to contain an additional subunit

of 50,000 to 70,000 daltons (44,45). The anomalous behavior of the α-polymerase on gel filtration is probably due to the molecule's asymmetry. The α-polymerase is an acidic protein. The isoelectric points of the polymerase from human lymphocytes, human KB cells, and calf thymus are in the range of 4.5 to 5.5 (46,47,48). The activity of α-polymerase in growing cells represents 80 to 90% of the total DNA polymerase activity (38).

The α-polymerase has often been referred to as the "cytoplasmic DNA polymerase" because it has been found predominantly in the cytoplasm when cells are subjected to normal extraction procedures. Many investigators feel that this cytoplasmic localization is an artifact. Lynch et al. (49) reported that when rat liver is homogenized in 0.3 M sucrose and 4 mM $CaCl_2$ only 1.4% of the total α-polymerase is found in the cytoplasmic fraction. If the solution used for the homogenization contained Tris buffer and/or KCl the α-polymerase leached out of the nucleus. When glycerol is used to prepare the nuclei approximately 85% of the α-polymerase is in the nucleus. Herrick et al. (50) have shown that 85% of the α-polymerase activity is associated with nuclei by subjecting mouse L cells to cytochalasin-B-induced enucleation. Foster and Gurney (51) isolated nuclei from mouse and human cells using a nonaqueous procedure of cell fractionation in which lyophilized cells were homogenized and centrifuged in 100% glycerol. They demonstrated that approximately 85% of the DNA polymerase activity of whole cells is in the nuclear fraction. The remaining 15% of the activity had been lost during fractionation.

The α-polymerase is particularly sensitive to high ionic strength (44) but significant enzyme activity can be observed in the presence

of 25 mM NaCl. Reagents such as p-chloromercuribenzoate and N-ethylmaleimide which block sulfhydryl groups strongly inhibit the α-polymerase (38). The α-polymerase is inhibited by high concentrations of actinomycin D and by low concentration of ethidium bromide (52). The inhibition of the α-polymerase by arabinofuranosyl cytosine-triphosphate may be due to the effect of this nucleotide analogue on DNA synthesis (53). Pyrophosphate is an inhibitor of the α-polymerase but only a minimal dNTP-pyrophosphate exchange reaction occurs (42,43). The α-polymerase from human KB cells promotes an exchange reaction which is about 0.8% of the polymerase activity. The α-polymerase from calf thymus has been shown by Herrick et al. (54) to be stimulated by two helix unwinding proteins isolated from calf thymus.

No nuclease activity has been found associated with the most highly purified α-polymerase preparations. With the α-polymerase from human KB cells, the limit of detection for nuclease activity was 0.003% of the polymerase activity based on production of acid soluble radioactive nucleotide fragments (43). Employing a highly sensitive endonuclease assay, which measured the conversion of supercoiled SV40 DNA to SV40 DNA with single strand nicks, to assay the most highly purified fraction of α-polymerase from regenerating rat liver, no nuclease activity was detected.

The α-polymerase requires 3'OH priming groups as initiators and utilizes gapped duplex DNA (activated DNA) at a high rate. Spadari and Weissbach (55) reported that α-polymerase from human HeLa cells can utilize DNA primed with natural RNA. The α-polymerase cannot use synthetic ribohomopolymers as templates.

There have been several reports that α-polymerase is heterogeneous (37,48,56,64). In each case several peaks were obtained by DEAE-cellulose or DEAE-Sephadex chromatography.

Spadari and Weissbach (57) used a rabbit antiserum to the HeLa α-polymerase to show that the α-polymerase from CHO cells is significantly inhibited by the antisera. Thus, in agreement with Chang and Bollum (58), it appears that α-polymerases of various species share some common peptide sequences.

DNA Polymerase β

The first recognition of a distinct nuclear DNA polymerase, separable from the predominant cytoplasmic DNA polymerase and which differed in its molecular weight and biochemical characteristics, was reported in HeLa cells by Weissbach et al. (22). Soon after this several other investigators reported low molecular weight DNA polymerases from other sources (59,60,61). Chang has purified the β-polymerase to homogeneity from calf thymus chromatin (62). The enzyme is homogeneous by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and by equilibrium sedimentation centrifugation.

When disaggregated the β-polymerase exhibits a sedimentation coefficient of 3 to 4S. In KB cells, the enzyme, is a single polypeptide of molecular weight 43,000 to 45,000 daltons (32). In low ionic strengths the β-polymerase forms aggregates comparable in size to the α-polymerase (32). Consequently, a mistaken structural relationship was first reported for these two DNA polymerases. The α- and β-polymerases have not been found to be antigenically related (63).

The β-polymerase is a basic protein. Isoelectric points for the enzyme from human lymphocytes, KB cells, calf thymus, and Novikoff hepatoma cells are in the range of 8.5 to 9.5 (46,32,65,44). The primary intracellular location of the β-polymerase is nuclear, although small amounts have sometimes been found in the cytoplasm. In growing cells it comprises about 5 to 15% of the total DNA polymerase activity.

The sensitivity to high ionic strength varies with the enzyme source. Activities in the presence of 0.2 M NaCl may be stimulated 0.5- to 4-fold above those in the absence of NaCl. The β-polymerase is insensitive to sulfhydryl group inhibitors. This serves as a distinguishing characteristic of the α- and β-polymerases. The β-polymerase from a variety of mammalian cells is more rapidly inactivated *in vitro* by elevated temperatures than the α-polymerase (36). Purified β-polymerase is rather insensitive to adverse conditions (62). For example, it is completely stable in 5 M urea and is not inhibited by 20% ethanol or 25% acetone. No detectable nuclease activity has been found to be associated with the β-polymerase and consequently it does not excise misplaced primer termini (32). The β-polymerase is inhibited by pyrophosphate but no detectable pyrophosphate exchange has been demonstrated (42,66).

Although the β-polymerase requires the four dNTPs for maximal activity, it does show activity in the absence of one or more dNTPs. This is probably a reflection of the fact that activated DNA may contain at least 10^{13} 3'OH termini per μg of DNA (38). The β-polymerase utilizes activated DNA at a high rate and can also utilize poly(rA)·oligo$(dT)_{12-18}$ to a lesser degree (67). It cannot use ribonucleotide primers.

A phylogenetic survey was conducted by Chang for β-polymerase activity using low molecular weight and insensitivity to N-ethylmaleimide as primary identifying characteristics (68). This type of polymerase has a widespread occurrence in multicellular animals beginning with sponge, the most primitive animal surveyed. The observation that the β-polymerase is absent from the nuclei of lower eukaryotes was made with yeast (69). This type of polymerase also has not been found in prokaryotes, protozoa, or plants.

The β-polymerase is immunologically distinct. This was demonstrated using antibody prepared in rabbit against the partially purified HeLa α-polymerase (63), reversing an earlier report by Chang and Bollum (58). The absence of any relationship between the α- and β-polymerases was confirmed using antiserum to α-polymerase prepared in rats, in both direct neutralization assays and additionally by immunoprecipitation of the α-polymerase antibody complex with goat anti-rat immunoglobulin (70). In addition, it has been shown that the α- and β-polymerases of chick embryo are immunologically unrelated (71).

DNA Polymerase γ

The separation and initial differentiation of γ-polymerase was first described by Fridlender et al. (72). Since then its presence has been reported from a number of sources. Originally the HeLa γ-polymerase was resolved into two forms by phosphocellulose and hydroxyapatite chromatography (73), but it has since been found to be a single form (29). The molecular weight for the HeLa γ-polymerase, purified over 60,000-fold, ranges from 160,000 to 333,000 (31). Optimal activity with poly(rA)·oligo$(dT)_{12-18}$ is in

the presence of 50 mM KPO_4 and 130 mM KCl. Under these conditions, the γ-polymerase copies poly(rA) twenty times more rapidly than activated DNA. These assay conditions also permit a clear distinction between γ- and β-polymerase, the latter of which is markedly inhibited by phosphate at this concentration. The γ-polymerase does not utilize natural RNA (73). It is not antigenically related to reverse transcriptase of the RNA tumor viruses (73,74). In addition, the cellular γ-polymerase and virus-induced reverse transcriptases can be clearly separated and identified in Type C virus infected cells (75,76). The γ-polymerase is an acidic protein and requires sulfhydryl groups for maximal activity (38). In growing cells it comprises about 1 to 2% of the total DNA polymerase activity (57). The apparent Michaelis constants for the dNTPs are 10-fold lower than those of the α- and β-polymerase. The γ-polymerase does not catalyze any significant amount of pyrophosphate exchange (31).

Ito et al. (77) have isolated a nuclear membrane complex from KB cells infected with adenovirus 2 that has some properties of an adenovirus DNA replication complex. The major DNA in this complex, which can synthesize viral DNA, is the γ-polymerase.

The original report from Fry and Weissbach (78) indicated that HeLa cell mitochondria contained two DNA polymerases. One resembled γ-polymerase and the other, designated mitochondrial DNA polymerase, was a unique enzyme unrelated to the other cellular DNA polymerases. Subsequently, tests for mycoplasma done under anaerobic conditions, revealed that the HeLa cells used in those experiments were contaminated with this organism (79). Mitochondria from rat liver and mycoplasma-free HeLa cells were reexamined and found to contain a single DNA

polymerase activity which closely resembles the γ-polymerase found in the corresponding cytoplasm of these cells.

DNA Polymerase Activities *in vivo*

The attempts to gain some insight into the physiological functions of the vertebrate DNA polymerases have involved analyses of the levels of activity of the various enzymes in cells at various stages of growth. The levels of DNA polymerase activity were measured in mouse L cells and baby hamster kidney cells in log and stationary phase (34,35). In both cell lines, there was a marked rise in the α-polymerase in log phase with the levels of activity being 5- to 12-fold higher than in stationary phase. The levels of β-polymerase remained unchanged. In two reports the levels of α-polymerase have been shown to increase in regenerating rat liver 18 to 30 hours post-hepatectomy (80,81). The levels of α-polymerase 48 hours post-hepatectomy were found to be increased 6- to 7-fold, whereas β-polymerase levels remained constant.

Chang and Bollum (42) have shown that L cells which are shifting from a stationary phase to a dividing state exhibit a rise in the α-polymerase levels within 48 hours with no change in the β-polymerase levels. The levels of DNA polymerase activities in synchronized HeLa cells have also been studied. Spadari and Weissbach (57) measured DNA polymerase activities in crude nuclear and cytoplasmic extracts of synchronized HeLa cells. They observed a rise in γ-polymerase in the early part of the S phase, a steady rise in α-polymerase during the entire S phase, and no change in β-polymerase. Chiu and Baril (82) have extended this approach by examining the complete cell cycle in synchronized HeLa cells. They measured

nuclear DNA polymerase activities which had been partially purified by phosphocellulose column chromatography. They observed a 7- to 10-fold increase in the α-polymerase activity in the nucleus and cytoplasm between the late G1 and mid-S phase and a subsequent rapid decline between late S and G2. No changes in the β-polymerase levels were observed and no reproducible variation in the γ-polymerase levels were observed. Addition of cycloheximide to cultures just prior to the G1 phase abolished the rise in α-polymerase levels, indicating that protein synthesis was necessary for the increase in enzymatic activity. The α-polymerase activity increased in the cell cycle even if DNA synthesis was blocked by hydroxyurea. Since it is known that hydroxyurea does not prevent synchronized G1 cells from entering the S phase (83), the finding that the rise and fall in α-polymerase activity is apparently independent of DNA synthesis signifies there is no simple correlation between DNA synthesis and DNA polymerase levels (39). Noy et al. (37) have found that cycloheximide leads to selective effects on the levels of the α-, β-, and γ-polymerases. The level of the β-polymerase fell rapidly to 30% of its initial value during the first seven hours of exposure to cycloheximide. While the level of the α-polymerase remained unchanged during the first seven hours, both the α- and γ-polymerases dropped to 50% of their original level after 24 hours.

In another study Bertazzoni et al. (23) measured DNA polymerase levels in phytohemagglutinin-stimulated, ultraviolet-irradiated human lymphocytes. The α- and β-polymerase levels were measured in crude extracts or after sucrose gradient centrifugation of crude extracts. In the stimulated lymphocytes a wave of DNA synthesis was observed which reached maximal levels after five days of stimulation

and later decreased to levels lower than one-tenth of the maximum. Two levels of repair synthesis were observed. One reached maximal levels approximately one day in advance of the peak in DNA replication rate and a second wave occurred three days later when the overall DNA synthesis rate was in the decreasing phase. The levels of α-polymerase paralleled the levels of DNA replication. A 20-fold increase in enzyme activity was observed after five days of stimulation. The β-polymerase levels increased more slowly and to a lesser extent and seemed to parallel the second wave of repair synthesis. After seven days of stimulation, maximal levels were about 7-fold greater than those in the unstimulated cells. From these results the investigators suggested a correlation between α-polymerase activity and DNA replication, and between β-polymerase activity and repair synthesis.

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

DNA Polymerases During Development

Differentiating systems have been studied in an attempt to find a pattern in the changes in DNA polymerase activity that occur as cells become specialized or mature. Spermatogenesis is one of the systems used to study changes in DNA polymerase during development. Chevaillier and Philippe (84) and Hecht et al. (85) examined the developing mouse testes. The nuclear β-polymerase activity increased

during meiotic prophase (84) and the α-polymerase activity diminished during the attainment of sexual maturity (85). Sherman and Kang (86) investigated α- and β-polymerase levels of midgestation mouse embryo, trophoblast, and decidua. The enzyme levels were highest in the rapidly dividing embryonic cells, significantly lower in DNA replicating but nondividing trophoblast cells, and lowest in the nonreplicating, nondividing decidual cells. Changes in the DNA polymerase levels have been followed during sea urchin development (87,88) and in *Xenopus laevis* (89,90,91). An increase in the polymerase activity was observed as the eggs matured.

In studies on the pattern of developmental changes of DNA polymerase in rat brain, Chiu and Sang (92) found that the α-polymerase showed higher activity than the β-polymerase in rat cerebellum immediately postnatal. Furthermore, the α-polymerase decreased rapidly with age and was much lower than that of the β-polymerase. The cerebellum of the rat grows rapidly for sixteen days after birth and shows rapid cell proliferation during this period. The DNA concentration continues to increase up to two weeks. The investigators found that in the rat cerebral cortex the α-polymerase showed higher activity than the β-polymerase only at the fetal stage. The α-polymerase was low after birth and essentially no activity was found in the adult. In the cerebral cortex of the rat, cell division is almost complete at birth. The DNA concentration decreases during maturation and the activity for DNA synthesis is very low. Therefore, the α-polymerase showed high activity in a proliferating stage and low activity when mitosis ceased.

Barton and Yang (93) examined the activities of α- and β-polymerase in spleens of Balb/c mice during aging. No change in the α-polymerase activity was noted, whereas the β-polymerase activity was decreased in the spleens of senescent animals compared with that of younger animals. DNA polymerase activity in normal and malnourished rat placentas has been measured and found to correlate with DNA synthesis. Thus, there is evidence from several laboratories that as cells progress toward maturity the level of DNA polymerase declines in parallel with the level of DNA synthesis.

Phosphonoacetate and Herpesvirus Infection

Initial Studies with Phosphonoacetate

Using a random testing of compounds with a tissue culture screen, workers at Abbott Laboratories discovered that phosphonoacetate was an effective inhibitor of the replication of herpes simplex virus types 1 and 2 (1). These initial studies using phosphonoacetate as an antiherpesvirus agent involved herpes simplex virus infection of mice and rabbits. Mice were infected with HSV-2 by topical application to denuded skin. The resulting herpes dermatitis caused death in untreated mice. However, if phosphonoacetate was administered orally or topically to infected mice two hours after infection, mortality was significantly reduced. Rabbits were infected with HSV-1 by topical application to the eyes. The resulting herpes keratitis caused corneal lesions in untreated rabbits. However, if an ointment of phosphonoacetate was applied to the eyes of infected rabbits two hours after infection, a reduction in the lesions was observed.

The inhibitory effect of phosphonoacetate on herpesvirus infections in tissue culture was first documented by investigators at Abbott Laboratories (5). The infection of Wi-38 cells with HSV-1 resulted in rounded, clumped, and multinucleated cells. However, when HSV-1-infected Wi-38 cells were cultured in the presence of 500 μM phosphonoacetate, cytopathic effects were not observed. The infected cells could not be distinguished from the uninfected cells. This suggested that phosphonoacetate prevented the replication of HSV-1.

Studies were also performed to determine at what stage of infection phosphonoacetate inhibited (5). Phosphonoacetate appeared to have no effect on absorption, penetration, or release of the virus. When phosphonoacetate was removed from the culture medium of HSV-1 infected Wi-38 cells, cytopathic effects reappeared. Thus, the viral genomes were still present and functional in the infected cells. No difference in RNA and protein synthesis was observed between phosphonoacetate-treated and untreated infected cells, but a reduction in DNA synthesis was noted in the phosphonoacetate-treated infected cultures. The inhibition of DNA synthesis was specific for viral DNA synthesis as determined by isolation and bouyant density centrifugation of DNA from infected cultures grown in the presence and absence of phosphonoacetate. Twelve hours post-infection two peaks of [^{14}C]thymidine, corresponding in density to HSV-1 DNA and cellular DNA were found in CsCl gradients for untreated cells. Only one peak of [^{14}C]thymidine, corresponding in density to cellular DNA, was found in CsCl gradients for phosphonoacetate-treated cells.

Overby et al. (5), were also interested in the effect of phosphonoacetate on uninfected cells. Phosphonoacetate was not toxic to contact inhibited Wi-38 cells. No morphological changes were observed when confluent Wi-38 cells were incubated in the presence of 1 mM phosphonoacetate for two days. No effect was noted on DNA, RNA, or protein synthesis. However, phosphonoacetate was toxic to growing cells. When 500 μM was present in the culture medium a 40% decrease in the number of cells in culture was observed, whereas no toxic effects were noted when 50 μM phosphonoacetate was present in the culture medium.

Mao et al. (6) first demonstrated that the inhibition of herpesvirus replication by phosphonoacetate is through an effect on the herpesvirus-induced DNA polymerases. When HSV-1-induced DNA polymerase was assayed in the presence of 1 μM phosphonoacetate, enzyme activity was inhibited by 50%.

Phosphonoacetate Inhibition of Herpesviruses and Other Viruses

Since the initial studies of Shipkowitz et al. (1) and Overby et al. (5) which demonstrated the effectiveness of phosphonoacetate in inhibiting the replication in cell culture of herpes simplex viruses, other herpesviruses have been found to be sensitive to phosphonoacetate. At a concentration of 500 μM, phosphonoacetate inhibited the replication of the avian herpesviruses, Marek's disease herpesvirus, herpesvirus of turkeys, and owl herpesvirus (3). Viral DNA synthesis as determined by CsCl equilibrium density gradient centrifugation or by DNA-cRNA hybridization was significantly reduced in viral-infected cells treated with phosphonoacetate. Inhibition of viral replication and DNA synthesis was also observed with

three types of cytomegalovirus, human, simian, and murine (2,95). Viral DNA synthesis resumed when phosphonoacetate was removed, indicating that the inhibition was reversible. Similar results were noted with herpesvirus saimiri in owl monkey kidney cells. Phosphonoacetate inhibited herpesvirus saimiri and the synthesis of virus-induced intracellular late antigens and membrane antigens, but not the synthesis of early antigens (96). The continued presence of phosphonoacetate was required to suppress replication (97). For example, maintenance of the infected cells on phosphonoacetate for 63 days had no effect on completely suppressing replication after removal. Phosphonoacetate also inhibited the replication of equine abortion herpesvirus (13,98), Epstein-Barr virus (4,10,99), and varicella zoster virus (13) in culture by inhibiting viral DNA synthesis. Although phosphonoacetate appears to be a universal inhibitor of herpesvirus DNA synthesis, inhibition has also been demonstrated for vaccinia virus to a lesser extent (13). The three RNA viruses, poliovirus, rhinovirus, and measles virus, are not significantly sensitive (13).

Phosphonoacetate blocked herpes simplex virus DNA synthesis in nuclei prepared from infected cells (12,100). In addition, an inhibition by phosphonoacetate of *in vitro* DNA synthesis by nuclei from Raji cells superinfected with EBV was noted (101). Both cellular and viral DNA synthesis were inhibited, although synthesis of cellular DNA may have been inhibited to a somewhat lesser degree than synthesis of EBV DNA.

Phosphonoacetate Inhibition of the Herpesvirus-Induced DNA Polymerase

Phosphonoacetate was a specific inhibitor of herpes simplex virus-induced DNA polymerase (6). The sensitivity of the herpesvirus type 1 and 2 DNA polymerases was similar. The K_i values for the HSV-2-induced DNA polymerase were 0.45 to 0.47 μM (7). Phosphonoacetate did not inhibit the herpesvirus-induced DNA polymerases because of the formation of a phosphonoacetate-DNA complex (2,6). The specificity of phosphonoacetate for the polymerase precludes this possibility. Moreover, phosphonoacetate inhibition was not decreased when DNA concentrations were increased in the DNA polymerization reaction mixtures. Finally, the presence of phosphonoacetate in HSV-1-induced polymerization reaction mixtures did not prevent formation of an enzyme-DNA complex as determined by glycerol density gradient sedimentation velocity centrifugation of these reaction mixtures (6). Phosphonoacetate was an effective inhibitor of both the Marek's disease herpesvirus and the herpesvirus of turkeys-induced DNA polymerase (9). The phosphonoacetate inhibition patterns and apparent inhibition constants for the DNA polymerization reaction catalyzed by the herpesvirus of turkeys-induced DNA polymerase were determined. Phosphonoacetate gave linear noncompetitive inhibition with the four dNTPs as the variable substrate and activated DNA at a saturating concentration of 200 μg/ml. 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 with activated DNA as the variable substrate and the four dNTPs at 100 μM each. The apparent inhibition constants were 1 to 2 μM. Phosphonoacetate was then shown to be a

competitive inhibitor of pyrophosphate in the dNTP-pyrophosphate exchange reaction. Multiple inhibition analysis showed that phosphonoacetate and pyrophosphate were mutually exclusive inhibitors. When taken together, the above results showed that phosphonoacetate inhibited the polymerase by interacting with it at the pyrophosphate binding site and functioned either as a dead-end inhibitor or as an alternate product (9, J. Boezi, personal communication).

Other herpesvirus-induced DNA polymerases have been shown to be inhibited by phosphonoacetate. The human cytomegalovirus-induced DNA polymerase (2,11), equine herpesvirus-induced DNA polymerase (98), and EBV-induced DNA polymerase (102) have all been shown to be sensitive.

Studies of phosphonoacetate-resistant mutants of herpesvirus support the hypothesis that phosphonoacetate inhibits herpesvirus replication through a specific inhibition of the herpesvirus-induced DNA polymerase. Hay and Subak-Sharpe (103) isolated mutants of HSV which formed plaques in the presence of 100 μg/ml phosphonoacetate. Three of the mutants induced viral DNA synthesis and viral DNA polymerase activity which was much less sensitive to phosphonoacetate than the wild type virus. Similar results were observed by Becker et al. (100) with HSV mutants isolated from 5-bromodeoxyuridine-mutagenized virus that were resistant to phosphonoacetate at high concentrations. Honess and Watson (104) passed the virus in the presence of phosphonoacetate to select resistant mutants. Stable clones of mutant viruses with a range of drug sensitivities were isolated. A higher level of drug resistance could be correlated with increased resistance of virus DNA synthesis, γ-protein synthesis

(late virus proteins), and resistance of the virus-induced DNA polymerase to the inhibitory effects of phosphonoacetate.

Studies of the analogues of phosphonoacetate indicate the structural requirements for antiherpesvirus action are rather narrowly defined (1,3,9,105, Reno et al., submitted for publication). Leinbach et al. (9) observed that 2-phosphonopropionate was an inhibitor but that the apparent inhibition constant for it was about 50 times greater than the corresponding apparent inhibition constant for phosphonoacetate. Herrin et al. (105) noted that propyl phosphonoacetate was as effective as phosphonoacetate in inhibiting the HSV-induced DNA polymerase and in animals with herpes dermatitis. The inhibitory effect of phosphonoformate was recently discovered by Reno et al. (submitted for publication). This compound was as effective as phosphonoacetate in inhibiting the herpesvirus of turkey-induced DNA polymerase. The inhibition constants and inhibition patterns were identical to those reported for phosphonoacetate. Phosphonoformate also inhibited the replication of Marek's disease herpesvirus, the herpesvirus of turkeys, and herpes simplex virus.

Effect of Phosphonoacetate on Other DNA Polymerases

The sensitivity of other DNA polymerases to phosphonoacetate has been examined. The α-polymerase from Wi-38 cells has been reported to be relatively resistant to phosphonoacetate inhibition (2,6,7,11). When the Wi-38 α-polymerase, partially purified by phosphocellulose chromatography, was assayed in the presence of 300 μM phosphonoacetate, only 10 to 15% inhibition of enzyme activity resulted (6). In contrast, Bolden et al. (12) found that the α-polymerase from HeLa cells was inhibited by 50% when assayed in

the presence of 30 μM phosphonoacetate. Phosphonoacetate was also an inhibitor of the α-polymerase from HEL (human embryonic lung) cells (33) and duck embryo fibroblasts (9) at concentrations which are 15 to 30 times higher than those which inhibit the herpesvirus-induced DNA polymerases. The β-polymerase from a number of cells has been found to be relatively insensitive to phosphonoacetate (2,6,7, 9,11,12,33), as has the γ-polymerase from HeLa cells (29).

The vaccinia virus-induced DNA polymerase was inhibited by 50% when assayed in the presence of 30 μM phosphonoacetate (12). Other viral DNA polymerases such as the reverse transcriptase from avian myeloblastosis virus (9) and Rous sarcoma virus (7), and hepatitis B virus DNA polymerase (7) were insensitive to phosphonoacetate. The prokaryotic DNA polymerases, *Escherichia coli* DNA polymerase I (9), *Micrococcus luteus* DNA polymerase (7), and the 6S mycoplasma DNA polymerase (33, J. Boezi, personal communication) are not inhibited by phosphonoacetate.

At concentrations of 100 μg/ml or less phosphonoacetate has been shown to have no obvious effect on cell growth and cellular DNA synthesis in Wi-38 cells (2,5), lymphocytes carrying the EBV genome (4,10), or duck embryo fibroblasts (3). Phosphonoacetate at concentrations of 500 to 1000 μg/ml causes inhibition of growth and cellular DNA synthesis, an increase in the diameter of the cell, and probably causes an arrest at interphase.

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

Epstein-Barr virus is a herpesvirus for which no completely permissive culture system exists, i.e., no cell type supports the efficient replication of EBV. There are, however, several well characterized human lymphoblastoid cell lines transformed by EBV.

These cell lines have been cultured in the presence of phosphonoacetate in an attempt to determine the mechanism of EBV DNA replication (4,99).

The Raji cell line is a transformed lymphoblastoid cell line derived from a tumor biopsy from a patient with Burkitt's lymphoma (106). It is a nonproducer cell line. Raji cells contain approximately 50 EBV genomes per cell but express only a single viral antigen, the EBV-associated nuclear antigen, and produce no viral particles. Replication of resident EBV genomes is under cellular control mechanisms and occurs only during early S phase (107). If Raji cells are superinfected with EBV, host cell DNA synthesis is completely inhibited and productive replication of EBV occurs (99).

The effect of phosphonoacetate on the EBV content in Raji cells before and after superinfection has been determined. When Raji cells were grown in the presence of 1 mM phosphonoacetate for three days or in the presence of 0.5 mM phosphonoacetate for three weeks, there was no change in the number of resident EBV genomes as determined by hybridization techniques (4,99). Therefore, the EBV DNA present in these transformed cells replicates by a mechanism which is insensitive to phosphonoacetate as might be expected since resident EBV DNA replication is under cellular control.

When Raji cells were superinfected, the amount of EBV DNA present in these cells increased to such levels that it could be detected in CsCl gradients if the superinfected cells were labeled with [^{3}H]thymidine (99). When Raji cells were superinfected and then cultured in the presence of 0.5 mM phosphonoacetate, EBV DNA could not be detected in CsCl gradients. Therefore, productive EBV DNA replication in the absence of cellular controls is sensitive to

phosphonoacetate. This was further demonstrated in D98/HR-1 cells (102). Under normal growth conditions the only EBV-specific antigen detectable in D98/HR-1 cells by the immunofluorescence assay is the EBV-associated nuclear antigen as in Raji cells. 5-Iodo-2'-deoxyuridine induces EBV-specific early antigen, viral capsid antigen, and membrane antigen, and DNA synthesis is initiated; the cells therefore resemble producer cells. The effect of phosphonoacetate on the induction of EBV antigens was determined by adding phosphonoacetate at the time of removal of IUdR. Phosphonoacetate (100 µg/ml) had little effect on the expression of EBV-specific early antigen, but markedly reduced the expression of the viral capsid antigen. This result suggests that an EBV-specific DNA polymerase might be induced by IUdR since the viral capsid antigen is dependent specifically upon viral DNA synthesis (108). Miller et al. (102) identified a salt-stimulated DNA polymerase in D98/HR-1 cells which resembled other herpesvirus-induced DNA polymerases.

The B95-8 cell line is a marmoset cell line derived by treatment with EBV from a human cell line established from a patient with acute infectious mononucleosis believed to be induced after blood transfusion (109). B95-8 cells release infectious virus particles and contain about 150 EBV genome equivalents per cell. The three virus specific antigens, EBV-associated nuclear antigen, early antigen, and viral capsid antigen, are expressed. The synthesis of EBV in B95-8 cells is completely inhibited by phosphonoacetate, as shown by the total inhibition of viral capsid antigen synthesis. This effect of phosphonoacetate on B95-8 cells and P3HR-1 cells, a human lymphoblastoid cell line, was also studied by Nyormoi et al. (10). Thorley-Lawson and Strominger (110) infected normal human peripheral

B lymphocytes with the transforming strain of EBV from the supernatant of a B95-8 marmoset lymphocyte culture. The B lymphocytes became a transformed cell line. Phosphonoacetate at a concentration of 100 to 200 μg/ml inhibited the transformation and the outgrowth of the EBV-infected cultures. Taken together, the above results are consistent with the hypothesis that EBV DNA is replicated by two mechanisms, one in the noninduced cell which is insensitive to phosphonoacetate and a different mechanism in the producer cell which is sensitive to phosphonoacetate. This second mechanism has been shown to involve an EBV-induced DNA polymerase which resembles the herpesvirus-induced DNA polymerases (102).

Animal Studies with Phosphonoacetate

The first studies using phosphonoacetate as an antiherpesvirus agent in animals, as reported by Shipkowitz et al. (1), involving herpes simplex virus infections of mice and rabbits, were previously discussed. Further studies in animal model systems demonstrate the efficacy of phosphonoacetate as an antiherpesvirus drug. The effectiveness of phosphonoacetate in the treatment of herpes dermatitis in mice was confirmed (111). Hairless mice infected percutaneously with inhibitor-resistant or with parental inhibitor-susceptible HSV strain were treated intraperitoneally with phosphonoacetate. Only the infection induced by the parental phosphonoacetate-susceptible virus was suppressed by phosphonoacetate. Both the phosphonoacetate-resistant and the phosphonoacetate-susceptible HSV-induced infections were suppressed by 9-β-D-arabinofurabosyl-adenine.

While the experimental model used by Abbott Laboratories in treating herpes keratitis was useful for screening purposes, it bore

little relationship to the problems faced in treating established herpes keratitis (112). To test the effect of phosphonoacetate and 5-iodo-2'-deoxyuridine (the only drug commercially available to treat herpetic keratitis) on established HSV corneal infections in rabbits, treatment was started three days postviral inoculation and was continued for five days. The effect of topically applied antiviral agent in one eye was compared to no treatment or placebo in the other eye of each animal. The effect of a 5% phosphonoacetate ointment was equivalent to that of a 0.5% 5-iodo-2'deoxyuridine ointment in the treatment of established herpetic eye infection. Similar studies were done to test the effect of phosphonoacetate against deep ocular herpetic infections (113). Treatment started three days after infection and continued for five days. Phosphonoacetate had a significant antiviral effect when applied topically in both liquid and ointment preparations on superficial herpes keratitis. Phosphonoacetate was not effective in the treatment of experimental herpes iritis when applied topically but was significantly effective when administered intravenously and subconjunctivally.

Genital infection of mice with herpesvirus hominis type 2 provides an experimental model for the testing of the antiviral potential of phosphonoacetate (114). Intravaginal treatment with phosphonoacetate (500 mg/kg in saline or as a 5% cream) initiated three hours after inoculation with herpesvirus hominis type 2 completely inhibited viral replication in the genital tract and prevented subsequent mortality. Therapy initiated 24 to 72 hours after infection significantly reduced titers of virus in vaginal secretions, but most mice eventually died of encephalitis. Treatment intraperitoneally had no effect on local viral replication or final mortality.

The antiviral activity of phosphonoacetate on mice with herpes encephalitis caused by HSV type 1 was investigated (115). The criteria used for judging were the rate of survival and the concentration of virus in the brain. Phosphonoacetate administered subcutaneously resulted in the long-term survival rate of approximately 15% of the infected, treated animals with four days of treatment. When treatment was extended to seven days, the overall survival rate was increased to 35%. There was also a reduction in the titer of virus in the brain with treatment. The ability of a topical application of phosphonoacetate after inoculation with HSV to prevent infection of dorsal root ganglia in mice, and whether such application would have an effect on established latent ganglionic infection, were also investigated (116). When administered 24 hours after HSV inoculation, phosphonoacetate markedly reduced skin lesions, mortality, and the development of a latent ganglionic infection. When given after 24 hours, phosphonoacetate reduced skin lesions and mortality but had little effect in preventing infection.

Phosphonoacetate was effective in the treatment of mice with lethal cytomegalovirus infections (117). Treatment was with 250 mg/kg of phosphonoacetate injected intraperitoneally twice daily for seven days. A marked reduction in viral titers in the brains and lungs of infected mice was observed. The major reason for efficacy appeared to be a complete inhibition of viral replication in the liver. Treatment of nonlethal cytomegalovirus infection with phosphonoacetate beginning 48 hours after viral challenge resulted in elimination of clinical signs of illness and reduction in viral titers in tissue.

Chicks were infected with Marek's disease herpesvirus after seven days of treatment with phosphonoacetate at a dosage level of

500 mg/kg/day (118). The birds were treated for an additional fourteen days and observed daily for paralysis and death. The incidence of paralysis in non-treated chicks was found to be four times greater than that in treated birds, and the first signs of paralysis occurred five days earlier in the non-treated chicks than in the treated. However, phosphonoacetate was not effective in inhibiting the replication of Marek's disease herpesvirus (3).

The efficacy of phosphonoacetate in the treatment of vaccinia virus has been investigated (119). The effectiveness was evaluated by the ability of phosphonoacetate to suppress the formation of tail lesions in mice. Other investigators studied the antiviral efficacy of phosphonoacetate in localized skin lesions of rabbits produced by the intradermal inoculation of vaccinia virus and of Shope fibroma virus (120). Complete suppression of the appearance of vaccinia virus-induced pustular lesions was achieved by a 2% phosphonoacetate ointment applied twice daily for four days. Phosphonoacetate was significantly effective against Shope fibroma virus-induced tumors when applied as an ointment beginning either 24 or 72 hours after virus inoculation.

The studies on toxicity and drug metabolism for phosphonoacetate have been carried out by Abbott Laboratories although little has been published. Phosphonoacetate was not mutagenic in the *Salmonella typhimurium* test (121). When administered orally, phosphonoacetate was poorly absorbed in rat, rabbit, and monkey as determined by urinary excretion (122). Absorption was somewhat higher in dogs but varied. When administered intravenously, a substantial amount of phosphonoacetate remained in the body. Whole body autoradiography

studies in rats demonstrated that it accumulated in bone and that the retention was long term.

Abbott Laboratories has recently applied for permission to have phosphonoacetate classified as an investigative drug for purposes of initiating clinical trials. The application was made to the Food and Drug Administration. Based on the toxicity of phosphonoacetate, the FDA did not sanction usage and requested Abbott Laboratories to undertake an additional series of toxicity tests. The results of these tests have not been reported. Therefore, the prospective clinical use of phosphonoacetate remains uncertain.

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, formerly 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 cells (CHO), 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 contaminated. HeLa and CHO cells were grown in suspension culture in F-14 and F-20 medium (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 acid 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 mid log 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 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 bovine serum albumin, 4 mM $MgCl_2$, 100 mM KCl, 50 µg/ml 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 cytoplasmic fraction according to the procedure of Chang et al. (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 (nmol of deoxyribonucleoside triphosphate incorporated into an acid insoluble form in 30 min at 37°C per milligram 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 7.5S 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 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%. Either in the presence or absence of phosphonoacetate, the rate of the reaction was linear for at least one hour. Phosphonoacetate gave linear noncompetitive inhibition with the four dNTPs as the variable substrate and 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 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. With activated DNA as the variable substrate and the 4 dNTPs at their apparent Michaelis constant concentrations of 7 μM each, phosphonoacetate gave linear noncompetitive inhibition (data not shown). A replot of the vertical intercepts yielded a K_{ii} of 34 μM and a replot of the slopes yielded a K_{is} of 94 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 (Figure 2).

Figure 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 pmol of ^{3}H-labeled dTMP incorporated into DNA per 30 min. Phosphonoacetate concentrations were 0 (●), 20 μM (○), 40 μM (□), and 60 μM (Δ). 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.

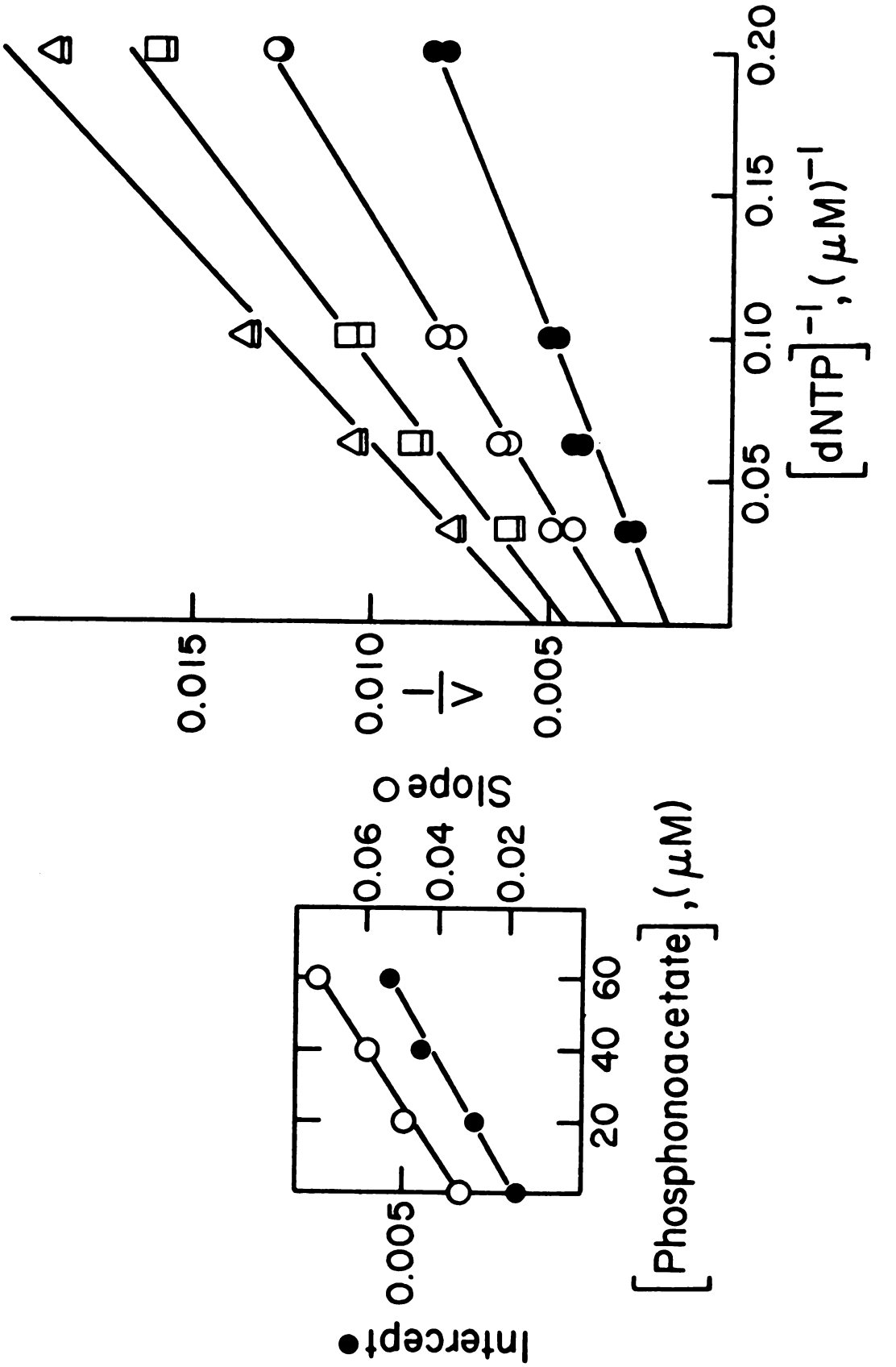
0.015
0.010
0.005
$\frac{1}{V}$
0.05
0.10
0.15
0.20
$[dNTP]^{-1}, (\mu M)^{-1}$
Slope
0.06
0.04
0.02
Intercept
0.005
20
40
60
$[Phosphonoacetate], (\mu M)$

Figure 1

Figure 2. Double reciprocal plots with activated DNA as the variable substrate and phosphonoacetate as inhibitor of the purified HeLa α-polymerase. The ^{3}H-labeled dTTP was at 20 μM and the three dNTPs were at 100 μM each. Phosphonoacetate concentrations were 0 (●), 20 μM (○), 40 μM (□), and 60 μM (Δ).

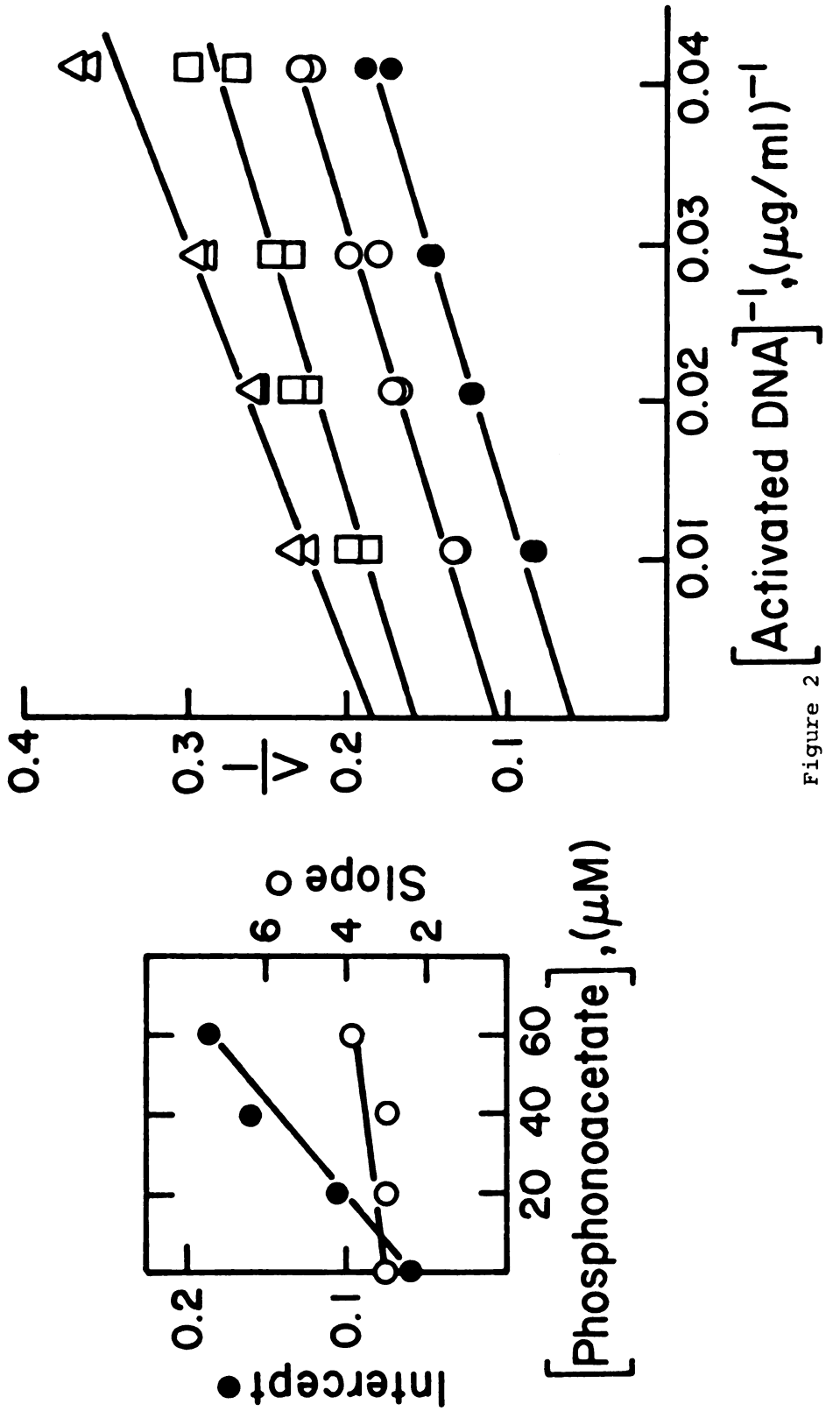
0.4
0.3
0.2
0.1
1/V
0.01
0.02
0.03
0.04
[Activated DNA]⁻¹, (μg/ml)⁻¹
Slope
6
4
2
Intercept
0.2
0.1
20
40
60
[Phosphonoacetate], (μM)

Figure 2

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).

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 (Figure 3). 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 phytohemagglutinin-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

Figure 3. Double reciprocal plots with activated DNA as the variable substrate and phosphonoacetate as inhibitor of the purified Wi-38 α-polymerase. The ^{3}H-labeled dTTP was at 20 μM and the three dNTPs were at 100 μM each. Phosphonoacetate concentrations were 0 (●), 20 μM (○), 40 μM (□), and 60 μM (Δ).

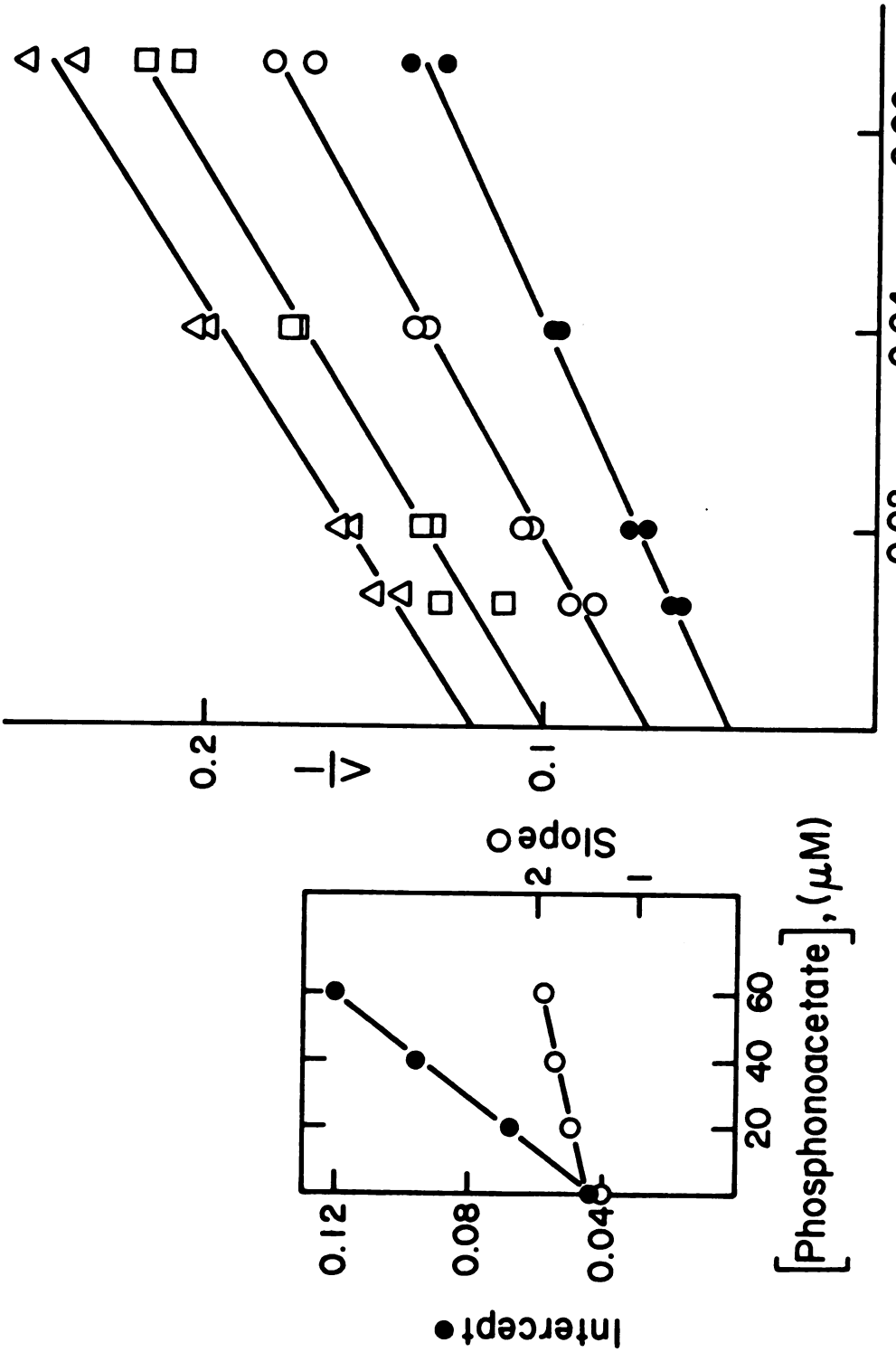
0.2
0.1
$\frac{1}{v}$
0.02
0.04
0.06
$\left[\text{Activated DNA}\right]^{-1}, (\mu g/ml)^{-1}$
Slope
2
1
Intercept
0.12
0.08
0.04
20 40 60
$\left[\text{Phosphonoacetate}\right], (\mu M)$

Figure 3

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 eukaryotic cells was not significantly inhibited by phosphonoacetate. 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 non-vertebrate eukaryotic 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 either DNA polymerase A or 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 (Figure 4). A replot of the vertical intercepts yielded a K_{ii} of 24 μM and a replot of the slopes a K_{is} of 59 μM. Phosphonoformate gave linear noncompetitive inhibition with activated DNA as the variable substrate and the four dNTPs at their apparent Michaelis constant concentrations of 7 μM each. A replot of the vertical intercepts gave a K_{ii} of 32 μM and a replot of the slopes a K_{is} of 176 μM (data not shown). Phosphonoformate, therefore, appears to be equally as 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 Figure 5, a plot of 1/v against phosphonoformate concentration resulted in a series of parallel lines, indicating the two phosphonate compounds 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

Figure 4. 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 µM (○), 40 µM (□), and 60 µM (Δ).

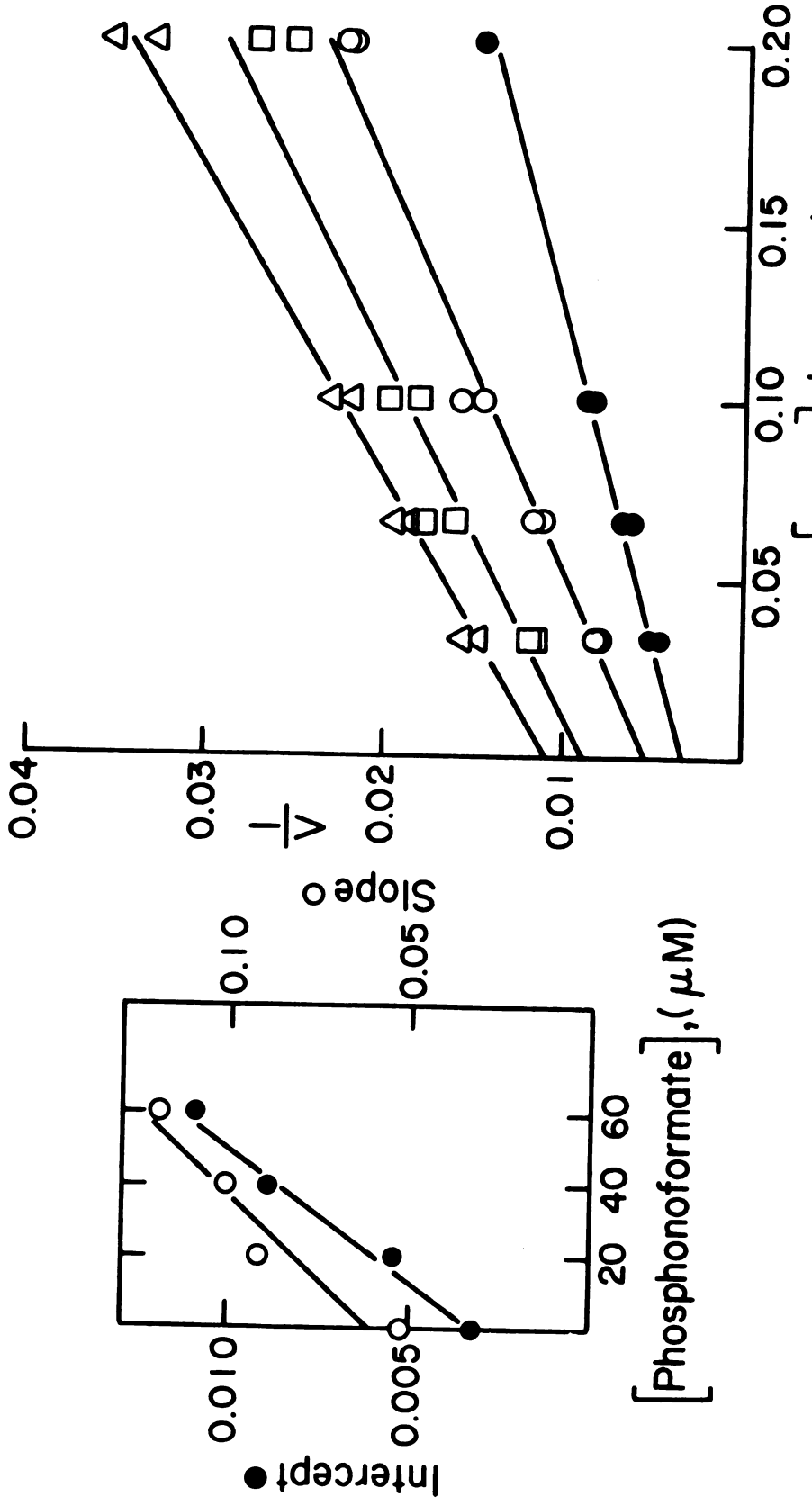
0.04
0.03
0.02
0.01
1/V
0.05
0.10
0.15
0.20
[dNTP]⁻¹,(μM)⁻¹
Slope ○
0.10
0.05
Intercept ●
0.010
0.005
20
40
60
[Phosphonoformate],(μM)

Figure 4

Figure 5. 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 µM (○), 20 µM (□), and 30 µM (Δ).

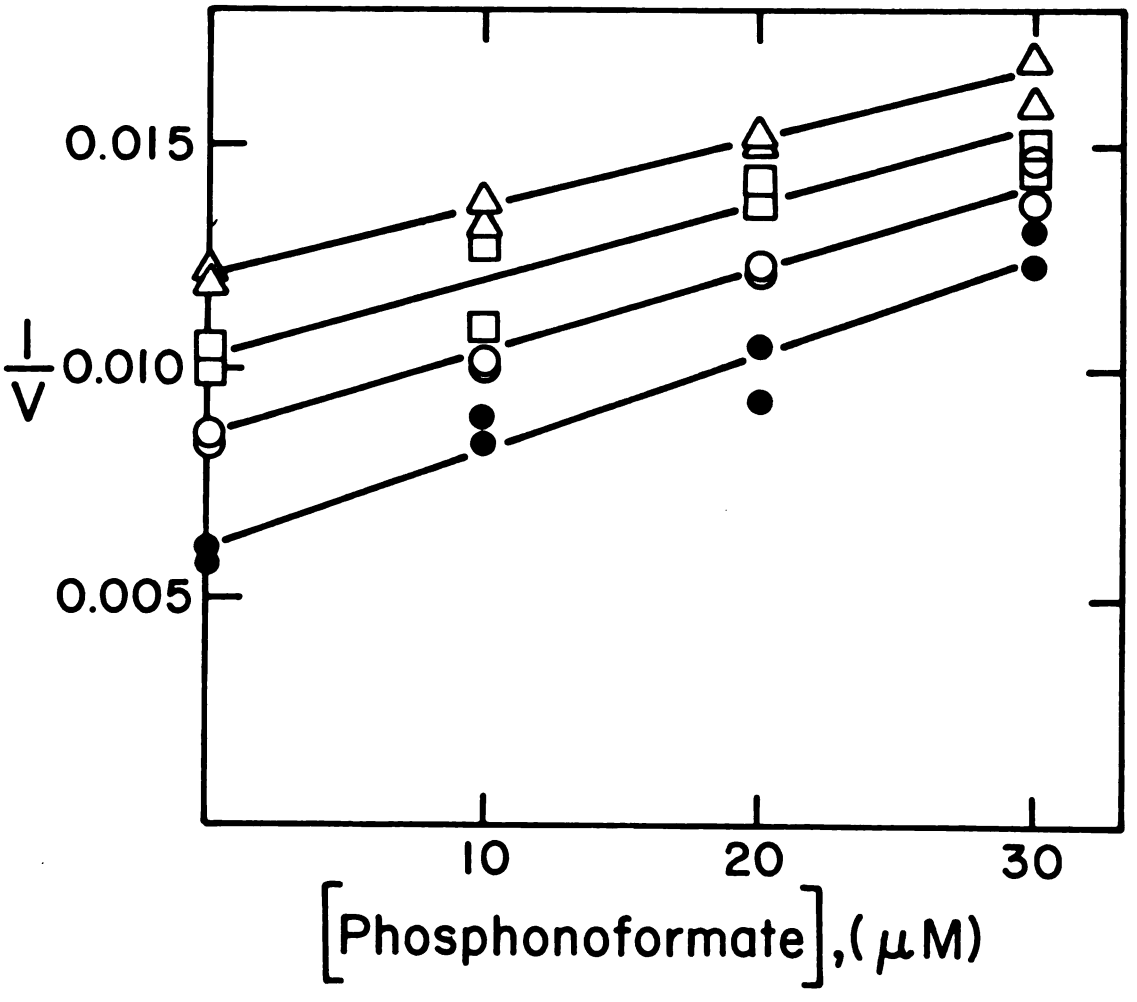

Figure 5

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-methyl phosphonoacetamide, and acetonyl phosphonate.

DISCUSSION

This report demonstrates that the α-polymerases from three human cells, HeLa, Wi-38, and phytohemagglutinin-stimulated lymphocytes, 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, the apparent inhibition constants and inhibition patterns for the α-polymerase are reported. In contrast to the results of Bolden et al. (12), who claimed that the α-polymerase and the herpesvirus-induced DNA polymerase were equally sensitive to phosphonoacetate, the results demonstrate that the α-polymerase is, in fact, 15 to 30 times less sensitive.

The results showing that the Wi-38 α-polymerase is sensitive to phosphonoacetate contradict the results reported by Mao et al. (6), Mao and Robishaw (7), Huang (2), and Hirai and Watanabe (11). Their reports are in error either because their putative α-polymerase preparations were contaminated with a mycoplasma DNA polymerase or because their putative α-polymerase preparations were, in fact, not α-polymerase preparations but possibly β-polymerase preparations (32). Mycoplasma, a common contaminant of eukaryotic cell cultures, produces a 6S DNA polymerase which is not inhibited by phosphonoacetate (33, J. Boezi, personal communication). The 6S mycoplasma polymerase could easily be confused with the 6-8S α-polymerase.

Indeed, the biphasic curves relating polymerase activity to phosphonoacetate concentration reported by Huang (2) could be interpreted to mean that his α-polymerase preparation contained two polymerase activities, one sensitive to phosphonoacetate which could be called the authentic Wi-38 α-polymerase and one insensitive to phosphonoacetate which would be labeled a possible mycoplasma contaminating activity.

The amount of α-polymerase found in a cell is highly dependent on the growth state of the cell (34,35). Extracts prepared from actively growing cells contain a large amount of α-polymerase activity and, under these growth conditions, the α-polymerase is the major polymerase activity found in the extracts. Extracts prepared from non-growing cells contain little or no α-polymerase activity and, under these growth conditions, the β-polymerase is the predominant polymerase activity of the extracts. Hirai and Watanabe (11) prepared their polymerase extracts from cells that had been mantained at confluency for 24 hours. Mao and Robishaw (7) used an unfractionated extract in their studies. Possibly, in contrast to their assumption, the predominant polymerase activity in their extract was β-polymerase rather than α-polymerase.

In addition to the α-polymerase from HeLa which is a continuous cell line that originated from cervical cancer tissue (15) and the α-polymerase from Wi-38 which is a human cell line with a limited capability to remain in culture and which originated from normal human embryonic lung tissue (14), the phosphonoacetate-sensitivity of the α-polymerase of human lymphocytes was checked. As was the case with HeLa and Wi-38, this α-polymerase was also sensitive. Recently, Miller and Rapp (33) reported that the α-polymerase of HEL,

a cell line with a finite life expectancy originating from normal human embryonic lung tissue, was also sensitive. Thus, it is clear, when all of these results are taken together, that the human α-polymerase is sensitive to phosphonoacetate.

Other vertebrate eukaryotic α-polymerases were also observed to be sensitive to phosphonoacetate. Calf thymus and CHO α-polymerase showed inhibition. Leinbach et al. (9) have reported that the α-polymerase from duck embryo fibroblasts is also inhibited by phosphonoacetate.

The β-polymerase of three human cells, HeLa, Wi-38 and lymphocytes, is relatively insensitive to phosphonoacetate. The result for HeLa is in agreement with that of Bolden et al. (12) and the result for Wi-38 agrees with those 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 appears to be a general characteristic of vertebrate polymerases.

Yeast, a simple eukaryote, contains two DNA polymerases in mitochondrial free cell extracts, designated A and B, rather than the three DNA polymerases, α, β, and γ, of vertebrate eukaryotes (24). In this report, both of the yeast polymerases were observed to be sensitive to phosphonoacetate and to about the same extent as α-polymerase. A single DNA polymerase in tobacco cells (unpublished

work) was identified. In this report, the DNA polymerase from tobacco cells was also shown to be sensitive to phosphonoacetate.

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 polymerase 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 ten to twenty times greater than that 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.

REFERENCES

REFERENCES

1. Shipkowitz, N. L., Bower, R. R., Appell, R. N., Nordeen, C. W., Overby, L. R., Roderick, W. R., Schleicher, J. B., and Von Esch, A. M. (1973) Appl. Microbiol. 26, 264-267.

2. Huang, E.-S. (1975) J. Virol. 16, 1560-1565.

3. Lee, L. F., Nazerian, K., Leinbach, S. S., Reno, J. M., and Boezi, J. A. (1976) J. Nat. Cancer Inst. 56, 823-827.

4. Summers, W. C., and Klein, G. (1976) J. Virol. 18, 151-155.

5. Overby, L. R., Robishaw, E. E., Schleicher, J. B., Rueter, A., Shipkowitz, N. L., and Mao, J. C.-H. (1974) Antimicrob. Agents Chemother. 6, 360-365.

6. Mao, J. C.-H., Robishaw, E. E., and Overby, L. R. (1975) J. Virol. 15, 1281-1283.

7. Mao, J. C.-H., and Robishaw, E. E. (1975) Biochemistry 14, 5475-5479.

8. Hay, J., and Subak-Sharpe, J. H. (1976) J. Gen. Virol. 31, 145-148.

9. Leinbach, S. S., Reno, J. M., Lee, L. F., Isbell, A. F., and Boezi, J. A. (1976) Biochemistry 15, 426-430.

10. Nyormoi, O., Thorley-Lawson, D. A., Elkington, J., and Strominger, J. L. (1976) Proc. Natl. Acad. Sci. USA 73, 1745-1748.

11. Hirai, K., and Watanabe, Y. (1976) Biochim. Biophys. Acta 447, 328-339.

12. Bolden, A., Aucker, J., and Weissbach, A. (1975) J. Virol. 16, 1584-1592.

13. Overby, L. R., Duff, R. G., and Mao, J. C.-H. (1977) Ann. N.Y. Acad. Sci. 284, 310-320.

14. Hayflick, L. (1961) Exp. Cell Res. 25, 585-586.

15. Gey, G. O., Coffman, W. D., and Kubicek, M. T. (1952) Cancer Res. 12, 264-265.

16. Nylen, P. (1924) Chem. Berichte 57B, 1023-1035.

17. Hayflick, L. (1965) Tex. Rep. Biol. Med. 23, (Suppl. 1), 285-303.

18. Filner, P. (1965) Exp. Cell Res. 39, 33-39.

19. Boyum, A. (1968) Scand. J. Clin. Lab. Invest. 21, 31-89.

20. Boezi, J. A., Lee, L. F., Blakesley, R. W., Koenig, M., and Towle, H. C. (1974) J. Virol. 14, 1209-1219.

21. Chang, L. M. S., and Bollum, F. J. (1972) Biochemistry 11, 1264-1272.

22. Weissbach, A., Schlabach, A., Fridlender, B., and Bolden, A. (1971) Nature (London), New Biol. 231, 167-170.

23. Bertazzoni, U., Stefanini, M., Noy, G. P., Guilotto, E., Nuzzo, F., Falaschi, A., and Spadari, S. (1976) Proc. Natl. Acad. Sci. USA 73, 785-789.

24. Wintersberger, U., and Wintersberger, E. (1970) Eur. J. Biochem. 13, 11-19.

25. Srivastava, B. I. S. (1973) Biochim. Biophys. Acta 299, 17-23.

26. Cleland, W. W. (1963) Biochim. Biophys. Acta 67, 104-137.

27. Cleland, W. W. (1963) Biochim. Biophys. Acta 67, 173-187.

28. Wilkinson, G. N. (1961) Biochem. J. 80, 324-332.

29. Knopf, K.-W., Yamada, M., and Weissbach, A. (1976) Biochemistry 15, 4540-4548.

30. Yonetani, J., and Theorell, H. (1964) Arch. Biochem. Biophys. 106, 243-251.

31. Segel, I. H. (1975) *Enzyme Kinetics -- Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems*, p. 465-504, John Wiley & Sons, Inc., New York.

32. Wang, T. S.-F., Sedwick, W. D., and Korn, D. (1975) J. Biol. Chem. 250, 7040-7044.

33. Miller, R. L., and Rapp, F. (1976) J. Virol. 20, 564-569.

34. Chang, L. M. S., Brown, M., and Bollum, F. J. (1974) J. Mol. Biol. 74, 1-8.

35. Craig, R. K., Costello, P. A., and Keir, H. M. (1975) Biochem. J. 145, 233-240.

36. Dube, D. K., Seal, G., and Loeb, L. A. (1977) Biochem. Biophys. Res. Commun. 76, 483-487.

37. Noy, G. P., and Weissbach, A. (1977) Biochim. Biophys. Acta 447, 70-83.

38. Weissbach, A. (1975) Cell 5, 101-108.

39. Weissbach, A. (1977) Ann. Rev. Biochem. 46, 25-47.

40. Wintersberger, E. (1977) Trends in Biol. Sci. 2, 58-61.

41. Bollum, F. J. (1960) J. Biol. Chem. 235, 2399-2403.

42. Chang, L. M. S., and Bollum, F. J. (1973) J. Biol. Chem. 248, 3398-3404.

43. Sedwick, W. D., Wang, T. S.-F., and Korn, D. (1975) J. Biol. Chem. 250, 7045-7056.

44. Bollum, F. J. (1975) Prog. Nucleic Acid Res. Mol. Biol. 15, 109-144.

45. Holmes, A. M., Hesslewood, I. P., and Johnston, I. R. (1976) Eur. J. Biochem. 62, 229-235.

46. Smith, R. G., and Gallo, R. G. (1972) Proc. Natl. Acad. Sci. USA 69, 2879-2884.

47. Sedwick, W. D., Wang, T. S.-F., and Korn, D. (1972) J. Biol. Chem. 247, 5026-5033.

48. Yoshida, S., Kondo, T., and Ando, T. (1974) Biochim. Biophys. Acta 353, 463-474.

49. Lynch, W. E., Surrey, S., and Lieberman, I. (1975) J. Biol. Chem. 250, 8179-8183.

50. Herrick, G., Spear, B. B., and Veomett, G. (1976) Proc. Natl. Acad. Sci. USA 73, 1136-1139.

51. Foster, D. N., and Gurney, T. (1976) J. Biol. Chem. 251, 7893-7898.

52. Byrnes, J. J., Downey, K. M., and So, A. G. (1973) Biochemistry 12, 4378-4384.

53. Momparler, R. L., Rossi, M., and Labitan, A. (1973) J. Biol. Chem. 248, 285-293.

54. Herrick, G., Delius, H., and Alberts, B. (1976) J. Biol. Chem. 251, 2142-2146.

55. Spadari, S., and Weissbach, A. (1975) Proc. Natl. Acad. Sci. USA 72, 503-507.

56. Hachmann, H. J., and Lezius, A. G. (1975) Eur. J. Biochem. 50, 357-366.

57. Spadari, S., and Weissbach, A. (1974) J. Mol. Biol. 86, 11-20.

58. Chang, L. M. S., and Bollum, F. J. (1972) Science 175, 1116-1117.

59. Chang, L. M. S., and Bollum, F. J. (1971) J. Biol. Chem. 246, 5835-5837.

60. Haines, M. E., Holmes, A. N., and Johnston, I. R. (1971) FEBS Letters 17, 63-67.

61. Berger, Jr. H., Huang, R. C., and Irwin, J. L. (1971) J. Biol. Chem. 246, 7275-7283.

62. Chang, L. M. S. (1973) J. Biol. Chem. 248, 3789-3795.

63. Spadari, S., Muller, R., and Weissbach, A. (1974) J. Biol. Chem. 249, 2991-2992.

64. Holmes, A. M., Hesslewood, I. P., and Johnston, I. R. (1974) Eur. J. Biochem. 43, 487-499.

65. Stalker, D. M., Mosbaugh, D. W., and Meyer, R. R. (1976) Biochemistry 15, 3114-3121.

66. Wang, T. S.-F., Sedwick, D., and Korn, D. (1974) J. Biol. Chem. 249, 841-850.

67. Chang, L. M. S. (1974) J. Biol. Chem. 249, 7441-7445.

68. Chang, L. M. S. (1976) Science 191, 1183-1185.

69. Wintersberger, U. (1974) Eur. J. Biochem. 50, 197-202.

70. Smith, R. G., Abrell, J. W., Lewis, B. J., and Gallo, R. C. (1975) J. Biol. Chem. 250, 1702-1709.

71. Brun, G. M., Assairi, L. M., and Chapeville, F. (1975) J. Biol. Chem. 250, 7320-7323.

72. Fridlender, B., Fry, M., Bolden, A., and Weissbach, A. (1972) Proc. Natl. Acad. Sci. USA 69, 452-455.

73. Spadari, S., and Weissbach, A. (1974) J. Biol. Chem. 249, 5809-5815.

74. Lewis, B. J., Abrell, J. W., Smith, R. G., and Gallo, R. C. (1974) Science 183, 867-869.

75. Lewis, B. J., Abrell, J. W., Smith, R. G., and Gallo, R. C. (1974) Biochim. Biophys. Acta 349, 148-160.

76. Weissbach, A., Bolden, A., Muller, R., Hanafusa, H., and Hanafusa, T. (1972) J. Virol. 10, 321-327.

77. Ito, K., Arens, M., and Green, M. (1975) J. Virol. 15, 1507-1510.

78. Fry, M., and Weissbach, A. (1973) Biochemistry 12, 3602-3608.

79. Bolden, A., Noy, G. P., and Weissbach, A. (1977) J. Biol. Chem. 252, 3351-3356.

80. Chang, L. M. S., and Bollum, F. J. (1972) J. Biol. Chem. 247, 7948-7950.

81. Baril, E. F., Jenkins, M. D., Brown, O. E., Laszlo, J., and Morris, H. P. (1973) Cancer Res. 33, 1187-1193.

82. Chiu, R. W., and Baril, E. F. (1975) J. Biol. Chem. 250, 7951-7957.

83. Walters, R. A., Tobey, R. A., and Hildebrand, C. E. (1976) Biochem. Biophys. Res. Commun. 69, 212-217.

84. Chevailler, P. H., and Phillippe, M. (1976) Exp. Cell Res. 99, 237-244.

85. Hecht, N., Farrel, D., and Davidson, D. (1976) Dev. Biol. 48, 56-66.

86. Sherman, M. I., and Kang, H. S. (1973) Dev. Biol. 34, 200-210.

87. Loeb, L. A. (1974) The Enzymes 10, 173-209.

88. De Petrocellis, B., and Vittorelli, M. L. (1975) Exp. Cell Res. 94, 392-400.

89. Grippo, P., and Lo Scavo, A. (1972) Biochem. Biophys. Res. Commun. 43, 280-285.

90. Benbow, R. M., Pestell, R. Q. W., and Ford, C. C. (1975) Dev. Biol. 43, 159-174.

91. Grippo, P., Locorotondo, G., and Caruso, A. (1975) FEBS Letters 51, 137-142.

92. Chiu, J. F., and Sung, S. C. (1972) Biochim. Biophys. Acta 269, 364-369.

93. Barton, R. W., and Yang, W.-K. (1975) Mech. Ageing Dev. 4, 124-136.

94. Velasco, E. G., and Brasel, J. A. (1975) J. Pediatr. 86, 274-279.

95. Huang, E.-S., Huang, C.-H., Huong, S.-M., and Selgrade, M. (1976) Yale J. Biol. Med. 49, 93-98.

96. Pearson, G. R., and Beneke, J. S. (1977) Cancer Res. 37, 42-46.

97. Barahona, H., Danill, M. D., Bekesi, J. G., Fraser, C. E. O., King, N. W., Hunt, R. D., Ingalls, J. K., and Jones, T. C. (1977) Proc. Soc. Exp. Biol. and Med. 154, 431-434.

98. Allen, G. P., O'Callaghan, D. J., and Randall, C. C. (1977) Virology 76, 395-408.

99. Yajima, Y., Tanaka, A., and Nonoyama, M. (1976) Virology 71, 352-354.

100. Becker, Y., Asher, Y., Cohen, Y., Weinberg-Zahlering, E., and Shlomai, J. (1977) Antimicrob. Agents Chemother. 11, 919-922.

101. Seebeck, T., Shaw, J. E., and Pagano, J. S. (1977) J. Virol. 21, 435-438.

102. Miller, R. L., Glaser, R., and Rapp, F. (1977) Virology 76, 494-502.

103. Hay, J., and Subak-Sharpe, J. H. (1976) J. Gen. Virol. 31, 145-148.

104. Honess, R. W., and Watson, D. H. (1977) J. Virol. 21, 584-600.

105. Herrin, T. R., Fairgrieve, J. S., Bower, R. R., Shipkowitz, N. L., and Mao, J. C.-H. (1977) J. Med. Chem. 20, 660-663.

106. Pulvertaft, R. J. V. (1964) Lancet 1, 238-240.

107. Hampar, B., Tanaka, A., Nonoyama, M., and Derge, J. G. (1974) Proc. Natl. Acad. Sci. USA 71, 631-633.

108. Gergely, L., Klein, G., and Ernberg, I. (1971) Int. J. Cancer 7, 293-302.

109. Miller, G., Shope, T., Lisco, H., Stitt, D., and Lipman, M. (1972) Proc. Natl. Acad. Sci. USA 69, 383-387.

110. Thorley-Lawson, D., and Strominger, J. L. (1976) Nature 263, 332-334.

111. Klein, R. J., and Friedman-Kien, A. E. (1975) Antimicrob. Agents Chemother. 7, 289-293.

112. Gerstein, D. D., Dawson, C. R., and Oh, J. O. (1975) Antimicrob. Agents and Chemother. 7, 285-288.

113. Meyer, R. F., Varnell, E. D., and Kaufman, H. E. (1976) Antimicrob. Agents Chemother. 9, 308-311.

114. Kern, E. R., Richards, J. T., Overall, J. C., and Glasgow, L. A. (1977) J. Infect. Dis. 135, 557-567.

115. Fitzwilliam, J. F., and Griffith, J. F. (1976) J. Infect. Dis. 133, A221-A225.

116. Wohlenberg, C. R., Walz, M. A., and Notkins, A. L. (1976) Infection and Immunity 13, 1519-1521.

117. Overall, J. C., Kern, E. R., and Glasgow, L. A. (1976) J. Infect. Dis. 133, A237-A244.

118. Schleicher, J. B., and Roderick, W. R. (1974) U. S. Patent Number 3,836,650.

119. Schleicher, J. B., and Roderick, W. R. (1973) U. S. Patent Number 3,767,794.

120. Friedman-Kien, A. E., Fondak, A. A., and Klein, R. J. (1976) J. Invest. Dermat. 66, 99-102.

121. Becker, B. A., Bopp, B. A., Brusick, D. J., and Lehrer, S. B. (1976) Federation Proceedings 35, 533.

122. Bopp, B. A., Estep, C. B., and Anderson, D. J. (1977) Federation Proceedings 36, 939.