Purification and Properties of Rauscher Leukemia Virus DNA Polymerase and Selective Inhibition of Mammalian Viral Reverse Transcriptase by Inorganic Phosphate*

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Rauscher leukemia virus RNA-directed DNA polymerase has been purified to near homogeneity (>90% pure) using affinity chromatography on polycytidylic acid-agarose with over 85% recovery of input enzymatic activity. The purified enzyme has a molecular weight of approximately 70,000 and appears to consist of a single polypeptide chain. The enzyme is free of DNase, but has RNase H activity. Analysis of the requirements for optimal rates of DNA synthesis by this enzyme using synthetic and natural template-primers has revealed template-specific variations in such requirements. During these studies it was observed that DNA synthesis catalyzed by Rauscher leukemia virus DNA polymerase is inhibited by the addition of inorganic phosphate. An analysis of the mechanism of phosphate inhibition was carried out using the synthetic template-primer poly(A)-(dT)$_n$. It appears that by some mechanism, possibly involving the substrate binding site of the enzyme, phosphate ions inhibit DNA synthesis with a more acute effect on the rate of chain growth than on that of initiation. The extension of these studies to DNA synthesis catalyzed by a variety of mammalian type C viral reverse transcriptases revealed that low levels (≤ 2 mM) of inorganic phosphate strongly inhibited DNA synthesis. The susceptibility to phosphate inhibition appears unique to mammalian type C viral enzymes since the type B viral enzyme, Escherichia coli DNA polymerase I, avian myeloblastosis virus and Mason-Pfizer monkey virus reverse transcriptase and cellular DNA polymerases α and γ are not inhibited by inorganic phosphate. This phenomenon of phosphate inhibition of various DNA polymerases, therefore, provides a new basis for the differentiation of the sources and nature of these enzymes.

* This study was supported by National Cancer Institute Grants CA-08748 and CA-18369. A preliminary report of these findings was presented at the 66th Annual Meeting of the American Association for Cancer Research, San Diego, May 7 to 11, 1975 as abstract 699.
viral origin did not exhibit a similar susceptibility to phosphatase inhibition. DNA polymerase (reverse transcriptase) isolated from each of the following mammalian type C viruses are included in these studies: Simian sarcoma virus, Rauscher leukemia virus, feline leukemia virus. Rauscher leukemia virus obtained by infecting a rat cell, and Wistar virus spontaneously produced by Wistar rat embryo fibroblast cells. The enzymes from murine mammary tumor virus, Mason-Pfizer monkey virus, avian myeloblastosis virus as well as Escherichia coli DNA polymerase I and mammalian cell DNA polymerases α, β, and γ are included for comparison.

**Experimental Procedures**

### Materials

All radioactive deoxyribonucleoside triphosphates were obtained from Amersham/Searle. Unlabeled triphosphates and template-primer were products of P-L Biochemicals, Inc. The molar ratio of template to primer in the case of poly(C):(dG)$_{15}$, and poly(dC):(dG)$_{15}$-assayed DNA was 1.5 as prepared described by Aposhan and Kornberg (9). Rabbit globin mRNA, purchased from Amersham/Searle, was annealed to oligo(dT)$_{12}$-18 in the molar ratio of 20:1 in 0.05 M Tris (pH 7.8) buffer (10). Avian RNA virus was kindly provided by Dr. J. Beard through the courtesy of Dr. M. A. Chigirov, and 70 S RNA from this was prepared as previously described (11).

Rauscher leukemia and Simian sarcoma viruses were kindly provided by Dr. J. Gruber. These were prepared from producer cells, isolated with the use of dodecyl sulfate (12), and freed of other proteins by gel filtration on a Sephadex G-50 column (7). The desired template-primer was 5'- oligo(dT)$_{12}$-18 and poly(dC):(dG)$_{15}$, respectively. The desired template-primer was poly(C):(dG)$_{15}$-15, and poly(dC):(dG)$_{15}$-15, respectively.

### Virus Disruption and Enzyme Purification

Virus disruption was achieved by suspending the virus preparation in a buffer containing 20 mM Tris/HCl (pH 7.8), 0.4 M KCl, 0.5% (v/v) NP-40, 0.2% (w/v) sodium deoxycholate, 10 mM dithiothreitol, and 20% (v/v) glycerol. In the crude, disrupted state, virus by Drs. Sawicki and Gomatos, murine mammary tumor virus and Mason-Pfizer monkey virus, and mouse mammary tumor virus were purified at least 60-fold using poly(rC)-agarose affinity chromatography. The purification procedures used to obtain these enzymes are identical to the one described herein for Rauscher leukemia virus. Enzymes are eluted with a linear salt gradient (0 to 0.5 M) and most of the enzymes were eluted at the salt concentration of 0.22 to 0.28 M. Enzyme preparations were extensively dialyzed and stored in buffer containing 0.1% (w/v) albumin, 20 mM Tris/HCl, pH 7.8, 5 mM dithiothreitol, and 20% (v/v) glycerol (w/v) at 70°C. Under these conditions, the enzymes are quite stable. DNA polymerases α and γ from HeLa cells (12) were the kind gift of Dr. A. Weissbach of the Roche Institute for Molecular Biology, while homogenous DNA polymerase β from Escherichia coli was generously contributed by Dr. H. K. Loeb of the Fox Chase Institute for Cancer Research. Calf thymus DNA polymerase β was prepared as described by Chang and Bellum (13).

### Template-Primers

Polyadenylic acid, poly(A)$_{100}$, and oligodeoxythymidyllic acid (dT)$_{100}$, or (dT)$_{15}$ were annealed before use at an equimolar ratio of nucleotides by heating the mixture to 60°C for 5 min and then allowing the solution to cool slowly to room temperature. Polyadenylic acid, poly(A)$_{100}$, was annealed to oligo(deoxyguanylyllic acid (dG)$_{15}$-15 at a molar ratio of nucleotides of 10:1, respectively. Annealing was carried out by heating the mixture to 80°C for 2 min followed by slow cooling. Poly(rC)$_{15}$, poly(dC)$_{15}$, and poly(cyl(dT)$_{12}$-18, were made in a similar fashion. The fresh annealing of template-primer, particularly for poly(C):(dG)$_{15}$-15, is required for optimal activity. In the absence of fresh annealing, 2- to 4-fold lower rates of substrate incorporation have been observed. A reported inconsistency in poly(C):(dG)$_{15}$-15-directed activity of some reverse transcriptase preparations (14) may be due to this effect.

### DNA Polymerase Assays

Reactions were carried out in a total volume of 0.1 ml and consisted of 50 mM Tris/hydrochloride, pH 7.8, 1 mM dithiothreitol, 50 mM NaCl, 1 mM MgCl$_2$, and 10% glycerol. Viral lysate containing 10 mg of protein was processed at a temperature in poly(C) (dG)$_{15}$-15-directed activity of some reverse transcriptase preparations (14) may be due to this effect.

### DNA Polymerase Assays

Reactions were carried out in a total volume of 0.1 ml and consisted of 50 mM Tris/hydrochloride, pH 7.8, 1 mM dithiothreitol, 50 mM NaCl, 1 mM MgCl$_2$, and 10% glycerol. Viral lysate containing 10 mg of protein was processed at a temperature of 37°C for 15 min for all templates except poly(A):(dT)$_{12}$ for which 25°C was used. Synthesis was terminated by the addition of 0.1 ml of acid (dG)$_{15}$-15, at a molar ratio of nucleotides of 10:1, respectively. Annealing was carried out by heating the mixture to 80°C for 2 min followed by slow cooling. Poly(rC)$_{15}$, poly(dC)$_{15}$, and poly(cyl(dT)$_{12}$-18, were made in a similar fashion. The fresh annealing of template-primer, particularly for poly(C):(dG)$_{15}$-15, is required for optimal activity. In the absence of fresh annealing, 2- to 4-fold lower rates of substrate incorporation have been observed. A reported inconsistency in poly(C):(dG)$_{15}$-15-directed activity of some reverse transcriptase preparations (14) may be due to this effect.
Characterization and Inhibition of C-type Viral DNA Polymerases

mm KCl, 0.02% (w/v) bovine serum albumin, 12 pmol of ϕX174 DNA- 
RNA hybrid (as total nucleotides), and optimal concentrations of 
Mg2+ or Mn2+. To measure single-stranded RNA degradation or 
DNase activity ([3H]poly(U) or -(C), and [3H]poly(dT), respectively, 
were used; reaction mixtures were identical with that described 
for RNase H except that all contained 5 mM Mg2+ and 30 mM KCl. 
Reactions were initiated by the addition of enzyme fraction and were 
incubated for various times at 37°. Reactions were terminated by 
the addition of trichloroacetic acid. and acid-insoluble counts deter-
mined as described in the DNA polymerase assay.

Labeling of Viral Proteins with [35S]-Solubilized virus or isolated 
polyproteins were iodinated using the lactoperoxidase procedure. To 
a 1-ml solution containing viral proteins 200 to 300 μCi of Na2[35S]I 
in 0.1 M NaOH (Amersham) was added at room temperature. Subse-
quent steps were identical with those described by Witte et al. (16).

Polyacrylamide Gel Electrophoresis — Sodium dodecyl sulfate-poly-
acylamide gel electrophoresis was performed as described previ-
ously (17) with the following modifications. Iodinated proteins were 
dialyzed for 4 to 5 days and then precipitated with 5% trichloroacetic 
acid using 100 μg of bovine serum albumin as a carrier. The protein 
precipitate was pelleted by centrifugation at 3000 × g, and washed 
twice. The pellet was dissolved in 50 μl of 0.1 mM sodium phosphate 
buffer, pH 7.8, containing 1% (w/v) sodium dodecyl sulfate and 1% 
(v/v) 2-mercaptoethanol. After heating the samples at 70-80° for 15 
min, the proteins were electrophoresed in gels containing 7.5% acryl-
amide for 16 h at 25 V, constant voltage. After running, gels were 
sliced into 0.5-mm fractions using a GIBSON gel fractionator and [35S] 
radioactivity determined using a gamma counter.

Molecular Weight Estimations

Glycerol Gradient—Velocity sedimentation was carried out in pre-
formed 10 to 30% (v/v) linear glycerol gradients in 0.01 M Tris/HCl 
buffer (pH 7.8) containing 1 mM ethylenediaminetetraacetic acid 
and 0.4 mM KCl. Rauscher leukemia virus polymerase from the peak column fraction 
was diluted 10-fold in the same buffer used to prepare the glycerol 
gradients and layered over a 5-ml gradient, which was then centri-
fuged at 45,000 rpm for 18 h at 4° using the SW 50.1 rotor. Fractions 
were collected from the bottom of the tube and assayed for DNA 
polymerase activity using poly(A)- (dT)₉ as well as poly(C)- (dG)₉ as. 
Parallel gradients were run using E. coli DNA polymerase I and its 
subtilisin-produced 70,000 molecular weight fragment to provide 
molecular weight markers. The marker gradients were assayed with 
poly(A)- (dT)₉ as described before (1).

RESULTS

Purification and Physical Properties of Rauscher Leukemia 
Virus DNA Polymerase

A typical purification protocol is summarized in Table I. Over 85% of 
the input enzyme activity is routinely recovered. Based on protein 
analysis by the Lowry procedure, greater 

<table>
<thead>
<tr>
<th>Enzyme fraction and volume</th>
<th>Total protein</th>
<th>Recovery of activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Disrupted virions (40 ml)</td>
<td>10.4</td>
<td>100</td>
<td>516</td>
</tr>
<tr>
<td>2. Poly(rc) column, flow through + wash (30 ml)</td>
<td>10.2</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>3. Poly(rc) column, eluate-peak (6 ml)</td>
<td>≥0.1</td>
<td>85</td>
<td>&gt;53,000</td>
</tr>
</tbody>
</table>

* One unit of activity is defined as that amount of enzyme incorporat-
ing 1 pmol of [α-32P]poly(dT) into acid-insoluble material with 
poly(A)·(dT)₉ as template-primer during 15 min incubation at 27°.

![Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel of purified, iodinated Rauscher leukemia virus DNA polymerase. The polymer-
ase from peak poly(rc)-agarose column fractions was iodinated, 
placed on a 12.5%-agarose gel, and processed as described under "Experi-
mental Procedures." The numbers at the top of the figure correspond to 
the molecular weight in thousands of standard marker proteins 
and exhibit almost identical catalytic properties with those of 
mammalian type C oncornaiviral reverse transcriptase. 

Transcription of this template-primer is most efficiently carried out 
in the presence of Mn2+. Addition of salt (with the exception of 
phosphate) to the reaction mixture containing Mn2+ stimulates 
poly(dT) synthesis severalfold. There appears to be no
specificity in the requirement for a particular monovalent cation since chlorides of sodium, potassium, ammonium, and lithium produced almost identical stimulation of poly(A) .(dT)$_{10}$-directed synthesis. This observation occurred under a variety of experimental conditions, and the optimal activity was found at an equimolar template-primer ratio (Table III). Polyamines, such as spermidine and spermine, as well as histone f, in the absence of salt stimulated poly(dT) synthesis. The stimulation by histone is dependent on the stoichiometry of histone to template ratio, 1:1 (w/w) being optimal. At incubation temperatures of 27° or 37°, histone stimulation appears to increase the rates of DNA synthesis, presumably through alteration(s) in the template structure.

There appears to be some discrepancy in the literature regarding the optimal temperature of incubation (2, 5, 8). The optimal temperature for poly(A) .(dT)$_{10}$-directed synthesis in the presence or absence of salt is 27°. Poly(A) .(dT)$_{10}$-directed synthesis allows maximal rates of synthesis at 27° in the absence of salt but exhibits maximal activity in the presence of salt at 37°.

The effect of changing template to primer ratio on the rate of Rauscher leukemia virus-catalyzed DNA synthesis was determined using a fixed concentration of poly(A)$_{1000}$ or poly(C)$_{1000}$ template and annealing various amounts of complementary DNA oligomers (Table III). For poly(A) as a template, optimal synthesis occurs at an equimolar template-primer nucleotide ratio. However, for poly(C) as a template, the best template-primer nucleotide ratio was approximately 20:1, and increasing the primer concentration reduced the rate of synthesis. The apparent $K_v$ values for substrate DNA precursors irrespective of template to primer ratio were approximately 20 µM for both dTTP and dGTP.

### Observations on Phosphate Inhibition of Rauscher Leukemia Virus DNA Polymerase

It may be seen from Table IV that inclusion of potassium phosphate (50 mM) in place of salt almost completely inhibited Rauscher leukemia virus polymerase activity in contrast to the stimulation observed with other salts. This inhibitory property of inorganic phosphate was further analyzed and the results are described below.

### Phosphate Inhibition Is Not due to Removal of Divalent Cation

Phosphate is known to form insoluble salts with several divalent cations, in particular Mn$^{2+}$, which is used in the assay of poly(A) .(dT)$_{10}$-directed DNA synthesis. It was therefore conceivable that the observed inhibition could result from removal of the divalent cation. However, the results shown in Table IV indicate that removal of Mg$^{2+}$ or Mn$^{2+}$ by phosphate in the assay medium did not account for the inhibition by phosphate. The inhibition by phosphate could be explained by the observation that dGTP, which is a high affinity substrate for Mn$^{2+}$, was reduced to a greater extent than dATP, which is a high affinity substrate for Mg$^{2+}$, in the presence of phosphate.

### Table II

Utilization of various template-primers by Rauscher leukemia virus DNA polymerase

The reaction mixture used for synthetic template-primer-directed synthesis contained, in addition to standard components, 0.5 µg of appropriate template-primer, 0.5 mM MnCl$_2$, and 50 mM KCl or 2.5 mM MgCl$_2$ (wherever indicated), and 20 µM of appropriate substrate and approximately 5 ng of enzyme. Incubations were for 15 min. For the reactions directed by natural template-primers, 0.5 µg of RNA or 2.5 µg of activated DNA (15) was used as a template, while unannealed dATP, dTTP, and dCTP were included at 80 µM each. Specific activity of dATP or dTTP was present at 10 µM was 2500 cpm/pmol. MgCl$_2$ or MnCl$_2$ was present at 10 mM while MnCl$_2$/KCl were present at 0.5 and 50 mM each, respectively. 50 ng of enzyme were used per assay, and incubations were at 37° for 60 min. Deletion of any one of the substrate phosphates resulted in almost total loss of synthesis.

<table>
<thead>
<tr>
<th>Template-primers</th>
<th>Divalent cation</th>
<th>$[^{3}H]$-labeled substrate</th>
<th>Incorporation pmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(A) .(dT)$_{10}$</td>
<td>Mn$^{2+}$ or Mg$^{2+}$</td>
<td>TTP</td>
<td>260</td>
</tr>
<tr>
<td>Poly(A) .(dT)$_{10}$</td>
<td>Mg$^{2+}$ (as KCl)</td>
<td>TTP</td>
<td>46</td>
</tr>
<tr>
<td>Poly(dA) .(dT)$_{10}$</td>
<td>Mn$^{2+}$/KCl</td>
<td>TTP</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Poly(dA) .(dT)$_{10}$</td>
<td>Mg$^{2+}$</td>
<td>TTP</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Poly(C) .(dG)$_{13-15}$</td>
<td>Mn$^{2+}$/KCl</td>
<td>dGTP</td>
<td>37</td>
</tr>
<tr>
<td>Poly(C) .(dG)$_{13-15}$</td>
<td>Mg$^{2+}$</td>
<td>dGTP</td>
<td>52</td>
</tr>
<tr>
<td>Poly(dC) .(dG)$_{13-15}$</td>
<td>Mg$^{2+}$</td>
<td>dGTP</td>
<td>207</td>
</tr>
<tr>
<td>Poly(c(Cm)) .(dG)$_{13-15}$</td>
<td>Mg$^{2+}$</td>
<td>dGTP</td>
<td>2</td>
</tr>
<tr>
<td>Poly(c(Cm)) .(dG)$_{13-15}$</td>
<td>Mn$^{2+}$</td>
<td>dGTP</td>
<td>10</td>
</tr>
<tr>
<td>Poly(U) .(A)$_{10}$</td>
<td>Mn$^{2+}$/KCl</td>
<td>dATP</td>
<td>10</td>
</tr>
<tr>
<td>Poly(A)$_{10}$</td>
<td>Mn$^{2+}$ or Mg$^{2+}$</td>
<td>TTP</td>
<td>0.5</td>
</tr>
</tbody>
</table>

- Poly(A) .(dT)$_{10}$
- Poly(dA) .(dT)$_{10}$
- Poly(dC) .(dG)$_{13-15}$
- Poly(c(Cm)) .(dG)$_{13-15}$
- Poly(U) .(A)$_{10}$
Characterization and Inhibition of C-type Viral DNA Polymerases

Table III

Effect of various template to primer molar nucleotide ratios of poly(A)·(dT)\textsubscript{10} and poly(C)·(dG)\textsubscript{10} on activity of Rauscher leukemia virus DNA polymerase

<table>
<thead>
<tr>
<th>Ratio of template to primer</th>
<th>Poly(A)·(dT)\textsubscript{10} incorporated</th>
<th>Poly(C)·(dG)\textsubscript{10} incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>100:0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>100:2</td>
<td>15.0</td>
<td>10.0</td>
</tr>
<tr>
<td>100:5</td>
<td>36.0</td>
<td>71.2</td>
</tr>
<tr>
<td>100:10</td>
<td>42.0</td>
<td>54.6</td>
</tr>
<tr>
<td>100:20</td>
<td>46.0</td>
<td>49.9</td>
</tr>
<tr>
<td>100:40</td>
<td>48.0</td>
<td>36.0</td>
</tr>
<tr>
<td>100:100</td>
<td>60.0</td>
<td>20.1</td>
</tr>
</tbody>
</table>

Table IV

Effect of various compounds on poly(A)·(dT)\textsubscript{10} directed synthesis of poly(dT) by Rauscher leukemia virus DNA polymerase

<table>
<thead>
<tr>
<th>Addition</th>
<th>dTMP incorporation/15 min pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>34</td>
</tr>
<tr>
<td>KCl or NaCl or (NH\textsubscript{4})Cl (50 mM)</td>
<td>480</td>
</tr>
<tr>
<td>(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} (50 mM)</td>
<td>300</td>
</tr>
<tr>
<td>Tris (100 mM)</td>
<td>200</td>
</tr>
<tr>
<td>KPO\textsubscript{4} (50 mM)</td>
<td>6</td>
</tr>
<tr>
<td>LiCl (50 mM)</td>
<td>433</td>
</tr>
<tr>
<td>Histone f\textsubscript{1} (0.5 µg)</td>
<td>200</td>
</tr>
<tr>
<td>Histone f\textsubscript{1} (0.5 µg) + KCl (50 mM)</td>
<td>600</td>
</tr>
<tr>
<td>Histone f\textsubscript{2a}, f\textsubscript{2b}, or f\textsubscript{3} (0.5 µg)</td>
<td>26</td>
</tr>
<tr>
<td>Spermidine or spermine (10 mM)</td>
<td>580</td>
</tr>
<tr>
<td>Spermidine (20 mM)</td>
<td>100</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate-sodium salt (10\textsuperscript{-3} M)*</td>
<td>2</td>
</tr>
<tr>
<td>O'Phanethroline (10\textsuperscript{-3} M)* + KCl (50 mM)</td>
<td>2</td>
</tr>
</tbody>
</table>

* Inhibition of enzyme activity by these compounds was also observed with activated DNA, poly(C)·(dG)\textsubscript{10}, and avian myeloblastosis virus RNA-templated reactions.

Discussion

Precipitation of Mn\textsuperscript{2+} ions, rendering them unusable by the enzyme. We have found that Rauscher leukemia virus DNA polymerase activity is inhibited to a similar extent by 0.5 to 5 mM phosphate at a range of concentrations of Mn\textsuperscript{2+} from below optimal (0.1 mM) to above optimal (2.5 mM). The concentrations of Mn\textsuperscript{2+} (0.5 to 1 mM) allowing optimal rates of synthesis remained unchanged at all phosphate concentrations tested. It was therefore concluded that phosphate inhibition was not due to the removal of Mn\textsuperscript{2+} as a required reaction component.

Comparison of Potassium Chloride and Phosphate Addition Effect

Addition of KCl to the reaction mixture stimulated Rauscher leukemia virus DNA polymerase-catalyzed DNA synthesis (Table IV). Maximum stimulation was found to occur at 120 mM KCl. The stimulatory effect due to the change in ionic strength can be achieved by the addition of any ionizable salt to the reaction mixture provided that the individual ion is not inhibitory to enzyme catalysis (Table IV). When potassium phosphate was the only salt added to the reaction mixture, no stimulatory effect was noted at any concentration. At very low phosphate concentrations (~1 mM), which do not significantly change the ionic strength, a marked inhibition of activity was observed. Increasing the potassium phosphate concentration above 1 mM paralleled the stimulatory effect on the enzyme activity observed with potassium chloride, although rates of synthesis did not rise above those observed in the absence of salt (data not shown).

Thus, it appears that, in the presence of potassium phosphate (but in the absence of KCl), the observed rate of DNA synthesis is the resultant rate produced by (a) a stimulatory effect due to increased ionic strength and (b) the direct inhibition by phosphate ions. This is further confirmed by the observation that, when reaction mixtures containing optimal KCl concentrations are used to test enzyme inhibition by increasing concentrations of phosphate, a dramatic inhibitory effect is observed at all phosphate levels.

The pH optimum (pH 7.8) for Rauscher leukemia virus DNA polymerase activity remains unchanged in the presence of phosphate, indicating that the inhibitory effect of phosphate is not due to pH change. A fixed phosphate concentration produced a consistent degree of inhibition regardless of the amount of enzyme used in the assay. In our studies, enzyme was always used at levels below saturation, and template-primer levels were at least 10-fold higher than the K\textsubscript{m} value. Further increases in template concentration, up to 50-fold the K\textsubscript{m} value, did not significantly alter the ability of low levels of phosphate to cause inhibition.

Effects of Phosphate Addition on Kinetics of Poly(dT) Synthesis

Changing the template to primer ratio used in the reaction mixture does not appear to alter the kinetics of phosphate inhibition (Fig. 3A). The amount of template-primer used was well above the concentration required for saturation, while enzyme was used at a concentration allowing rates of synthesis at which we could critically compare the early stages of DNA synthesis. Much less inhibition by phosphate occurs during the first 10 min of synthesis, relative to that which is observed in the latter 20 min of synthesis. Fig. 3B illustrates the kinetics of poly(dT) synthesis in the presence and absence of inorganic phosphate. In this experiment, phosphate was added to a standard reaction mixture at various times during the course of the reaction, and the subsequent course of synthesis was followed. The data clearly indicate that phosphate inhibits the rate of DNA synthesis, regardless of the time of addition. Addition of phosphate at early time points, however, results in less inhibition than does phosphate addition at the later stages of DNA synthesis. At limiting template-primer concentrations (5 to 10 ng), addition of phosphate causes a rapid cessation of DNA synthesis (data not shown), presumably due to the limited number of initiation points. As the concentration of template-primer is increased to levels well above saturation (~50 ng), the rate of product synthesis in the presence of phosphate is relatively increased.

Apparent Mechanism of Phosphate Inhibition of DNA Synthesis

Neither increasing the concentration of enzyme nor the amount of template-primer used in the reaction mixture significantly lessen the ability of inorganic phosphate to inhibit DNA synthesis by Rauscher leukemia virus DNA polymerase. Increasing concentrations of substrate deoxynucleoside triphosphates, however, appear to prevent or decrease inhibition by phosphate. Fig. 4 is a double reciprocal plot of data from an experiment to examine the effect of varying dTTP concentration at 0, 2.5, and 10 mM phosphate on rates of poly(A)·(dT)\textsubscript{10} directed synthesis. The results indicate that inhibition by inorganic phosphate is competitive with respect to dTTP concentration. The K\textsubscript{1} for inorganic phosphate using poly(A)·(dT)\textsubscript{10} directed synthesis was determined to be 1 mM.
Characterization and Inhibition of C-type Viral DNA Polymerases

Effects of Exogenous Addition of Inorganic Phosphate on DNA Synthesis by Other RNA- and DNA-directed DNA Polymerases

It was of interest for us to determine whether inhibition by inorganic phosphate was specific to the Rauscher leukemia virus enzyme or whether other DNA polymerases would be similarly affected. From the results summarized in Table V, it is clear that addition of low concentrations of phosphate (1 to 5 mM) causes significant inhibition of poly(A)·(dT)$_{12-18}$-directed DNA polymerase activity of all the mammalian type C viruses tested. In contrast to these results, the catalytic activity of *Escherichia coli* DNA polymerase I, HeLa DNA polymerase γ, avian myeloblastosis virus, murine mammary tumor virus, and Mason Pfizer monkey tumor virus reverse transcriptases is either unaffected or only slightly affected by the addition of phosphate at any Mn$^+$ concentration (Table V). The DNA polymerases from avian myeloblastosis virus, murine mammary tumor virus, and Mason Pfizer monkey tumor virus are known to prefer Mg$^+$ for optimal activity (5, 14, 15, 19) and hence the phosphate susceptibility of these enzymes was also examined in the presence of optimal concentrations of Mg$^+$. As seen from data presented in Table V, these enzymes are resistant to phosphate inhibition irrespective of the divalent cation used. In the presence of Mn$^+$, poly(A)·(dT)$_{12-18}$-directed synthesis catalyzed by all enzymes tested requires the addition of salt for maximum rates of activity. Inclusion of KCl, NaCl, or NH$_4$Cl at 50 mM concentration stimulates poly(dT) synthesis severalfold. Among the cellular enzymes tested, DNA polymerase α, γ, and *E. coli* DNA polymerase I appear resistant to phosphate inhibition, while DNA polymerase β (Table V) exhibits susceptibility to inhibition.

To rule out the possibility that enzymes purified by affinity chromatographic procedures using poly(rC)-agarose are somehow altered to become susceptible to phosphate inhibition, crude disrupted virions of Rauscher leukemia virus, feline leukemia virus, and Wistar virus as well as enzymes purified by DEAE-cellulose and phosphocellulose chromatography (Rauscher leukemia virus and Simian sarcoma virus) were tested for their response to inorganic phosphate. In all cases, inhibition of enzyme activity comparable to that of poly(rC) agarose purified enzyme preparations was observed (Table V).

**Poly(C)·(dG)$_{12-18}$, Poly(dC)·(dG)$_{12-18}$, Activated DNA, and mRNA-directed Synthesis**

Rates of DNA synthesis directed by poly(C)·(dG)$_{12-18}$, poly(dC)·(dG)$_{12-18}$, activated DNA, and globin mRNA-oligo(dT)$_6$ by various DNA polymerases in the presence and

**Fig. 4.** Double reciprocal plot of $1/v$ vs $1/dTTP$ concentration. Units of velocity are expressed as picomoles of substrate incorporated/30 min under standard assay conditions (see "Experimental Procedures") except that KCl was present at only 50 mM concentration. Effect of 2.5 mM (△) or 10 mM (○) phosphate present in the reaction mixture on the reaction kinetics indicates inhibition competitive with dTTP.
Characterization and Inhibition of C-type Viral DNA Polymerases

TABLE V

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>No KCl</th>
<th>No KCl +KPO₄ (2 mM)</th>
<th>-KCl (50 mM)</th>
<th>+KCl (50 mM) +KPO₄ (2 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLV</td>
<td>3.0</td>
<td>0.6</td>
<td>22.0</td>
<td>2.3</td>
</tr>
<tr>
<td>RLV (crude)</td>
<td>2.8</td>
<td>0.53</td>
<td>19.0</td>
<td>3.1</td>
</tr>
<tr>
<td>SSV</td>
<td>0.6</td>
<td>0.18</td>
<td>1.6</td>
<td>0.6</td>
</tr>
<tr>
<td>SSV (crude)</td>
<td>2.1</td>
<td>0.35</td>
<td>8.3</td>
<td>1.1</td>
</tr>
<tr>
<td>FeLV</td>
<td>1.3</td>
<td>0.18</td>
<td>6.5</td>
<td>0.7</td>
</tr>
<tr>
<td>FeLV (crude)</td>
<td>5.0</td>
<td>0.59</td>
<td>68.0</td>
<td>18.0</td>
</tr>
<tr>
<td>Wistar virus</td>
<td>1.3</td>
<td>0.27</td>
<td>3.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Wistar virus (crude)</td>
<td>0.9</td>
<td>0.2</td>
<td>4.8</td>
<td>0.7</td>
</tr>
<tr>
<td>RRT virus</td>
<td>1.1</td>
<td>0.22</td>
<td>4.7</td>
<td>0.7</td>
</tr>
<tr>
<td>AMV</td>
<td>10.3</td>
<td>10.5</td>
<td>33.4</td>
<td>17.5</td>
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<tr>
<td>AMV (Mg⁺⁺ system)</td>
<td>50.5</td>
<td>52.8</td>
<td>60.6</td>
<td>58.7</td>
</tr>
<tr>
<td>MuMTV</td>
<td>1.1</td>
<td>1.2</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>MuMTV (Mg⁺⁺ system)</td>
<td>20.6</td>
<td>21.8</td>
<td>18.6</td>
<td>19.2</td>
</tr>
<tr>
<td>MPMV</td>
<td>3.6</td>
<td>3.5</td>
<td>10.6</td>
<td>11.1</td>
</tr>
<tr>
<td>MPMV (Mg⁺⁺ system)</td>
<td>30.1</td>
<td>32.2</td>
<td>35.2</td>
<td>37.7</td>
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<td>E. coli DNA polymerase I</td>
<td>26.0</td>
<td>51.0</td>
<td>86.0</td>
<td>76.0</td>
</tr>
<tr>
<td>DNA polymerase α</td>
<td>1.2</td>
<td>0.4</td>
<td>10.8</td>
<td>1.2</td>
</tr>
<tr>
<td>DNA polymerase γ</td>
<td>1.7</td>
<td>2.9</td>
<td>8.0</td>
<td>12.0</td>
</tr>
</tbody>
</table>

a RLV, Rauscher leukemia virus; SSV, Simian sarcoma virus; FeLV, feline leukemia virus; RRT, Rauscher leukemia virus grown in rat cells; AMV, avian myeloblastosis virus; MuMTV, murine mammary tumor virus; MPMV, Mason Pfizer monkey tumor virus.

b In these experiments enzyme purified by DEAE-cellulose and phosphocellulose chromatography was used.
c In this experiment enzyme purified by DEAE-cellulose and phosphocellulose chromatography was used.
d In these experiments MgCl₂ (10 mM) was used as an effective divalent cation in place of Mn⁺⁺.

absence of inorganic phosphate are shown in Table VI. Similar to the results obtained with poly(A)-(dT)₁₀, synthesis directed by all of these template-primer, and catalyzed by type C viral polymerases is severely inhibited by phosphate addition. In contrast, the DNA polymerase from avian myeloblastosis virus, murine mammary tumor virus and Mason Pfizer monkey tumor virus are not affected by phosphate irrespective of the divergent cation used in the reaction mixture. Among the cellular DNA polymerases, polymerase β appears to be more sensitive while DNA polymerases α and γ, as well as E. coli DNA polymerase I, are insensitive.

DISCUSSION

Recently several reports on the purification and properties of murine leukemia virus DNA polymerase have appeared in the literature (2-7). Most of the purification procedures used are somewhat lengthy, and require large amounts of starting material. The protocol that we have standardized using poly(rC) cosawnly linked to agarose as an affinity matrix appears to be an efficient method since a single passage of disrupted virions through the column yields a near homogeneous preparation of polymerase with over 85% recovery of input activity. The purified enzyme was found to possess RNase H activity. An additional low molecular weight RNase H activity is detected in disrupted virions (Fig. 2) and in poly(rC) column flow-through material. The RNase H activities also differ with respect to divalent cation preference in agreement with a previous report by Gerard and Grandgenett (6). The low molecular weight nuclease is not present to any significant degree in purified enzyme preparations and may therefore be of cellular rather than viral origin.

Our findings on the catalytic properties of the purified enzyme are generally in agreement with those reported in the literature (2-7), except for some new observations we have made which may be important regarding the expression of optimal activity with a given template-primer. To briefly summarize these findings: poly(A)-(dT)₁₀ and mRNA-directed DNA synthesis requires Mn⁺⁺/KCl (no preference for salt), while poly(C)-(dG)₁₂₋₁₈, poly(C)-(dG)₁₂₋₁₈, and DNA-directed DNA synthesis prefer Mg⁺⁺ for optimal activity, although the optimum Mg⁺⁺ concentration for DNA-directed synthesis is 4- to 5-fold higher than that required for the synthetic template-primer (2 mm). The optimal temperature for all of the polycytidylylate and DNA-targeted reactions is 43°, and synthesis at the traditionally used temperature of 37° amounts to only 60% of that at 43°. For poly(C)-(dG)₁₂₋₁₈ and poly(C)-(dG)₁₂₋₁₈, a template to primer ratio of 20:1 is optimally used by this enzyme and increasing the amount of primer results in inhibition (Table III). Natural RNA (both viral 70 S RNA and globin mRNA initiated with oligo(dT)₁₀) is utilized rather poorly.

The use of the synthetic template-primer, poly(A)-oligo(dT), to measure reverse transcriptase activity is quite common (14). We have critically analyzed the reaction conditions that would affect the synthesis directed by this template primer. Furthermore, this analysis was also expected to reveal some viral enzyme-specific response, thereby providing new information by which to further distinguish it from cellular DNA polymerases y (6). The pertinent observations of this investigation are presented below.

1. Unlike poly(C)-(dG)₁₂₋₁₈, the template to primer ratio for optimal activity was 1:1 and up to 10-fold lower concentrations of primer (dT)₁₀ reduced the synthesis only 25%.

2. Manly (20) has reported stimulation of poly(A)-(dT)₁₀ directed synthesis by histone f₂, with detergent-disrupted Moloney murine leukemia virus virions and our results obtained with purified enzyme are in agreement with his findings. The specificity of histone f₂ for this stimulatory effect is shown by the fact that other histones, namely, f₁₈, f₂₆, and f₂₉, do not stimulate the reaction (Table IV). Furthermore, this stimulatory effect appears to be restricted to only this template-primer since reactions directed by other template-primer were either unaffected or slightly inhibited (data not shown).

3. The length of oligomeric(dT) used by different investigators ranges in size from (dT)₁₀ to (dT)₁₂₋₁₈. Waters and Yang (5), using (dT)₁₀ as a primer and poly(A) as a template, found that synthesis on this template-primer combination required a low temperature (15°-20°) for optimal synthesis. Most of the other investigators in this field have used an incubation temperature of 37° irrespective of the length of primer used. We have found 27° to be the optimal temperature for poly(A)-(dT)₁₀ directed synthesis in the presence or absence of salt, while poly(A)-(dT)₁₂₋₁₈ shows optimal activity at 37° only in the presence of salt. Apparently, the structural stability of the ribo(A) polymer-deoxy(T) oligomer duplex may be critical for optimal enzyme activity (2).
polymerase. In addition to this type of inhibition, phosphate ions compete with deoxynucleoside triphosphates for a site on the template-primer concentrations (limiting numbers of initiation in the later stages. Addition of phosphate to the reaction mixture caused the most drastic inhibition in the presence of limiting template-primer concentrations, the initial stages of DNA synthesis (0 to 15 min). was more profound when phosphate was added at later stages of synthesis (15 to 30 min) rather than at early stages of synthesis catalyzed by mammalian type C viral enzymes, but not by cellular DNA polymerase γ, and directed by all of the template-primer combinations studied.  

Effect of phosphate addition on DNA synthesis directed by different template-primers and catalyzed by various DNA-polymerases  

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Poly(C)·(dG)₉₋₁₅, Poly(dC)·(dG)₉₋₁₅</th>
<th>Activated DNA</th>
<th>mRNA + oligo(dT)ₙ</th>
<th>Incorporation of [³H]dGTP with following template primers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PO₄⁻</td>
<td>+ PO₄⁻</td>
<td>PO₄⁻</td>
<td>+ PO₄⁻</td>
</tr>
<tr>
<td>Wistar</td>
<td>5.1</td>
<td>1.4</td>
<td>3.1</td>
<td>1.0</td>
</tr>
<tr>
<td>SSV</td>
<td>2.1</td>
<td>0.8</td>
<td>2.1</td>
<td>0.7</td>
</tr>
<tr>
<td>RRTC</td>
<td>3.5</td>
<td>0.8</td>
<td>3.9</td>
<td>1.4</td>
</tr>
<tr>
<td>FeLV</td>
<td>19.0</td>
<td>2.2</td>
<td>11.4</td>
<td>3.9</td>
</tr>
<tr>
<td>AMV</td>
<td>3.5</td>
<td>1.5</td>
<td>2.4</td>
<td>0.6</td>
</tr>
<tr>
<td>AMV⁺</td>
<td>12.5</td>
<td>10.1</td>
<td>7.9</td>
<td>6.0</td>
</tr>
<tr>
<td>MuMTV</td>
<td>4.1</td>
<td>4.7</td>
<td>18.2</td>
<td>16.7</td>
</tr>
<tr>
<td>MuMTV⁺</td>
<td>48.0</td>
<td>49.0</td>
<td>68.6</td>
<td>70.4</td>
</tr>
<tr>
<td>MP-MV</td>
<td>1.2</td>
<td>1.4</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>MP-MV⁺</td>
<td>10.2</td>
<td>11.0</td>
<td>25.0</td>
<td>24.9</td>
</tr>
</tbody>
</table>
| E. coli DNA polymerase I  
E. coli DNA polymerase II  
DNA polymerase α⁺  
DNA polymerase β  
DNA polymerase γ  | 0.7  | 1.2   | 69.0 | 66.0  | 30.0 | 35.0  | 0.7  | 0.7   |

4. The observed inhibition by inorganic phosphate of poly(A)·(dT)₉ directed poly(T) synthesis was quite unexpected since phosphate buffers have routinely been employed in the purification of several DNA polymerases (19–25) including murine leukemia virus DNA polymerase (3). Since a similar degree of inhibition is obtained with phosphate at various divalent cation concentrations, phosphate does not appear to precipitate the Mn²⁺ from the reaction mixture. Phosphate also apparently does not exert its inhibition through an alteration in the ionic environment of the reaction mixture. Further experiments were carried out to determine the mechanism of phosphate inhibition.  

Studies performed on the kinetics of DNA synthesis by the Rauscher leukemia virus DNA polymerase to examine the effect of phosphate addition at various times after the initiation of DNA synthesis (Fig. 3B) indicate that phosphate is inhibitory at all stages of synthesis. The inhibition, however, was more profound when phosphate was added at later stages of synthesis (15 to 30 min) rather than at early stages of synthesis (0 to 15 min).  

In the experiment illustrated in Fig. 3A it is seen that regardless of the template to primer ratio used at saturating template-primer concentrations, the initial stages of DNA synthesis are indeed less affected by phosphate addition than the later stages. Addition of phosphate to the reaction mixture caused the most drastic inhibition in the presence of limiting template-primer concentrations (limiting numbers of initiation sites). We interpret the kinetic studies described above as suggesting that phosphate ions affect the process of chain elongation more acutely than that of initiation, although additional studies will be necessary to clarify this point.  

Examination of the nature of phosphate inhibition by classical methods (Fig. 4) revealed that phosphate ions apparently compete with deoxynucleoside triphosphates for a site on the polymerase. In addition to this type of inhibition, phosphate ions in the presence of low triphosphate concentration appear to cause deviations in the linearity of the double reciprocal plot, suggesting that additional mechanisms of inactivation of the Rauscher leukemia virus enzyme may exist. Therefore, phosphate appears to exert its inhibitory effect through interactions involving the substrate binding site of Rauscher leukemia virus DNA polymerase.  

Phosphate inhibition of DNA polymerases—We have also demonstrated that addition of inorganic phosphate to reaction mixtures strongly inhibits the DNA synthesis catalyzed by several mammalian type C virus DNA polymerases in contrast to its lack of action on the reverse transcriptases from other sources. During the purification and characterization of enzymes from unknown (or mixed) viral sources, the criterion of phosphate inhibition may serve to distinguish between the enzymes from type C and type B viruses. Similarly, in studies on type C virus-infected cells, a clear distinction between cellular DNA polymerase γ and the viral enzyme may be achieved by analyzing phosphate sensitivity. Since DNA synthesis catalyzed by mammalian type C viral enzymes, but not by cellular DNA polymerase γ, and directed by all of the template-primer combinations, is inhibited by the addition of inorganic phosphate, the finding may be utilized in the final analysis to confirm that the enzyme under study is of viral origin. At present, distinction among cellular DNA polymerases α, β, and γ is chiefly dependent upon analysis of their respective template-primer utilization preferences and molecular weight determinations (20). The phosphate sensitivity of β polymerase may serve as an additional distinguishing characteristic, particularly during purification of DNA polymerase γ which is monitored with the synthetic template-primer, poly(A)-oligo(dT). Similar results for cellular polymerases have been obtained by Weissbach and colleagues.¹ The inhibitory  

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¹ A. Weissbach, personal communication
action of inorganic phosphate on calf thymus DNA polymerase β has already been documented in the literature (13). These observations suggest that caution should be exercised in employing phosphate buffer in the purification or assay of DNA polymerases.

Our finding that inorganic phosphate inhibits DNA synthesis catalyzed by several type C mammalian viral reverse transcriptases suggests that compounds containing phosphate may possess the potential to serve as more specific inhibitors of reverse transcriptases than those previously described (27). Preliminary results which we have obtained (28) indicate that pyridoxal phosphate may be one such compound that demonstrates such specificity.

Acknowledgments—We thank Drs. J. Gruber for Simian sarcoma virus and Rauscher leukemia virus, J. Beard and M. A. Chirigos for avian myeloblastosis virus, W. Hardy for feline leukemia virus, D. Sawicki and P. J. Comatos for Wistar and Rauscher leukemia virus grown in rat cells, N. Sarkar for murine mammary tumor virus and Mason Pfizer monkey tumor virus, L. Loeb for homogeneous Escherichia coli DNA polymerase I, A. Weissbach for HeLa DNA polymerase α and γ, and V. S. Sethi for a phosphocellulose-purified preparation of Simian sarcoma virus-DNA polymerase. We thank Dr. Rollin D. Hotchkiss for helpful discussions and critical evaluation of this manuscript. We thank Drs. P. J. Comatos, L. J. Old, and R. A. Good for their continued support and encouragement. The expert assistance of Scott Soldner and Steven W. Smith in this investigation is appreciated.

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Purification and properties of Rauscher leukemia virus DNA polymerase and selective inhibition of mammalian viral reverse transcriptase by inorganic phosphate.

M J Modak and S L Marcus


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