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

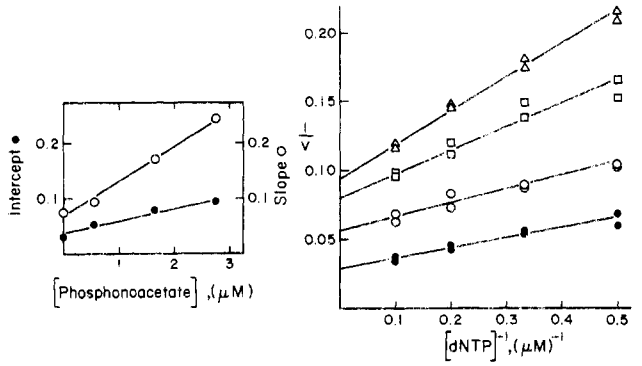

FIGURE 1: Double reciprocal plots with the four dNTPs as the variable substrate and phosphonoacetate as inhibitor. Activated DNA was at 200 μg/ml. The initial velocities were expressed as pmol of ^{3}H-labeled dCMP incorporated into DNA per 30 min. Phosphonoacetate concentrations were 0 (●), 0.55 μ*M* (○), 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.

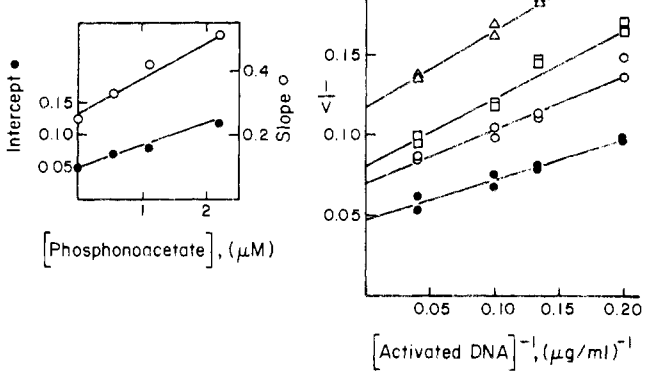

FIGURE 2: Double reciprocal plots with activated DNA as the variable substrate and phosphonoacetate as inhibitor. The four dNTPs were at 2.5 μ*M* each. Phosphonoacetate concentrations were 0 (●), 0.55 μ*M* (○), 1.1 μ*M* (□), and 2.2 μ*M* (△).

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

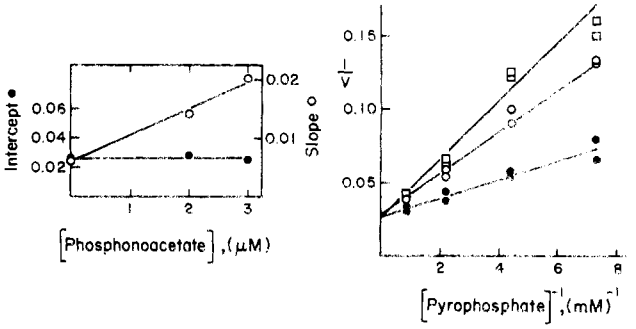

FIGURE 3: Double reciprocal plots of the dNTP–pyrophosphate exchange reaction with pyrophosphate as the variable substrate and phosphonoacetate as inhibitor. The initial velocities were expressed as picomoles of ^{32}P-labeled pyrophosphate converted to a Norit-adsorbable form per 30 min. Phosphonoacetate 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.

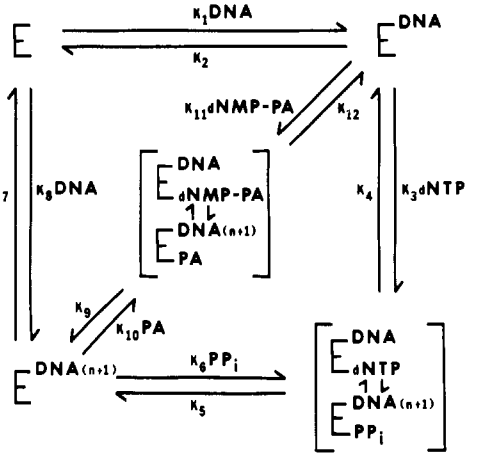

FIGURE 4: Proposed mechanism of phosphonoacetate inhibition of herpesvirus-induced DNA polymerase. The basic reaction mechanism in the absence of phosphonoacetate (PA) is a modified ordered bi-bi mechanism (Kornberg, 1969; McClure and Jovin, 1975). Initial velocity studies (S. S. Leinbach et al., unpublished 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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Influence of Electric Dichroism on the Temperature-Jump Relaxation Study of Proflavine–DNA Complexes†

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ABSTRACT: The temperature-jump relaxation kinetics of proflavine–DNA complexes has been reinvestigated with a standard apparatus equipped for absorption detection of plane-polarized light in order to discriminate between chemical relaxation and transient orientation effects. Under low ionic strength conditions (0.015 *M* Na^+), these effects may represent the major contribution to the signal when the T-jump apparatus is used without a polarizer. They have been improperly assigned to chemical relaxation in previous work. The actual relaxation times are smaller than 30 μsec at 10°C. Under medium ionic strength conditions (0.2 *M* Na^+) it is shown that: (i) the "instantaneous" change of transmission reported in earlier work (Li, H. J., and Crothers, D. M. (1969), *J. Mol. Biol. 39,* 461–477; Schmechel, D. E. V., and Crothers, D. M. (1971), *Biopolymers 10,* 465–480) is due to orientation effects; (ii) an intermediate exists whose absorption spectrum resembles somewhat that of proflavine aggregates on a linear polyanion; (iii) the rate constants for outside binding may be significantly larger than previously reported. The new kinetic data are consistent with a modified mechanism derived from equilibrium studies (Ramstein, J., Hogrel, J. F., Dourlent, M., Leng, M., and Hélène, C. (1973), in Dynamic Aspects of Conformation Changes in Biological Macromolecules, Sadron, C., Ed., Dordrecht, Holland, Reidel Publisher, pp 333–347; Dourlent, M., and Hogrel, J. F. (1976), *Biopolymers* (in press)), but, however, do not unambiguously prove it. From these studies, it is inferred that orientation effects can perturb relaxation data on systems containing linear polymers in many cases.

The dynamics of dye–nucleic acid interactions have been extensively studied by means of temperature-jump relaxation and stopped-flow techniques (Li and Crothers, 1969; Schmechel and Crothers, 1971; Ramstein et al., 1972, 1973; Thusius et al., 1973; Steenbergen and Mohr, 1973; Akasaka et al., 1970; Sakoda et al., 1971, 1972; Bittman, 1969; Tritton and Mohr, 1971; Tritton and Mohr, 1973). Simultaneously the mechanism of cooperative binding to other linear polyanions has also been investigated (Hammes and Hubbard, 1966a,b; Schwarz et al., 1970; Schwarz and Balthazar, 1970; Schwarz and Klose, 1972; Vitagliano, 1973). In a recent work (Dourlent et al., 1974) it was suspected that the very fast component of the relaxation signal observed with solutions containing proflavine (Li and Crothers, 1969; Schmechel and Crothers, 1971) could be an artefact resulting from a transient orientation of the polymer molecules in the electric field of the temperature-jump

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