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Observations on the Pyridoxal 5′-Phosphate Inhibition of DNA Polymerases†

Mukund J. Modak

ABSTRACT: Pyridoxal 5′-phosphate at concentrations >0.5 mM inhibits polymerization of deoxynucleoside triphosphate catalyzed by a variety of DNA polymerases. The requirement for a phosphate as well as aldehyde moiety of pyridoxal phosphate for inhibition to occur is clearly shown by the fact that neither pyridoxal nor pyridoxamine phosphate are effective inhibitors. Since the addition of nonenzyme protein or increasing the amount of template primer exerted no protective effect, there appears to be specific affinity between pyridoxal phosphate and polymerase protein. The deoxynucleoside triphosphates, however, could reverse the inhibition. The binding of pyridoxal 5′-phosphate to enzyme appears to be mediated through classical Schiff base formation between the pyridoxal phosphate and the free amino group(s) present at the active site of the polymerase protein. Kinetic studies indicate that inhibition by pyridoxal phosphate is competitive with respect to substrate deoxynucleoside triphosphate(s).

DNA polymerases from a wide variety of sources, ranging from viruses to eukaryotes, appear to share several basic characteristics with respect to requirements and mechanism of polymerization (Kornberg, 1974; Loeb, 1974; Saxinger et al., 1975). However, even though catalytic activities, functions, and to some degree structure of several of these enzymes is known, the exact enzyme mechanisms involved in catalysis and the actual structure of the active site of these enzymes are poorly understood. We have found that a naturally occurring coenzyme pyridoxal 5′-phosphate is a specific competitive inhibitor that offers the possibility of labeling the active site. The effect of pyridoxal phosphate was discovered during our search for oncornaviral reverse transcriptase specific inhibitors. Earlier, we had observed that inorganic phosphate may specifically inhibit DNA synthesis catalyzed by variety of mammalian C-type viral reverse transcriptases (Modak and Marcus, submitted for publication) and hence the examination of several phosphate-containing compounds was carried out. However, the studies with pyridoxal phosphate indicated a lack of specificity toward a particular class of DNA-polymerizing enzymes, since DNA polymerases from both B- and C-type oncornaviruses, as well as prokaryotic and eukaryotic cell enzymes, are equally susceptible to inhibition. We now demonstrate that inhibition by pyridoxal phosphate is competitive with nucleotide substrates and is expressed via the formation of a Schiff base. This offers the prospect of labeling the active site of DNA polymerase and deciphering the geometry of the active site on these enzymes.

Materials and Methods

Materials. All radioactive deoxyribonucleoside triphosphates were obtained from Amersham Searle, Inc. Unlabeled triphosphates and template primers were products of P. L. Biochemicals, Inc. The molar ratio of template to primer was 1:1 in the case of poly(rA)·$(dT)_{10}$ and 10:1 in the case of poly(rC)·$(dG)_{12-18}$ and poly(dC)·$(dG)_{12-18}$. Activated DNA was

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prepared as described by Aposhian and Kornberg (1962). Rabbit globin mRNA, purchased from Amersham Searle, Inc., was annealed to oligo(dT)$_{10}$ in the molar ratio of 20:1 in 0.05 M Tris (pH 7.8) buffer (Modak et al., 1974). Pyridoxine-HCl, pyridoxal, pyridoxal 5′-phosphate, pyridoxamine, and pyridoxamine 5′-phosphate were obtained from Sigma Chemical Co. All other chemicals were of analytical grade.

Enzymes. Avian myeloblastosis virus (AMV)[1] and Rauscher leukemia virus (RLV) DNA polymerases used in these studies were partially purified using affinity chromatography on polycytidylate–agarose columns (Marcus et al., 1974). The enzymes from simian sarcoma virus (SSV), feline leukemia virus (FeLV), spontaneously produced type-C virus from Wistar rat embryo fibroblast (Wistar Virus), Mason Pfizer monkey virus (MPMV), and mouse mammary tumor virus (MuMTV) were purified at least 60-fold using poly(rC)–agarose affinity chromatography. The details of the purification procedures used to obtain these enzymes together with their characterization will appear elsewhere (Marcus et al., 1976a; Modak and Marcus, to be published). Briefly, the concentrated virus suspension (approximately 2–5 mg of protein) is treated with high salt and nonionic detergent in the presence of dithiothreitol to solubilize the virion-associated RNA-dependent DNA polymerase. The soluble extract, after dilution with buffer, is passed through a Pasteur pipet column of poly(rC)–agarose, washed, and the enzyme is eluted either with a linear salt gradient or 0.4 M salt. Enzyme is stored in a buffer containing 1% serum albumin, 20 mM Tris, pH 7.8, 5 mM dithiothreitol, and 20% glycerol at −70 °C. Under these conditions enzymes are quite stable. DNA polymerase α and γ from HeLa cells were the kind gift of Dr. A. Weissbach of Roche Institute for Molecular Biology, while homogeneous DNA polymerase I from *E. coli* was generously provided by Dr. L. A. Loeb of Fox Chase Institute for Cancer Research. Terminal deoxyribonucleotidyl transferase from human leukemic lymphocytes was purified according to the procedure described by McCaffrey et al. (1975) except that the phosphocellulose fraction enzyme was further purified by glycerol velocity gradient centrifugation (Marcus et al., 1976b).

Enzyme Assays. Enzyme reactions, except for terminal deoxyribonucleotidyl transferase, were carried out in a final volume of 100 μl and contained the following components: 50 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, 50 μg of bovine serum albumin, 20 μM appropriate tritiated nucleoside triphosphate, adjusted to a final specific activity of 1000 cpm/pmol, and 0.5 μg of template-primer. The type of divalent cation (Mg^{2+} or Mn^{2+}) and its concentration together with additional components, whenever required, for various template primers and enzymes are listed below:

(i) poly(rA)·(dT)$_{10}$ directed synthesis: AMV, MPMV, and MuMTV DNA polymerases require 10 mM $MgCl_2$ for optimal activity, while all other polymerase reactions were carried out in the presence of 0.5 mM $MnCl_2$ and 100 mM KCl.

(ii) poly(rC)·(dG)$_{12-18}$ and poly(dC)·(dG)$_{12-18}$ directed synthesis: For this template primer-directed reaction most of the enzymes reported herein preferred Mg^{2+} (2.5–5 mM) as a divalent cation.

(iii) Activated DNA and globin mRNA·oligo(dT)$_{10}$-directed synthesis: The amount of DNA used as a template primer was 2.5 μg/assay. The reaction mixture in addition to standard components also contained 100 μM of each of three unlabeled nucleoside triphosphates and 10 μM labeled triphosphate (specific activity adjusted to 2500 cpm/pmol). $MgCl_2$ at 10 mM concentration was used for DNA-directed DNA synthesis catalyzed by all the enzymes, while 1 mM $MnCl_2$ and 50 mM KCl was used for globin mRNA-directed synthesis catalyzed by RLV DNA polymerase.

Terminal deoxyribonucleotidyl transferase was assayed as described by McCaffrey et al. (1975) using oligo(dA)$_{12-18}$ as a primer, in the presence of 0.6 mM $MnCl_2$ and [^{3}H]dGTP as a substrate.

Incubations, unless indicated otherwise, were carried out at 37 °C for 30 min and were terminated by addition of 5% (w/v) trichloroacetic acid containing 0.01 M sodium pyrophosphate. The acid-insoluble material was collected on Whatman GF/B filters, washed extensively with Cl_3CCOOH-containing sodium pyrophosphate, water, and finally ethanol, dried and counted in toluene-based scintillation fluid.

Results

Effect of Pyridoxal 5′-Phosphate (PyP) on the Activity of Various DNA Polymerases

The response of reactions catalyzed by purified DNA polymerases of oncornaviral, bacterial, and mammalian origin to the addition of pyridoxal phosphate (0.5 mM) is described in Table I. The synthetic ability of the various DNA polymerases except terminal transferase was monitored using poly(rA)·(dT)$_{10}$ and activated DNA as synthetic and natural template primers, respectively. It is clear from Table I that all of the DNA polymerases tested, irrespective of their source, are significantly (70–85%) inhibited in the presence of 0.5 mM PyP. Poly(rA)·(dT)$_{10}$ directed synthesis was severely inhibited (~80–85%) compared to DNA directed synthesis (~60–70%).

A detailed investigation of this inhibitory property of PyP was carried out using RLV DNA polymerase as representative of deoxyribonucleoside triphosphate polymerizing enzymes.

Inhibition Is Not Due to Removal of Divalent Cation

Since the divalent cation requirement for the enzymatic synthesis of DNA is obligatory (Loeb, 1974), the possibility that pyridoxal phosphate may form an insoluble complex with metal ion and thus deplete the reaction of the required metal ion was tested. The response of RLV DNA polymerase to increasing concentrations of pyridoxal phosphate (PyP) in the presence of four different Mn^{2+} concentrations is depicted in Figure 1. RLV DNA polymerase activity is inhibited to a similar extent by 0.5–5 mM pyridoxal phosphate at Mn^{2+} concentrations ranging from below optimal (0.25 mM) to well above saturating levels (2.5 mM). Furthermore, the concentration of Mn^{2+} (0.5 mM) allowing optimal rates of synthesis remains unchanged at all pyridoxal phosphate concentrations tested (see inset to Figure 1). Identical results were obtained with Mg^{2+} as a divalent cation (data not shown). It is therefore concluded that PyP inhibition was not due to the removal of the effective divalent cation from the reaction mixture.

Effect of Pyridoxal Phosphate on the DNA Synthesis Catalyzed by RLV DNA Polymerase under the Direction of Various Template-Primers

RLV DNA polymerase can catalyze DNA synthesis under

[1] Abbreviations used are: PyP, pyridoxal 5′-phosphate; AMV, avian myeloblastosis virus; RLV, Rauscher leukemia virus; SSV, simian sarcoma virus; FeLV, feline leukemia virus; Wistar virus, rat leukemia virus derived from a spontaneous tumor; MPMV, Mason Pfizer monkey tumor virus; MuMTV, murine mammary tumor virus; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

TABLE I: Effect of Pyridoxal Phosphate (0.5 mM) on the Activity of Various DNA Polymerases Using Poly(rA)·$(dT)_{10}$ and Activated DNA as Template.[a]

Enzyme Source	pmol of Tritiated Precursor Incorporation					
	Poly(rA)·$(dT)_{10}$		%	Activated DNA		%
	Control	+PyP	Inhibition	Control	+PyP	Inhibition
AMV	16.2	3.9	76	8.8	2.1	76
Wistar virus	8.6	1.4	84	1.3	0.3	77
RLV	20.9	3.1	85	7.4	2.8	63
SSV	6.9	.9	87	1.8	0.5	72
FeLV	21.6	3.2	85	13.3	4.1	69
MPMV	10.6	1.7	84	7.4	2.8	62
MuMTV	4.8	.6	83	1.9	0.6	68
HeLa DNA Polymerase α	–	–		25.7	4.0	84
HeLa DNA Polymerase γ	38.6	7.2	81	5.2	1.6	69
E. coli DNA Polymerase I	17.6	4.0	77	28.1	13.7	51
Terminal deoxyribonucleotide transferase[b]	12.6	2.8	78	–	–	–

[a] Assay conditions for the determination of activity of various enzymes are described under Materials and Methods. [b] For this enzyme, assays were carried out using oligo(dA) as a primer and [^{3}H]dGTP as a precursor.

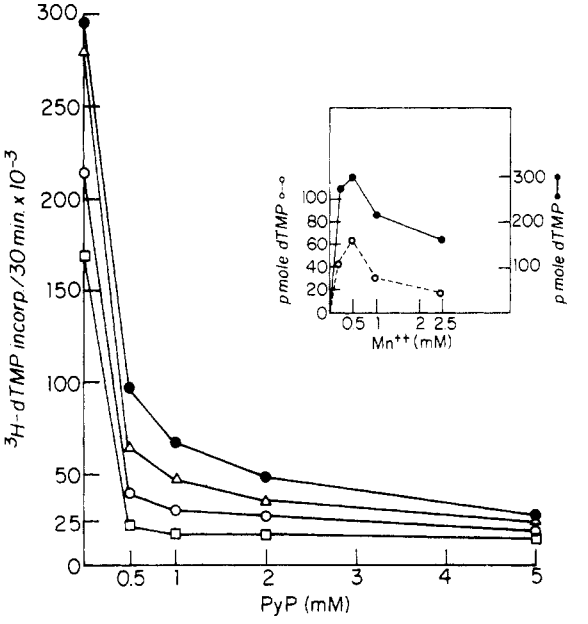

FIGURE 1: Effect of varying concentrations of pyridoxal 5′-phosphate on the poly(rA)·$(dT)_{10}$ directed synthesis in the presence of fixed concentrations of $MnCl_2$. The assay mixture contained standard components as described under Materials and Methods. The concentration of $MnCl_2$ was 0.2 mM (△—△), 0.5 mM (●—●), 1 mM (○—○), or 2.5 mM (□—□). The inset describes the Mn^{2+} optimum in the presence (○- - -○) and absence (●- - -●) of pyridoxal 5′-phosphate (0.5 mM).

the direction of several ribo- and deoxyribonucleotide polymers. However, the preference for required metal ion (Mg^{2+} or Mn^{2+}) depends strictly on the template-primer used to direct the synthesis (Modak and Marcus, submitted for publication). The effect of pyridoxal phosphate on the synthesis of DNA was, therefore, examined using a variety of synthetic and natural template primers and optimal concentratons of individual metal ions for that reaction. The results of this experiment are summarized in Table II. It is clearly demonstrated that, irrespective of the nature of the template-primer or metal ion used in the reaction mixture, addition of pyridoxal phosphate (0.5 mM) significantly inhibits DNA synthesis.

Characterization of the Inhibitory Effect of PyP

Specificity of Inhibition. In order to determine the specificity of inhibition by pyridoxal phosphate, four other related compounds were tested for their inhibitory effect on the RLV DNA polymerase. As seen in Table III, pyridoxine, pyridoxal, pyridoxamine, or pyridoxamine phosphate at a concentration of 1 mM have almost no effect on the poly(dT) synthesis catalyzed by RLV DNA polymerase under the direction of poly(rA)·$(dT)_{10}$, although at the concentration of 5 mM these compounds exert some inhibitory effect. However, as little as 0.1 mM pyridoxal 5′-phosphate severely inhibits catalysis.

To rule out the possibility that the inhibitory effect of PyP was restricted to purified enzyme, the response of crude enzyme preparations (detergent-disrupted RLV virions) to various PyP concentrations was examined and compared to that obtained with purified enzyme. The activity of both crude and purified enzyme was monitored with exogenously added template-primer, since the endogenous activity of crude disrupted virions is extremely poor and cannot be readily compared with the purified enzyme. The purpose of this experiment was to examine whether the several proteins present in crude disrupted virions would protect the resident enzyme against inhibition. Results are presented in Figure 2 that clearly indicate that both crude and purified enzyme exhibit almost identical susceptibility to PyP inhibition, suggesting a specific affinity of PyP for DNA polymerase(s).

Nature of Inhibition. The observation that pyridoxal phosphate but *not* pyridoxal exerted the inhibitory effect suggested that the observed inhibition is probably not mediated through Schiff base formation between PyP and free amino groups in the enzyme protein. However, to rule out this possibility, the effects of addition of excess albumin (100 μg), Tris buffer (50 mM), and lysine-HCl (20 mM) on the inhibition of RLV polymerase by PyP was examined (see Table IV). None of these compounds, all of which contain reactive amino groups, were able to relieve the inhibition caused by addition of PyP. Similarly, preincubation of enzyme with PyP in a reaction mixture lacking substrate at 4 as well as 27 °C for 15 min did not enhance the inhibitory effects of PyP (Table III).

Mode of PyP Inhibition. In order to gain some insight into

TABLE II: Effect of Pyridoxal Phosphate (0.5 mM) on the DNA Synthesis Directed by Various Template Primers and Catalyzed by RLV DNA Polymerase.

Template Primer	Substrate and Metal Ion	Substrate Incorporation (pmol/30 min)		% Inhibition
		Control	+PyP	
Poly(rA)·$(dT)_{10}$	dTTP-Mn^{2+}	20.9	3.1	85
	dTTP-Mg^{2+}	1.6	0.2	83
Poly(dC)·$(dG)_{12-18}$	dGTP-Mn^{2+}	2.8	0.9	68
	dGTP-Mg^{2+}	16.3	4.7	78
Poly(rC)·$(dG)_{12-18}$	dGTP-Mn^{2+}	4.1	0.9	76
	dGTP-Mg^{2+}	12.0	2.6	79
Activated DNA	dATP-Mg^{2+}	4.0	1.2	68
Globin mRNA + oligo$(dT)_{10}$	dGTP-Mn^{2+}	1.2	0.27	79

[a] Reaction mixtures are identical to those described under Materials and Methods. For poly(rC)·$(dG)_{12-18}$- and poly(dC)·$(dG)_{12-18}$-directed synthesis, reactions were also carried out in the presence of optimal concentrations of Mn^{2+} (1 mM), although it is not a preferred cation. Similarly for poly(rA)·$(dT)_{10}$-directed synthesis, values at optimal concentrations of both Mn^{2+} (preferred) and Mg^{2+} (2.5 mM) are presented.

TABLE III: Effects of Various Pyridoxine Derivatives on the Activity of RLV DNA Polymerase.[a]

Addition	dTMP Incorporation/ 30 min (pmol)	% Inhibition
None	30.0	None
0.1 mM pyridoxal 5′-phosphate	9.8	66.0
0.2 mM pyridoxal 5′-phosphate	6.6	78.0
0.5 mM pyridoxal 5′-phosphate	4.3	85.7
1.0 mM pyridoxal	29.0	3.0
5.0 mM pyridoxal	21.0	30.0
1.0 mM pyridoxine	29.8	0.6
5.0 mM pyridoxine	25.5	15.0
1.0 mM pyridoxamine	29.5	1.6
5.0 mM pyridoxamine	26.5	11.6
1.0 mM pyridoxamine phosphate	29.0	3.0
5.0 mM pyridoxamine phosphate	22.5	25.0
0.5 mM pyridoxal phosphate (preincubated with enzyme for 15 min at 27 °C)	4.3	85.7

[a] The enzyme activity is measured using poly(rA)·$(dT)_{10}$ as a template primer as described under Materials and Methods.

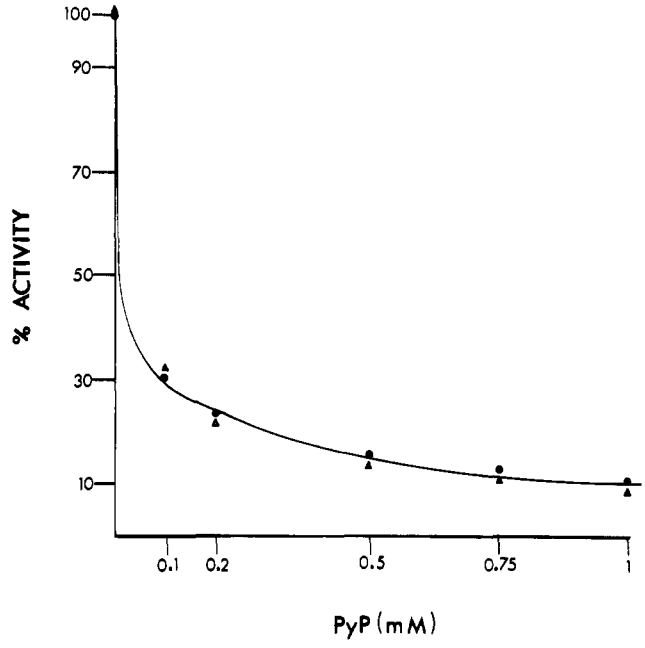

FIGURE 2: Dose response of pyridoxal 5′-phosphate on the poly(rA)·$(dT)_{10}$-directed activity of purified RLV DNA polymerase (Δ—Δ) and detergent disrupted virions of RLV (●—●). 100% activity in each case was approximately 70 pmol of TTP incorporation in 30 min.

the molecular level at which the catalysis of DNA synthesis is affected by pyridoxal phosphate, the response of RLV DNA polymerase to poly(rA)·$(dT)_{10}$-directed synthesis was measured in the presence and absence of inhibitor under a variety of conditions detailed in Table IV. It may be noted that the observed percentage of inhibition remains constant with changing enzyme concentrations. Up to fivefold the standard concentration of poly(rA)·$(dT)_{10}$, which represents approximately 50-fold the apparent K_m value for this template primer, affords no protection from PyP inhibition, indicating that the template primer was not reacting with PyP to produce inhibition. Similarly, addition of poly(dA)·$(dT)_{10}$ to reaction mixtures containing poly(rA)·$(dT)_{10}$ does not alter the degree of inhibition. Increasing the substrate concentration reduces PyP inhibition. At a 1:1 molar ratio of substrate dTTP and inhibitor PyP, approximately 40% inhibition of DNA synthesis still persists. Nonsubstrate triphosphates (e.g., dATP, or dGTP, or dCTP) at high concentrations partially protect the enzyme, albeit not as effectively as substrate triphosphates (Table IV). Addition of ribonucleoside triphosphate, deoxynucleoside di- or monophosphate and activated DNA does not protect the enzyme from PyP inhibition.

Kinetic Studies of Mechanism of PyP Inhibition. Rates of DNA synthesis, under our assay conditions, in the absence of PyP are linear for at least 1 h. Addition of PyP to reaction mixtures prior to or following initiation of synthesis results in an immediate reduction in the rates of synthesis (Figure 3). Kinetic studies on the synthesis of DNA directed by poly(rA)·$(dT)_{10}$ and poly(dC)·$(dG)_{12-18}$ and varying substrate and PyP concentrations were then carried out. Results are presented as double-reciprocal plots in Figure 4. (Data for poly(dC)·$(dG)_{12-18}$-directed synthesis are not shown.) The results indicate that pyridoxal phosphate inhibition is competitive with respect to dTTP or dGTP concentrations, respectively, when poly(rA)·$(dT)_{10}$ and poly(dC)·$(dG)_{12-18}$ are used as template primers. The apparent K_i value is calculated to be 0.1 and 0.9 mM.

Reversal of Inhibition by Deoxynucleoside Triphosphate.

TABLE IV: Effect of Changing Nucleoside Triphosphate, Template Primers, and Enzyme Concentrations on the Inhibition of Catalysis by PyP, Using Poly(rA)·$(dT)_{10}$ as Template Primer.[a]

Addition	dTMP Incorporation (pmol)	dTMP Incorporation in the Presence of PyP (pmol)	% Inhibition
None	30.0	4.3	85.7
5 μl of Enzyme	60.0	8.5	85.9
20 μl of Enzyme	151.0	21.0	86.1
0.5 μg of poly(rA)·$(dT)_{10}$	30.8	4.2	86.4
2.0 μg of poly(rA)·$(dT)_{10}$	31.1	4.5	85.5
0.5 μg of poly(dA)·$(dT)_{10}$	15.2	1.8	88.0
80 μM dTTP	50.1	11.0	78.0
100 μM dTTP	66.0	19.1	71.0
250 μM dTTP	75.2	39.0	48.0
500 μM dTTP	73.6	42.6	42.0
1000 μM dTTP	75.0	60.0	20.0
1000 μM dGTP	29.0	13.6	53.0
1000 μM dATP	32.0	12.8	60.0
1000 μM dCTP	27.0	12.8	60.0
50 mM Tris-HCl (pH 7.8)[b]	30.8	4.4	85.0
20 mM Lysine-HCl	20.6	4.0	80.0

[a] The standard reaction mixture, as described under Materials and Methods, contained 5 μl of enzyme fraction, 20 μM dTTP, and 0.5 μg of poly(rA)·$(dT)_{10}$. The additions were made to the standard reaction mixture in the presence or absence of 0.5 mM PyP. [b] This addition brings the total Tris concentration to 100 mM in the reaction mixture.

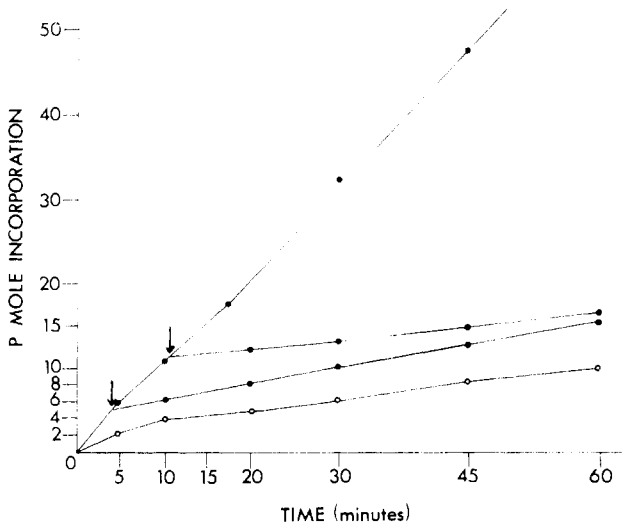

FIGURE 3: Kinetics of poly(dT) synthesis in the presence (●—●) and absence of pyridoxal 5′-phosphate (0.5 mM). The arrow indicates addition of pyridoxal phosphate (0.5 mM) to ongoing reaction.

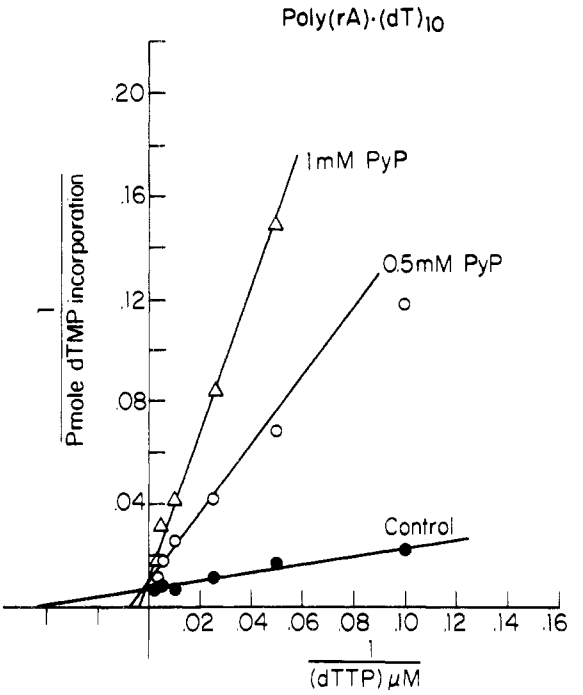

FIGURE 4: Double-reciprocal plot of 1/velocity vs. 1/dTTP concentration. Units of velocity are expressed as pmol of substrate incorporated in 30 min under standard assay conditions. Effect of 0.5 mM (○—○) and 1 mM (△—△) pyridoxal phosphate on the reaction directed by poly(rA)·$(dT)_{10}$.

As seen in Table IV the deoxyribonucleoside triphosphates are able to reduce the inhibition caused by addition of PyP. This observation together with the kinetic data on the mode of inhibition suggests that binding of PyP to enzyme, although specific, is reversible. This is further confirmed by the fact that addition of deoxynucleoside triphosphate (data for dGTP is shown) to a reaction mixture containing PyP and catalyzing synthesis at a reduced rate results in a prompt increase in the rate of DNA synthesis (Figure 5). In order to understand the nature of PyP–enzyme binding, the effect of addition of a reducing agent, sodium borohydride, to a reaction proceeding in the presence of PyP was examined. It was expected that if binding of PyP to enzyme involved a Schiff base, the addition of $NaBH_4$ will reduce this base to a more stable, irreversible bond. Subsequent addition of deoxynucleoside triphosphate to such a reaction would *not* relieve the enzyme inhibition. The experiment described in Figure 5 indeed confirms that addition of dGTP, after the putative "Schiff base" has been reduced to a stable enzyme–PyP complex with $NaBH_4$, has no ability to reverse the inhibition. It may be pointed out that neither dGTP nor $NaBH_4$ at the concentration used either individually or in combination have any effect on polymerization reaction directed by poly(rA)·$(dT)_{10}$.

Discussion

The inhibitory effect of pyridoxal phosphate on DNA polymerizing enzymes isolated from a wide variety of sources may be interpreted as indicating all of these enzymes have a common mechanism by which they accept and polymerize deoxyribonucleoside triphosphates. The nature of the inhibitory effect of PyP was studied in greater detail using RLV DNA polymerase as a test enzyme, since it functions as both a DNA- and RNA-directed polymerase. The possibility that PyP inhibiton may result from complexing between the required divalent cation (Mg^{2+} or Mn^{2+}) and PyP had to be ruled out. From the data shown in Figure 1, it is clear that PyP does not affect the Mn^{2+} optima of the reaction directed by poly(rA)·$(dT)_{10}$. Similarly, experiments performed with reactions buffered at various pH's showed no alteration in pH optima in the presence of PyP and a constant degree of inhibition was noted at all the pH (data not shown). It is, therefore, concluded that the inhibition of several DNA polymerases (Table I) by PyP is, indeed, directed against the catalytic process and not due to nonspecific effects. Further support for this interpretation is obtained from a study of RLV DNA polymerase response to PyP utilizing a variety of synthetic and natural template primers with both Mg^{2+} and Mn^{2+} as effective divalent cations (Table II). The DNA synthesis directed by all the template primers irrespective of the metal ion used in the reaction mixture was inhibited significantly in the presence of PyP.

Pyridoxal phosphate has been shown to be a strong inhibitor of both prokaryotic and eukaryotic RNA polymerases (Bull et al., 1975; Venegas et al., 1973; Martial et al., 1975). The apparent mode of inhibition in this case was attributed to Schiff base formation between the aldehyde group of PyP and the ϵ-amino group of lysine, presumably located at the active center of these enzymes. The inhibition of DNA polymerases by pyridoxal phosphate also appears to be due to an interaction at the active site that presumably involves a lysine or arginine residue. Furthermore, analogous to the RNA polymerase system, substrate triphosphates are also shown to reverse the inhibitory effect of PyP at least partly. However, the affinity of amino acid residues of DNA polymerases seems to be much greater than that shown by RNA polymerases for the following reasons:

(i) Tris buffer (up to 100 mM) used in the reaction mixture or addition of 20 mM lysine does not protect the enzyme against PyP inhibition. A protective effect of these compounds (due to the availability of free NH_2 groups to form Schiff base with PyP) has been demonstrated in the RNA polymerase system (Martial et al., 1975). (ii) Addition of protein, such as albumin, does not reduce the degree of inhibition caused by PyP. If PyP exerted its effect via reactivity with lysine residues in the protein, addition of albumin would be expected to compete for PyP with enzyme protein and would bring about a reduction of the observed inhibition. Similarly, preincubation of enzyme protein with PyP for varying periods of time does not enhance the degree of inhibition as has been observed with RNA polymerase protein (Martial et al., 1975). (iii) The site of action appears to be enzyme specific, since an increase in template concentration has no effect on inhibition unlike the RNA polymerase system where DNA has been shown to protect the enzyme from inhibition (Martial et al., 1975).

At the present time, it has not been possible to determine the amount of PyP bound to enzyme due to restriction on the availability of quantity of enzyme protein required for such studies. It is also for the same reason that the actual identification of amino acid residue reacting with PyP has not been accomplished. However, the fact that pyridoxal phosphate has long been known to form a Schiff base with lysine and/or arginine and that both of these residues are functionally implicated at the active site of DNA polymerases (see below) may allow us to tentatively assume the involvement of lysine and/or arginine in the inhibitory action of PyP.

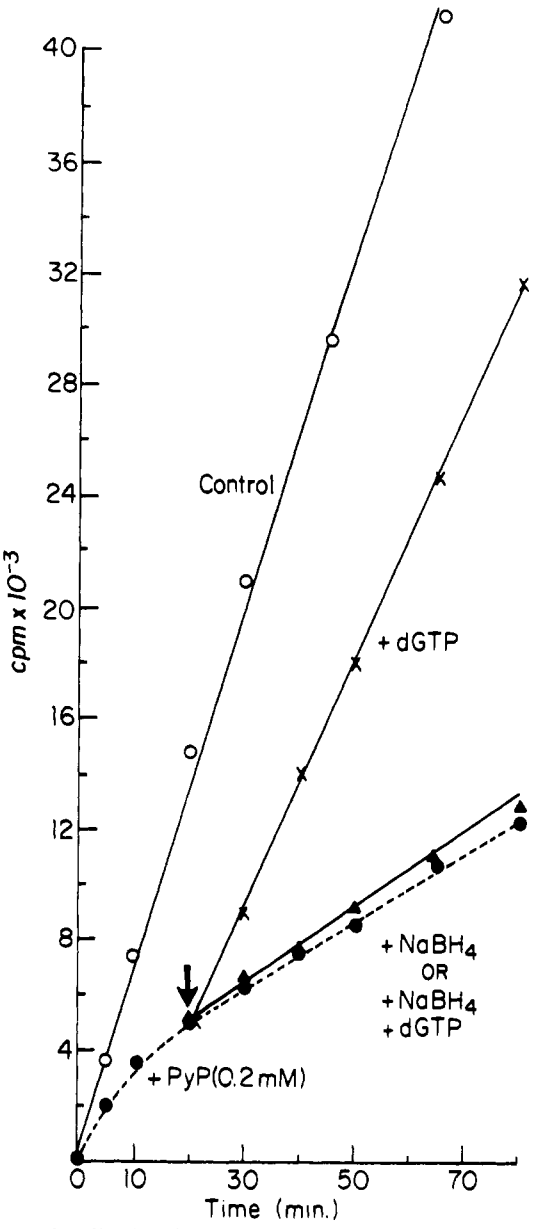

FIGURE 5: Kinetics of reversal of inhibition of dTTP incorporation by pyridoxal phosphate. Rates of control reaction are shown by (O—O) while the rates of synthesis in the presence of PyP (0.2 mM) is denoted by (●—●). At 20 min (position shown by arrow) the reaction mixture containing PyP was divided in four parts. One part received dGTP at the final concentration of 1 mM (x—x), the second part received $NaBH_4$ to the final concentration of 1 mM (▲—▲), to the third part both dGTP and $NaBH_4$ (final concentration 1 mM each) were added (▲—▲), and the fourth and final part served as a control. Aliquots of various reaction mixtures were removed at desired time and synthesis of poly(dT) monitored as described under Materials and Methods. The addition of both $NaBH_4$ and dGTP to the control reaction (absence of PyP) either individually or in combination has no effect on the incorporation of dTTP.

From the above discussion and the kinetic data presented in Figure 4, it appears that PyP exerts its inhibitory effect by competing with the substrate deoxynucleoside triphosphate, presumably for the substrate binding site on the enzyme. Furthermore, the fact that DNA polymerase, even in crude

extracts (Figure 2), is susceptible to PyP inhibition indicates a specific affinity of this compound towards DNA polymerases. However, due to the actual structural disparity between pyridoxal phosphate and deoxynucleoside triphosphate, the observed inhibition may very well be of an allosteric nature, which might implicate PyP as playing a regulatory role in DNA synthesis.

The observation that both the phosphate and aldehyde groups of pyridoxal phosphate are required for the inhibitory action (Table III) suggests that a phosphate moiety may be required for proper orientation of inhibitor with respect to substrate binding site followed by formation of Schiff base between lysine and/or arginine and reactive aldehyde of PyP at that site. The evidence for Schiff base formation at the active site is provided by the fact that addition of $NaBH_4$ to the enzyme–PyP complex prevents the normal reversal of inhibition by subsequent addition of deoxynucleoside triphosphate (Figure 5). The presence of a lysine residue at the catalytic site of *E. coli* DNA polymerase I has been shown by Jovin et al. (1969). Recently, Salvo et al. (1976), using affinity-labeling techniques, have demonstrated the presence of both lysine and arginine at the active center of the same enzyme. Similarly, the occurrence of arginine residues at the active site of several oncornaviral DNA polymerases has been reported by Borders et al. (1975). The high affinity of PyP for these residues present at the active site of DNA polymerases provides a useful reagent for the dissection of the structure and probably the sequence of amino acids at the active site of these enzymes. Furthermore, since both DNA and RNA polymerases are susceptible to PyP inhibition through the reactivity of (at least) lysine residue situated at the enzyme active center, PyP may prove to be an excellent probe for the study of nucleoside triphosphate polymerization. The observation that both substrate and nonsubstrate deoxyribonucleoside triphosphate can protect the enzyme against PyP inhibition suggests that the amino acid residues(s) that are reacting with PyP may also be involved in recognition and binding of all four deoxyribonucleoside triphosphates. However, the fact that the substrate triphosphate is more effective than its nonsubstrate counterpart in reversing inhibition lends support to the notion of a triphosphate-specific subsite (Burd and Wells, 1970; Miller and Wells, 1972; Marcus et al., 1974; Battula and Loeb, 1974; Battula et al., 1975; Travaglini et al., 1975) within the active center of polymerase(s). However, due to the ability of nonsubstrate triphosphates to protect and at least partly reverse the PyP inhibition, it appears plausible that these subsites may share some common amino acid residue(s) that have the ability to react with PyP.

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