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Cleavage of Deoxyribonucleic Acid by the 1,10-Phenanthroline–Cuprous Complex. Hydrogen Peroxide Requirement and Primary and Secondary Structure Specificity†

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ABSTRACT: 1,10-Phenanthroline (OP) coordinated to copper ion cleaves DNA in a reaction requiring hydrogen peroxide and thiol. The alternating copolymer poly([^{3}H]dA-[^{3}H]T) (10 μg/mL) is made 95% acid soluble in less than 30 s with 20 μM OP, 2 μM copper ion, 7 mM 3-mercaptopropionic acid, and 7 mM hydrogen peroxide under both aerobic and anaerobic conditions. In the absence of added hydrogen peroxide, oxygen is required for the thiol-mediated scission of DNA by 1,10-phenanthroline and copper ion because it serves as a precursor for hydrogen peroxide formed as a result of the in situ oxidation of thiol catalyzed by the 2:1 phenanthroline–cupric complex. In the absence of 1,10-phenanthroline and copper ion, hydrogen peroxide does not measurably degrade poly(dA-dT). In the presence of the ligand and metal ion, the reaction is much faster when both hydrogen peroxide and thiol are present than when either is present alone. The involvement of the cuprous complex is inferred from the requirement for reducing agent and from the inhibition of the reaction by cuprous specific ligands. Intercalation of 1,10-phenanthroline into the DNA during the course of the reaction is suggested by (1) inhibition of DNA scission by intercalating agents such as ethidium bromide and (2) a strong, if not absolute, preference for double-stranded [poly(dA)·poly(T)] as compared to single-stranded [poly(dA) or poly(T)] DNA as a substrate for the cleavage. Electrophoresis of a fragment of *E. coli lac* operon reacted with the coordination complex revealed scission of nearly equal intensity at every base, suggesting little, if any, primary sequence specificity in the reaction. Because of this lack of specificity, this chemistry may be useful in studying the primary sequence specificity of other agents in their interactions with DNA.

1,10-Phenanthroline inhibits the poly(dA-T)-directed polymerization catalyzed by *E. coli* DNA polymerase I via a mechanism dependent on cupric ion and thiol (D'Aurora et al., 1977, 1978). Recently, we have reported that the 2:1 1,10-phenanthroline–cuprous complex [$(OP)_2Cu^+$][1] formed under these conditions cleaves DNA in an oxygen-dependent reaction and that the products of this reaction are effective inhibitors of Pol I (Sigman et al., 1979). The structure(s) of the inhibitory product(s) are not yet known.

Direct physical evidence for the scission reaction has been provided by electrophoretic analysis of poly(dA-T) and SV40 supercoiled DNAs which had been incubated with OP, Cu^{2+}, and thiol under aerobic conditions (Sigman et al., 1979). Downey et al. (1980), working independently, have confirmed that $(OP)_2Cu^+$ reacts with DNA in an oxygen-dependent reaction by showing that the coordination complex alters the sedimentation properties of φX174-RF DNA and releases acid-soluble counts from ^{3}H-labeled poly(dA-T). Since the production of acid-soluble counts from radioactively labeled poly(dA-T) is a more rapid assay than electrophoresis and better reflects the cleavage chemistry than the production of inhibitors of *E. coli* Pol I, we had been using it to study the role of molecular oxygen in the cleavage chemistry and to examine the effectiveness of free-radical traps and intercalating agents as inhibitors of this novel scission reaction.

Previously, we reported that hydrogen peroxide must be involved in the cleavage because catalase blocked the reaction between the coordination complex and poly(dA-T). In the present paper, we demonstrate that the exclusive role of molecular oxygen is to serve as a precursor for hydrogen peroxide in the $(OP)_2Cu^{2+}$-catalyzed oxidation of thiol (Graham et al.,

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[1] Abbreviations used: OP, 1,10-phenanthroline; $(OP)_2Cu^+$, 2:1 1,10-phenanthroline–cuprous complex; $(OP)_2Cu^{2+}$, 2:1 1,10-phenanthroline–cupric complex; 4′,6-diamidino-2-phenylindole bis(hydrochloride); SOD, superoxide dismutase; Pol I, *E. coli* DNA polymerase I.

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1980). In the presence of excess thiol, $(OP)_2Cu^+$ accumulates and together with hydrogen peroxide reacts with DNA. We further show that the cleavage reaction is relatively nonspecific with respect to primary structure but that double-stranded DNA is greatly preferred to single-stranded DNA as a substrate for this DNase-like activity. The requirement for double helical DNA suggests that the coordination complex may intercalate into the DNA during the reaction. This conclusion is strongly supported by the potent inhibition of the cleavage reaction by intercalating agents and by the known DNA binding properties of the phenanthroline nucleus.

Experimental Procedures

Materials

Poly([^{3}H]dA-[^{3}H]T) was prepared by using 4 units of *E. coli* DNA polymerase I (Boehringer) in a polymerization reaction primed with unlabeled poly(dA-T). In addition to the enzyme, the reaction mixture contained poly(dA-T) (2 μg/mL), [8-^{3}H]dATP, tetrasodium salt (0.3 μmol/mL, specific activity 25 μCi/μmol), and [*methyl*-^{3}H]TTP, tetrasodium salt (0.3 μmol/mL, specific activity, 25 μCi/μmol) in a total volume of 2.0 mL buffered at pH 7.0 with 0.1 M Tris acetate and 5 mM magnesium acetate. It was equilibrated at 37 °C prior to the addition of 100 μL of enzyme and then incubated for 14 h at 37 °C. The reaction was monitored by following the incorporation of radioactivity into an acid-precipitable form (see below). When no further significant increase was observed, the enzyme was inactivated by adding 2 mL of 0.4 M NaCl to the incubation mixture and then heating at 75 °C for 15 min and cooling in an ice bath. Unincorporated mononucleotides were removed by dialysis against 0.2 M NaCl with several changes of dialysate and finally against deionized distilled water. The specific activity of the product was 5×10^{10} cpm/mmol of mononucleotide. Poly([^{3}H]dA-[^{3}H,^{32}P]T) was prepared similarly with the exception that [^{3}H,α^{32}P]TTP (specific activity ^{32}P, 47 μCi/μmol, ^{3}H, 25 μCi/μmol) was included in the reaction mixture; poly(dA-[^{3}H]T) was prepared by using [^{3}H]TTP (specific activity 50 μCi/μmol) as the only labeled nucleotide.

Poly(dA)–poly([^{3}H]dT) was prepared by adding 15 units of *E. coli* polymerase I to a 0.250-mL assay mixture buffered at pH 7.0 with 0.1 M Tris acetate and 5 mM magnesium acetate and containing 50 μg of poly(dA) and 0.3 μmol of [^{3}H]TTP (specific activity 1.77×10^{10} cpm/mmol). The mixture was incubated with the enzyme for 28 h at 37 °C and monitored by assaying incorporation of counts into an acid-precipitable form.

The cleavage of the polynucleotide product was carried out without heat inactivation of the enzyme or removal of the untreated triphosphates by dialysis. Poly([^{3}H]dA)–poly(T) was similarly prepared except that a 0.500-mL assay mixture contained 100 μg of poly(T) and 0.6 μmol of [^{3}H]dATP (specific activity 1.75×10^{10} cpm/mmol).

A 60-base-pair fragment of the *E. coli lac* operon was a gift of Dr. J. Gralla, J. Stefano, and A. J. Carpousis (Stefano & Gralla, 1979). It is prepared from a *lac*-containing plasmid (pMB 9-UV5) which contains a 203-base-pair *E. coli lac* control region which can be excised by digestion with *Eco*R1 and isolated on an 8% polyacrylamide gel. After cleavage by *Hha*I, the 60-base-pair fragment can be separated by polyacrylamide gel electrophoresis. The 5′ end was labeled with [γ-^{32}P]ATP and T-4 polynucleotide kinase.

The chemicals listed below were obtained from the following suppliers: 1,10-phenanthroline (Aldrich); 3-mercaptopropionic acid (Aldrich); cupric sulfate (Mallinckrodt); ascorbic acid (Eastman); deoxynucleotide triphosphates (Sigma); radioactive deoxynucleotide triphosphates (ICN); poly(dA-T) (Miles); poly(dA) and poly(dT) (P-L Biochemicals); xanthine, xanthine oxidase, and bovine erythrocyte superoxide dismutase (Sigma). [*methyl*-^{3}H]Polythymidylate (s_{20} = 5.5 s; specific activity 17.4 μCi/μmol of P) and [8-^{3}H]polydeoxyadenylate (s_{20} = 7.4 s; specific activity 46.4 μCi/μmol of P) were obtained from Miles Laboratories, Inc.

Methods

Anaerobic experiments were performed in a Plas-Labs XPL 555 Anaerobic Chamber. Two assays of the DNA cleavage reaction are relevant to the present communication. The assay involving the inhibition of *E. coli* DNA Pol I has been previously described (Sigman et al., 1979).

The assay involving the formation of acid-soluble products was carried out in the following manner. Solutions of the coordination complex were prepared by adding aliquots of stock solutions of OP and $CuSO_4$, both dissolved in 0.1 M Tris acetate, pH 7.0, buffer containing 5 mM magnesium acetate, to solutions containing reducing agents in the same buffer. Unless otherwise indicated, OP was present in 10-fold excess relative to added copper ion. When a concentration of $(OP)_2Cu^+$ is designated, it refers to the concentration of added copper ion. This approximation is based on the assumption that OP, at ten times the concentration, will sequester all the metal as $(OP)_2Cu^{2+}$ which, in turn, will be quantitatively reduced to $(OP)_2Cu^+$. The concentration of $(OP)_2Cu^+$ is overestimated because (a) polynucleotides are effective ligands and will compete with the OP for copper and (b) $(OP)_2Cu^+$ will be oxidized either by molecular oxygen or by hydrogen peroxide. $(OP)_2Cu^+$ prepared in situ by reduction of $(OP)_2Cu^{2+}$ was used instead of crystalline $(OP)_2Cu^+$ because of this susceptibility to oxidation under the conditions of the assay. Only micromolar amounts of the complex are required for cleavage, and it is not practical to reduce oxygen to these levels. Solutions of the poly(dA-T) were prepared separately by adding 0.05 mL of a 60–100 μg/mL solution of poly(dA-T) to 0.20 mL of the pH 7.0 Tris acetate buffer containing 5 mM magnesium acetate. Any other reaction component (e.g., hydrogen peroxide, ethanol, 2-propanol, xanthine, xanthine oxidase) was added to the buffer prior to the DNA. The two solutions were equilibrated with air and incubated at 37 °C for 5 min.

The reactions were initiated by adding 225 μL of the $(OP)_2Cu^+$ solution to 225 μL of the DNA solution. Aliquots (50 μL) were removed at the time intervals indicated and spotted on Whatman glass microfiber filters (2.4 cm). The filters were then washed twice in an ice-cold solution containing 5% trichloroacetic acid and 1.5% sodium pyrophosphate and then dried in 95% ethanol for 15 min and diethyl ether for 15 min. The dried filters were counted in 3.0-mL scintillation cocktail (Research Products International 3A20).

The *lac* operon restriction fragment was cleaved by adding 4 μL of a solution containing OP (40 μM), $CuSO_4$ (4.0 μM), and mercaptopropionic acid (14 mM) buffered with 0.1 M Tris acetate–5 mM $Mg(OAc)_2$, pH 7.0, to a 4-μL solution containing the hot fragment and 20 μg/mL carrier poly(dA-T) in the same buffer. The reaction was incubated at 37 °C and stopped after 11, 27, and 60 min by the addition of 2 μL of a solution containing 1.5 mM 2,9-Me_2OP and 25 mM EDTA. The DNA was precipitated by the addition of 3 volumes of absolute ethanol. For DNA denaturation, the pellets were resuspended in 12 μL of 4 M urea, 50 mM NaOH, 1 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol, heated 5 min at 95 °C, and quick chilled in ice water. The

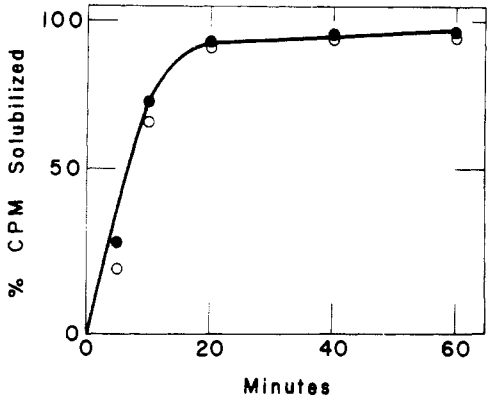


FIGURE 1: Acid solubilization of ^{32}P- and ^{3}H-labeled poly(dA-T). Poly([^{3}H]dA-[^{3}H,^{32}P]T) (11.9 μg/mL) was incubated at 37 °C with 30 μM OP, 3 μM $CuSO_4$, and 7 mM mercaptopropionic acid in an air-saturated 0.1 M Tris acetate, pH 7.0, buffer containing 5 mM magnesium acetate and assayed as described under Methods. ^{32}P-labeled products (○); ^{3}H-labeled products (●).

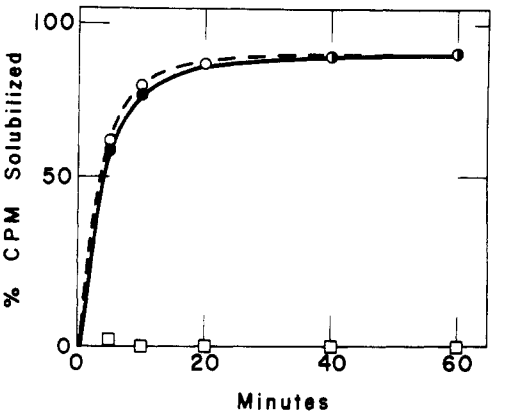


FIGURE 2: Blockage of cleavage reaction by catalase. Poly(dA-[^{3}H]T) (8.0 μg/mL) was incubated at 37 °C with 40 μM OP, 4 μM $CuSO_4$, and 7 mM mercaptopropionic acid in air-saturated 0.1 M Tris acetate buffer, pH 7.0, containing 5 mM magnesium acetate and the following additional components: none (○); 50 μg/mL catalase (□); 50 μg/mL catalase and 1 mM sodium azide (●).

cleavage products were then electrophoresed on 20% polyacrylamide gels at 1000 V (in 7 M urea, 50 mM Tris borate, pH 8.3, and 1 mM EDTA) and autoradiographed on Cronex 4 X-ray film using an intensifying screen (Du Pont Quanta II at −70 °C).

Results

Cleavage Based on Acid-Soluble Products. Acid-soluble radioactive products are generated when labeled poly(dA-T) is incubated with OP, cupric ion, and thiol under aerobic conditions (Figure 1). If ^{32}P,^{3}H-labeled poly(dA-T) prepared from [α-^{32}P]TTP, [^{3}H]TTP, and [^{3}H]dATP is used as the substrate in the cleavage reaction, the rate of formation of ^{32}P- and ^{3}H-labeled acid-soluble products is identical. The coincident release of both labels is consistent with the phosphodiester bond being the primary locus of attack on the DNA. However, it is also possible that the adenine or thymine β-deoxyriboside bond or any other locus on the deoxyriboside is the initial reaction site and that the phosphodiester backbone cleavage occurs rapidly thereafter.

Congruence of Conditions for Pol I Inhibition and Polynucleotide Cleavage. The conditions which lead to the formation of acid-soluble products of the polynucleotide are identical with those which resulted in the inhibition of the poly(dA-T)-directed polymerization of *E. coli* Pol I. Previously, it has been demonstrated that thiol, copper ion, and OP, all essential for inhibition, also must be present to cause the depolymerization observed by gel electrophoresis (Sigman et al., 1979). All three reactants are also required for the loss of acid-precipitable poly(dA-T). 2,9-Dimethyl-1,10-phenanthroline, a cuprous specific chelating agent which blocks the enzyme inhibition by $(OP)_2Cu^+$, has also been shown to stop the digestion of the poly(dA-T).

The reaction of $(OP)_2Cu^+$ with DNA leading to the inhibition of the *E. coli* Pol I DNA polymerase activity is blocked by catalase (Sigman et al., 1979). The cleavage of DNA measured by the appearance of acid-soluble counts is also effectively blocked by catalase (Figure 2). To exclude the trivial explanation that catalase retards the digestion by competing with 1,10-phenanthroline for the copper ion, sodium azide, an effective inhibitor of catalase (Schonbaum & Chance, 1976), was added to the incubation mixture. Since sodium azide allows the cleavage of DNA to proceed in the presence of catalase, the catalytic activity of the enzyme—destruction of hydrogen peroxide—must be responsible for the inhibition of the DNA cleavage reaction. All the data presently available indicate that the acid solubilization of poly(dA-T) and the inhibition of *E. coli* Pol I by $(OP)_2Cu^+$ reflect a common process.

$(OP)_2Cu^+$ and H_2O_2 Are Essential for DNA Cleavage. Inhibition of the DNA cleavage reaction by catalase prompted our investigation into the role of hydrogen peroxide. Since it is formed under aerobic conditions by the oxidation of $(OP)_2Cu^+$ by molecular oxygen (eq 1) (Crumbliss & Poulos,

$$2H^+ + O_2 + 2(OP)_2Cu^+ \rightarrow H_2O_2 + 2(OP)_2Cu^{2+} \quad (1)$$

1975; Crumbliss & Gestaut, 1976), its concentration in the inhibition mixture cannot significantly exceed 0.2 mM, the solubility of O_2 in aqueous solution at pH 7.0. The failure of 70 mM hydrogen peroxide alone to cleave DNA on the same time scale as $(OP)_2Cu^+$ under aerobic conditions indicates that its involvement in the cleavage reaction must be mediated by either $(OP)_2Cu^+$ or $(OP)_2Cu^{2+}$.

Although exogenously added H_2O_2 can potentiate the cleavage of DNA by $(OP)_2Cu^{2+}$, it dramatically enhances the rate of the cleavage reaction if it is added to a reaction mixture containing a reducing agent capable of generating $(OP)_2Cu^+$ from $(OP)_2Cu^{2+}$. For example, the addition of 7 mM H_2O_2 to a solution of 2 μM $(OP)_2Cu^{2+}$ and 7 mM mercaptopropionic acid leads to complete solubilization of poly(dA-T) within 30 s (Figure 3). The rates of the cleavage reaction potentiated by H_2O_2 and mercaptopropionic acid separately are only a fraction of the rate observed when they are both present.

The cleavage of DNA by $(OP)_2Cu^{2+}$ and H_2O_2 without added reducing agent also proceeds through $(OP)_2Cu^+$. This conclusion is based on the inhibition of this reaction by 2,9-dimethyl-1,10-phenanthroline, a cuprous ion specific chelating agent (Hall et al., 1962). The $(OP)_2Cu^+$ could be formed in trace levels by a reducing impurity which is initially present in the solution or is generated by H_2O_2 via the oxidation of an organic component. Since copper complexes have catalase-type activity, it is also possible that the cuprous complex may form by direct reduction of $(OP)_2Cu^{2+}$ by hydrogen peroxide (Sigel, 1969).

If diffusible hydrogen peroxide is essential for cleavage, molecular oxygen serving as a precursor of H_2O_2 would explain the oxygen dependence of the reaction. According to this view, the addition of hydrogen peroxide to an anaerobic reaction mixture containing $(OP)_2Cu^+$ should lead to a rapid cleavage reaction in an anaerobic chamber under a 85% N_2, 5% CO_2, 15% H_2 atmosphere. The data summarized in Figure 4 dem-

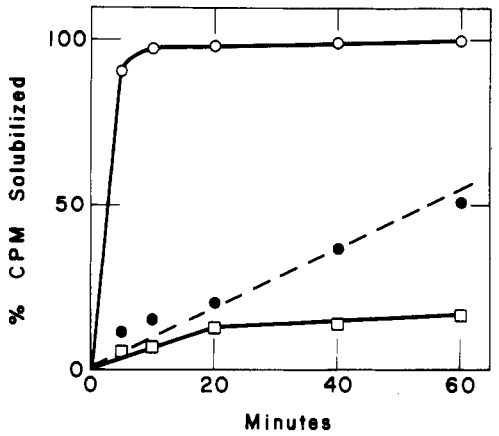


FIGURE 3: Synergistic effect of H_2O_2 and mercaptopropionic acid on cleavage. Poly(dA-[^{3}H]T) (6.8 μg/mL) was incubated in air-saturated 0.1 M Tris acetate, pH 7.0, buffer containing 5 mM magnesium acetate, 10 μM OP, 1 μM $CuSO_4$, and the following additional components: 7 mM mercaptopropionic acid (□); 7 mM H_2O_2 (●); 3.5 mM mercaptopropionic acid and 3.5 mM H_2O_2 (○). There is no detectable degradation of poly(dA-[^{3}H]T) incubated with 7.0 mM mercaptopropionic acid and 7.0 mM H_2O_2 in the absence of $(OP)_2Cu^{2+}$.

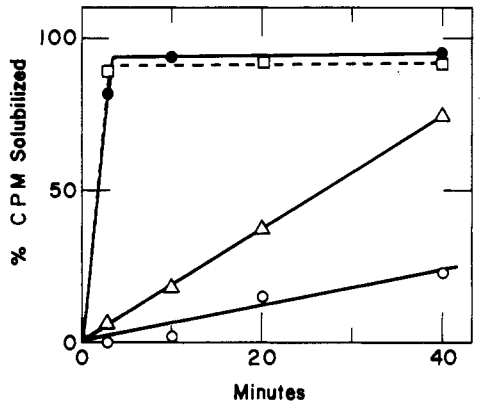


FIGURE 4: DNA cleavage by hydrogen peroxide in the presence and absence of oxygen. Poly([^{3}H]dA-[^{3}H]T) (3.9 μg/mL) was incubated at 37 °C in 0.1 M Tris acetate, pH 7.0, buffer with 30 μM OP and 3 μM $CuSO_4$ in the presence of (a) 7 mM H_2O_2 and 7 mM mercaptopropionic acid anaerobically (●) and aerobically (□) and (b) 7 mM mercaptopropionic acid anaerobically (○) and aerobically (△).

onstrate that if hydrogen peroxide is added to the reaction mixture containing 3-mercaptopropionic acid, the cleavage proceeds as rapidly under anaerobic conditions as under aerobic conditions. On the other hand, the cleavage reaction is markedly slower under anaerobic conditions in the absence of added hydrogen peroxide because molecular oxygen is not available to generate the peroxide.

Stoichiometry of Coordination Complex in Cleavage Reaction. If H_2O_2 is not added to the reaction, $(OP)_2Cu^+$ plays two essential roles in the cleavage reaction under aerobic conditions. First, it is responsible for generating hydrogen peroxide. Second, it reacts with the generated H_2O_2 to cleave DNA. The stoichiometry of the coordination complex responsible for both these steps is not necessarily the same. By adding hydrogen peroxide to the reaction mixture, it is possible to determine the stoichiometry of the coordination complex directly responsible for the cleavage reaction. Poly(dA-T) was incubated with 10 μM OP–1 μM cupric sulfate and 1 μM OP–10 μM cupric sulfate in solutions containing 7 mM H_2O_2 and 7 mM 3-mercaptopropionic acid. Whereas in the former case rapid cleavage was observed (e.g., Figure 2), only marginal cleavage was obtained in the latter case. The data clearly show that the 2:1 complex and not the 1:1 complex is directly

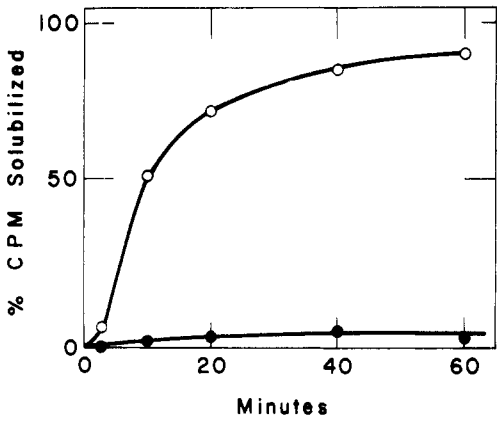


FIGURE 5: Potentiation of the DNA cleavage reaction by $(OP)_2Cu^{2+}$ with the superoxide generator, xanthine, and xanthine oxidase. Poly(dA-[^{3}H]T) (8.0 μg/mL) was incubated under aerobic conditions in a 0.1 M Tris acetate, pH 7.0, buffer containing 5 mM magnesium acetate, 0.1 mM xanthine, and 6.7×10^{-3} unit/mL xanthine oxidase (●) and, in addition, 30 μM OP and 3 μM $CuSO_4$ (○).

involved in the cleavage even when hydrogen peroxide does not have to be generated in situ from the oxidation of $(OP)_2Cu^+$ by O_2. This result is intriguing because the 1:1 complex is probably planar and the 2:1 complex tetrahedral.

Reactive Oxygen Intermediates. It is possible that H_2O_2 and $(OP)_2Cu^+$ are essential for the reaction because they serve as precursors for reactive oxygen intermediates such as singlet oxygen or hydroxyl radicals. Previously we have demonstrated that superoxide anion is not solely responsible for the cleavage reaction even though it is formed during the oxidation of $(OP)_2Cu^+$ by molecular oxygen (Graham et al., 1980). Evidence for excluding superoxide acting alone in its diffusible form includes (1) the failure of xanthine and xanthine oxidase, a superoxide-generating system (McCord & Fridovich, 1969), to cause detectable cleavage of poly(dA-T) and (2) the inability of the cuprous complexes of 2,2′-bipyridine and 2,2′,2″-terpyridine to cleave DNA under aerobic conditions even though superoxide is also an intermediate in their oxidation (Graham et al., 1980).

Xanthine and xanthine oxidase potentiate the cleavage reaction in the presence of $(OP)_2Cu^{2+}$ (Figure 5). This observation is consistent with $(OP)_2Cu^+$ and H_2O_2 being required for cleavage because superoxide produced by xanthine and xanthine oxidase will increase the concentration of H_2O_2 by spontaneous dismutation (eq 2) and will increase the con-

$$O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \qquad (2)$$

centration of $(OP)_2Cu^+$ by reducing $(OP)_2Cu^{2+}$ (Valentine & Curtis, 1975; Valentine, 1979) (eq 3). As expected, cat-

$$(OP)_2Cu^{2+} + O_2^- \rightarrow O_2 + (OP)_2Cu^+ \qquad (3)$$

alase blocks the superoxide anion potentiated cleavage of poly(dA-T).

Superoxide dismutase alters the rate of the DNA cleavage reaction in a manner which is consistent with superoxide affecting the steady-state levels of $(OP)_2Cu^+$ and H_2O_2 but not playing a direct role in the chemistry of the cleavage. Most importantly, superoxide dismutase has no effect on the rate of the cleavage when thiol and peroxide are present in the same reaction mixture. In the absence of reducing agent but in the presence of H_2O_2, superoxide dismutase enhances the rate of cleavage. This rate increase could be due to the dynamic reversal of the dismutation reaction by SOD to produce superoxide which in turn could reduce the cupric complex to $(OP)_2Cu^+$ (Hodgson & Fridovich, 1973). The cleavage re-

Table I: Hydroxyl Radical and Singlet Oxygen Traps Which Fail to Block Cleavage Reaction[a]

compound	concn (mM)
ethanol	10
mannitol	10
sodium formate	10
potassium iodide	10
2,6-di-*tert*-butyl-4-methylphenol	10
1,4-diazabicyclo[2.2.2]octane	10

[a] Poly(dA[^{3}H]T) (10 μg/mL) was cleaved by 20 μM OP, 2 μM $CuSO_4$, 7 mM H_2O_2, and 7 mM mercaptopropionic acid.

Table II: Intercalating Agents Which Completely Block Cleavage[a]

compound	concn (μM)
4',6-diamidino-2-phenylindole bis(hydrochloride) (DAPI)	1.5
ethidium bromide	50
actinomycin D	200

[a] DNA (10 μg/mL) was cleaved by 10 μM OP, 1 μM $CuSO_4$, 7 mM H_2O_2, and 7 mM mercaptopropionic acid.

action would be enhanced because of the increased concentration of the coordination complex. A trivial explanation for the rate enhancement, which cannot be excluded, is that the enzyme preparation contains reducing components which reduce the 1,10-phenanthroline–cupric complex to the cuprous complex.

Hydroxyl radical and singlet oxygen traps were incorporated into incubation mixtures to test for the involvement of these species in the cleavage reaction (Foote, 1979; Misra, 1974; Bodaness & Chan, 1977). None of them were effective in inhibiting the rate (Table I). Reactive diffusible species are unlikely to play a central role in the cleavage reaction.

Inhibition by Intercalators. The only compounds which inhibited the cleavage of poly(dA-T) without decreasing the concentration of $(OP)_2Cu^+$ and H_2O_2 were intercalating agents (Table II). A particularly effective inhibitor of the cleavage reaction is ethidium bromide, which shares the same tricyclic structure as OP (Paoletti et al., 1977). These observations suggest that the cleavage reaction may proceed through an intercalative complex. Further evidence of the binding of the coordination complexes to the DNA prior to cleavage is that the mononucleotides TTP and dATP are not altered by $(OP)_2Cu^+$ and H_2O_2 under conditions where complete digestion of poly(dA-T) is observed.

Secondary Structure Specificity. An important feature of the cleavage reaction is its specificity with respect to primary and secondary structure of the DNA. The poly(dA-T) is heterogenous in size containing hairpin turns and gaps even though it predominantly exists in double-helical form (Kornberg, 1980; Wells et al., 1977). Clearer indication of the secondary structure preference of the reaction was obtained by studying the relative rates of digestion of ^{3}H-labeled poly(dA), and poly(dT), poly([^{3}H]dA)–poly(dT), and poly(dA)–poly([^{3}H]dT) (Table III). The last two DNA's were prepared in a Pol I directed polymerization rather than by annealing in order to ensure that the resulting products were in double-helical form. The data summarized in Table III show that neither single-stranded poly(dA) nor single-stranded poly(dT) is cleaved by $(OP)_2Cu^+$ and hydrogen peroxide under conditions where poly(dA)–poly(dT), like poly(dA-T), is completely degraded.

A 60-base-pair restriction fragment of the *lac* operon prepared from the *lac*-containing plasmid (pMB 9-UV5) was digested with $(OP)_2Cu^+$ in order to investigate the primary

Table III: Secondary Structure Specificity of Cleavage Reaction[a]

substrate	[OP] (μM)	[Cu^{2+}] (μM)	% cpm solubilized
^{3}H-labeled poly(T) (20.3 μg/mL)	30	3	0
^{3}H-labeled poly(dA) (10 μg/mL)	50	5	0
poly(dA)–poly([^{3}H]T) (13.9 μg/mL)	30	3	85
poly([^{3}H]dA)–poly(T) (0.73 μg/mL)	30	3	100
poly([^{3}H]dA-[^{3}H]T) (11.3 μg/mL)	30	3	97

[a] 7 mM mercaptopropionic acid and 7 mM H_2O_2 were present in all incubation mixtures; pH 7.0 Tris acetate–5 mM magnesium acetate buffer. $T = 37$ °C. Incubation time, 60 min.

sequence specificity of the cleavage reaction. The sequence of the 5'-labeled strand (Schmitz & Galas, 1979) is

5' AATTCCGATTCAATTAATGCAGCT
GGCACGACAGGTTTCCCGACT
GGAAAGCGGGCAGTGAC 3'

The gel pattern obtained after limited cleavage shows no apparent sequence specificity. Discrete bands are observed for each base in the sequence. More subtle analysis of this apparently nonselective cleavage reaction must await determination of the chemical structure of the termini and evaluation of its effect on the electrophoretic migration of the oligonucleotides.

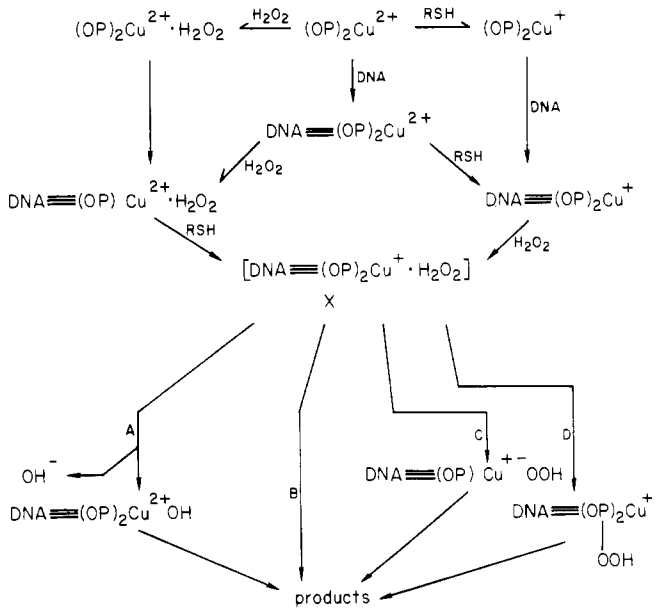
Discussion

H_2O_2 is an essential reactant in the DNA cleavage observed with $(OP)_2Cu^+$. Molecular oxygen serving as a precursor for hydrogen peroxide fully explains the oxygen dependence of the reaction. The essential role for hydrogen peroxide, first suggested by the catalase blockage of the cleavage reaction (Sigman et al., 1979), is strongly supported by the demonstration that the addition of hydrogen peroxide to a reaction mixture containing $(OP)_2Cu^+$ eliminates the requirement for oxygen in the cleavage reaction. With added peroxide, the cleavage reaction is as rapid anaerobically as aerobically. In the absence of added hydrogen peroxide, $(OP)_2Cu^+$ serves a dual function under aerobic conditions. The first is to generate H_2O_2. This step must be rate limiting in order to account for the substantial rate increase when exogenous H_2O_2 is added. The second is to participate directly in the cleavage reaction with the hydrogen peroxide it has produced. In both steps, the optimal stoichiometry of the complex is two 1,10-phenanthrolines and one cuprous ion.

The simultaneous appearance of ^{32}P- and ^{3}H-labeled products in the digestion of ^{32}P- and ^{3}H-labeled poly(dA-T) demonstrates that the phosphodiester bond cleavage readily proceeds at pH 7.0 and 37 °C. These conditions are mild in comparison to the cleavage chemistry used in DNA sequencing (Maxam & Gilbert, 1977) and more closely resemble the scission reactions of bleomycin and neocarzinostatin (Kappan & Goldberg, 1978; Sausville et al., 1978; Burger et al., 1979).

The rate of the cleavage reaction is remarkable. With 7 mM hydrogen peroxide present in the assay mixture, the poly(dA-T) is completely solubilized within 30 s by micromolar levels of the coordination complex. More detailed kinetic measurements have not yet been carried out because the constants of the equilibria which affect the concentration of $(OP)_2Cu^+$ under the conditions of the cleavage reaction are not available. These include the stability constants for the formation of $(OP)_2Cu^+$ (James & Williams, 1961) and for the formation of the copper complexes of DNA and mercap-

Scheme I[a]



[a] Lines between OP and DNA indicate intercalation.

topropionic acid. The competition of 5 mM magnesium acetate with copper for 1,10-phenanthroline will also affect the concentrations of $(OP)_2Cu^+$.

An important feature of the cleavage reaction may be the binding of the coordination complex to the DNA during the course of the reaction. This possibility is suggested by the structural similarity of OP and ethidium bromide, a well-studied intercalator [e.g., Paoletti et al. (1971)], and the X-ray structure of the *N*-methyl-3,4,7,8-tetramethylphenanthrolium salt of the dinucleotide, GpA (Jain et al., 1979), which indicates that the quaternary salt of phenanthroline forms favorable stacking interactions with the bases. Several lines of evidence suggest binding, perhaps in an intercalative mode. For example, the pronounced and perhaps absolute preference for double-helical DNA as a substrate for the cleavage reaction is most readily understood in terms of the formation of a kinetically competent complex between the DNA and $(OP)_2Cu^+$. Single-stranded poly(T) and poly(dA) may not be cleaved because an intercalative complex cannot form. The effective inhibition of the cleavage reaction by intercalating agents is also consistent with the binding of the cleavage reagent to the DNA during the course of the reaction. Ethidium bromide, 4′,6-diamidino-2-phenylindole bis(hydrochloride) (DAPI), and actinomycin D (Table II) may either occupy intercalative sites essential for cleavage or block the access of the coordination complex to an alternate site. Finally, the formation of reactive complexes with DNA can explain the inability of the cuprous complexes of 2,2′-bipyridine and 2,2′,2″-terpyridine to cleave DNA even though the coordination chemistry of these ligands is similar to that of 1,10-phenanthroline. Complexes of terpyridine and bipyridine may bind to DNA (Howe-Grant & Lipard, 1980), but OP complexes must be unique in achieving orientations consistent with cleavage.

Possible reaction pathways which can account for the thiol and hydrogen peroxide dependent cleavage are summarized in Scheme I. Because of the thiol and peroxide requirement, the reaction must proceed through an activated complex with composition of X where $(OP)_2Cu^+$ is probably intercalated into the DNA. It is not known whether H_2O_2 is noncovalently bound to the metal ion or DNA in the initial state leading to X or which equilibria are quantitatively most important on the pathway to X. Four pathways leading from X have been included. Pathway A, which involves the reduction of hydrogen peroxide to hydroxyl radical by a metal ion, has ample precedent in the chemistry of Fenton's reaction (Walling, 1975). Iron-bound hydroxyl radicals have been proposed as central intermediates in liver microsomal cytochrome P-450 hydroxylases (Groves et al., 1978). Hydroxyl radicals generated by, or coordinated to, the 1,10-phenanthroline–copper complex intercalated into DNA should be effective at hydrogen atom abstraction or hydroxyl insertion which could lead to the cleavage of DNA. The inability of hydroxyl radical traps to block the reaction indicates that if hydroxyl radicals form, they are not freely diffusible. The second pathway (B) would utilize the tetrahedral $(OP)_2Cu^+$ to intercalate at two loci and possibly strain the DNA structure so that the phosphodiester bond or some other locus becomes sensitive to nucleophilic attack by hydrogen peroxide external to the coordination sphere of the metal ion. A third pathway (C) would involve formation of a pentacoordinate cuprous complex with hydrogen peroxide serving as a ligand and then attacking the DNA by an intracomplex nucleophilic attack. Coordinated alcohols react readily with esters and ATP by a similar mechanism (Sigman & Jorgensen, 1972; Sigman et al., 1972). Finally, pathway D indicates that the hydrogen peroxide may react with the OP intercalated into the DNA which in turn will react with the DNA, causing cleavage.

The precise chemical structures of the termini are currently under investigation and must be determined before further studies on the mechanism of the cleavage are undertaken. The discrete bands obtained on gel electrophoresis indicate that a limited number of species are formed. Even without a complete description of the chemistry of the cleavage, the lack of primary sequence specificity of the nuclease-like activity of $(OP)_2Cu^+$ and H_2O_2 can be utilized to identify DNA sequences which are protected from cleavage by other agents which interact with DNA, such as proteins or intercalating agents. Since the antitumor agent bleomycin also cleaves DNA, $(OP)_2Cu^+$ and H_2O_2 may have useful cytotoxic and pharmacological properties.

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Quantitation of the Interaction of *Escherichia coli* RNA Polymerase Holoenzyme with Double-Helical DNA Using a Thermodynamically Rigorous Centrifugation Method†

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ABSTRACT: The nonspecific interaction of *E. coli* RNA polymerase holoenzyme with native bacteriophage P22 DNA was characterized by means of a rigorous centrifugation technique. A bound polymerase molecule was found to cover 42 base pairs, which implies that the configuration of the protein on DNA is similar for both specific and nonspecific binding. The data at each ionic strength studied can be fit well with single average association constants, $K = 4.7 \times 10^5$ M^{-1} at 0.15 M Na^+ and $K = 7.8 \times 10^4$ M^{-1} at 0.20 M Na^+. (These values for K are for DNA concentrations in moles of base pairs per liter.) Binding to phage T7 DNA was found to be similar. About seven ionic interactions are involved in the binding. At least 500 polymerase molecules can bind simultaneously to P22 DNA (or to T7 DNA) at 0.15 M Na^+; these 500 sites appear to have approximately the same affinity for holoenzyme. The sedimentation approach indicates that the G-C-rich DNA from *M. luteus* has fewer nonspecific binding sites for RNA polymerase. The notion that holoenzyme binds preferentially to A-T-rich regions was investigated by using synthetic DNAs. Very tight binding was seen to both poly[d(A-T)] and poly(dA)·poly(dT). However, a considerable (and unexpected) interaction was also observed with poly(dG)·poly(dC) at 0.20 M Na^+, indicating that variations in the affinity of polymerase for different "natural" DNA sequences are not trivially related to G-C content. The results presented here agree very well with those obtained previously by deHaseth et al. [deHaseth, P. L., Lohman, T. M., Burgess, R. R., & Record, M. T., Jr. (1978) *Biochemistry 17*, 1612], using DNA–cellulose chromatography. The magnitudes and salt dependences of K are similar, and we too find little effect on nonspecific binding of temperature or pH. The binding characterized here resembles the "tight-binding" complexes of Kadesch et al. [Kadesch, T. R., Williams, R. C., & Chamberlin, M. J. (1980) *J. Mol. Biol. 136*, 79] in some but not all respects. The large number of relatively high-affinity nonspecific binding sites must be accounted for in proposed mechanisms of promoter site selection by RNA polymerase.

RNA polymerase binds tightly to its specific functional sites on DNA (promoters) and displays a lesser but marked affinity for other DNA regions as well. Such "nonspecific" binding apparently modulates transcription in vivo; studies with an *E. coli* minicell producing strain show that there is no active RNA polymerase in the cytoplasm, implying that all enzyme molecules are chromosome bound in the bacterium (Cohen et al., 1968; Rünzi & Matzura, 1976). Nonspecific binding thus plays a role in the mechanism whereby polymerase seeks and finds promoter sites at which to initiate transcription, and characterization of this binding is essential for interpretation

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