Vol. 115, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 15, 1983 Pages 567-576

MECHANISM OF o-PHENANTHROLINE MEDIATED INHIBITION OF E.COLI DNA POLYMERASE I : Formation of Template-Primer-Metal-Phenanthroline complexes with resultant loss of catalytic activity

Kakkudiyil I.Abraham and Mukund J.Modak*

Sloan Kettering Institute for Cancer Research,Walker Laboratory
145 Boston Post Road, Rye, New York 10580

Received August 2, 1983

ABSTRACT : Inhibition of E.coli DNA polymerase I activity by 1,10 phenanthroline in the absence of reducing agents requires a high concentration of inhibitor(1-10 mM) depending upon the template primer used to direct the synthesis. We find that o-phenanthroline,unlike its non-chelating analogue, forms a divalent cation mediated complex with template-primers. Enzyme bound to such complexes is unable to catalyse either polymerization or nuclease functions.

INTRODUCTION: Nucleic acid polymerases have been thought to be zinc-metalloenzymes based on two empirical findings: a) A zinc directed reagent,1,10-phenanthroline(o-phe)[†], but not its non-chelating analogue,m-o-phe,strongly inhibits catalysis of both RNA and DNA synthesis by respective polymerases (1-3) and B) DNA polymerases from E.coli and Avian Myeloblastosisvirus(AMV) have been shown to contain stoichiometric quantities of zinc(4-5). However, inhibition of polymerase activity by o-phe described above was subsequently found to occur through template-primer strand scission mechanism(6-9). The recent re-examination of the zinc content of E.coli DNA polymerase I (pol I) revealed the presence of sub-stoichiometric quantities of zinc in fully active preparations of pol I (10-11). These observations have casted serious doubts on the long held belief that DNA polymerases are zinc metalloenzymes. We have earlier reported that the ribonuclease H activity associated with AMV reverse transcriptase and pol I was resistant to o-phe treatment,although the polymerase activity was sensitive(12).The concentration of o-phe that we found essential for inhibitory activity,however, ranged from 50-100 fold higher than that reported to inhibit these DNA polymerases(1-5,9,13,14) involving the DNA strand scission mechanism. Therefore the inhibition of DNA synthesis requiring high concentrations of o-phe was effected via a mechanism, other than that of DNA strand scission. We therefore examined the mechanism of inhi-

* To whom to address the correspondence.
† Abbreviations Used: pol I, E.coli DNA polymerase I; o-phe, 1,10,o-phenanthroline; dNTP, deoxynucleoside triphosphate; TP, Template-primer.

0006-291X/83 $1.50

bition of pol I by o-phe under these conditions and found that a) both the polymerization and nuclease functions of pol I are inhibited, b) the sensitivity to o-phe inhibition varies with the template primer used to direct synthesis and c) the formation of template-primer-o-phe complexes, which is dependent on the presence of divalent cation, was responsible for the inhibition of catalysis.

MATERIALS AND METHODS :

Materials: Tritiated deoxynucleoside triphosphates(dNTPs) were obtained from New England Nuclear Corporation.Synthetic template-primers and non-radioactive dNTPs were purchased from P.L.Biochemicals Inc.1,10,o-phenanthroline, 2,9 dimethyl o-phenanthroline and m-phenanthroline were the products of Matheson,Coleman and Bell Inc.Preparation of activated DNA and annealing of template primers was essentially as described before(15-16). The homogeneous E.coli DNA polymerase I isolated from E.coli (11) was a generous gift of Dr.L.Loeb of University of Washington,Seattle,Wa.For comparison,we had also included a preparation isolated from a lysogen carrying heat inducible pol I gene,which was kindly donated by Dr.Behnke of University of Cincinnati,Cincinnati,Ohio.Both enzymes gave identical results.

Methods: The conditions for polymerase,3'-5' and 5'-3' exonuclease assays are described below.

DNA polymerase assay: DNA polymerase assays were carried out in a final volume of 100 µL containing : 20 mM Hepes-KOH buffer(pH=7.8),10µg bovine serum albumin,20 µM tritiated dNTP adjusted to a final specific activity of 1000 cpm/pmol, 0.5 µg of template primer,100 mM KCl and 10-20 mM $MgCl_2$. or 1 mM Mn^{2+}. Reactions were initiated by the addition of enzyme and were incubated for 30 min.at 37 C. Acid insoluble radioactivity was determined as described before(15).

3'-5' Exonuclease assay: This assay measures the formation of acid soluble radioactivity formed from 3H-labeled poly(dT)$_{100}$. A radiolabeled poly(dA).(dT) using standard pol I catalysed synthetic reaction also serves as an excellent substrate for 3'-5' exonuclease assay(17).The reaction components are similar to those used for polymerase assay except that no dNTPs are added and 3H-labeled poly(dT) or poly(dA).poly(dT) representing about 50,000 cpm served as a substrate.

5'-3' Exonuclease assay: This assay measures the release of 5',^{32}P-labeled nucleotide (as an acid soluble radioactivity) from a Hinf I fragment of pBR 322 DNA which was labeled at its 5'end by polynucleotide kinase and gamma-32P-labeled ATP. The restriction nuclease digetsed DNA fragments and all the labeling reagents were purchased from New England Nuclear Corp, in a form of a ready to use kit. The labeling protocol was essentially as described by Maxam and Gilbert(18).Reaction mixture composition to measure the 5'-3' exonuclease activity of pol I was identical to that used for 3'-5' exonuclease assay,except that 5 ng of 5',32P-labeled restriction fragment of pBR 322 DNA served as a substrate.

Nitrocellulose - filter binding assay for the formation of Template-primer-o-phe complexes: The protocol to determine the formation of TP-o-phe complex is based on the earlier protocol that we used to measure the formation of Enzyme-TP complexes(19-20).It is described in the legend to figure 2.

RESULTS

o-phe mediated inhibition of pol I is not due to reduced Copper-o-phe complex: o-phe mediated inhibition of pol I as well as other DNA polymerases requires only micromolar concentration of inhibitor and has been shown to result from formation of a reduced Cu-o-phe complexes(6-9). Traces of Cu^{2+} are usually

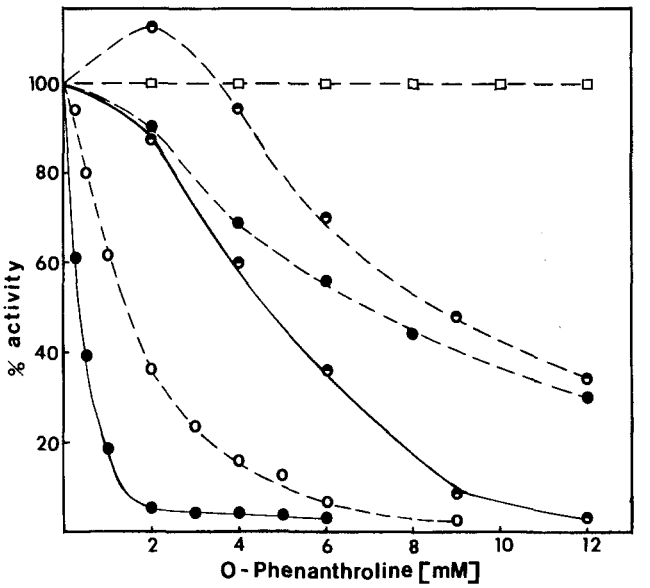


Fig 1: Inhibition of E.coli DNA polymerase I by o-phe with different template-primers: DNA polymerase activity of pol I was measured using poly(rA).(dT)●------●; poly(dC).(dG)○-----○; poly(dA).(dT)◓-----◓;poly(dAT) ○-----○;and activated DNA ◓----◓ as described in materials and methods. Freshly prepared solution of o-phe was added to individual reaction at the desired concentrations. 20 mM Mg^{2+} was used to prevent metal depletion in the assays that used poly(dA).(dT),poly(dAT) and activated DNA as template primers.No inhibition of polymerase activity was observed when o-phe was replaced by 2,9 dimethyl o-phe with any one of the above template-primers(□-----□).One hundred percent activity with various templates, in the order described above, amounted to incorporation of 69,175,148,24 and 60 picomoles of appropriate substrate.

present as contaminant in various reagents and addition of reducing agents, such as mercaptoethanol,to standard reaction mixtures favored the formation of an inhibitory complex(13-14). This complex was shown to cleave template-primers, resulting in the inhibition of catalysis(6-9).The results summarised in Fig.1 and Table 1 clearly indicate that the inhibitory effect of o-phe that we have observed with pol I was not due to the formation of the Cu-o-phe species described above or by the deprivation of essential divalent cation required for the catalytic activity. These conclusions were based on the observations that: i) All of our reagents, except for the divalent metal salts,were chelex treated and the concentration of o-phe required for comparable inhibition of pol I was at least 50-100 fold higher than that observed,when o-phe was provided in the form of its Cu-complex(13-14).ii) The presence or absence of reducing agent,i.e.mercaptoethanol,had no effect on the concentration of o-phe required for inhibition and iii) Addition of exogenous Cu^{2+}, at concentrations as low as 2.5 μM, potentiated the inhibitory effect of o-phe, requiring two orders of magnitude lower concentration, an observation which is in agreement with previous reports (13-14). Since in the absence of Cu^{2+},the concentration of o-phe required to

Table 1: The concentration of o-phe required to inhibit 50% activity of pol I using different template primers and in the presence reducing agent and Cu ions.

Template -primer	Conc.of o-phe needed to effect 50% inhibition	Conc.of o-phe needed to effect 50% inhibition in the presence of 5 μM CuSO4 and 1 mM Mercaptoethanol
Poly(rA).$(dT)_{12-18}$	0.4 mM	40 μM
Poly(dC).$(dG)_{12-18}$	1.5 mM	40 μM
Poly(dA).$(dT)_{12-18}$	4.5 mM	50 μM
Polyd(AT)	7.0 mM	20 μM
Activated DNA	8.0 mM	40 μM

A typical dose response of o-phe concentration on the activity of pol I with different template primers was determined in the presence or absence of exogenously added Cu ions and mercaptoethanol. From these results,the concentration of o-phe required to effect 50% inhibition of activity with desired template primer was computed.

inhibit pol I is significantly higher,the possibility of reduction in the optimal concentration of essential divalent cation by chelation with o-phe had to be ruled out. The finding that o-phe inhibition remained constant, over two fold increase (from 10 mM to 20 mM) in the Mg^{2+} concentration in the assay mixture, ruled out the above possibility(Data not shown).

Effect of o-phe on DNA synthesis directed by various template primers: Pol I has been shown to use a variety of synthetic template primers for catalysis of DNA synthesis.If the inhibition in the presence of o-phe was effected by its reactivity with enzyme protein,the extent of inhibition of DNA synthesis would be identical regardless of the type of template-primer used to direct the synthesis.When sensitivity of DNA synthesis catalysed by pol I to o-phe was examined as a function of template-primer, distinct differences in the degree of sensitivity became apparent(figure 1). There was as much as 20 fold difference in the concentration of o-phe required for the inhibition of synthesis directed by various template-primer, with poly(rA).(dT) being the most sensitive while activated DNA directed synthesis being the least sensitive.Such differences in the senitivity to o-phe were not apparent when traces of Cu^{2+} and mercaptoethanol were included in the reaction mixture(Table 1). Furthermore, a non-chelating analogue of o-phe, m-o-phe as well as 2,9 dimethyl o-phe had no adverse effect on the catalysis directed by any one of the five template-primers, suggesting that the chelating activity of o-phe may be required for the inhibitory effect of o-phe.

Possible targets for o-phe action: Since the zinc-metalloenzyme status of pol I has been seriously questioned(10-11),target site for o-phe,requiring its

chelating function,could conceivably occur in enzyme protein(in the form of as yet unidentified metal),a Mg-dNTP or Mg-template-primer complexes.The possibility of enzyme protein as a target was ruled out by the fact that an extensive(7days) dialysis of enzyme protein against 20 mM o-phe did not inactivate the enzyme(Data not shown). Reactivity of Mg-dNTP and Mg-template primer complexes with o-phe may be expected to occur via the divalent metal portion of the complex,which may conceivably serve as a bridge between the nucleotide(s) and o-phe.The formation of such a structure at the substrate nucleotide level was ruled out by the observations that synthesis directed by two template primers, namely, poly(rA).(dT) and poly (dA).(dT), both of which require dTTP as a substrate, showed distinctly different sensitivity to o-phe (fig 1).Additional support for this interpretation was also obtained by the following experiment: In a reaction mixture containing poly(rA).(dT)together with all other standard components and 1 mM o-phe, no polymerization of dTTP occurs. However, poly (dA).(dT) is added to this reaction mixture,polymerization of dTTP is instantly initiated.Similarly rescue from inhibition may also be observed by the addition of poly(dC).(dG) and dGTP to inhibited reaction containing poly(rA).(dT) and dTTP, as judged by the prompt resumption of dGTP polymerization(Table 2).To inhibit the resumed reactions by the rescuing template-primer, further addition of o-phe, at concentrations known to inhibit catalysis of synthesis directed by the directing template-primer,is required(Table 2). These results rule out both substrate and enzyme as possible targets of o-phe

Table 2: o-phe mediated inhibition of poly(rA).(dT) directed synthesis catalysed by pol I and its rescue by addition of poly(dC).(dG) or poly(dA).(dT).

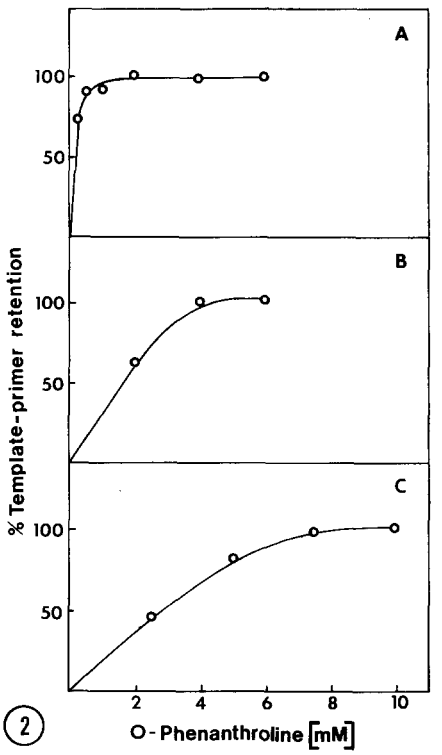
Template primer	concentration of o-phe	^{3}H-dTMP Incorp. (CPM)	^{3}H-dGMP incorp. (CPM)
Poly(rA).(dT)	-	22,000 (100%)	-
"	1 mM	2,600 (12%)	-
" +(dC).(dG)[a]	-	-	110,000(100%)
" + "	1 mM		102,000(93%)
" + "	3 mM		21,800(20%)
Poly(rA).(dT) + poly(dA).(dT)	-	162,000(100%)	
"	1 mM	155,000(96%)	
"	6 mM	16,000(10%)	

DNA polymerase assays were initiated by addition of poly(rA).$(dT)_{12-18}$ to a complete reaction mixture that contained indicated amount of o-phe.The addition of second template primer to this reaction mixture was made after 5 min and incubation was further continued till 30 min.
a : 20 μM 3H-dGTP was also added together with this template primer.

action and suggested that template-primer specific reactivity of o-phe may be responsible for the template specific inhibitory effects of o-phe.

Formation of divalent cation dependent template-primer-o-phe complexes and binding of enzyme to these complexes: In a standard polymerization reaction, binding of enzyme to template-primer occurs quite efficiently.However, the addition of o-phe to the reaction mixture,presumably converts the Mg-TP complex to an o-phe-Mg-TP complex which is likely to interfere in the efficient binding of enzyme to templates due to steric hindrances. To test such a possibility,we employed a classical nitrocellulose filter binding assay which quantitates the ability of enzyme to bind to template primer(19-20). We found that the nitrocellulose filter binding assay could not be used in the presence of o-phe since, in the presence of o-phe,metal-TP complexes,in the absence of added enzyme, were quantitatively retained on the filter.If the divalent cation was deleted from the reaction mixture,no retention of template-primer occurred.Substitution of o-phe by its non chelating analogues, m-ophe and 2,9 dimethyl o-phe, did not permit retention of metal-TP complexes on the nitrocellulose filter(figure 2).Furthermore, nitrocellulose filter bound o-phe-Mg-TP complex could be readily released from filter by washing it with 20 mM EDTA solution, presumably by removal of divalent cation from the complex. Thus, divalent mediated complex formed between TP and o-phe could be specifically quantitated by their ability to bind to nitrocellulose filter.We therefore, determined the formation of o-phe-Mg-TP complex as a function of o-phe concentration using 3 different template-primers. Results presented in figure 2 clearly show that with increasing concentration of o-phe,an increased quantity of o-phe-Mg-TP complex is formed as judged by its retention on the filter until most of the added template-primer is retained on the filter(fig 2). Furthermore, different template-primers required different concentration of o-phe to effect their complete retention on filter.The concentration of o-phe required for the complete inhibition of synthesis directed by a particular template-primer was also the concentration needed to convert that template-primer to its nitocellulose filter binding form.

Binding of enzyme to o-phe-Mg-TP complex:To assess the ability of pol I to to bind to o-phe-Mg-TP complexes, we employed a gel filtration technique using a Sephacryl S200 column. The columns were equilibrated with 50mM Hepes buffer(pH=7.8) containing 0.1% albumin,10 mM Mg^{2+} in the absence or presence of 10 mM o-phe.The enzyme-TP complexes were formed using poly(dA).(dT) in standard reaction mixture in the presence or absence of 10 mM o-phe and the extent of E-TP complex formation was determined by their relative migratory patterns upon gel-filtration.Results presented in figure 3 clearly show that o-phe does not interfere in the binding of pol I to template-primer as



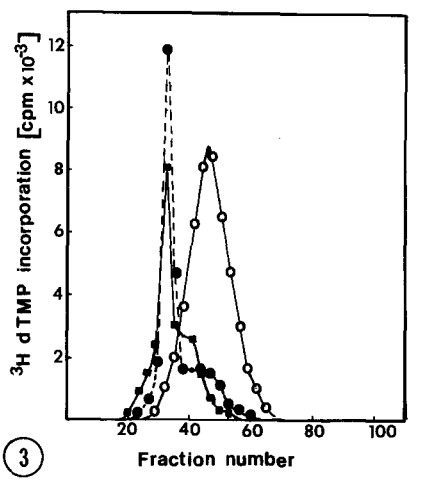


Fig 2: Retention of o-phe-Mg-Template primer complexes on Nitrocellulose filters as a function of o-phe concentration: A standard reaction mixture containing 0.5 µg of 3H-labeled template-primer representing about 50,000 cpm were incubated with increasing concentration of o-phe,in the absence of enzyme and substrate.After 10 min at 20°C,reaction mixtures were filtered through a presoaked nitrocellulose membranes(BA-85 ,0.45µ,a product of Schleicher & Scheull) at a flow rate of 15ml/hr.Filter was washed once with 0.5 ml of reaction mixture lacking template-primer, dried and counted for radioactivity(19-20). Less than 3% retention of template-primers on the filter was noted when o-phe was replaced by m-phe or 2,9 dimethyl o-phe or when Mg^{2+} was deleted from the reaction mixture. The retained template-primer-ophe complex could also be released by a single wash with 20 mM EDTA soln. Frames A,B and C represent % retention of 3-H-labeled poly(rA).(dT),poly(dC).(dG) and poly(dA).(dT) on nitrocellulose filter.

Fig 3: Gel-filtration profile of Enzyme-Template primer complex in the presence and absence of o-phe: Enzyme-template primer complexes were formed in a standard reaction mixture that contained 50 mM Hepes buffer(pH=7.8),20mM MgCl2, 2 µg of poly(dA).(dT) and 50 ng pol I. The extent of complex formation was determined by applying the preincubated mixture to a pre-equilibrated column(14x0.9cm) of Sephacryl S200. Flow rate was maintained at 20 ml/h and aliquots of 200 µL were collected. An assay with poly(dA).(dT) was carried out to determine the position of free enzyme(O----O) and enzyme bound to poly(dA).(dT) denoted by (■----■). Effect of addition of 10 mM o-phe on the formation of E-TP complexes is denoted by (●----●). o-phe addition has no effect on the elution pattern of free enzyme(data not shown).

judged by the identical elution profile of E-TP complexes in the presence or absence of o-phe.

Effect of o-phe on the exonuclease activities associated with pol I :

Since o-phe mediated inhibition of polymerization reaction appeared to be

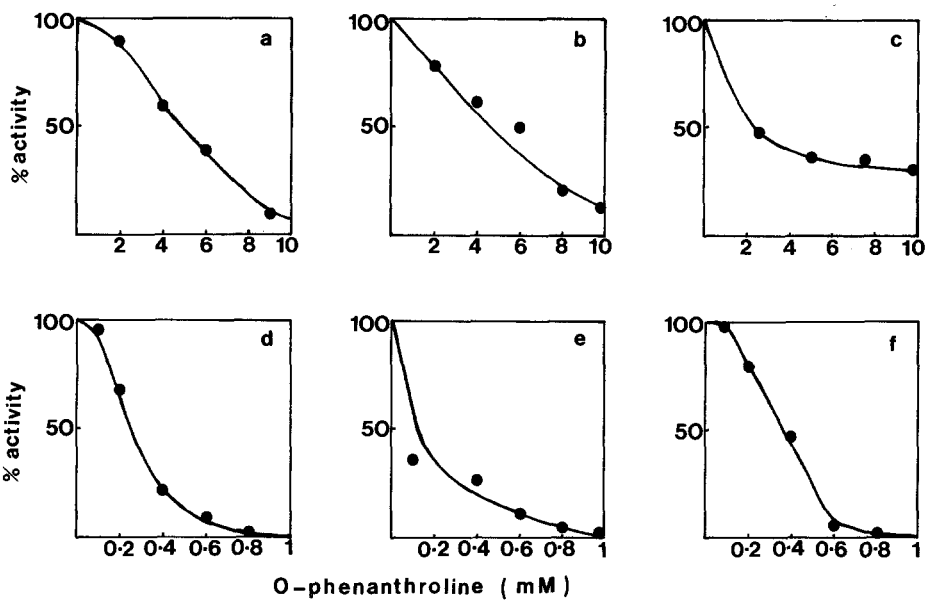


Fig 4: Effect of o-phe on the polymerase,3'-5' and 5'-3'exonuclease activities of pol I in the presence of Mg^{2+} or Mn^{2+} : The assay conditions for exonuclease assays are described in materials and methods.Polymerase reaction was carried out in the presence of poly(dA).(dT) as a template-primer.A dose response of o-phe on the various activities in the presence of 20 mM Mg (Frames A,B and C) or 2 mM Mn is shown in frames D,E and F.

due to the divalent dependent complex formation between TP and o-phe and since TP-metal complexes also serve as substrate for exonuclease activities associated with pol I, it was important to determine if nuclease activity could be observed with o-phe complexed substrates.Results presented in fig 4 show that both 3'-5' and 5'-3' exonuclease activities are sensitive to o-phe addition and we conclude that catalysis of nuclease activities associated with pol I is blocked by o-phe complexed to polynucleotide substrates.

DISCUSSION

In our studies dealing with o-phe mediated inhibition of DNA polymerases and associated activities(12,21,22),we had noted the need for unusually high concentrations of o-phe in order to observe the inhibitory effect. These observations suggested that a mechanism distinct from the one requiring low concentrations of o-phe and presence of reducing agents, was involved in the observed inhibition.It had been our tentative assumption that zinc,thought to be an intrinsic part of many DNA polymerases until recently,was the probable target of o-phe. With two recent reports indicating a lack of zinc in pol I (10,11),a detailed investigation was begun to determine the actual mechanism of inhibition of pol I by o-phe.There appeared to be 3 possible targets for o-phe action which are: a) enzyme protein itself via some hydrophobic interaction with o-phe or through a hitherto unknown metal component present in

pol I, b) metal-dNTP chelate and c) metal-template-primer complex. The last two presumably react with o-phe through their (divalent) metal moiety. Involvement of enzyme protein as a possible target for o-phe reactivity was ruled out since no loss of enzyme activity could be observed upon prolonged dialysis against 20 mM o-phe and the inhibitory effect of o-phe was different with different template primers.The observations that the inhibition of polymerization of the same dNTP by pol I required vastly different concentrations of o-phe,depending upon the template-primer, ruled out metal-dNTPs as a target for o-phe reactivity.The possibility that the template-primer itself may react with o-phe was investigated.Requirement for the presence of a divalent cation for the persistant inhibitory effect was also implied by the fact that the non-chelating analogues of o-phe were ineffective inhibitors.Detailed examination of the dose response of o-phe on the metal-TP complexes indeed revealed that the various template-primers required varying concentration of o-phe in order to form a stable complex as judged by the selective retention of such complexes on nitrocellulose filter(fig 2).The formation of such complexes has not been known before and conceptually it resembles to a tricomponent complex involving Mn^{2+}, dNTP and Pi,which we have reported before(23).The tricomponent complex involving o-phe, divalent cation and Template-primer, was tentatively concluded to be the species responsible for the inhibition of catalysis.When binding ability of pol I to template-primer-o-phe-metal complex was examined,we were surprised to note that the enzyme was fully capable of binding to template primers complexed to o-phe(fig 3). We therefore postulate that enzyme bound to o-phe complexed template-primers may not be able to continue its movement along the template strand due to the steric interference caused by o-phe bound to metal-TP complexes.Some support for this hypothesis is provided by the fact that both 3'-5' and 5'-3' exonuclease activities associated with pol I , which also require movement of enzyme along the metal-phosphate backbone of the polynucleotide substrate, are sensitive to o-phe addition.

ACKNOWLEDGEMENTS: This research was supported by a grant from NIH and by a research career development award# 1 K04 CA-545 to MJM.

REFERENCES:

1) Slater,J.P.,Mildvan,A.S. and Loeb,L.A.(1971) Biochem.Biophys.Res.Commun.44:37
2) Springgate,C.F.,Mildvan,A.S.,Abramson,R.,Engle,J.F. and Loeb,L.A. (1973) J.Biol.Chem. 248: 5987
3) Poiesz,B.J.,Battula,N. and Loeb,L.A.(1974)Biochem.Biophys.Res.Commun.56:959
4) Poiesz,B.J.,Seal,G. and Loeb,L.A.(1974) Proc.Natl.Acad.Sci.71:4892
5) Auld,D.S.,Kawaguchi,S.,Livingston,D.M. and Vallee,B.L.(1974) Proc.Natl.Acad.Sci. 71:2091
6) Que,B.G.,Downey,K.M. and So,A.G.(1980) Biochemistry 19:5987
7) Downey,K.M.,Que,B.G. and So,A.G.(1980) Biochem.Biophys.Res.Commun.93:264
8) Sigman,D.S.,Graham,D.R.,D'Aurora,V. and Stern,A.M.(1979)J.Biol.Chem.254:12269

9) Marshall,L.E.,Graham,D.R.,Reich,K.A. and Sigman,D.S.(1981)Biochemistry 20:244.
10) Walton,K.E.,FitzGerald,P.C.,Herrman,M.S. and Behnke,W.D.(1982) Biochem.Biophys.Res.Commun. 108: 1353
11) Ferrin,L.J.,Mildvan,A.S. and Loeb,L.A.(1983) Biochem.Biophys.Res.Commun. 112: 723.
12) Modak,M.J. and Srivastava,A.(1979) J.Biol.Chem.254: 4756.
13) D'Aurora,V.,Stern,A.M. and Sigman,D.S.(1977) Biochem.Biophys.Res.Commun.78:170
14) D'Aurora,V.,Stern,A.M. and Sigman,D.S.(1978) Biochem.Biophys.Res.Commun.80:1025
15) Marcus,S.L.and Modak,M.J.(1976) Nucleic Acids Res. 3:1473
16) Modak,M.J. and Marcus,S.L.(1977) J.Biol.Chem.252:11
17) Modak,M.J.,Marcus,S.L.and Cavalieri,L.F.(1974) J.Biol.Chem.249:7373
18) Maxam,A.M. and Gilbert,W.(1977) Proc.Natl.Acad.Sci. 74:560
19) Srivastava,S.K.and Modak,M.J.(1982) Biochemistry 21:4633
20) Srivastava,S.K.and Modak,M.J.(1983) Biochemistry 22:1983
21) Srivastava,A. and Modak,M.J.(1980) J.Biol.Chem. 255:2000
22) Srivastava,A. and Modak,M.J.(1980) Biochemistry 19:3270
23) Modak,M.J.,Rao,K. and Marcus,S.L.(1982) Biochem.Biophys.Res.Commun.107:811