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Inhibition of *Euglena gracilis* and Wheat Germ Zinc RNA Polymerases II by 1,10-Phenanthroline Acting as a Chelating Agent†

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ABSTRACT: Copper complexes of 1,10-phenanthroline (OP–Cu) hydrolyze DNA [D'Aurora, V., Stern, A. M., & Sigman, D. S. (1978) *Biochem. Biophys. Res. Commun. 80*, 1025–1032; Marshall Pope, L., Reich, K. A., Graham, D. R., & Sigman, D. S. (1982) *J. Biol. Chem. 257*, 12121–12128]. This reaction has been studied to determine whether the 1,10-phenanthroline (OP) inhibition of the activity of RNA and DNA polymerases is the result of template hydrolysis or the chelation of a metal associated with and essential to the function of these enzymes. Addition of 4′,6-diamino-2-phenylindole dihydrochloride (DAPI) to DNA generates a fluorescence signal with a linear increase of the intensity over a broad range of DNA concentrations from 0 to 100 μg/mL. The progress of hydrolysis of DNA by DNase I or OP (2 mM) is monitored by the time-dependent decrease in DAPI-induced fluorescence. In the presence of OP, the rate of hydrolysis increases as the Cu^{2+} concentration in the reaction mixture rises from 10^{-8} to 10^{-5} M. The rate differs for each nucleic acid template used; hydrolysis of poly(dA-dT) > denatured DNA > double-stranded DNA. However, millimolar amounts of OP do not hydrolyze the template even in the presence of Cu^{2+} (10^{-6} M) when DNA is complexed with either *Escherichia coli* DNA polymerase I or *Euglena gracilis* or wheat germ RNA polymerase II. Under the same conditions, OP inhibits the activity of both varieties of RNA polymerase II with pK_i's of 3.4 and 3.0, respectively. The addition of neocuproine from 10^{-5} to 10^{-3} M to chelate any Cu^{2+} present in the reaction mixture does not change this inhibition. In contrast, OP does not affect DNA polymerase I activity. Thus, OP inhibits enzyme activity of a complex of RNA polymerase with nucleic acid template by chelation of metal atoms essential for the function of these polymerases rather than by hydrolysis of their template. Zinc is the only enzymatically active metal associated with RNA polymerases. This functional role is confirmed further by the demonstration that these enzymes are also inhibited by other chelating agents whose structures differ distinctively from that of OP: dipicolinic acid, 8-hydroxyquinoline, α,α′-bipyridyl, and 8-hydroxyquinoline-5-sulfonic acid.

The chelating agent 1,10-phenanthroline (OP) has frequently been used to study the functional role of zinc in enzymes (Chang & Bollum, 1970; Scrutton et al., 1971; Auld et al., 1974; Slater et al., 1972; Springgate et al., 1973). It has a particularly high affinity for zinc, and its high solubility permits the use of concentrated solutions (Sillen & Martell, 1964). Further, its relatively lower affinity for Mg^{2+}, a metal required for activation of all the nucleotidyl transferases (Mildvan, 1979), makes it particularly suitable for studies of this class of enzymes. The occurrence of OP inhibition of enzyme activity generally leads to the inference that chelation of a functional metal is the underlying mechanism. This assumption is usually tested by using nonchelating analogues of OP which are known not to inhibit activity.

However, recent reports have provided clear evidence that this generalization is not valid. Thus, in the presence of thiols, micromolar quantities of OP form complexes with Cu^{2+} which hydrolyze DNA (Marshall et al., 1982). OP, in millimolar amounts, also forms complexes with the Mg primer essential for DNA polymerase activity (Abraham & Modak, 1983).

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Clearly, OP inhibits the activity of DNA-dependent polymerases by mechanisms other than zinc chelation and potentially could pertain to others which are RNA dependent. Therefore, the nature of the mechanism of OP inhibition of the activity of enzymes requiring DNA (or other nucleic acids) as templates must be examined in each case. Furthermore, the recent demonstration that *Escherichia coli* DNA polymerase I is not a zinc metalloenzyme (Walton et al., 1982; Ferrin et al., 1983), as previously assumed partially on the basis of OP inhibition (Slater et al., 1971), raises questions both as to the proposed fundamental role of zinc in the function of other nucleotidyl transferases (such as the RNA polymerases) and as to the suitability of OP as a chelating agent for use with enzymes that require nucleic acid templates.

Our studies demonstrate that OP inhibition of eukaryotic RNA polymerase II from two different sources is by chelation of a metal essential for their function and confirm that these enzymes are zinc metalloenzymes (Falchuk et al., 1976; Jendrisak & Burgess, 1975).

Materials and Methods

The nucleotides [^{3}H]UTP (specific activity 23.7 Ci/mmol) and [^{3}H]TTP (specific activity 18.7 Ci/mmol) were obtained from New England Nuclear; ATP, CTP, UTP, GTP, calf thymus DNA, dithiothreitol, dipicolinic acid, and wheat germ (type II) were from Sigma Chemical Co.; 8-hydroxyquinoline and α,α'-bipyridyl were from G. F. Smith Co.; 1,10-phenanthroline and 2,9-dimethyl-1,10-phenanthroline (neocuproine) were from Aldrich Chemical Co.; 4′,6-diamino-2-phenylindole dihydrochloride (DAPI) was from Polysciences Inc.; Specpure $MgCl_2$ was from Johnson-Matthey Co.; and ultrapure ammonium hydroxide (Alfa Division) and the protein assay kit were from Bio-Rad Laboratories, Inc. *E. coli* DNA dependent DNA polymerase I was purchased from Boehringer-Mannheim, and the four deoxynucleotide triphosphates, poly(dA-dT), and DNase I were obtained from P-L Biochemicals, Inc.

Euglena gracilis and wheat germ RNA polymerases II were purified according to the procedures of Falchuk et al. (1976) and Jendrisak and Burgess (1975), respectively. The specific activities of the purified enzymes were approximately 6.0×10^7 cpm/mg of protein. Protein concentration was measured by using the Bio-Rad protein assay kit with bovine serum albumin as a standard (Bladford, 1976).

DNA-Dependent RNA Polymerase Assay. The DNA-directed incorporation of [^{3}H]UTP into acid-insoluble material by RNA polymerase II was measured as described previously (Falchuk et al., 1976). The total volume of the assay mixture was 0.1 mL. It contained 50 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) buffer, pH 7.9, 5 μCi of [^{3}H]UTP, 50 μM each of ATP, CTP, and GTP, 5 μM UTP, 10 mM $MgCl_2$, 1 μg of template consisting of poly(dA-dT) or calf thymus DNA, 1 mM dithiothreitol (DTT), 10 μg of bovine serum albumin (BSA), 10% glycerol, and 10 μg of RNA polymerase II. Assay mxitures were incubated 20 min at 30 °C. An aliquot of 75 μL was taken from each example, applied to a DE-81 filter paper disk, washed with 5% trichloroacetic acid (Cl_3CCOOH)/1% pyrophosphate, dried, and counted in Econofluor by using a Packard scintillation counter.

DNA-Dependent DNA Polymerase Assay. DNA polymerase assays were carried out as described by Abraham and Modak (1983). The total volume of the assay mixture was 0.1 mL. It contained 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid–potassium hydroxide (Hepes–KOH) buffer, pH 7.8, 10 μg of BSA, 1 mM DTT, 10 mM $MgCl_2$, 100 mM KCl, 1 μCi of [^{3}H]TTP, 50 μM each of deoxynucleotide triphosphates dATP, dGTP, and dCTP, 5 μM TTP, and 0.6 μg of DNA polymerase I together with 1 μg of DNA template-primer. The mixture was incubated for 30 min at 30 °C. Acid-insoluble product was determined as described for RNA polymerase.

DNA Analysis. The concentration of template [double-stranded or denatured DNA or poly(dA-dT)] in samples was measured by the quantitative fluorometric micro-method (Kapuscinski & Skoczylas, 1977). Standards consisted of DAPI at a concentration of 2 μg/mL in 1:1 ratio with template. The fluorescence of the template dye complex was analyzed with a MPF-3 Hitachi Perkin-Elmer fluorescence spectrophotometer with a 43 filter by exciting at 372 nm and reading the fluorescence at 454 nm. This method can detect DNA in amounts as low as 5×10^{-10} g. The amount of template used in our experiments was in the range of $(1.0–30) \times 10^{-6}$ g. The effect of OP, in the presence of $10^{-8}–10^{-5}$ M Cu^{2+}, on the fluorescence generated by a DAPI–DNA or a DAPI–poly(dA-dT) complex was analyzed. A sample of 10^{-6} g of either DNA or poly(dA-dT) was mixed with a solution of DAPI, OP (2 mM), and Cu^{2+} (1 μM), and the fluorescence was measured for periods of up to 10 min after various time intervals.

Polymerase Assays with Chelating Agents. In the studies with OP, either DNA or poly(dA-dT) was preincubated first with DNA polymerase I or RNA polymerase II for 10 min at 30 °C in 5% glycerol, 40 mM KCl, and 1 mM $MgCl_2$ in 50 mM Tris-HCl buffer, pH 7.9. The other assay components were added after the enzyme–template binary complex had been cooled in an ice bath. The chelating agent was the component added last before the samples were incubated. The template concentration in assay mixtures (with or without OP) was measured by the DAPI method described above. Aliquots were removed from the assay mixture, and the enzymatic reaction was stopped by the addition of sufficient ethylenediaminetetraacetic acid (EDTA) to provide a final concentration of 1 mM. The samples were mixed with DAPI solution (1:1), and the fluorescence was measured.

In studies with dipicolinic acid, 8-hydroxyquinoline-5-sulfonic acid, and α,α'-bipyridyl, the chelating agents were preincubated with the enzyme, and the incubation mixture was added to the reaction mixture. All chealting solutions were adjusted to pH 7.9 with ultrapure ammonium hydroxide.

The reversibility of inhibition by either OP or a structurally different chelator, dipicolinic acid, was examined with both varieties of RNA polymerase II. Enzymes were preincubated at 30 °C with 2 mM OP or 4 mM dipicolinic acid. Aliquots were removed after time intervals of 0–120 min and placed in one of two reaction mixtures. In the first, the chelator concentration was diluted 10-fold. In the second, it was maintained at 2 mM OP or 4 mM dipicolinic acid. Each mixture was then incubated for 20 min at 30 °C and its activity measured as described previously.

Results

Figure 1 shows the relationship between the activity of DNA-dependent RNA polymerase II and DNA concentration. Enzyme activity increases as the concentration of DNA is raised from 0 to 20 μg/mL. At higher concentration, enzyme activity actually decreases slightly. Similar relationships were observed between the concentration of poly(dA-dT) or denatured DNA and enzyme activity (not shown). Therefore, a concentration of 10 μg of template/mL was chosen for all subsequent enzyme assays.

The fluorescence generated by a DAPI–DNA complex increases linearly with increasing template concentration from

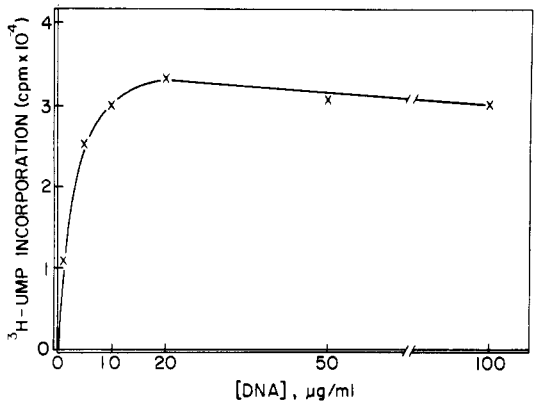

FIGURE 1: Effect of increases in DNA concentration on RNA polymerase II activity.

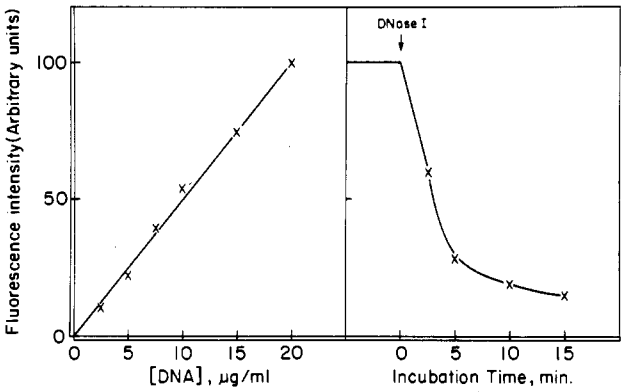

FIGURE 2: Quantitative analysis of DNA with DAPI. The relative fluorescence intensity of a DNA–DAPI complex increases linearly with the DNA concentration (left panel). The fluorescence intensity decreases following incubation of DNA with DNase I (right panel).

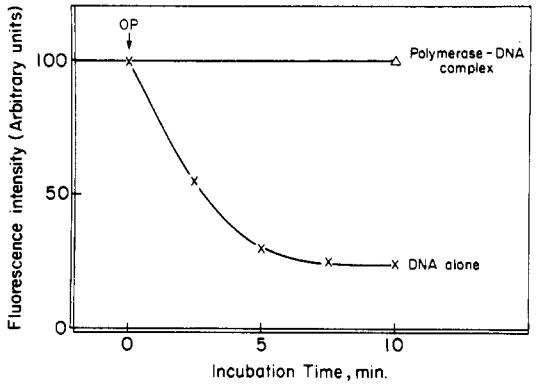

FIGURE 3: Comparison of the effects of 1,10-phenanthroline (OP) on DNA alone and a polymerase–DNA complex. Preincubation of DNA with RNA or DNA polymerase prevents hydrolysis of the template by 2 mM OP and 1 μM Cu^{2+}.

0 to 20 μg/mL (Figure 2, left panel). Linearity persists with further increases up to a template concentration of 100 μg/mL (not shown). The minimal effects on the fluorescence signal produced by OP and Cu^{2+} were corrected for in each assay. Following addition of DNase I (Figure 2, right panel) or OP (Figure 3) to a solution containing DNA, there is a time-dependent decrease in fluorescence which signals template hydrolysis. The rate of hydrolysis by OP increases when the concentration of Cu^{2+} is raised from 10^{-8} to 10^{-5} M. The total extent of hydrolysis is different for double- and single-stranded templates including natural DNAs as well as synthetic ones such as poly(dA-dT) (Table I). Thus, OP-induced hydrolysis depends both upon the presence of Cu^{2+} (Ferrin et al., 1983) and upon the type of template used.

Table I: Effect of [Cu^{2+}] on DNA Hydrolysis by OP[a]

	% hydrolysis		
[Cu^{2+}] (M)	dsDNA	ssDNA	poly(dA-dT), ss
0	0	0	0
10^{-8}	6	23	2
10^{-7}	8	23	16
10^{-6}	22	39	65
10^{-5}	38	58	77

[a] [OP] = 2 mM. Abbreviations: ds, double stranded; ss, single stranded.

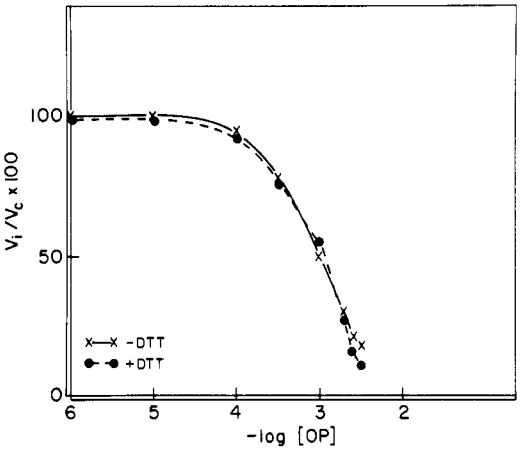

FIGURE 4: Effect on enzyme activity of incubating RNA polymerase II with 1,10-phenanthroline (OP) at concentrations between 10^{-6} and 5×10^{-3} M. V_i is the velocity in the presence of inhibitor and V_c the velocity in its absence. Enzyme assays were performed in the presence (●) or absence (×) of dithiothreitol.

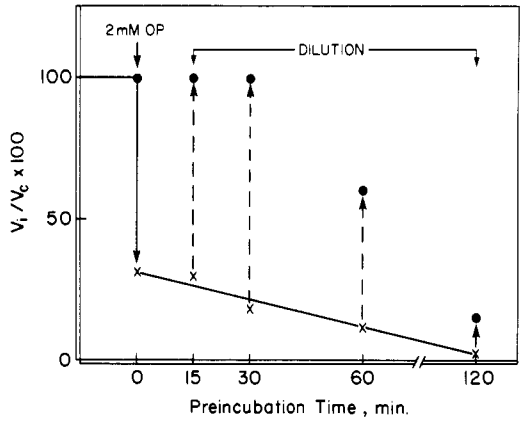

FIGURE 5: Effect on the activity of RNA polymerase II from wheat germ of varying the time of preincubation with 1,10-phenanthroline (OP). Ten micrograms of enzyme was preincubated with 2 mM OP for varying time periods up to 120 min (×). At 0, 15, 30, 60, and 120 min, aliquots of enzyme were diluted with 0.1 M Tris, PH 8.0, buffer, and the activity was assayed. The resultant activity (●) was corrected for dilution.

Hydrolysis of DNA is prevented if the template is incubated with either RNA polymerase II or DNA polymerase I *before* mixing the template with OP. In that case, there is no decrease in fluorescence (Figure 3).

When there is no template hydrolysis, addition of from 10^{-4} to 5×10^{-3} M OP to the assay mixture containing a DNA–RNA polymerase II complex instantaneously inhibits enzyme activity (Figures 4 and 5). In the presence of 10 mM Mg^{2+}, the pK_i's for OP inhibition are 3.4 and 3.0 for *E. gracilis* and wheat germ RNA polymerase II, respectively. There is no inhibition at concentrations of OP below 10^{-4} M. The calculated value for $\bar{n}$ is 1.1, suggesting formation of a 1:1 enzyme–chelate complex.

Table II: Effect of OP and Neocuproine on DNA and RNA Polymerase Activities[a]

	act. $[(V_i/V_c) \times 100]$	
	RNA polymerase II (*E. gracilis*)	DNA polymerase I (*E. coli*)
control	100	100
+neocuproine	90	114
+neocuproine and OP	45	104

[a] [OP] = 2 mM; [neocuproine] = 1 mM.

Inhibition by OP is unchanged even when trace amounts of Cu^{2+} are prevented from forming a complex with the chelating agent. For example, the inhibitory effect of 1 mM OP is unchanged when neocuproine is present (Table II). Neocuproine alone inhibits enzyme activity by only 10%.

Preincubation of the *E. coli* DNA-dependent DNA polymerase I with DNA prevents template hydrolysis by OP–Cu complexes. However, OP does not inhibit this enzyme as is the case for RNA polymerase II (Table II).

The reversibility of inhibition by OP of RNA polymerase II activity depends on the length of time the enzyme is exposed to the chelator. Inhibition is instantaneous and completely reversible when the two are preincubated (in the absence of template) for up to 30 min. Activity, in this case, is determined after dilution of the chelator to a noninhibitory concentration (Figure 5). Longer periods of preincubation, however, result in irreversible inhibition. The inhibition by another chelator, dipicolinic acid, is also instantaneous and completely reversible up to 30 min (not shown).

These experiments show that under the assay conditions described the inhibition by OP of RNA polymerase II is due to chelation of catalytically essential metal atoms associated with the enzymes rather than to hydrolysis of their template. Zinc is the only metal known to be bound tightly to these enzymes; Cu^{2+}, Mn^{2+}, Mg^{2+}, or Fe^{2+} is not present (Falchuk et al., 1976; Jendrisak et al., 1976). The functional role of zinc in the activity of these enzymes is confirmed further by studies with various other chelating agents which inhibit enzyme activity when present in millimolar concentrations (Table III). The nonchelating analogues of OP do not inhibit enzyme activity.

Discussion

The present studies make use of the fluorescence generated by a complex of DAPI with DNA to monitor directly the effects of OP on a DNA template.

The suitability of this approach has been confirmed by a number of observations. Fluorescence intensity has been shown to be dependent on the amount of DNA and to decrease when the DNA is hydrolyzed by a known nuclease, e.g., DNase I (Figure 2). In addition, OP interferes with the fluorescence signal to a miniscule extent. Finally, the method is sensitive enough to allow for the quantitative analysis of the DNA present in assays of both the DNA and RNA polymerases (Table II).

The present results confirm that OP hydrolyzes DNA (Figure 3) and that the rate of breakdown is dependent on the presence of Cu^{2+} as well as on the physical structure of the template. It is greater for synthetic than for native templates and for single-stranded than for double-stranded DNA (Table I). Hydrolysis occurs during the time required for assay of nucleotidyl transferase activity; hence, it is possible that inhibition occurs both by this mechanism and by chelation of the functional metal atoms in these enzymes. The present results show, however, that the nucleolytic complex OP–Cu does not hydrolyze a polymerase–DNA complex during the time interval studied (Figure 3). A mechanism for this protective effect can be suggested from observations with other nucleases. Thus, the rate of hydrolysis of DNA by micrococcal nuclease or DNases I or II is reduced if the nucleic acid is associated with histones or protamines or with either DNA or RNA polymerases, all of which hinder access of the nucleases to the template (Chamberlin, 1976). The polymerases may also hinder access to the sequences in the DNA which seem to be hydrolyzed by OP–Cu. Since both DNA and RNA polymerases bind to and prevent OP hydrolysis of DNA, it was possible to test whether the OP inhibition reported by us for both these RNA polymerases (Falchuk et al., 1976, 1977) and for DNA polymerases by others (Springgate et al., 1973; Slater et al. 1971) occurred in the absence of template breakdown. OP–Cu does inhibit RNA polymerase II activity in the absence of DNA hydrolysis (Figure 4). Therefore, the mode of inhibition in this case must be through chelation of a functional metal atom. In these enzymes, zinc is the only metal present. The inhibition is not due to an effect on the polymerase-activating Mg^{2+} since this metal is present in excess (relative to OP) in the reaction mixture. The functional nature of zinc in the RNA polymerases is consistent with the inhibition studies with structurally different chelating agents (Table I). In contrast, the *E. coli* DNA polymerase I, which is not a metalloenzyme (Walton et al., 1982; Ferrin et al., 1983), is not inhibited by OP (Table II).

It appears that when template is protected by enzyme, OP inhibition can be used to demonstrate the presence of functional zinc atoms in DNA-dependent enzymes. The usefulness of OP as a chelating agent extends beyond this, however. We have shown recently that the pK_i's for OP with the three RNA polymerases found in *E. gracilis* respond characteristically to this inhibitor (Falchuk et al., 1985). Response to OP inhibition might potentially provide another criterion in the recognition and differentiation of these important enzymes.

It is over a decade now since Chang and Bollum (1970) first identified terminal deoxynucleotidyl transferase as a zinc enzyme. This finding led both ourselves and others to examine

Table III: Effect of Different Chelating Agents on RNA Polymerase II Activity

agent	[chelator] (mM)	wheat germ polymerase $[(V_i/V_c) \times 100]$	[chelator] (mM)	*E. gracilis* polymerase $[(V_i/V_c) \times 100]$
control		100		100
dipicolinic acid	4	48		
EDTA			1	25
8-hydroxyquinoline	1	46	3	60
8-hydroxyquinoline-5-sulfonate	1	25	5	60
α,α'-bipyridyl	1	36	4	57
1,10-phenanthroline	1	50	0.3	58
1,10-phenanthroline	5	18	5.0	0
1,7-phenanthroline	1	100	0.3	100
4,7-phenanthroline	1	98	0.3	98

the DNA and RNA polymerases as well as the reverse transcriptases and to identify them as zinc metalloenzymes (Slater et al., 1971; Falchuk et al., 1976, 1977; Auld et al., 1974) and to generalize that zinc was essential for the function of these enzymes in *all* organisms. The recent reexamination of the zinc content of the *E. coli* DNA polymerase and the demonstration that it was not, in fact, a zinc metalloenzyme (Walton et al., 1982; Ferrin et al., 1983) raised questions about the presence and function of zinc in RNA polymerases and reverse transcriptases which merit consideration in the context of the present data.

The data in support of a functional role for zinc in the RNA polymerases are manifold. Many analyses of RNA polymerases from *E. coli*, *E. gracilis*, yeast, and wheat germ by different investigators using different methods consistently have confirmed that these enzymes contain tightly bound zinc (Miller et al., 1979; Falchuk et al., 1976; Auld et al., 1976; Jendrisak et al., 1976). RNA polymerases, from both prokaryotes and eukaryotes, are inhibited by a number of different chelating agents, including OP (Vallee & Falchuk, 1981). The present results show that OP inhibition, in this case, is not due to template hydrolysis.

In the case of *E. coli* RNA polymerase, the zinc atoms can be removed by extensive dialysis with OP but, unlike the case of the corresponding eukaryotic enzymes, activity can be recovered on zinc addition. The specific binding site for zinc in this enzyme has been identified (Miller et al., 1979; Wu et al., 1977). Together, the data permit the conclusion that, as a class, the RNA polymerases are zinc metalloenzymes.

Registry No. OP, 66-71-7; RNA polymerase, 9014-24-8; 8-hydroxyquinoline, 148-24-3; 8-hydroxyquinoline-5-sulfonic acid, 84-88-8; α,α'-bipyridyl, 366-18-7; dipicolinic acid, 499-83-2; Zn, 7440-66-6.

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