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

(Received for publication, February 24, 1976)

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Rauscher leukemia virus RNA-directed DNA polymerase has been purified to near homogeneity (>90% pure) using affinity chromatography on polycytidylate-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),. 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 tumor 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.

We have described a one-step procedure for the purification of RNA-dependent DNA polymerase (reverse transcriptase) from avian myeloblastosis virus using polycytidylic acid (poly(C)), covalently linked to agarose (1). In this procedure for the purification of avian myeloblastosis virus DNA polymerase, phosphate buffer was found to be quite satisfactory and was, therefore, included in our protocol as a standard component. However, direct application of this protocol to the purification of Rauscher leukemia virus DNA polymerase and many other mammalian RNA tumor virus enzymes repeatedly resulted in low recoveries of enzyme activity, a finding which we, at first, attributed to the possible higher lability of the Rauscher leukemia virus enzyme relative to its avian myeloblastosis virus counterpart. The substitution of Tris/HCl buffer in place of phosphate buffer was subsequently found to stabilize Rauscher leukemia virus DNA polymerase activity, and inclusion of this buffer in the purification protocol has enabled us to obtain purified preparations of Rauscher leukemia virus DNA polymerase, with over 85% recovery of starting activity. At the time of the preparation of this manuscript, several reports on the purification and properties of murine leukemia virus DNA polymerases have appeared in the literature (2-7). However, in most cases somewhat lengthy purification procedures have been used and yields have amounted to 40 to ~55% of input activity. The use of a one-step affinity chromatographic procedure described in this manuscript offers an excellent method for the rapid high yield purification of oncornaviral DNA polymerases.

We have also examined catalytic properties of the purified Rauscher leukemia virus enzyme with particular emphasis on the requirements for optimal synthesis under the direction of a synthetic template-primer, poly(A)·(dT), with the aim of unraveling those characteristics of the viral enzyme which distinguish it from cellular DNA polymerase γ (8). During the course of these studies, we discovered that low concentrations of phosphate ions (1 to 5 mM) greatly inhibited DNA synthesis catalyzed by Rauscher leukemia virus DNA polymerase. The inhibitory effect was noted in the reactions directed by both synthetic and natural RNA and DNA templates and appears to be effected by competition with substrate triphosphate. Extension of these studies to other mammalian type C virus reverse transcriptases revealed that all the mammalian viral enzymes studied are inhibited by inorganic phosphate. However, several other DNA polymerases of cellular as well as...
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viral origin did not exhibit a similar susceptibility to phosphate 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 mixtures were products of P-L Biochemicals, Inc. The molar ratio of nucleotides was varied by using both synthetic and commercial nucleoside-glycosyl compounds, and stored at 70°C as a concentrate (approximately 1011 particles/ml) until use. The other virus stocks were similarly prepared and were kindly donated to us by our colleagues at Sloan-Kettering as follows: feline leukemia virus (Gardner strain) by Dr. W. B. Huber, Rauscher leukemia virus in rat cells and Wistar virus by Drs. Sawicki and Gomatos, murine mammary tumor virus and Mason Pfizer monkey virus tumor virus by Dr. N. Sarkar. Antiserum against purified Rauscher leukemia virus DNA polymerase and preimmune sera were kindly donated by Dr. D. W. B. Huber, Rauscher leukemia virus in rat cells and Wistar virus. The purification procedures used to obtain these enzymes were quite similar to that described herein for Rauscher leukemia virus. Enzymes are eluted with a linear salt gradient (0 to 0.5 M KCl) and most of the enzyme was 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/Cl, pH 7.8, 5 mM dithiothreitol, and 20% (v/v) glycerol. In the crude, disrupted state, little enzyme activity was measured before use at an equimolar ratio of nucleotides by heating the mixture to 1:65° for 5 min and then allowing the solution to cool slowly to room temperature. Polycytidylic acid, poly(rC)m, was annealed to oligo(dG)18 at a molar ratio of nucleotides of 1:1, respectively. Annealing was carried out by heating the mixture to 80°C followed by slow cooling. Poly(rC)m and poly(rC)m dithiothreitol were carried out in a similar manner. The fresh annealing of template-primer, particularly for poly(rC) - (dG)18-directed activity of some reverse transcriptases (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 diithiothreitol, 50 μg/ml of ribonuclease, and an appropriate amount of divalent cation. Mg2+ was used for activated DNA and polycytidylic acid (dG)18, and poly(rC)m or (dC)m templates while a Mn2+/KCl combination was employed for polycytidylic acid (dG)18-directed synthesis (see table and figure legends for concentrations used). The desired template-primer was present at 0.5-μg concentration while tritiated triphosphate was added as unlabeled substrate (specific activity 250 cpm/μmol) was used at 20 μM concentration. Reactions were initiated by the addition of reaction mixture to enzyme fractions and synthesis was carried out at 37° for 10 min for all templates except poly(A) (dT)18 for which 37° was used. Synthesis was terminated by the addition of 0.5% (w/v) trichloroacetic acid containing 0.1% sodium pyrophosphate. Acid-insoluble material was collected onto Whatman glass fiber filters (GF/B), washed extensively, and after drying, radioactivity on the filters was counted using toluene-based scintillation fluid. Assays using avian myeloblastosis virus 70 S RNA, globin mRNA + poly(rC)m (annealed just prior to use), and activated DNA containing 0.5 μg of RNA or 2.5 μg of DNA in place of synthetic template-primer. In addition, the reaction mixture contained all four deoxyribonucleoside triphosphates at a concentration of 80 μM except for the labeled precursor, which was present at 10 μM concentration. Usually higher amounts of enzyme had to be used to measure activity with natural RNA templates than for studies with synthetic RNA. The synthesis in all cases was linear with respect to time of incubation up to 1 h.

Nuclease Assays - RNase and DNase activities were assayed by monitoring the degradation of labelled isolated nucleotides to acid-soluble material. The substrate used for detection of RNAse H activity was 5'-dX74 DNA:5'-dHtRNA hybridized as described by Verma (7) with some minor modifications (15). RNA-DNA hybrids were purified by isopycnic banding in cesium sulfate (10) and deaggregated by chromatography on Sephadex G-75. The reaction mixture for assay of RNAse H activity contained, in a volume of 0.1 ml, 20 mM Tris/Cl, pH 7.8, 10 mM dithiothreitol, 150
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mm KCl, 0.02% (w/v) bovine serum albumin, 12 pmol of φX174 DNA-
RNA hybrid (as total nucleotides), and optimal concentrations of Me2+ or Mn2+. To measure single-stranded RNA degradation or DNase activity [3H]poly(U) or [C]-, and [H]poly(dT), respectively, were used; reaction mixtures were identical with that described for RNase H except that all contained 5 mM Me2+ 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 determined as described in the DNA polymerase assay.

Labeling of Viral Proteins with 35S-Solubilized virus or isolated polypeptides were iodinated using the lactoperoxidase procedure. To a 1-ml solution containing viral proteins 200 to 300 μCi of Na125I in 0.1 M NaOH (Amersham) was added at room temperature. Subsequent steps were identical with those described by Witte et al. (16).

Polyacrylamide Gel Electrophoresis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described previously (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 M 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% acrylamide and 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 M 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% acrylamide for 16 h at 25 V, constant voltage. After running, gels were sliced into 2-mm fractions using a Gilson gel fractionator and “1

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 diithiothreitol and 0.4 M 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 centrifuged 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)18 as well as poly(C)·(dT)18. 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)18 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 than 100-fold purification has been achieved. Due to the very low protein concentration in purified enzyme fractions, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis was carried out after labeling the protein with 35S. The purified enzyme protein shows a single band corresponding to a molecular weight of approximately 70,000 (Fig. 1). Glycerol gradient analysis of the purified native enzyme in high salt also confirms this molecular weight value, consistent with the notion that Rauscher leukemia virus DNA polymerase is a single polypeptide. The purified enzyme activity is inhibited by p-chloromercuribenzoate implying a requirement for reactive sulfhydryl group(s) at the active center of the enzyme. The inhibition observed in the presence of O-phenanthroline indicates that Rauscher leukemia virus reverse transcriptase may be a zinc-containing metalloenzyme (Table IV). Purified en-

zyme was stored at −70° in 0.05 M Tris/HCl (pH 7.8) containing 20% glycerol and 0.1% (w/v) bovine serum albumin and under these conditions was quite stable, retaining more than 90% of the original activity over a period of 6 months.

Catalytic Properties

Nuclease Activities—While DNase, RNase, and RNase H activities are present in disrupted virions as well as in the flow through of the poly(rC)-agarose column, the purified enzyme preparation is free of all nuclease activities except for RNase H. This ribonuclease H activity in the crude and purified enzyme fractions was further analyzed by glycerol centrifugation (Fig. 2). In crude disrupted virions, the presence of two molecular weight species is quite evident. The larger molecular weight species corresponds to that found associated with purified DNA polymerase and preferred Mn2+ for optimal activity (optimum = 0.5 mM). The low molecular weight species (Mr, ≈30,000) preferred 5 mM Mg2+ for optimal activity and, unlike the larger species, is not inhibited by antisera prepared against Rauscher leukemia virus DNA polymerase.

Utilization of Various Template-Primers—The enzyme can utilize a variety of synthetic RNA and DNA template-primers. However, conditions for optimal synthesis varied for different template-primers (see Table II) and are in general agreement with previous reports (2-7). We have, however, investigated optimal conditions for the utilization of poly(A) as a template by the enzyme in more detail, since a cellular enzyme, DNA polymerase γ (8), utilizes this template-primer most efficiently and exhibits almost identical catalytic properties with those of mammalian type C oncncoravirus 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

![Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel of purified, iodinated Rauscher leukemia virus DNA polymerase. The polymerase from peak poly(rC)-agarose column fractions was iodinated, placed on a 120-mm gel, and processed as described under "Experimental Procedures." The numbers at the top of the figure correspond to the molecular weight in thousands of standard marker proteins run at the same time as the experimental gel. Direction of protein migration is from left to right.](http://www.jbc.org/)

![Fig. 2.](http://www.jbc.org/)
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)₉₀-directed synthesis. The stimulation by histone f, as well as histone h, in the absence of salt stimulated poly(dT) synthesis. The stimulation by histone f is dependent on the stoichiometry of histone to template ratio. 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)₉₀-directed synthesis in the presence or absence of salt is 27°. Poly(A)·(dT)₉₀-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)₁₀₀₀₀ template and annealing various amounts of complementary DNA oligomer primers (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 highest synthesis occurred at a 20:1 nucleotide ratio. Increasing the primer concentration reduced the rate of synthesis. The apparent Kᵣ values for substrate DNA precursors irrespective of template to primer ratio were approximately 20 μM for both dTTP and dGTP.

DNA oligomer primers (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 20:1, and increasing the primer concentration reduced the rate of synthesis. The apparent Kᵣ 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)₉₀-directed DNA synthesis. It was therefore conceivable that the observed inhibition could result from phosphate inhibition of 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 MnCl₂, and 50 mM KCl or 2.5 mM MgCl₂ (wherever indicated), and 20 μM of substrate and approximately 5 μg 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 unlabelled dATP, TTP, and dCTP were included at 80 μM each. Specific activity of [*³²P]dGTP which was present at 10 μM was 2500 cpm/μmol. MgCl₂ was present at 10 mM while Mn⁺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 triphosphates resulted in almost total loss of synthesis.

### Table II

<table>
<thead>
<tr>
<th>Template-primers</th>
<th>Divalent cation</th>
<th>[*³²P]dGTP Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(A)·(dT)₁₀₀₀</td>
<td>Mn⁺² (KCl)</td>
<td>TTP 37</td>
</tr>
<tr>
<td>Poly(A)·(dT)₁₀₀₀</td>
<td>Mg⁺² (*KCl)</td>
<td>TTP 46</td>
</tr>
<tr>
<td>Poly(dA)·(dT)₁₀₀₀</td>
<td>Mn⁺²/KCl</td>
<td>TTP &lt;1</td>
</tr>
<tr>
<td>Poly(dA)·(dT)₁₀₀₀</td>
<td>Mg⁺²</td>
<td>TTP &lt;1</td>
</tr>
<tr>
<td>Poly(C)·(dG)₁₀₀₀</td>
<td>Mn⁺²/KCl</td>
<td>dGTP 20</td>
</tr>
<tr>
<td>Poly(C)·(dG)₁₀₀₀</td>
<td>Mg⁺²</td>
<td>dGTP 10</td>
</tr>
<tr>
<td>Poly(A)·(dA)₁₀₀₀</td>
<td>Mn⁺²</td>
<td>dATP 10</td>
</tr>
<tr>
<td>Poly(A)·(dA)₁₀₀₀</td>
<td>Mn⁺² or Mg⁺²</td>
<td>TTP 0.5</td>
</tr>
</tbody>
</table>

**Synthetic**

**Natural**

- Activated salmon Mn⁺² | dGTP 15.5
- Sperm DNA Mn⁺²/KCl | dGTP 3.0
- Avian myeloblastosis virus 70 S RNA Mg⁺² | dGTP 0.6
- Rauscher leukemia virus 35 S RNA Mn⁺²/KCl | dGTP 0.38
- Globin mRNA + Oligo(dT)₁₀₀₀ Mn⁺²/KCl | dGTP 2.1
- Globin mRNA + Oligo(dT)₁₀₀₀ Mn⁺²/KCl | dGTP <0.01

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

DNA oligomer primers (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 20:1, and increasing the primer concentration reduced the rate of synthesis. The apparent Kᵣ values for substrate DNA precursors irrespective of template to primer ratio were approximately 20 μM for both dTTP and dGTP.
which do not significantly change the ionic strength, a marked reaction mixture, no stimulatory effect was noted at any concentration. When potassium phosphate was the only salt added to the reaction mixture, the ionizable salt to the reaction mixture provided that the individual ion is not inhibitory to enzyme catalysis (Table IV).

To standard reaction mixture containing MnCl₂ (0.5 mM), 10 ng of enzyme, and poly(A) - (dT), as a template-primer, various additions were made as described. Incubation was for 15 min at 27°C. The 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 Kₘ value. Further increases in template concentration, up to 50-fold the Kₘ value, did not significantly alter the ability of low levels of phosphate to cause inhibition.

### Table III

<table>
<thead>
<tr>
<th>Ratio of template to primer</th>
<th>Poly(A) - (dT)₆₀ incorporated</th>
<th>Poly(C) - (dG)₆₀ 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>
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### Table IV

<table>
<thead>
<tr>
<th>Addition</th>
<th>dTMP incorporated 15 min</th>
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</thead>
<tbody>
<tr>
<td>Noise</td>
<td>34</td>
</tr>
<tr>
<td>KCl or NaCl or (NH₄)Cl (50 mM)</td>
<td>480</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (50 mM)</td>
<td>300</td>
</tr>
<tr>
<td>Tris (100 mM)</td>
<td>200</td>
</tr>
<tr>
<td>KPO₄ (50 mM)</td>
<td>6</td>
</tr>
<tr>
<td>LiCl₂ (50 mM)</td>
<td>433</td>
</tr>
<tr>
<td>Histone f₁ (0.5 µg)</td>
<td>200</td>
</tr>
<tr>
<td>Histone f₁ (0.5 µg) + KCl (50 mM)</td>
<td>600</td>
</tr>
<tr>
<td>Histone f₂a, f₂b, or f₃ (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 (10⁻² M) × + KCl (50 mM)</td>
<td>2</td>
</tr>
<tr>
<td>O'-Phenanthroline (10⁻² 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)₆₀, and avian myeloblastosis virus RNA-transcribed templates.

### Apparent Mechanism of Phosphate Inhibition of DNA Synthesis

1. **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).

2. **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.

3. **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 lessens 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)₆₀-directed synthesis. The results indicate that inhibition by inorganic phosphate is competitive with respect to dTTP concentration. The Kᵢ for inorganic phosphate using poly(A) - (dT)₆₀-directed synthesis was determined to be 1 mM.
DNA polymerase activity of all the mammalian type C viruses

mM) causes significant inhibition of poly(A) . (dT),,-directed

is clear that addition of low concentrations of phosphate (1 to 5

inorganic phosphate was specific to this Rauscher leukemia

virus enzyme or whether other DNA polymerases would be

simply due to nonspecific complex formation by triphosphate

DNA synthesis, nor do they prevent inhibition caused by

addition of inorganic phosphate. This result suggests that the

inhibition by phosphate is probably truly competitive and not

higher concentration added to reaction mixtures do not inhibit

the apparent

Kapparent

values for d'I TP and

PM).

Non-

complementary deoxynucleoside triphosphates up to lo-fold

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).

Values of DNA synthesis directed by 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),o by various DNA polymerases in the presence and
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Effect of phosphate on synthesis of poly(dT) catalyzed by various DNA polymerases in presence and absence of KC1

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>No KC1</th>
<th>No KC1 + KPO4 (2 mm)</th>
<th>-KCl (50 mm)</th>
<th>+KCl (50 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLV*</td>
<td>3.0</td>
<td>1.0</td>
<td>1.6</td>
<td>2.5</td>
</tr>
<tr>
<td>RLV*</td>
<td>2.8</td>
<td>0.4</td>
<td>0.5</td>
<td>4.0</td>
</tr>
<tr>
<td>RLV (crude)*</td>
<td>0.16</td>
<td>0.2</td>
<td>2.0</td>
<td>2.0</td>
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<td>SSV</td>
<td>2.1</td>
<td>1.5</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>SSY*</td>
<td>1.3</td>
<td>0.2</td>
<td>0.3</td>
<td>10.0</td>
</tr>
<tr>
<td>FeLV</td>
<td>16.0</td>
<td>14.0</td>
<td>9.0</td>
<td>12.0</td>
</tr>
<tr>
<td>FeLV (crude)*</td>
<td>2.8</td>
<td>1.7</td>
<td>0.4</td>
<td>0.9</td>
</tr>
<tr>
<td>Wistar virus</td>
<td>1.7</td>
<td>1.2</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Wistar virus (crude)*</td>
<td>0.9</td>
<td>0.2</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>RRTC virus</td>
<td>1.1</td>
<td>0.2</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>AMV</td>
<td>10.3</td>
<td>9.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>AMV (Mg+2 system)*</td>
<td>50.5</td>
<td>50.5</td>
<td>10.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>
| Rauscher leukemia virus, SeV, Simian sarcoma virus; FeLV, feline leukemia virus; RRTC, Rauscher leukemia virus grown in rat cells; AMV, avian myeloblastosis virus; MuMTV, murine mammary tumor virus; MPMV, Mason Pfizer monkey virus. In this experiment enzyme purified by DEAE-cellulose and phosphocellulose chromatography was used. In this experiment, NP-40 disrupted virions in place of purified enzyme were used. In these reactions MgCl2 (10 mm) was used as an effective divalent cation in place of Mn+2.

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) covalently 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 those findings: poly(A) - (dT)10, and mRNA-directed DNA synthesis requires Mn+2/KCl (no preference for salt), while poly(C) - (dG)12-18, poly(dC) - (dG)12-18, and DNA-directed DNA synthesis prefer Mg+2 for optimal activity, although the optimum Mg+2 concentration for DNA-directed synthesis is 1-5-fold higher than that required for the synthetic template-primer (2 mm). The optimal temperature for all of the polycytidylyl and DNA-directed reactions is 45°, and synthesis at the conventionally used temperature of 37° amounts to only 60% of that at 45°. For poly(C) - (dG)12-18 and poly(dC) - (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)16) 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 which by far distinguished it from cellular DNA polymerase 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)16-directed synthesis by histone f1, with detergent-disrupted Moloney murine leukemia virus virions and our results obtained with 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. This catalytic property is shown by the fact that other histones, namely, f2, f3, and f4, 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-primers were either unaffected or slightly inhibited (data not shown).

3. The length of oligomeric (dT) used by different investigators ranged in size from (dT)5 to (dT)10. Waters and Yang (5), using (dT)6 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 21° to be the optimal temperature for poly(A) - (dT)16-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).
The observed inhibition by inorganic phosphate of poly(A)·(dT)$_{10}$-directed polymerase 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 Mn$^{2+}$ 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. 3A) 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. 3B it is seen that regardless of the template to primer ratio used at saturating template-primer concentrations, the initiation 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 $\gamma$ 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 $\gamma$, and directed by all of the template-primers, 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 $\alpha$, $\beta$, and $\gamma$ is chiefly dependent upon analysis of their respective template-primer utilization preferences and molecular weight determinations (26). The phosphate sensitivity of $\beta$ polymerase may serve as an additional distinguishing characteristic, particularly during purification of DNA polymerase $\gamma$ which is monitored with the synthetic template-primer, poly(A)·oligo(dT). Similar results for cellular polymerases have been obtained by Weissbach and colleagues. The inhibitory

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**Table VI**

**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)$_{10}$,15</th>
<th>Poly(dC)·(dG)$_{10}$,15</th>
<th>Activated DNA</th>
<th>mRNA + oligo(dT)$_{10}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PO$_4^-$ + PO$_4^-$</td>
<td>PO$_4^-$ + PO$_4^-$</td>
<td>PO$_4^-$ + PO$_4^-$</td>
<td>PO$_4^-$ + PO$_4^-$</td>
</tr>
<tr>
<td>Wistar</td>
<td>5.1 1.4</td>
<td>3.1 1.0</td>
<td>4.8 1.1</td>
<td>N.D. N.D.</td>
</tr>
<tr>
<td>SSV$^a$</td>
<td>2.1 0.8</td>
<td>2.1 0.7</td>
<td>1.8 0.6</td>
<td>0.92 0.5</td>
</tr>
<tr>
<td>RRTC</td>
<td>3.5 0.8</td>
<td>3.9 1.4</td>
<td>1.3 0.5</td>
<td>0.92 0.5</td>
</tr>
<tr>
<td>FeLV</td>
<td>12.0 2.2</td>
<td>11.4 3.9</td>
<td>5.8 2.5</td>
<td>0.9 0.9</td>
</tr>
<tr>
<td>AMV</td>
<td>3.5 1.5</td>
<td>2.4 0.6</td>
<td>1.3 0.6</td>
<td>0.8 0.3</td>
</tr>
<tr>
<td>AMV$^a$</td>
<td>12.5 10.1</td>
<td>7.9 6.0</td>
<td>3.5 3.0</td>
<td>1.2 1.1</td>
</tr>
<tr>
<td>MuMTV</td>
<td>4.1 4.7</td>
<td>18.2 16.7</td>
<td>2.4 2.5</td>
<td>N.D. N.D.</td>
</tr>
<tr>
<td>MuMTV$^a$</td>
<td>48.0 49.0</td>
<td>68.6 70.4</td>
<td>20.4 22.1</td>
<td>N.D. N.D.</td>
</tr>
<tr>
<td>MPMV</td>
<td>1.2 1.4</td>
<td>1.0 1.3</td>
<td>0.75 0.85</td>
<td>N.D. N.D.</td>
</tr>
<tr>
<td>MPMV$^a$</td>
<td>10.2 11.0</td>
<td>25.0 24.9</td>
<td>15.0 16.8</td>
<td>N.D. N.D.</td>
</tr>
<tr>
<td><em>E. coli</em> DNA polymerase I</td>
<td>0.7 1.2</td>
<td>69.0 66.0</td>
<td>28.0 32.0</td>
<td>N.D. N.D.</td>
</tr>
<tr>
<td><em>E. coli</em> DNA polymerase II</td>
<td>N.D. N.D.</td>
<td>162.0 168.0</td>
<td>144.0 152.0</td>
<td>1.3 1.5</td>
</tr>
<tr>
<td>DNA polymerase $\alpha$</td>
<td>N.D. N.D.</td>
<td>69.0 73.0</td>
<td>28.0 32.0</td>
<td>N.D. N.D.</td>
</tr>
<tr>
<td>DNA polymerase $\beta$</td>
<td>N.D. N.D.</td>
<td>72.0 12.0</td>
<td>22.0 10.0</td>
<td>N.D. N.D.</td>
</tr>
<tr>
<td>DNA polymerase $\gamma$</td>
<td>1.4 2.5</td>
<td>5.3 6.5</td>
<td>3.7 3.5</td>
<td>N.D. N.D.</td>
</tr>
</tbody>
</table>

* N.D., enzyme activity was not detectable.

* SS, Simian sarcoma virus; RRTC, Rauscher leukemia virus grown in rat cells; RLV, Rauscher leukemia virus; FeLV, feline leukemia virus; AMV, avian myeloblastosis virus; MuMTV, murine mammary tumor virus; MPMV, Mason-Pfizer monkey tumor virus.

* In these reactions MgCl$_2$ (10 mM) was used as an effective divalent cation and replaced MnCl$_2$-KCl.

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

Our finding that inorganic phosphate inhibits DNA synthe-
sis catalyzed by several type C mammalian viral reverse trans-
scriptases 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 demon-
strates such specificity.

Acknowledgments—We thank Drs. J. Gruber for Simian
carcinoma 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 homogenous Escherichia coli DNA
polymerase I, A. Weissbach for HeLa DNA polymerase \( \alpha \) and
\( \gamma \), and V. S. Sethi for a phosphocellulose-purified preparation
of Simian sarcoma virus-DNA polymerase. We thank Dr.
Rollin D. Hothkiss for helpful discussions and critical evalua-
tion of the manuscript. We thank Drs. P. J. Comatos, L. J.
Old, and R. A. Good for their continued support and encour-
gagement. The expert assistance of Scott Soldner and Steven
W. Smith in this investigation is appreciated.

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