The Influence of Divalent Cations on the Activity of the Ribonucleic Acid Polymerase of *Micrococcus lysodeikticus*

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**SUMMARY**

The dependence of *Micrococcus lysodeikticus* RNA polymerase on divalent cations was studied in vitro with the use of synthetic polynucleotides as templates. It was found that the various combinations of manganese or magnesium cofactors with polyuridylic, polycytidylic, and polyadenylic acid templates had characteristic activity profiles for the chain initiation and elongation steps. To distinguish between the initiation of chain synthesis and its elongation, appropriate pentanucleotides were added to bypass the rate-limiting chain initiation reaction. In the copying of polycytidylic acid, the divalent cation requirement was independent of substrate concentration. The synthesis of polyadenylate and polyuridylate, however, evinced sharp metal ion optima at levels approximately equimolar with the total ligand concentration (i.e. nucleoside triphosphate plus other chelating species).

Whereas manganese stimulated chain initiation and elongation in all functioning polymer systems, magnesium ion exhibited great selectivity in the initiation step. Thus, Mg$^{2+}$ failed to promote the initiation of polyadenylic and polyuridylic acid synthesis, but still stimulated the elongation of the corresponding exogenous pentanucleotide chains. Poliguanolic acid was the only homopolymer the synthesis of which was initiated by Mg$^{2+}$. The relevance of this model system to the problem of the initiation of RNA synthesis in vivo is discussed.

In copying nucleic acid templates, bacterial RNA polymerase (EC 2.7.7.6) utilizes ribonucleoside triphosphate substrates and a divalent cation cofactor to yield a polynucleotide product and pyrophosphate. Co$^{2+}$ (1), Mn$^{2+}$, and Mg$^{2+}$ in vitro (1-3) satisfy this requirement. In addition, Mn$^{2+}$ enables microbial RNA polymerases to catalyze several nonphysiological reactions which occur far less readily, or not at all, with Mg$^{2+}$ as cofactor. These include the synthesis of homopolymers by the reiterated copying of DNA templates (4) with one NTP$^*$ as substrate (1, 5, 6); the slow formation of polyadenylic and polyuridylic acids in systems lacking template (7); the transcription of fragmented Q$\beta$ RNA or foreign RNA by Q$\beta$ RNA replicase, which is specific for intact Q$\beta$ RNA in the presence of Mg$^{2+}$ (8); and the copying of poly A and poly U homopolymers which are inactive in Mg$^{2+}$-promoted systems (9, 10). Manganese also promotes the misincorporation of deoxynucleotides into the RNA product of *Micrococcus lysodeikticus* RNA polymerase at a frequency many times that found with magnesium.$^2$

The copying of synthetic polyribonucleotide templates by bacterial RNA polymerases has been studied in detail (9-12). Of interest was the observation that the reaction catalyzed by the *Escherichia coli* enzyme showed kinetics greater than first order (10). In addition, the divalent cation optima were found to differ for each metal and each homopolymer template, although a single enzyme appeared to be involved in all cases (3, 9).

As in the polymerization of other macromolecules, the synthesis of RNA can be considered to consist of a slow initiation step wherein the initial phosphodiester bonds are formed, followed by the rapid extension of the initiated chains. Consonant with this premise, Niyogi and Stevens (13, 14) have reported that synthetic oligonucleotides complementary to homopolymer templates stimulate the synthesis of poly A and poly U by *E. coli* RNA polymerase. The oligomers appeared to be incorporated at the 5'-terminus of the polymerized product. A free 3'-hydroxyl group was essential for their action, while their 5'-end could be phosphorylated without inhibiting activity. These data, plus the effect of the oligonucleotides on the substrate dependence and time course of polymerization, indicated that the oligonucleotides served as initiators of chain synthesis. It thus appeared that the complex kinetics of over-all polymerization could be resolved into two component reactions, chain initiation and chain elongation.

The present study sought to investigate the role of divalent cations in the initiation and elongation of RNA synthesis.
Incorporation of designated tritiated NTP's was assayed under standard conditions. Each reaction mixture (0.3 ml) contained 2
mM metal ion, 2 mM NTP (divided equally among the NTP's present), and 50 pg of all polymers except for poly d(A-T) and poly dG.
poly dC, in which case 5 pg of polymer were used. As indicated (+ or -), 10 pg of complementary pentanucleotide were added or
omitted, its base component being identical with the tritiated substrate in each case.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Substrate</th>
<th>Mn&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Mg&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Co&lt;sup&gt;2+&lt;/sup&gt;</th>
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<tr>
<td>Poly A</td>
<td><em>H</em>-UTP</td>
<td>32.6</td>
<td>43.0</td>
<td>&lt;0.01</td>
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<tr>
<td>Poly U</td>
<td><em>H</em>-ATP</td>
<td>15.3</td>
<td>49.2</td>
<td>&lt;0.01</td>
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<tr>
<td>Poly C</td>
<td><em>H</em>-GTP</td>
<td>8.6</td>
<td>4.3</td>
<td>0.03</td>
</tr>
<tr>
<td>Poly G</td>
<td><em>H</em>-CTP</td>
<td>&lt;0.01</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Poly dG-dC</td>
<td><em>H</em>-GTP + CTP</td>
<td>0.15</td>
<td>0.20</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Poly d(A-T)</td>
<td><em>H</em>-UTP + ATP</td>
<td>10.0</td>
<td>8.8</td>
<td>13.4</td>
</tr>
<tr>
<td>DNA</td>
<td><em>H</em>-GTP + 3 NTP's</td>
<td>3.0</td>
<td>1.3</td>
<td>2.9</td>
</tr>
</tbody>
</table>

EXPERIMENTAL PROCEDURE

Materials—M. lysodeikticus cells, poly G, penta uridy late, and pentaadenylate were obtained from Miles Chemical Company. Poly C, poly U, and poly A were obtained from Schwarz BioResearch, Inc. The polymers were purified by phenol extraction, precipitation with 2-propanol, and dialysis overnight against 0.2 M NaCl. Poly d(A-T) and poly dG-poly dC were obtained from Biopolymers, Inc. Calf thymus DNA was purchased from Worthington. All *H*, *P*, and *T*-nucleotides were obtained from Schwarz BioResearch. Pentacytidylic acid was the generous gift of Dr. M. Lipsett. All pentanucleotides were of the dephosphorylated form, (Np)<sub>N</sub>

Methods

Preparation of Enzyme—RNA polymerase was isolated from dried M. lysodeikticus cells by Procedure A of Nakamoto, Fox, and Weiss (15). The enzyme was stored in 50% glycerol at -15°C.

Assay of RNA Polymerase—The assay of RNA polymerase activity was a modification of that of Fox et al. (9). Each tube contained 67 mM Tris-Cl, pH 7.5, MgCl<sub>2</sub>, MnCl<sub>2</sub>, or CoCl<sub>2</sub>, template, appropriate NTP's, 60 pg of enzyme, and, where specified, 5 to 10 pg of pentanucleotide, in a final volume of 0.3 ml. After incubation for 20 min at 30°C, the samples were chilled, and 0.1 ml of 0.1 M EDTA, pH 7, 0.4 ml of 0.1 M sodium pyrophosphate, and 0.1 ml of 100% trichloracetic acid were immediately added. After the mixture had stood for 5 min in ice, 2 ml of 5% trichloroacetic acid were added, and the samples were collected on Millipore filters and washed three times with 2 ml of 5% trichloroacetic acid. The filters were dried, placed in a toluene 2,5-diphenyloxazole (PPO)-1,4-bis[2-(5-phenyloxazolyl)]-benzene (POP) scintillation solution, and the radioactivity was determined in a Packard scintillation counter (16). Controls lacked either divalent cation or template and, in general, incorporated less than 0.5% of the radioactivity found in the samples. Rates of polymerization were expressed as millimicro- moles of *H*-NTP incorporated per 20 min incubation.

Base Composition of Ribonucleic Polyribonucleotides—Approximately 500 pg of each polymer were hydrolyzed in 0.5 ml of 0.3 N KOH for 18 hours at 37°C. The solution was neutralized with 6 N HClO<sub>4</sub> at 0°C, and the KClO<sub>4</sub> precipitate was removed by centrifugation. The separation of the 3'(2')-nucleotides was performed by ion exchange chromatography according to the method of Katz and Comb (17).

RESULTS

Stimulation of Copying of Various Templates by Mn<sup>2+</sup>, Mg<sup>2+</sup>, and Co<sup>2+</sup>—Several synthetic ribo- and deoxyribopolynucleotides, as well as calf thymus DNA, were assayed for template activity in the presence of Mn<sup>2+</sup>, Mg<sup>2+</sup>, and Co<sup>2+</sup> ions. While vigorous polymerization was generally obtained, poor rates of synthesis were observed with certain template-metal combinations. To establish whether this low activity resulted from a block in the initiation of polymer synthesis or from limitation in the elongation of growing chains, appropriate pentanucleotides were added to bypass the chain initiation step. Results of such an experiment are summarized in Table I.

Templates containing guanylic or deoxyguanylic acid residues stimulated polymer syntheses very poorly. The addition of pentacytidylic acid as an initiator did not improve incorporation rates, which suggests a block, at least in the chain elongation step. Preliminary observations indicated that increasing the mole fraction of guanylate residues in synthetic RNA copolymers inhibited their template activity proportionately. When poly G was added to a poly C-directed system, the synthesis of the poly G product was inhibited proportionately. In contrast, associated
FIG. 1. Time course of polymerization at high and low substrate concentrations. A, poly G synthesis: standard incubation mixtures contained 50 µg of poly C, optimum MnCl₂ (4 mM), and 3H-GTP as indicated. B, poly U synthesis: as in A, except that 50 µg of poly A and 3H-UTP plus optimum MnCl₂ were used as indicated. C, poly A synthesis: as in A, except 30 µg of poly U and 3H-ATP plus optimum MnCl₂ were used as indicated.

FIG. 2. Time course of poly A synthesis at high and low Mn²⁺ concentration. Standard incubation mixtures contained 30 µg of poly U, 3 mM ³H-ATP, and MnCl₂ as noted.

FIG. 3. The influence of pentanucleotides on the time course of poly U synthesis at high and low Mn²⁺ concentrations. Standard incubation mixtures contained 50 µg of poly A, 1 mM ³H-UTP, and 9 µg of pentanucleotidic acid and MnCl₂ as indicated.

that poly U and poly A were not copied by RNA polymerase when magnesium was the metal cofactor. We found (Table I) that the addition of their corresponding pentanucleotide initiators conferred activity on these otherwise inactive systems; this suggests a block in the initiation step for poly A and poly U synthesis. Poly G was the only homopolymer the synthesis of which was initiated by the magnesium-stimulated RNA polymerase system. DNA and poly d(A-T) were also readily initiated in the presence of Mg²⁺.

Time Course of Polymerization Reaction—The time course of homopolymer synthesis was examined as a function of substrate and divalent cation concentration. In general, an accelerating time course was observed at low substrate and low metal ion concentrations (Figs. 1 and 2). Increasing the levels of these reaction components sufficiently led to linear rates of reaction. The addition of the appropriate pentanucleotide initiators also converted the slowly accelerating time course found at low concentrations of metal or substrate to a rapid, linear polymerization (Fig. 3). In contrast, pentanucleotides did not significantly

strands of poly A and poly U directed the copying of both component polymers in this system (9, 18).

The other polymers tested showed good template activity. Manganese and cobalt ions, in contrast to magnesium, stimulated some degree of chain initiation with all templates. Magnesium ion appeared more selective. Others (9, 10, 19) have reported
enhance the synthetic rates obtained at high reactant concentrations. Similar effects have been reported by Niyogi and Stevens (14) for the NTP dependence of E. coli RNA polymerase.

The accelerating kinetics could have arisen from a two-step mechanism for the polymerization reaction, a slow chain initiation step upon which the rapid elongation reaction depended. Thus, the number of growing chains would have increased with time, each, in turn, being elongated at a constant rate. It was clear that kinetic parameters derived from such nonlinear initial rates would ambiguously reflect the two rate processes. Linear plots could be anticipated whenever chain initiation was not rate-limiting, namely, at high substrate and metal ion concentrations, in the presence of exogenous oligonucleotide initiator, or following the endogenous initiation of polymer synthesis. Decreasing the substrate and metal ion levels appeared to affect chain initiation more profoundly than the chain elongation step. (It was considered unlikely that the lag observed in these reactions resulted from a failure of short nascent chains to precipitate, since the same small extent of incorporation followed a linear time course when sufficient substrate was present.)

The reason for a slowing of the reaction despite an excess of reactants (e.g., Figs. 1A and 3) is unclear, but may be related to a failure of the enzyme to release template and completed polynucleotide product (20). The copying of short (degraded) template polymers would be especially subject to such effects.

**Dependence of Polymerization on Substrate Concentration**—The rate of polynucleotide synthesis directed by the three active homopolymer templates was examined, at fixed levels of metal ion, as a function of substrate concentration (Fig. 4). All three systems were found to display sigmoid kinetics. Hill plots (21) were linear and revealed interaction coefficients of 

\[ n = 2 \]

in each of the three systems. This value was consistent with a rate-determining initiation step, second order in substrate concentration. However, other interpretations are possible in this nonlinear, two-step, metal ion-dependent reaction. These coefficients were thus regarded as a descriptive index of cooperativeness, without clear-cut physical significance. The addition of pentanucleotide initiators was seen to shift the kinetics toward a hyperbolic (first order) form, which further supports Niyogi and Stevens' suggestion (34) that exogenous oligonucleotides serve to bypass the rate-limiting chain initiation step. It should be noted that in the poly A and poly U systems, but not in the case of poly G synthesis, a peak in incorporation rate was observed at substrate levels approximately equimolar with the manganese ion concentration. The relationship between these two reaction components is considered next.

**Dependence of Polymerization on Metal Ion Concentration**—Characteristic metal ion profiles have been reported for each of the various homopolymer-directed E. coli (10) and *M. lysodeikticus* (9) RNA polymerase systems. The optimum divalent cation concentration was not considered to be a function of the NTP levels present. In the present study, on the other hand, optimum divalent cation levels were found to vary with the total nucleoside triphosphate concentration in certain systems. The metal dependence profile varied with the several divalent cation template combinations and differed for the chain initiation and elongation steps. Manganese-catalyzed reactions are considered first.

The most complex of the metal ion dependence patterns was that seen for poly A synthesis (Fig. 5A). Three features were of interest: (a) optimum Mn\(^{++}\) concentration varied with the amount of ATP present; polymerization proceeded best when the Mn\(^{++}\) level exceeded that of ATP by about 50%; (b) Mn\(^{++}\) in further excess of nucleotide was inhibitory; and (c) ATP in excess of Mn\(^{++}\) was inhibitory, as well. The last effect was shown by the profound loss of activity observed at ATP concentrations exceeding those of Mn\(^{++}\), although both reactants were present at levels otherwise adequate for polymerization.

The synthesis of poly U was maximum when Mn\(^{++}\) and UTP levels were approximately equal (Fig. 5B). Sharp inhibition by excess Mn\(^{++}\), but not by excess UTP, was observed. The inflection seen in these curves at low Mn\(^{++}\) levels was independent of UTP concentration and was therefore considered to reflect the complex kinetics of the rate-limiting chain initiation step. Poly C synthesis, although proceeding at a very slow rate, appeared to obey a similar equimolar relationship between the CTP concen-
Fro. 5. Dependence of polymerization rate on Mn\textsuperscript{2+} concentration. A, poly A synthesis: standard incubation mixtures contained 50 µg of poly U, and MnCl\textsubscript{2} and \textsuperscript{3}H-ATP as specified. B, poly U synthesis: as in A, except that 30 µg of poly A and \textsuperscript{3}H-UTP were used as indicated. C, poly G synthesis: as in A, except that 50 µg of poly C and \textsuperscript{3}H-GTP were used as indicated.

Fig. 6. Dependence of polymerization rate on the concentration of equimolar mixtures of NTP and Mn\textsuperscript{2+}. Standard incubation mixtures contained either 50 µg of poly A or 30 µg of poly U, and equimolar concentrations of Mn\textsuperscript{2+} and either \textsuperscript{3}H-UTP or \textsuperscript{3}H-ATP, inhibition, seen in Fig. 4, at high NTP levels (where nucleotide exceeded metal concentration). Furthermore, plotting polymerization rate against the calculated metal-nucleotide complex concentration, assuming a \( K \) of \( 5 \times 10^4 \) M\textsuperscript{-1} (see Reference 23), did not eliminate the inflections seen in Fig. 6. It was concluded that making divalent cation and nucleotide levels equivalent minimized, but did not abolish, the sigmoid concentration dependence in these systems. Therefore, the early inflection in standard substrate dependence curves (Fig. 4) probably reflected both inhibition by excess metal ion and the complex kinetics of chain initiation.
FIG. 7. Influence of divalent cations and oligonucleotides on polymerization rate. A, poly U synthesis: standard incubation mixtures contained 50 pg of poly A, 2 mM 3H-UTP, and 9 pg of pentauridylic acid, MnCl₂ or MgCl₂ as indicated. B, poly A synthesis: as in A, except that 30 pg of poly U, 2 mM 3H-ATP, and 7 pg of pentauridylic acid, MnCl₂ or MgCl₂ were used as specified. Oligo U, oligouridylic acid; Oligo A, oligoadenylic acid.

Dependence of Chain Initiation and Elongation on Metal Ion Concentration—Thus far, we have demonstrated that the synthesis of RNA homopolymers was sensitive to inhibition by imbalance between substrate and available divalent cation concentrations. It remained to be established whether such effects occurred at the chain initiation or chain elongation step, or both.

To evaluate these alternatives, the metal ion dependence of polymerization was examined in the presence and absence of pentanucleotide initiators. It was found for the synthesis of poly U (Fig. 7A) that pentauridylic acid did not obviate the metal ion requirement but did abolish the early inflection seen in the Mn⁺-stimulated reaction. Optimum synthesis still occurred at a Mn⁺:UTP ratio near unity in the presence of pentauridylic acid, owing to persistent inhibition by excess manganese. It was clear that at a suboptimal metal to nucleotide ratio (e.g. 0.5 μM and 2 mM UTP), the presence of pentauridylic acid effected a dramatic stimulation. At a Mn⁺:UTP ratio of 1, however, little enhancement by pentanucleotide was seen, which suggests that the chain initiation step was then only slightly limiting of velocity. Fig. 3 illustrated the time course for such effects.

The kinetics of poly A synthesis (Fig. 7B) was subject to similar modification by the addition of pentaadenylic acid. The sigmoid takeoff of polymerization rate seen at low Mn⁺ concentration was abolished by oligornucleotide, which suggests that chain initiation, rather than elongation, was profoundly sensitive to inhibition by excess ATP. Furthermore, other studies showed that, in the presence of pentaadenylc acid, the activity optimum ceased to vary with ATP concentration. Thus, the elongation step of poly A synthesis was nearly hyperbolic in form. Since chain initiation was probably rate-limiting in the usual conditions of assay, it was not surprising that the over-all rates of polymerization reflected the complex kinetics of chain initiation.

The magnesium dependence of poly A and poly U formation was also analyzed. Fig. 7 clearly indicates that no synthesis of these polymers occurred at any Mg²⁺ concentration. The addition of their corresponding pentanucleotide initiators, however, conferred polymerization activity on these otherwise unreactive systems. Optimum synthetic rates were found near a Mg²⁺:NTP ratio of 1:1. The previously discussed inhibitory effects of excess metal and NTP were also observed. It was of interest that the elongation of such preinitiated chains was still poorly stimulated by magnesium as compared with manganese.

The stimulation of poly G synthesis by Mg²⁺ occurred in the absence of oligonucleotide. The Mg²⁺ dependence of this reaction resembled that found for manganese (Fig. 5C); it reached a maximum at around 4 mM, and was free of inhibition by excess metal or GTP. The Mg²⁺ dependence plots, however, manifested a slight sigmoid curvature.

The stimulation of polymerization by cobalt conformed to the same types of metal nucleotide effects illustrated for Mn²⁺ and Mg²⁺-catalyzed systems.

Effects of Nonincorporated Nucleoside Triphosphates on Polymer Synthesis—Niyogi and Stevens (10) observed that nucleoside triphosphates which were not complementary to the homopolymer template inhibited the polymerization of the substrate. It has also been shown that these noncomplementary nucleotides are not incorporated into the homopolymer product to any significant extent (9, 10). We therefore sought to investigate the relationship between inhibition by nonincorporated nucleotides and the metal ion requirement for polymerization.

Fig. 8 illustrates the effects of noncomplementary nucleotides on homopolymer synthesis at fixed substrate and manganese ion levels. Deoxy- and ribonucleoside triphosphates were both found to inhibit the three polymer systems studied. A sole exception was the observation (Fig. 8C) that ATP and dATP consistently stimulated, about a-fold, the synthesis of poly G. In this regard, AMP and dAMP were found to constitute about 1% of the poly G product, as determined by both direct label incorporation and nearest neighbor analysis. Base composition studies of the poly C templates revealed a small (approximately 1%) uridylate component. However, Brenneman and
Fig. 8. Influence of noncomplementary NTP's on polymerization rate. A, NTP's and poly U synthesis: standard incubation mixtures contained 50 μg of poly A, 2.5 mM 3H-UTP, 2.5 mM MnCl₂, and GTP, ATP, and CTP as indicated. B, dNTP's and poly A synthesis: as in A, except with 30 μg of poly U, 2.5 mM 3H-ATP, 3.3 mM MnCl₂, and the four dNTP's as noted. C, NTP's and poly G synthesis: as in A, except with 50 μg of poly C, 2.5 mM 3H-GTP, 4 mM MnCl₂, and ATP, UTP, and CTP as indicated.

Fig. 9. Influence of noncomplementary nucleotides and PPᵢ on the metal ion dependence of polymerization rate. A, NTP's, Mn²⁺, and poly A synthesis: standard incubation mixtures contained 30 μg of poly U, 2.8 mM 3H-ATP, 2.8 mM NTP or PPᵢ effector, and MnCl₂ as noted. B, NTP's, Mg²⁺, and poly A synthesis: as in A except with 7 μg of pentadecylc acid, and MgCl₂ as noted. C, adenine nucleotides and poly U synthesis: as in A, except with 50 μg of poly C, 1.5 mM 3H-UTP, 1.0 mM adenine nucleotide or PPᵢ effector, and MnCl₂ as noted.

Singer (24) have shown that small amounts of UMP are formed by the alkaline deamination of cytidylic acid under the digestion conditions used here. The nature of the participation of ATP and dATP in poly C-directed synthesis thus remains unresolved.

The mechanism of inhibition by noncomplementary NTP's was elucidated by experiments represented in Fig. 9. The addition of these nucleotides was found to shift the divalent cation optimum to a higher level by an amount roughly equimolar with the NTP added. It appeared that the additional nucleoside triphosphates inhibited the polymerization reaction by forming inactive chelate complexes with the metal ion cofactor. Thus, synthetic activity at a divalent cation level held equal to substrate concentration was diminished as metal was titrated by the competing NTP species.

The proposed mechanism implies that the phosphate groups constitute the important portion of the noncomplementary NTP's and that the base moiety is not responsible for the inhibition. Supporting this interpretation was the finding (Fig. 9C) that adenosine monophosphate, diphosphate, and triphosphate, and inorganic pyrophosphate shifted the manganese dependence curves (i.e., inhibited at a fixed metal concentration) in proportion to their metal-binding affinities; the association constants of AMP, ADP, ATP, and PPᵢ for Mn were on the order of 200, 8,700, 56,000, and 250,000 M⁻¹ respectively (23). Similarly, EDTA and citrate ion were able to inhibit polymerization at fixed manganese concentrations by a similar shift in the metal dependence profile. These inhibitory effects applied equally to Mn²⁺- and Mg²⁺-stimulated systems. Such metal ion chelation
might have been the mechanism for the "low efficiency inhibition" observed by Chamberlin and Berg for nonincorporated NTP's (4).

The Mn²⁺-stimulated synthesis of poly A was subject to an additional inhibitory effect by nonincorporated nucleotides. Fig. 9A shows that, even when the added nucleotide had been matched by additional metal, the new activity optimum was significantly lower than in the uninhibited system. This inhibitory action could have been due to competition of the spurious NTP-Mn complex with the true metallosubstrate for enzyme sites. The marked inhibition by pyrophosphate could have derived from a "product inhibition" mechanism, assuming Mn-PP₁ to be the true cleavage product. In contrast, EDTA and citrate shifted, but did not reduce, the optimum enzyme activity; this evidence supports the premise that Mn-PP₁ inhibited by binding specific enzyme sites. None of the other systems tested appeared susceptible to this form of inhibition by nonsubstrate NTP's or PP₁.

Just as the presence of nonincorporated nucleoside triphosphates inhibited polymerization by titrating metal ion away from the optimum, the reaction was stimulated by extra nucleotides whenever they served to titrate the metal ion concentration toward the optimum point. For example, Fig. 10 represents the effect of the addition of CTP on the synthesis of poly U in the presence of excess Mn²⁺. CTP initially enhanced activity as it bound the excess Mn²⁺. Beyond the equimolar NTP:Mn²⁺ ratio, increasing the nonincorporated NTP led to inhibition as it competed with UTP for the manganese ion. Such effects illustrated the profound influence that metal-nucleotide balance exerted on over-all RNA polymerase activity.

**DISCUSSION**

We have observed two types of effects by which divalent cations influenced the activity of the *M. lysodeikticus* RNA polymerase. One of these was related to the specificity of the metal cofactor in the recognition of chain initiator sites by the enzyme. The other category dealt with the influence of the metal-nucleotide balance on the kinetics of the polymerization reaction.

Manganese and, to a lesser extent, cobalt catalyzed the formation of the initial phosphodiester bonds in all active systems tested. Magnesium, on the other hand, conferred great selectivity on the enzyme's ability to initiate polymer synthesis, while no such specificity was seen in the elongation of exogenous oligonucleotides. Since the levels of unbound Mn²⁺ in microorganisms are probably so low that only a minute fraction of the nucleoside triphosphates present forms its complex (25), it was useful to regard Mg²⁺ as the physiologically important cation for this microbial RNA polymerase, and to consider what insight into the regulation of RNA transcription in vivo such a hypothesis might afford.

In the presence of magnesium, neither poly U nor poly A was initiated. It may be presumed that the 5'-terminal pppApA and pppUpU phosphodiester linkages could not be formed. However, the synthesis of poly (A-U) on poly d(A-T) templates was readily initiated in the presence of Mg²⁺ (Table I), an indication that a pppApU or pppUpA 5'-terminal linkage was readily formed. Poly C was the only homopolymer capable of promoting chain initiation with Mg²⁺ ion. It would appear that the pppGpG-terminal linkage could be readily synthesized in this system. The possibility that a small uridylic contamination of the poly C template was involved in the initiation of poly G synthesis must be considered. However, poly G initiation occurred in the absence of ATP. Furthermore, preliminary studies performed with β,γ⁻³²P-ATP and β,γ⁻³²P-GTP indicated that the 5'-terminus of this copolymer product was exclusively guanylic acid. Nevertheless, it is possible that the presence of uridylic residues in the template may have been sufficient to signal initiation points, while not being copied themselves. Further investigation is need to clarify these points.

In connection with these observations, a series of synthetic copolymers was studied. It was found that systems directed by copolymers containing adenylate and uridylate in various proportions showed poor chain initiation in Mg²⁺-stimulated systems, in contrast to the excellent initiation seen with poly d(A-T) under identical conditions. Mixing small amounts (i.e. 5 to 20%) of a second nucleotide base into template polymers of either A or U did not enhance their capacity to support chain initiation in the presence of Mg²⁺, with the sole exception of poly (A,C) (10:1) which was moderately active. Further studies with this model system particularly with deoxyribopolynucleotide templates, should help to clarify these relationships.

It appears that of the four homopolymer systems, poly C-directed synthesis most closely resembles that found for DNA-dependent polymerization (1) in initiation properties, time course, and substrate, Mn²⁺, and Mg²⁺ dependence profiles. This suggests that cytidylate residues, perhaps in clusters, might be the determinants of the over-all kinetics of DNA-directed RNA polymerization in this system. A similar hypothesis regarding
the role of cytidylate tracts in the initiation of DNA transcription has been detailed by Szybalski, Kubinski, and Sheldrick (26) from complexes in rapid equilibrium. If the 5'-terminus of RNA synthesized in vitro by E. coli RNA polymerase. In addition, Anthony, Zeszotek, and Goldthwait (28) found that guanylate and adenylate residues predominated at the 5'-terminus of RNA synthesized in vitro by E. coli RNA polymerase. If the use of Mn$$^{2+}$$ or Mg$$^{2+}$$ alone as cofactor would have altered the specificity of the initiation step. In this regard, a recent report has indicated a roughly similar 5'-terminal nucleotide distribution for a Mg$$^{2+}$$-stimulated RNA polymerase isolated from Azotobacter vinelandii (30).

In contrast to the bacterial studies with divalent cation mixtures, the Q5 RNA polymerase has been examined with magnesium as the sole metal cofactor. A high degree of specificity for $$\gamma$$-ATP-GTP incorporation at the 5'-terminus was observed by Banerjee et al. (31). Furthermore, Q5 "replicase" preparations synthesized poly G in the presence of poly C and Mg$$^{2+}$$, while the other homopolymer templates were inactive (19). It seems likely that the metal ion conferred initiator specificity on this system, since earlier studies (8) showed that the presence of Mn$$^{2+}$$ allowed this enzyme to copy fragmented Q5 RNA and foreign RNA templates.

The mechanism by which Mn$$^{2+}$$ and GTP$$^{2+}$$ obliterated the Mg$$^{2+}$$-related specificity of chain initiation in the present study is unexplained. The mode of action here may be similar to that by which Mn$$^{2+}$$ promotes various types of "nonphysiological" activity in RNA polymerase (1, 7, 10) and DNA polymerase (32) systems in vitro, and may also resemble the mechanism of manganese-induced mutagenesis in vivo (33). Through magnetic resonance studies, manganese and cobalt have been found to interact with all three phosphates as well as the hydrogen S atom on the purine ring of ATP, whereas magnesium associated only with the 1- and 3-phosphate groups (34).

In addition to qualitatively influencing the specificity of chain initiation, divalent cations exerted a complex effect on the kinetics of RNA polymerization. The metal dependence was found to vary as a function of the substrate concentration, metal species, and template used; it also differed for the initiation and elongation steps of polymerization. Since we presumed to be observing the activity of a single enzyme (9), an attempt was made to relate the variety of substrate and metal dependence curves to quantitative differences in the kinetic parameters of a single reaction pattern.

A most general formulation (35) assumes that the metal ion and the NTP substrate are each capable of binding to the enzyme as well as to each other and forming $$M_{s}$$, $$EM$$, $$ES$$, and $$RMS$$ complexes in rapid equilibrium. If the MS complex is the true substrate, and the incomplete substrate species, M and S, both compete with it for binding sites on the enzyme-template complex, then the various patterns observed in Figs. 4 through 7 are compatible with a single rate equation. This may be most simply expressed as

$$V = \frac{K_{M_M(M)}S}{K_{M_M(M)} + K_{M_S(S)} + K_{M_M(M)} + 1}$$

where each $$K$$ denotes the association constant for the corresponding species and the enzyme. From this paradigm, metal and substrate dependence curves can be generated which exhibit no inflection point (i.e. hyperbolic in form), one inflection point (i.e. sigmoid in either the ascending or descending arm of the curve), or two inflection points (i.e. sigmoid curvature in both the ascending and descending limbs), depending on whether neither, one, or both of the incomplete species are inhibitory. Each metal-NTP template combination would engender a set of association constants for the chain initiation and the elongation steps, and thereby determine the reactant dependence profiles for that system. Several less complex metal-nucleotide-enzyme systems have been analyzed in this context (36).

Other possibilities for inhibition by excess metal or nucleotide exist. One example is the formation of inactive $$M_{s}$$ or $$S_{M}$$ complexes. The existence of such species has been questioned by Bock (23). Noncompetitive metal ion inhibition and the influence of metal on the reaction equilibrium (e.g. by binding PP$$^{i}$$) could also have influenced the polymerization reaction profoundly. Complexes of divalent cation with the template or product polynucleotide chains (37) might participate in the inhibition seen with excess metal ion. In view of these many uncertainties, no attempt was made to derive kinetic parameters or to consider a mechanism of reaction.

The substrate and metal dependence curves, as well as the inhibition plots for nonincorporated nucleotides, formally resemble the homotropic and heterotropic interactions postulated for allosteric systems (38), as well as the solutions proposed by Morales et al. (39) for the kinetics of multivalent enzymes possessing interacting binding sites and for enzymes subject to ligand-induced deformations. Although other enzymes requiring nucleoside triphosphates and Mg$$^{2+}$$ have been evaluated in this context (40, 41), there is no compelling reason to favor an allosteric model in this system. Rather, our observations appear related to the complex interactions long noted in enzyme systems dependent on divalent cations and nucleotides (e.g. References 23, 36, and 42-46).

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