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DEGRADATION OF DNA BY 1,-10-PHENANTHROLINE

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SUMMARY

The metal chelator 1,10-phenanthroline, in the presence of a reducing agent and a copper salt, causes the degradation of double-stranded DNA to acid soluble fragments. The degradation of DNA is dependent on the presence of O_2. The concentrations of 1,10-phenanthroline which are effective in degrading DNA are of the same order of magnitude as those that result in inhibition of nucleotidyl transferase reactions. The requirement for a copper salt can only be demonstrated when all reagents are treated with Chelex to remove metal contaminants. It is proposed that the degradation of DNA in the presence of 1,10-phenanthroline may account for the *in vitro* inhibition of DNA and RNA synthesis seen with this metal chelator, rather than any effect on nucleotidyl transferases.

INTRODUCTION

The metal chelator 1,10-phenanthroline has been reported to inhibit the activity of a wide variety of nucleotidyl transferases, e.g., DNA polymerases (1-7), RNA polymerases (6, 8-16), reverse transcriptases (9, 17-23) and terminal transferase (24,25). Many of these enzymes have been shown to contain zinc, in some cases in stoichiometric amounts (2,3,10,12,15,17,19,21).

Until recently, it was generally believed that the inhibitory effect of 1,10-phenanthroline on nucleotidyl transferases was the result of either removal of zinc from the protein or binding of phenanthroline to protein-bound zinc; and it was postulated that zinc was involved in catalysis (2,3,26). However, D'Aurora *et al.* (5,6) have demonstrated that the inhibition of several DNA and RNA polymerases by 1,10-phenanthroline is

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dependent upon the presence of a reducing agent and that inhibition is potentiated by copper salts. They have suggested that a phenanthroline-Cu(I) complex, which is fortuitously complementary to the active site of virtually all nucleotidyl transferases, is the true inhibitory species. They have further suggested that the trace amounts of Cu(II) present in reagent grade chemicals would be reduced to Cu(I) by the sulfhydryl compounds usually included in polymerase reaction mixtures.

We have recently extended the observations of D'Aurora et al. and have demonstrated that, of the two enzymatic activities associated with the "large fragment" of DNA polymerase I, only the polymerase activity is inhibited by 1,10-phenanthroline whereas the 3' to 5' exonuclease activity is not inhibited (7). This inhibition required 2-mercaptoethanol and was potentiated by the addition of $CuSO_4$. The ribonuclease H activity associated with AMV reverse transcriptase has also been reported to be resistant to 1,10-phenanthroline although the polymerase activity is extremely sensitive to this inhibitor (23).

In this communication we shall present evidence that 1,10-phenanthroline causes rapid and extensive degradation of double-stranded DNA under conditions similar to those used to measure the activity of nucleotidyl transferases.

MATERIALS AND METHODS

[^{3}H]ϕX174-RF DNA, 4×10^4 cpm/µg, was purchased from Bethesda Research Labs and [^{3}H]dTTP from ICN. 1,10-phenanthroline and 4,7-phenanthroline were obtained from K & K Chemicals. Unlabeled nucleotides and the "large fragment" of DNA polymerase I were from Boehringer Corp. Chelex was purchased from BioRad.

Distilled water and all solutions other than metals salts were Chelex-treated as previously described (7). [^{3}H]poly (dA-dT) was synthesized with the "large fragment" of DNA polymerase I using 33 µM dATP and [^{3}H] dTTP, 130 cpm/pmole, as described by Modrich and Lehman (27).

Degradation of [^{3}H] poly (dA-dT) was followed by acid solubilization. The reaction mixture contained in a final volume of 0.1 ml; 50 µM 1,10-phenanthroline; 50 mM Hepes Buffer, pH 7.4; 20 µM [^{3}H]poly (dA-dT); 1 mM 2-mercaptoethanol and 2.5 µM $CuSO_4$. After 30 min at 25° C, the reaction

was stopped by the addition of 0.01 ml 1 M EDTA, pH 8.0; 0.1 ml salmon sperm DNA, 2 mg/ml; and 0.5 ml 1 M perchloric acid containing 10 mM sodium pyrophosphate. After standing in ice for 10 min, the solution was centrifuged at 7,500 rpm for 10 min and a 0.1 ml aliquot of the supernatant was counted in 10 ml of Biofluor in a liquid scintillation spectrometer.

Degradation of [^{3}H]ϕX174-RF DNA was followed by neutral sucrose density gradient sedimentation. The reaction mixture contained in a final volume of 0.1 ml; 50 mM Hepes, pH 7.4; 0.5 µg [^{3}H]ϕX174-RF DNA; 100 µM 1,10-phenanthroline; 50 µM EDTA; 0.5 mM 2-mercaptoethanol; and 1 µM $CuSO_4$. After 50 min at 25°C, the reaction was stopped by the addition of 0.01 ml 1M EDTA, pH 8.0. The solution was layered on a 5 to 20% sucrose gradient containing 10 mM Tris·HCl, pH 7.8; 0.3 M NaCl; 0.1 mM EDTA and 0.15% Sarkosyl, and centrifuged in a SW-65 rotor at 49,000 rpm for 240 min at 4°C. The gradient was collected in 40 fractions and aliquots of 0.02 ml were spotted on 2.4 cm GF/B glass fiber filters, dried and counted in 10 ml of toluene-Omnifluor scintillant in a liquid scintillation spectrometer.

RESULTS AND DISCUSSION

The degradation of [^{3}H]poly (dA-dT) by 1, 10-phenanthroline is shown in Figure 1, where the degradation of DNA as a function of time is followed by increased acid solubility. The degradation of DNA by 1,10-phenanthroline requires the presence of $CuSO_4$ and a reducing agent, e.g., 2-mercaptoethanol. Non-chelating analogs such as 4,7-phenanthroline did not cause degradation of [^{3}H]poly (dA-dT). Chelex-treatment of all reagents to remove contaminating metals was necessary to demonstrate a requirement for $CuSO_4$.

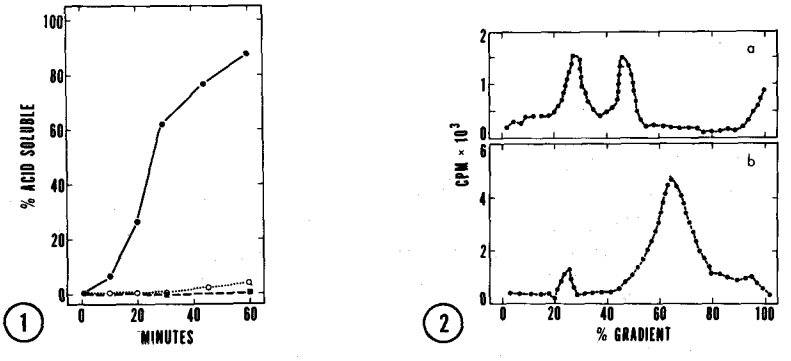


Figure 1: Degradation of [^{3}H]poly (dA-dT) as a function of time in the presence of 50 µM 1,10-phenanthroline, 1 µM $CuSO_4$ and 1 mM 2-mercaptoethanol (●). Controls were in the absence of $CuSO_4$ (O) and in the absence of 2-mercaptoethanol (■).

Figure 2: Neutral sucrose gradient sedimentation of [^{3}H]ϕX174-RF DNA either untreated (panel A) or treated with 100 µM 1,10-phenanthroline, 1 µM $CuSO_4$ and 0.5 mM 2-mercaptoethanol (panel B). The direction of sedimentation is from right to left.

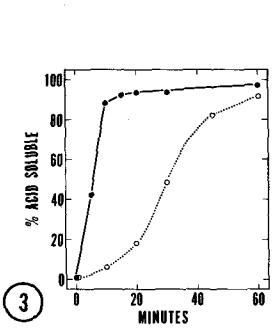
The degradation of [^{3}H]poly (dA-dT) is non-linear in time when followed by acid solubility (Figure 1), suggesting that the initial products of the reaction may be DNA fragments that are not acid soluble. That this is the case as seen in Figure 2 where the effect of 1,10-phenanthroline on [^{3}H]ϕX174-RF DNA was followed by sedimentation in a neutral sucrose gradient. Following incubation with 1,10-phenanthroline, $CuSO_4$ and 2-mercaptoethanol, nearly all of the full length molecules (21S supercoiled form and 16S relaxed circular and linear forms) were converted to slowly sedimenting species; however, under the reaction conditions, little of the [^{3}H]ϕX174-RF DNA was rendered acid soluble.

Table I shows the concentrations of 1,10-phenantroline, $CuSO_4$ and 2-mercaptoethanol which are effective in causing the degradation of DNA. In the presence of optimal concentrations of the other two reagents, the rate of DNA degradation is maximal above 50 μM 1,10-phenanthroline, 2.5 μM $CuSO_4$

Table 1: Effective Concetrations of 1,10-Phenanthroline, $CuSO_4$ and 2-Mercaptoethanol

1,10-phenanthroline conc. (μM)	$CuSO_4$ conc. (μM)	2-mercaptoethanol conc. (mM)	% acid soluble
0	2.5	1.0	<1
10	2.5	1.0	1
30	2.5	1.0	45
50	2.5	1.0	90
100	2.5	1.0	97
50	0	1.0	3
50	0.5	1.0	21
50	1.0	1.0	51
50	2.5	1.0	97
50	5.0	1.0	99
50	2.5	0	<1
50	2.5	0.1	15
50	2.5	0.5	85
50	2.5	1.0	85

Except for the concentrations of 1,10-phenanthroline $CuSO_4$ and 2-mercaptoethanol which were as indicated, the reaction conditions were as described in Materials and Methods for the degradation of [^{3}H]poly (dA-dT).



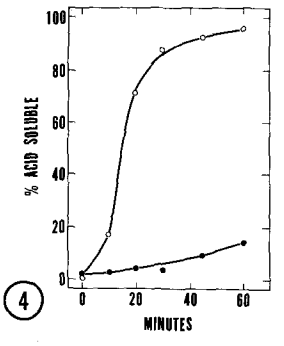


Figure 3: Degradation of [^{3}H]poly (dA-dT) by 100 μM 1,10-phenanthroline as a function of time at 37° C in the presence (●) and absence (O) of 2.5 μM $CuSO_4$. Reaction mixtures contained in a final volume of 0.1 ml: 67 mM potassium phosphate, pH 7.5; 6.7 mM $MgCl_2$; 33 μM dATP and dTTP; 1 mM 2-mercaptoethanol; and 20 μM [^{3}H]poly (dA-dT). Solutions were not treated with Chelex.

Figure 4: Degradation of [^{3}H]poly (dA-dT) as a function of time in the presence of either air (O) or nitrogen (●). Reaction conditions were as described in Materials and Methods.

and 0.5 mM 2-mercaptoethanol. These optimal concentrations are of the same order of magnitude as those which have been found to be effective in inhibiting nucleotide polymerization reactions (5-7).

It is likely that degradation of DNA by 1,10-phenanthroline can occur under conditions usually employed to assay DNA and RNA polymerases. As shown in Figure 3, under typical assay conditions for DNA polymerase I, [^{3}H]poly (dA-dT) degradation occurs in the absence of added $CuSO_4$, although the addition of 2.5 μM $CuSO_4$ markedly increases the rate of degradation. The degradation of DNA in the absence of added $CuSO_4$ is probably due to contamination of reagent grade chemicals with Cu(II) (5), since with Chelex-treated reagents no degradation was observed (Figure 1). The requirement for a reducing agent for DNA degradation by 1,10-phenanthroline is absolute (Figure 1), as is the case for inhibition of nucleotide polymerization (5-7). Since most DNA and RNA polymerases require added sulfhydryl compounds for activity, this requirement for DNA degradation is almost always present in assays for 1,10-phenanthroline inhibition.

In addition to a reducing agent and a copper salt, degradation of DNA by 1,10-phenanthroline is also dependent upon the presence of molecular oxygen.

As shown in Figure 4 the degradation of DNA is markedly inhibited when the reaction is carried out in the presence of nitrogen rather than air. The absolute requirement for a reducing agent (2-mercaptoethanol) and oxygen for degradation of DNA suggests that a reduced form of oxygen, most likely either superoxide radical or hydroxyl radical, may be the reactive species responsible for the degradation of DNA. This appears plausible since a phenanthroline-Cu(II) complex has been shown to catalyze the air oxidation of sulfhydryl groups in proteins (28) as well as other sulfhydryl compounds (29), and the autooxidation of thiols has been shown to result in the generation of free radicals of oxygen (30).

The present studies demonstrate that, under conditions where 1,10-phenanthroline has been found to inhibit nucleotide polymerization reactions, extensive degradation of DNA occurs. Furthermore, the requirements for DNA degradation are virtually identical to the requirements for inhibition of nucleotide polymerization. It is postulated that DNA degradation is the mechanism of inhibition of nucleotide polymerization, rather than any effect on nucleotidyl transferases. The fact that most nucleotidyl transferases are zinc-containing enzymes and are apparently inhibited by 1,10-phenanthroline has led to the widely accepted hypothesis that zinc is present at the active site of these enzymes and is mechanistically involved in nucleotide polymerization (26). Although the present studies do not rule out a functional role for zinc in nucleotide polymerization, the data supporting this hypothesis that are based on 1,10-phenanthroline inhibition studies must clearly be re-evaluated.

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