Mechanisms of the Reactions of Some Copper Complexes in the Presence of DNA with O_2^-, H_2O_2, and Molecular Oxygen

Sara Goldstein and Gidon Czapski*

Contribution from the Department of Physical Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel. *Received July 15, 1985*

Abstract: The kinetics and the reaction mechanism of some copper complexes of 1,10-phenanthroline, 5-nitro-1,10-phenanthroline, and 2,2'-bipyridine with O_2^-, H_2O_2, and O_2 in the presence of calf thymus DNA have been investigated with use of the pulse radiolysis technique. We have found that both copper(II) and copper(I) complexes bind to DNA. The ternary complexes react very slowly with O_2^- relative to the free complexes, while the rates of the oxidation of free and bound cuprous complexes by H_2O_2 are almost the same. Therefore these ternary copper complexes turned out to be good catalysts of the reaction between O_2^- and H_2O_2.

A complex between the chelating agent 1,10-phenanthroline (OP) and copper(II) is able to induce the degradation of DNA in the presence of a reducing agent.[1-7] No primary sequence specificity is apparent in the scission reaction[8] which proceeds under a variety of experimental conditions. These include incubtation of DNA, OP, and copper(II) ions with the following: (a) reducing agents such as thiol or ascorbate in the presence of molecular oxygen;[1-6] (b) systems generating the superoxide radical in the presence of molecular oxygen;[4,6] (c) NADH and hydrogen peroxide;[6,7] (d) reducing agents and hydrogen peroxide.[5]

The degradation of DNA was inhibited by intercalating agents and any reagent which reduced the concentration of either the cuprous complex (e.g., neocuproine)[2,4,5] or hydrogen peroxide (e.g., catalase).[4-6] The sensitivity of the reaction to other inhibitors depended on the pathway for the generation of the cuprous complex and hydrogen peroxide (e.g., superoxide dismutase (SOD) inhibited the reaction potentiated by NADH and hydrogen peroxide but had no effect where thiol and hydrogen peroxide were present.[4-7]

5-NO_2-OP and 5-Cl-OP were more effective than OP in cleaving DNA while 5-CH_3-OP was less effective than OP under comparable conditions.[1,7] The cuprous complex of 2,2'-bipyridine (bpy) was unable to degrade DNA at similar concentrations used for OP,[3-5] although the coordination chemistry, the kinetics, and the mechanism of the oxidation of this complex by oxygen and hydrogen peroxide are similar to that of OP.[9,10] Moreover, it is known that complexes of bpy as well as those of OP bind to DNA.[11]

The reaction mechanism for this process has not yet been determined. It is believed that the cuprous complex intercalates with DNA and that the subsequent oxidation by hydrogen peroxide causes the damage due to the formation of OH· at the binding site.[4-6]

The binding constants of the various copper complexes to DNA and the kinetics and mechanism of the oxidation of the ternary complexes by oxygen, hydrogen peroxide, and superoxide radicals have not yet been determined. The understanding of the kinetics and mechanism of these reactions may shed light on the mechanism of DNA cleavage initiated by the various copper complexes.

Experimental Section

Materials. All chemicals employed were of analytical grade and were used as received: calf thymus DNA, type I, 2,2'-bipyridine, and sodium formate (Sigma Chemical Co.), 1,10-phenanthroline, 5-nitro-phenanthroline (Fluka), H_2O_2 (Merck), SOD (Diagnostic Data Int.), cupric sulfate, monosodium and disodium phosphate (Mallinckrodt).

All solutions were prepared in distilled water which was further purified by a Millipore reagent grade water system. A stock solution of DNA was prepared as 1 mg/mL containing 1 mM sodium phosphate buffer at pH 7. The concentration of DNA per nucleic acid phosphate was determined spectrophotometrically at 260 nm with $\epsilon = 6875\ M^{-1}\ cm^{-1}$.[12]

The cuprous complexes were generated by using the pulse radiolysis technique in oxygenated solutions containing 0.02 M sodium formate and 1 mM sodium phosphate buffer at pH 7. Under these conditions all the radicals formed by irradiation reduce the cupric complexes.[13-14] Kinetic studies were followed at 435 nm, where the various cuprous complexes absorb.[9,10]

The concentration of H_2O_2 was determined with ferrous sulfate.[15]

Apparatus. UV-visible absorption spectra were recorded with a Bauch and Lomb Model Spectronic 2000 spectrophotometer. The pulse radiolysis setup consisted of a Varian 7715 linear accelerator. The pulse duration ranged from 0.1 to 1.5 μs with a 200 mA current of 5 MeV electrons. The total concentration of the various cuprous complexes produced per pulse (1-15 μM) was evaluated with the use of a $(OP)_2Cu^{2+}$ dosimeter. The yield of $(OP)_2Cu^+$ in oxygenated formate solution was assumed to be $G = 6.05$ and $\epsilon = 6770\ M^{-1}\ cm^{-1}$ at 435 nm.[9]

Irradiation was carried out in a 2 cm long optical spectrosil cell with use of three light passes. A 150-W xenon lamp was used as the analytical light source and appropriate light filters were used to avoid photochemistry and to eliminate any scattered light. The detection system included a grating monochromator and an IP28 photomultiplier. The signal was transferred to a Nova 1200 minicomputer via either a Biomation 8100 or an analog-to-digital converter. The analysis of the data was carried out with the Nova 1200 minicomputer.

Results and Discussion

A. The Reduction of Copper(II) by O_2^- in the Presence of DNA. In the irradiation of aqueous solutions containing formate ions and oxygen, the superoxide radical is produced.[13,14] As the pK of HO_2 is 4.8,[14,16] the reducing radical is mainly O_2^- at pH 7.

When the cupric complexes of OP, 5-NO_2-OP, or bpy (CuL_2^{2+}) are present in excess relative to $[O_2^-]_0$, reaction 1 takes place:

$$CuL_2^{2+} + O_2^- \xrightarrow{k_1} CuL_2^+ + O_2 \qquad (1)$$

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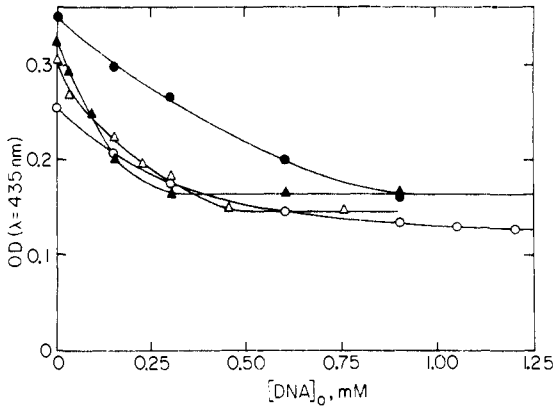

Figure 1. The effect of the concentration of DNA on the absorbance measured at the end of reaction 1. The initial concentrations of CuL_2^{2+} are the following: (▲) 30 μM $(OP)_2Cu^{2+}$; (●) 100 μM $(OP)_2Cu^{2+}$; (△) 30 μM $(5\text{-}NO_2\text{-}OP)_2Cu^{2+}$; (○) 30 μM $(bpy)_2Cu^{2+}$. The oxygenated solutions contained 0.02 M HCO_2Na at pH 7. The optical path length was 6.2 cm and the pulse duration 0.5 μs.

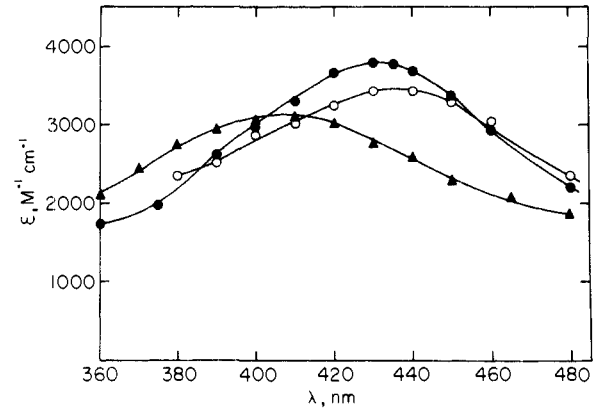

Figure 2. Spectra of $DNA{\equiv}CuL_2^{+}$. The initial concentrations of CuL_2^{2+} and DNA per nucleic acid phosphate are the following: (●) 20 μM $(OP)_2Cu^{2+}$, 300 μM DNA; (○) 30 μM $(5\text{-}NO_2\text{-}OP)_2Cu^{2+}$, 300 μM DNA; (▲) 20 μM $(bpy)_2Cu^{2+}$, 1.2 mM DNA. The oxygenated solutions contained 0.02 M HCO_2Na at pH 7 and the pulse duration was 0.5 μs.

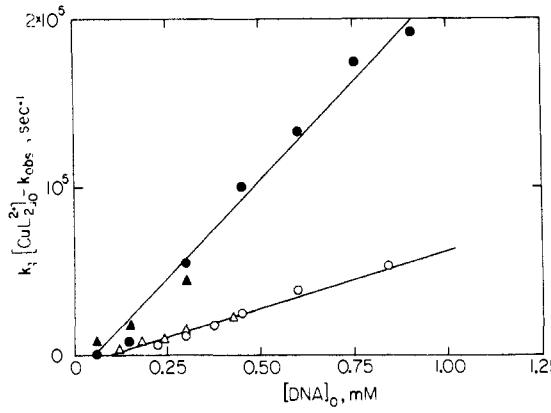

Figure 3. A plot of $k_1[CuL_2^{2+}]_0 - k_{obsd}$ vs. $[DNA]_0$. The initial concentrations of CuL_2^{2+} are the following: (●) 100 μM $(OP)_2Cu^{2+}$; (▲) 30 μM $(OP)_2Cu^{2+}$; (○) 50 μM $(5\text{-}NO_2\text{-}OP)_2Cu^{2+}$; (△) 30 μM $(5\text{-}NO_2\text{-}OP)_2Cu^{2+}$. The oxygenated solutions contained 0.02 M HCO_2Na at pH 7.

where $k_1 = (1.9 \pm 0.05) \times 10^9$ M^{-1} s^{-1}, $(1.5 \pm 0.05) \times 10^9$ M^{-1} s^{-1}, and $(2.8 \pm 0.2) \times 10^9$ M^{-1} s^{-1}, respectively.[9,10] The formation of CuL_2^{+} was followed at 435 nm and the absorption change (ΔOD) was followed until reaction 1 was completed. ΔOD decreased as the concentration of DNA increased until it reached a plateau (Figure 1). It has been demonstrated that DNA-Cu^{+} does not absorb in the visible region[17] and therefore we conclude that the decrease in the absorption, which reached a constant value at high [DNA], is due to the fact that CuL_2^{+} binds to DNA and that $\epsilon_{CuL_2^+} > \epsilon_{DNA{\equiv}CuL_2^+}$. The point where the plateau was reached depended not only on DNA concentration but also on the initial concentrations of CuL_2^{2+} (Figure 1). From these observations we conclude that both CuL_2^{2+} and CuL_2^{+} bind to DNA. As $[CuL_2^{2+}] > [CuL_2^{+}]$, at low [DNA] there may not be sufficient binding sites for CuL_2^{+}, and therefore as the concentration of CuL_2^{2+} increased, high concentrations of DNA were needed to reach the plateau. From the [DNA] where the plateau was reached, we estimated the number of binding sites per nucleic acid phosphate (n) for CuL_2^{2+}. We have calculated for OP $0.07 < n < 0.125$, for $5\text{-}NO_2\text{-}OP$ $0.055 < n < 0.0625$, and for bpy $0.009 < n < 0.011$. These values are for double stranded calf thymus DNA and may differ for circular or supercoiled and of course single-stranded DNA.

The rate of the reduction of the cupric complexes by O_2^{-} decreased as the concentration of DNA increased. It reached a constant value only in the case of $5\text{-}NO_2\text{-}OP$. This confirms the assumption that CuL_2^{2+} binds to DNA as we assume that bound CuL_2^{2+} reacts very slowly with O_2^{-} relative to free CuL_2^{2+}. We suggest the following reaction mechanism for the formation of bound CuL_2^{+}:

$$CuL_2^{2+} + O_2^{-} \xrightarrow{k_1} CuL_2^{+} + O_2 \quad (1)$$

$$CuL_2^{2+} + DNA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} DNA{\equiv}CuL_2^{2+} \quad (2)$$

$$DNA{\equiv}CuL_2^{2+} + O_2^{-} \xrightarrow{k_3} DNA{\equiv}CuL_2^{+} + O_2 \quad (3)$$

$$CuL_2^{+} + DNA \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} DNA{\equiv}CuL_2^{+} \quad (4)$$

We assume that $k_1 > k_3$ and that processes 2 and 4 reach rapid equilibrium. The equilibrium between free and bound molecules to a polymer is given by the well-known Scatchard equation[18]

$$K = \frac{r}{c(n - r)}$$

where in the present case r is the ratio of bound copper complex per nucleic acid phosphate, n is the number of binding sites per nucleic acid phosphate, c is the concentration of free copper complex, and K is the intrinsic association constant to a site.

At high [DNA], we assume that there are sufficient free binding sites for CuL_2^{+} and the concentration of bound CuL_2^{+} is given by

$$[DNA{\equiv}CuL_2^{+}] = \frac{[O_2^{-}]_0 K_4 [DNA] n_4}{1 + K_4[DNA]n_4}$$

At high concentration of DNA where $K_4[DNA]n_4 \gg 1$, the concentration of $DNA{\equiv}CuL_2^{+}$ equals $[O_2^{-}]_0$ and one can follow the absorption spectra of the various $DNA{\equiv}CuL_2^{+}$ (Figure 2).

Processes 1–4 lead to the following rate equation (Appendix A):

$$\frac{dOD}{dt} = (k_1[CuL_2^{2+}] + k_3[DNA{\equiv}CuL_2^{2+}])OD = k_{obsd}OD$$

Under the conditions where $n_2[DNA]_0 < [CuL_2^{2+}]_0$ and K_2 is high enough so that $k_2[CuL_2^{2+}] \gg k_{-2}$, $[DNA{\equiv}CuL_2^{2+}] = n_2[DNA]_0$, and $k_{obsd} = k_1[CuL_2^{2+}] + k_3 n_2[DNA]_0 = k_1[CuL_2^{2+}]_0 - (k_1 - k_3)n_2[DNA]_0$. In Figure 3, $k_1[CuL_2^{2+}]_0 - k_{obsd}$ is plotted vs. $[DNA]_0$ for OP and $5\text{-}NO_2\text{-}OP$. We did not get a straight line in the case of bpy and therefore we assumed that K_2 in this case is too low, and equilibrium 2 takes place even at low [DNA]. Assuming that $k_1 > k_3$ we obtained from the slope of the lines $n_2 = 0.125 \pm 0.014$ and 0.0525 ± 0.005 for OP and $5\text{-}NO_2\text{-}OP$, respectively.

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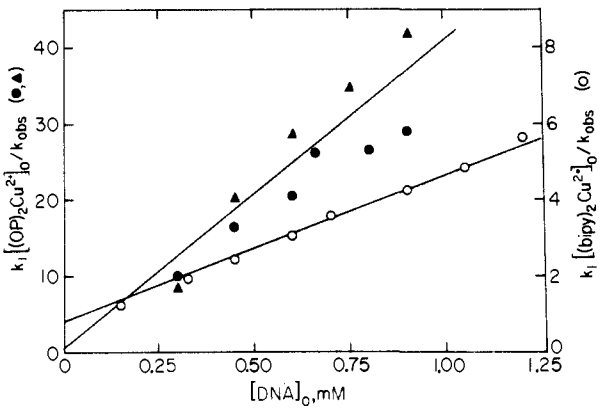

Figure 4. A plot of $k_1[CuL_2^{2+}]_0/k_{obsd}$ vs. the concentration of DNA. The initial concentrations of CuL_2^{2+} are the following: (●) 20 μM $(OP)_2Cu^{2+}$; (▲) 30 μM $(OP)_2Cu^{2+}$; (○) 20 μM $(bpy)_2Cu^{2+}$. The solutions contained 0.02 M HCO_2Na at pH 7 and were oxygen saturated.

In the case of OP and 5-NO_2-OP, at high [DNA], where $n_2[DNA]_0 > [CuL_2^{2+}]_0$ and at all [DNA] in the case of bpy, the concentrations of free and bound CuL_2^{2+} are given by

$$[CuL_2^{2+}] = \frac{[CuL_2^{2+}]_0}{1 + K_2[DNA]_0 n_2 + K_L[L]}$$

and

$$[DNA{\equiv}CuL_2^{2+}] = \frac{[CuL_2^{2+}]_0 K_2[DNA]_0 n_2}{1 + K_2[DNA]_0 n_2 + K_L[L]}$$

where K_L is the association constant of CuL_3^{2+}, which does not bind to DNA nor react with O_2^- (results not shown).

Thus, we obtain eq 5 for k_{obsd}. Only in the case of 5-NO_2-OP

$$k_{obsd} = \frac{k_1 + k_3 K_2[DNA]_0 n_2}{1 + K_2[DNA]_0 n_2 + K_L[L]}[CuL_2^{2+}]_0 \quad (5)$$

did k_{obsd} reach a constant value at high [DNA]. We assume that in this case $k_2[DNA]_0 n_2 + K_L[L] > 1$ and $k_3 K_2[DNA]_0 n_2 > k_1$ and at low concentration of free 5-NO_2-OP we get $k_{obsd} = k_3 = (1.1 \pm 0.2) \times 10^8$ M^{-1} s^{-1}. In the case of OP and bpy we assume that $k_1 \gg k_3$ and at low [L] eq 6 is obtained

$$k_1[CuL_2^{2+}]_0/k_{obsd} = 1 + K_2[DNA]_0 n_2 \quad (6)$$

In Figure 4 we plotted $k_1[CuL_2^{2+}]_0/k_{obsd}$ vs. $[DNA]_0$. From the slope of the line we determined $K_2 n_2$ which is $(3.7 \pm 0.5) \times 10^4$ M^{-1} and $(4 \pm 0.4) \times 10^3$ M^{-1} for OP and bpy, respectively.

At low concentration of DNA the initial OD of the reduced form was higher than that reached at high $[DNA]_0$. In the case of OP and 5-NO_2-OP we observed a fast decay to the same OD reached at high $[DNA]_0$ (Figure 5a,b). The order of the reaction with respect to ΔOD was one and the observed rate constant was independent of $[CuL_2^{2+}]_0$ and linear dependent on $[DNA]_0$ (Figure 6). We attribute this behavior to reaction 7 which takes place together with reaction 4 at low [DNA], where there are not sufficient free binding sites for CuL_2^+:

$$DNA{\equiv}CuL_2^{2+} + CuL_2^+ \rightarrow DNA{\equiv}CuL_2^+ + CuL_2^{2+} \quad (7)$$

The rate of the absorption decay, due to reaction 7, is given by the equation

$$-\frac{d\Delta OD}{dt} = k_7[DNA{\equiv}CuL_2^{2+}]\Delta OD = k_7 n_2[DNA]_0\Delta OD = k_{obsd}\Delta OD \quad (8)$$

From the slope of the lines in Figure 6, $n_2 k_7$ is obtained. Knowing the values of n_2 we calculated $k_7 = (2 \pm 0.3) \times 10^8$ M^{-1} s^{-1} and $(4.4 \pm 0.6) \times 10^8$ M^{-1} s^{-1} for OP and 5-NO_2-OP, respectively.

In the case of bpy, the absorption decayed to a lower value than that reached at high DNA (Figure 5c). Therefore we assume that process 7 cannot be separated from the subsequent process 9 that also causes the absorption to decay. We mentioned earlier that

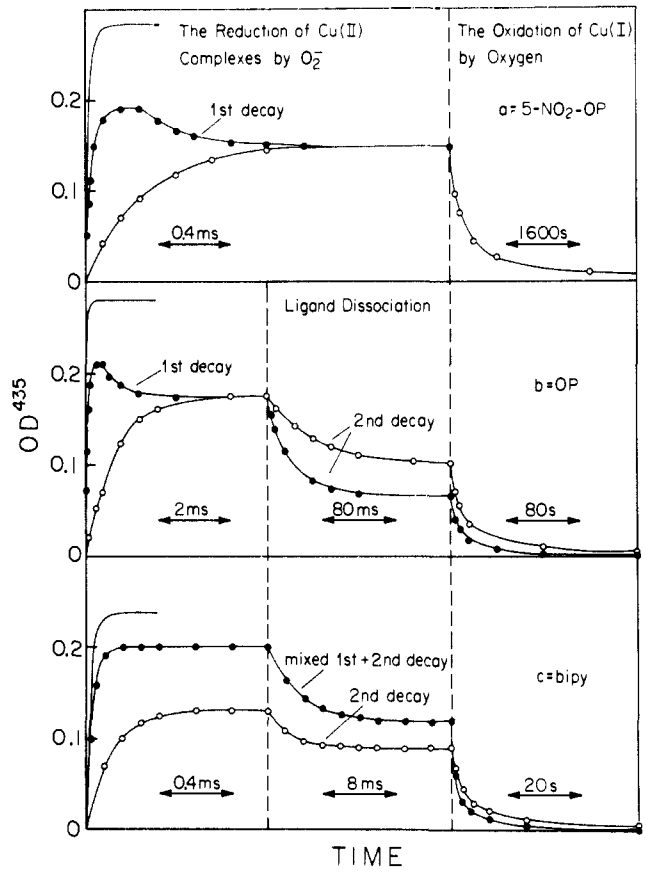

Figure 5. The consecutive steps in the reduction and reoxidation of the various copper complexes. All solutions were saturated with oxygen and contained 0.02 M HCO_2Na at pH 7. The optical path length was 6.2 cm and the pulse duration 0.5 μs. (a) $[(5\text{-}NO_2\text{-}OP)_2Cu^{2+}]_0 = 30$ μM, (—) [DNA] = 0, (●) [DNA] = 0.005%, (○) [DNA] = 0.02%. (b) $[(OP)_2Cu^{2+}] = 20$ μM, (—) [DNA] = 0, (●) [DNA] = 0.003%, (○) [DNA] = 0.02%. (c) $[(bpy)_2Cu^{2+}] = 20$ μM, (—) [DNA] = 0, (●) [DNA] = 0.005%, (○) [DNA] = 0.04%.

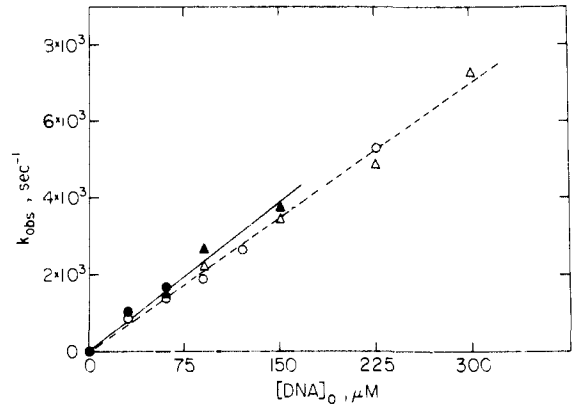

Figure 6. The dependence of the observed rate constant of reaction 7 on $[DNA]_0$. The initial concentrations of CuL_2^{2+} are the following: (●) 20 μM $(OP)_2Cu^{2+}$; (▲) 100 μM $(OP)_2Cu^{2+}$; (○) 30 μM $(5\text{-}NO_2\text{-}OP)_2Cu^{2+}$; (△) 50 μM $(5\text{-}NO_2\text{-}OP)_2Cu^{2+}$. All solutions contained 0.02 M HCO_2Na at pH 7 and were either oxygen or air saturated.

in the case of bpy $[DNA{\equiv}CuL_2^{2+}] < n_2[DNA]_0$ and therefore we expect reaction 7 to be slower in this case as compared to that of OP or 5-NO_2-OP.

We conclude that the formation of $DNA{\equiv}CuL_2^+$ takes place through the reduction of free copper(II) complexes by O_2^-. The CuL_2^+ being formed yields $DNA{\equiv}CuL_2^+$ through reactions 4 and 7 depending on the concentration of DNA.

After the formation of $DNA{\equiv}CuL_2^+$ was completed, a second slow decay to a new plateau was observed in the case of OP (Figure 5b). In the case of bpy this decay was separated from the pre-

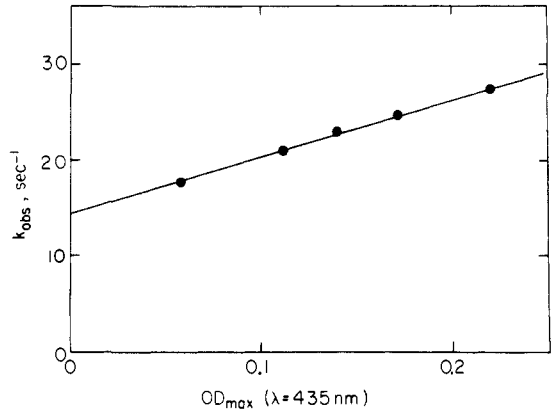

Figure 7. The dependence of k_{obsd} of the second decay on OD_{max}, where $OD_{max} = [O_2^-]_0/\epsilon_{DNA\equiv CuL_2^+}$. The oxygenated solutions contained 0.02 M HCO_2Na at pH 7, $[DNA]_0$ = 225 μM, $[CuSO_4]_0$ = 20 μM, $[OP]_0$ = 48 μM. The optical path length was 6.2 cm and the pulse duration ranged from 0.1 to 1.5 μs.

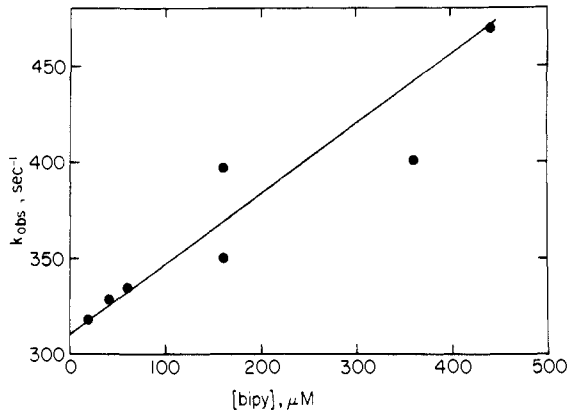

Figure 8. The dependence of k_{obsd} of the second decay on the concentration of free bpy in oxygenated solution containing 20 μM $CuSO_4$, 1.05 mM DNA, and 0.02 M HCO_2Na at pH 7.

ceding decay only at high [DNA], where process 7 was completed (Figure 5c). The height of this plateau depended strongly on the concentration of the free ligand and in the case of OP it also depended on [DNA]. The decay was first order with respect to ΔOD. In the case of OP the observed rate constant was linearly dependent on $[O_2^-]$ (Figure 7) and was slightly dependent on the concentration of OP and DNA. In the case of bpy the observed rate constant was independent of [DNA] and linearly dependent on the concentration of the free ligand in the solution (Figure 8). This decay disappeared at relatively low concentration of free OP in comparison to free bpy. We attribute this behavior to process 9, where a ligand dissociation from bound CuL_2^+ takes place:

$$DNA\equiv CuL_2^+ \underset{k_{-9}}{\overset{k_9}{\rightleftharpoons}} DNA\equiv CuL^+ + L \qquad (9)$$

In the case of OP the observed rate constant and the height of the plateau were dependent on [DNA] and therefore we assume that two different binding sites for $OPCu^+$ exist in rapid equilibrium and only one of them, $DNA\equiv CuOP^+$, reacts with OP to yield $DNA\equiv Cu(OP)_2^+$.

$$DNA\equiv CuOP^+ \underset{k_{-10}}{\overset{k_{10}}{\rightleftharpoons}} DNA\equiv CuOP^{+*} + DNA \qquad (10)$$

As process 10 reaches equilibrium fast and [OP] was not in excess relative to $[O_2^-]_0$, rate eq 11 is obtained.

$$-\frac{d\Delta OD}{dt} = [k_9 + 2k_{-9}[DNA\equiv CuOP^+]_{eq}]\Delta OD = \left[k_9 + \frac{2k_{-9}[O_2^-]_0}{1 + [OP]/K_9 + K_{10}/[DNA]}\right]\Delta OD = k_{obsd}OD \qquad (11)$$

From the intercept and the slope of the line in Figure 7, we obtained $k_9 = (14.7 \pm 0.3)$ s^{-1} and $k_{-9} \geq 6.72 \times 10^5$ M^{-1} s^{-1}

The concentration of bpy was in excess relative to $[O_2^-]_0$ and therefore $k_{obsd} = k_9 + k_{-9}[bpy]$. From the intercept and the slope of the line in Figure 8 we obtained $K_9 = (8.6 \pm 3) \times 10^{-4}$ M.

We conclude that at high concentrations of DNA the reduced form in the case of 5-NO_2-OP is $DNA\equiv CuL_2^+$, while in the case of OP and bpy an equilibrium between $DNA\equiv CuL_2^+$ and $DNA\equiv CuL^+$ is achieved, which depends mainly on the concentration of the free ligand in the solutions.

B. The Oxidation of the Cuprous Complexes by Oxygen in the Presence of DNA. The final decay of OD to zero was very slow (Figure 5). In the case of 5-NO_2-OP it was too slow to be measured under the time limitation of the detection system. Therefore, immediately after the pulse, the cell was removed to a spectrophotometer where the decay was followed. This decay which depends on the concentration of oxygen, DNA, free ligand, and CuL_2^{2+} indicates a complex mechanism. However, we can conclude the following:

(a) The decay was first order with respect to $[O_2]$ and second order with respect to OD.

(b) The rate of the oxidation decreased as the concentration of DNA increased and the observed second order rate constant was linearly dependent on $1/[DNA]_0$. This suggests the involvement of both free and bound cuprous complexes in the mechanism of the two-electron reduction of O_2 to H_2O_2. We were not surprised as it is almost impossible for a bimolecular interaction to occur between two cuprous complexes bound to a polymer.

(c) The rate of the oxidation increased as the concentration of the free ligand increased. This suggests that $DNA\equiv CuL^+$ reacts very slowly with O_2 in comparison to $DNA\equiv CuL_2^+$. The fact that k_{obsd} continued to increase even at high [OP], where no ligand dissociation occurs, suggests the involvement of $(OP)_2Cu^{2+}$ and/or $DNA\equiv Cu(OP)_2^{2+}$ in the mechanism.[9,10] Therefore we assume that the one-electron reduction of O_2 to O_2^- takes place and probably through free CuL_2^{2+} since $DNA\equiv CuL_2^{2+}$ reacts very slowly with O_2^-.

(d) We confirmed the assumption that the one-electron reduction of O_2 to O_2^- takes place by adding SOD to the solutions. In the presence of SOD the decay of the signal turned from second order to first order with respect to OD. In this case there was a little dependence of k_{obsd} on [DNA] and it was within the experimental error.

We assume that the one-electron reduction of oxygen to O_2^- and H_2O_2 proceeds through free CuL_2^+ while the two-electron reduction of oxygen to hydrogen peroxide proceeds through free and bound CuL_2^+:

$$CuL_2^+ + O_2 \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} CuL_2^{2+} + O_2^- \qquad (1)$$

$$CuL_2^+ + O_2^- + 2H^+ \xrightarrow{k_{12}} CuL_2^{2+} + H_2O_2 \qquad (12)$$

$$CuL_2^+ \text{ or } DNA\equiv CuL_2^+ + O_2 \underset{k_{-13}}{\overset{k_{13}}{\rightleftharpoons}} CuL_2O_2^+ \text{ or } DNA\equiv CuL_2O_2^+ \qquad (13)$$

$$CuL_2O_2^+ + DNA\equiv CuL_2^+ + 2H^+ \xrightarrow{k_{14}} DNA\equiv CuL_2O_2^+ + CuL_2^+$$

$$DNA\equiv CuL_2^{2+} + CuL_2^{2+} + H_2O_2 \qquad (14)$$

In the presence of SOD, only the one-electron reduction of oxygen (reaction 1) takes place. Assuming steady state for the concentration of O_2^- rate eq 15 is obtained (Appendix B):

$$-\frac{dOD}{dt} = \frac{1}{1 + K_4[DNA](1+\alpha)n_4}\frac{k_{-1}k_{cat}[SOD][O_2]}{k_1[CuL_2^{2+}] + k_{cat}[SOD]}OD = k_{obsd}OD \qquad (15)$$

k_{cat} is the "turnover" rate constant of SOD. As the values of k_{cat} are slightly different for SOD from different sources and batches,[19-21] we measured it directly with our SOD and we determined

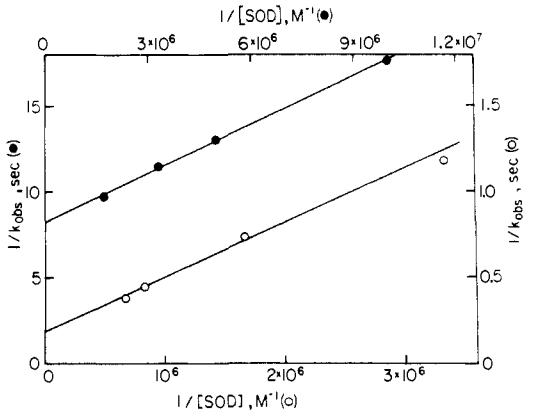

Figure 9. Reciprocal of the observed rate constant of the reaction of the oxidation of the cuprous complexes by oxygen vs. 1/[SOD]. The initial concentrations of $CuSO_4$, L, and DNA are the following: (●) 20 μM $CuSO_4$, 200 μM OP, 260 μM DNA; (○) 20 μM $CuSO_4$, 200 μM bpy, 1.05 mM DNA. The oxygenated solutions contained 0.02 M HCO_2Na at pH 7.

$k_{cat} = (3 \pm 0.3) \times 10^9$ M^{-1} s^{-1}. For 5-NO_2-OP, $\alpha = 0$ and $k_{-1} = 580$ M^{-1} s^{-1}.[10] For OP, $\alpha = K_9K_{10}/[OP][DNA]$ and $k_{-1} = 5 \times 10^4$ M^{-1} s^{-1}.[9] For bpy, $\alpha = K_9/[bpy]$ and $k_{-1} = 5.82 \times 10^4$ M^{-1} s^{-1}.[10] In Figure 9, $1/k_{obsd}$ is plotted vs. 1/[SOD]. From such a plot one can determine the value of K_4n_4 which is $(2 \pm 0.2) \times 10^5$, $(1.22 \pm 0.2) \times 10^5$, and $(5.4 \pm 0.5) \times 10^3$ M^{-1} for OP, 5-NO_2-OP, and bpy, respectively.

C. The Oxidation of Cuprous Complexes by H_2O_2 in the Presence of DNA. When $[H_2O_2]$ was present in excess relative to $[O_2^-]$ and $[O_2]$ in solutions containing DNA and CuL_2^{2+}, the oxidation of the cuprous complexes by H_2O_2 competed with that by O_2 and the final decay of OD to zero was first order with respect to $[H_2O_2]$ and first order with respect to OD. The observed first order rate constant increased as the concentration of the free ligand increased and in the case of OP also when the concentration of DNA increased. In the case of 5-NO_2-OP, where no dissociation of the ligand from bound CuL_2^+ occurs, k_{obsd} was independent of DNA and free ligand concentrations.

From these observations we conclude that the rate of the oxidation of bound CuL^+ by H_2O_2 is relatively very slow in comparison to that of bound CuL_2^+. (It is easy to show that the oxidation of bound CuL^+ and the oxidation of a trace amount of free CuL_2^+ in these experiments are negligible.) We assume that reaction 16 takes place. The OH· being formed at the binding

$$DNA{\equiv}CuL_2^+ + H_2O_2 \xrightarrow{k_{16}} DNA{\equiv}CuL_2^{2+} + OH^- + OH\cdot \quad (16)$$

site reacts with DNA and therefore cannot initiate a chain reaction. If the oxidation of DNA≡CuL^+ and free CuL_2^+ can be neglected, rate eq 17 is obtained, where α is defined as in eq 15.

$$-\frac{dOD}{dt} = \frac{k_{16}}{1+\alpha}[H_2O_2]OD = k_{obsd}OD \quad (17)$$

For 5-NO_2-OP and at high [OP] where no ligand dissociation takes place ($\alpha = 0$), k_{16} was determined directly to be (270 ± 30) and (1450 ± 100) M^{-1} s^{-1} for 5-NO_2-OP and OP, respectively. When free [OP] was low ($\alpha > 0$), k_{obsd} depended on DNA and OP concentrations. In Figure 10 the reciprocal of k_{obsd} is plotted vs. $1/[DNA]_0$, yielding a straight line. From the intercept we obtained $k_{16} = (1150 \pm 200)$ M^{-1} s^{-1}. From the slope and the intercept we determined $K_9K_{10}/[OP] = (3.4 \pm 0.4) \times 10^{-4}$ M^{-1}. In Figure 11 the reciprocal of k_{obsd} is plotted vs. 1/[bpy]. From the intercept we determined $k_{16} = (1240 \pm 100)$ M^{-1} s^{-1} and from the slope and the intercept $K_9 = (1.3 \pm 0.1) \times 10^{-4}$ M. This value differs from that which was obtained directly from Figure 8 ((8.6 ± 3) × 10^{-4} M). To explain this discrepancy, we must assume that another process which cannot be observed takes place. We attribute this discrepancy to the existence of two isomers of DNA≡CuL_2^+ which have almost the same absorption at 435 nm:

$$(DNA{\equiv}Cu(bpy)_2^+)_I \underset{k_{-18}}{\overset{k_{18}}{\rightleftharpoons}} (DNA{\equiv}Cu(bpy)_2^+)_{II} \quad (18)$$

We assume that process 18 is in rapid equilibrium and that both isomers are oxidized by H_2O_2 with k_{16} and k_{16}' for the first and second isomer, respectively. With these assumptions, eq 17 is modified into eq 19. If the ligand dissociation 9 takes place with

$$-\frac{dOD}{dt} = \frac{k_{16} + k_{16}'K_{18}}{1 + K_{18} + \gamma}[H_2O_2]OD = k_{obsd}OD \quad (19)$$

isomer I, γ equals α, and α was defined earlier for eq 15 and 17. From the measured intercept and slope in Figure 10 and using K_9, which was determined directly, we obtained $K_{18} = 5.6 \pm 0.8$. If the ligand dissociation takes place with isomer II, $\gamma = \alpha K_{18}$ and $K_{18} = 0.178 \pm 0.03$. Thus, the value of k_{18} depends on whether isomer I or II dissociates. For both cases K_2 is the same and $k_{16} + k_{16}'K_{18} = 1240 \pm 100$ M^{-1} s^{-1}. If process 18 occurs, the value obtained from the slope of the line in Figure 9 for bpy is not K_4n_4 but $K_4(1 + K_{18})n_4$ if isomer I dissociates and $K_4(1 + 1/K_{18})n_4$ if isomer II dissociates and hence $K_4n_4 = 818 \pm 150$ M^{-1}.

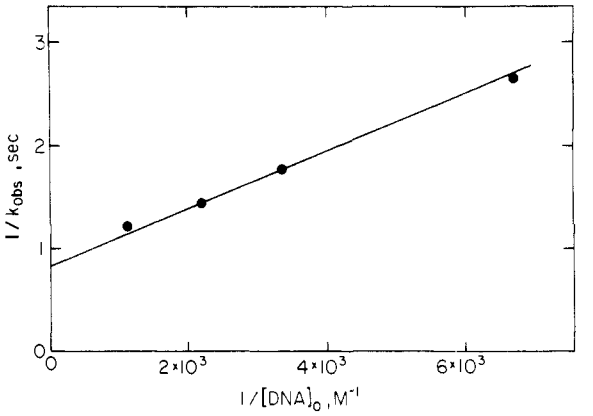

Figure 10. Reciprocal of the observed rate constant of the oxidation of the ternary cuprous complex of OP by H_2O_2 as a function of $1/[DNA]_0$. The solutions contained 20 μM $CuSO_4$, 48 μM OP, 1 mM H_2O_2, and 0.02 M HCO_2Na at pH 7 and were air saturated.

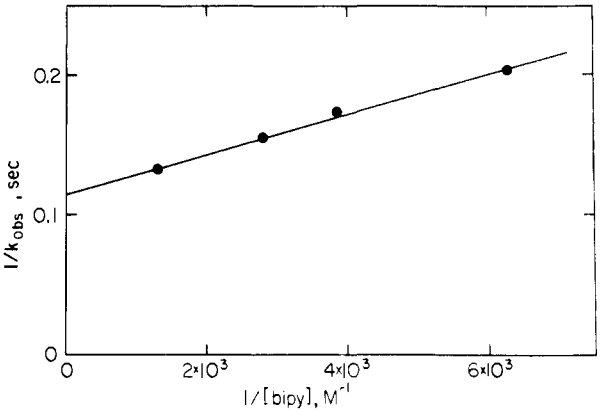

Figure 11. Reciprocal of the observed rate constant of the oxidation of the ternary cuprous complex of bpy by H_2O_2 as a function of 1/[bpy]. The solutions contained 20 μM $CuSO_4$, 1.05 mM DNA, 8 mM H_2O_2, and 0.02 M HCO_2Na at pH 7 and were air saturated.

D. The Mechanism of the Degradation of DNA in the Presence of CuL_2^{2+}, O_2^-, and H_2O_2. We have demonstrated that copper complexes of OP, 5-NO_2-OP, and bpy bind to DNA. The ternary complexes react very slowly with O_2^- in comparison to the free complexes, while the rates of the oxidation of free and bound CuL_2^+ by H_2O_2 are almost the same (Table I). We will show why the free complexes catalyze the dismutation of O_2^- and the

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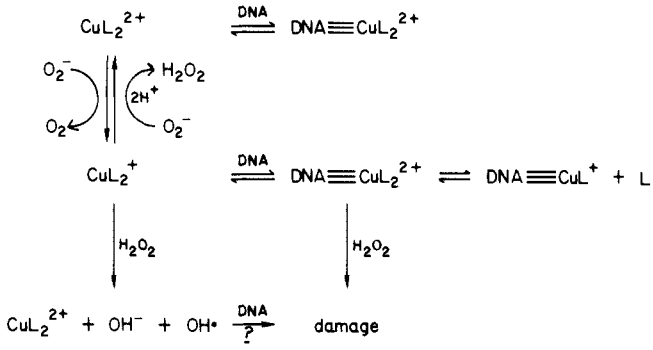
Table I. Kinetic Parameters of the Various Copper Complexes in the Absence and Presence of DNA

	OP	5-NO_2-OP	bpy
$k_1 = k_{CuL_2^{2+}+O_2^-}$, M^{-1} s^{-1}	1.93×10^9 [9]	1.5×10^9 [10]	2.8×10^9 [10]
$k_{CuL_2^{+}+O_2^-}$, M^{-1} s^{-1}	2.95×10^8 [9]	8.3×10^8 [10]	1.8×10^8 [10]
K_2n_2, M^{-1}	3.70×10^4		4.0×10^3
($DNA + CuL_2^{2+} \rightleftharpoons DNA{\equiv}CuL_2^{2+}$)			
$k_{DNA\equiv CuL_2^{2+}+O_2^-}$, M^{-1} s^{-1}	too slow to be measured	1.1×10^8	too slow to be measured
K_4n_4, M^{-1}	2×10^5	1.22×10^5	818
($DNA + CuL_2^{+} \rightleftharpoons DNA{\equiv}CuL_2^{+}$)			
K_9, M	$\leq 2.2 \times 10^{-5}$		8.6×10^{-4}
($DNA{\equiv}CuL_2^{+} \rightleftharpoons DNA{\equiv}CuL^{+} + L$)			
K_9K_{10}, M^2	$\simeq 5 \times 10^{-9}$		
($DNA{\equiv}CuL_2^{+} \rightleftharpoons DNA{\equiv}CuL^{+} + L + DNA$)			
$k^0_{H_2O_2} = k_{CuL_2^{+}+H_2O_2}$, M^{-1} s^{-1}	930,[9] 2×10^3 [22]	440[10]	1540,[10] 850[23]
$k_{16} = k_{DNA\equiv CuL_2^{+}+H_2O_2}$, M^{-1} s^{-1}	1450	270	≤ 1240

ternary complexes catalyze the Haber–Weiss reaction.

The mechanism of the degradation of DNA by CuL_2^{2+} in the presence of O_2^- and H_2O_2 is summarized in the following scheme:



We can divide these systems into two extreme cases:

(a) CuL_2^{2+} and CuL_2^{+} do not bind to DNA. In this case CuL_2^{2+} would catalyze the dismutation of O_2^- [9,10] because the rate constant of the oxidation of CuL_2^{+} by O_2^- is about 10^5-fold higher than the rate constant of the oxidation by H_2O_2 (Table I). Even if in a system CuL_2^{+} would react with H_2O_2 to yield OH· homogeneously in the bulk, this OH· would most probably react with other entities and would be far less harmful than an OH· which is formed at the binding site via a site-specific mechanism.

(b) CuL_2^{2+} and CuL_2^{+} do bind to DNA. If CuL_2^{2+} binds to DNA, the direct reduction of the various ternary complexes by O_2^- is very slow and it can be neglected (Table I). The reduction of Cu(II) by O_2^- occurs through free CuL_2^{2+} and the reduction rate is slowed down by increasing the concentration of DNA. If the binding constant of CuL_2^{2+} to DNA is too high, then the reduction of free CuL_2^{2+} by O_2^- would be too slow in comparison to the dismutation of O_2^- at pH 7 and no reduction of free CuL_2^{2+} by O_2^- would occur and the complex would not damage DNA.

If CuL_2^{2+} is reduced in the presence of DNA, the CuL_2^{+} binds to DNA to form a ternary complex with it. The ternary cuprous complex reacts very slowly with O_2^- as compared to its reaction with H_2O_2. Therefore, in this case the ternary copper complex does not catalyze the dismutation of O_2^- but rather catalyzes the Haber–Weiss reaction. In this case OH· is formed at the binding site and would degrade DNA. Therefore, as the binding constant of CuL_2^{+} to DNA increases and the ligand dissociation constant from bound CuL_2^{+} decreases, the majority of the OH· radicals would be formed at the binding site.

It is essential to know the concentrations of bound and free CuL_2^{+} and from this one would be able to predict and compare the damage to DNA due to the various copper complexes.

Que et al.[4] and Marshall et al.[5] found that under the same conditions OP cleaved DNA while bpy did not. Their explanation was that cuprous phenanthroline binds to DNA in a unique orientation and that the produced OH· via the oxidation of the ternary complex by H_2O_2 causes the damage. We can explain the different efficiency between OP and bpy in cleaving DNA on kinetic grounds. Que et al.[4] and Marshall et al.[5] used excess concentrations of the reducing agents, DNA, and free ligand relative to cupric ions. Using their concentrations together with the values of K_4n_4, K_9, K_{10}, K_{18}, and k_{16} determined in this work and the rates of the oxidation of free CuL_2^{+} by H_2O_2 ($k^\circ_{H_2O_2}$),[9,10,22,23] all of which are summarized in Table I, we have calculated the concentrations of bound and free cuprous complexes. We have also calculated the effective oxidation rate constant of the total bound cuprous complex by H_2O_2 which is $k_{eff} = k_{16}/(1 + K_9K_{10}/[OP][DNA])$ and $k_{eff} = (k_{16} + k_{16}'K_{18})/(1 + K_{18} + K_9/[bpy])$ for OP and bpy, respectively. We have found that for OP, $k_{eff}[DNA{\equiv}CuL^{+} + DNA{\equiv}CuL_2^{+}]$ is about 4–10 times higher than $k^\circ_{H_2O_2}[CuL_2^{+}]$, while for bpy it is about 30–60 times lower. Thus, with OP, most of the OH· radicals were formed at the binding site, while with bpy the majority of the OH· radicals were formed in the bulk and therefore the damage in the case of bpy was less effective. It is possible that the methods used by Que et al.[4] and Marshall et al.[5] for detection of DNA cleavage were not sensitive enough and that the extent of damage due to bpy could not be observed.

The oxidation of the ternary complexe by O_2 may compete with that by H_2O_2. We assume that as the rate of the oxidation of the ternary cuprous complex by O_2 decreases, the copper complex will be more effective in cleaving DNA. We assume that this is the reason why 5-NO_2-OP is more effective than OP in cleaving DNA under comparable conditions.

The formation of ternary complexes betwen copper compound and biological targets may change the ability of the copper compound from catalyzing the dismutation of O_2^-, which protects systems from O_2^- toxicity, into sensitizing the toxicity of O_2^-. If the ternary complex is reduced by O_2^- and then reacts with H_2O_2, the OH· is formed at the binding site and can be very efficient in causing damage. Such a mechanism operates with several antibiotics in their chemotherapeutic action.

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Appendix A

The Kinetics of the Formation of the Absorbance of the Cuprous Complexes Due to the Reduction of Copper(II) Complexes by O_2^- in the Presence of DNA:

$$CuL_2^{2+} + O_2^- \xrightarrow{k_1} CuL_2^{+} + O_2 \quad (1)$$

$$DNA + CuL_2^{2+} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} DNA{\equiv}CuL_2^{2+} \quad (2)$$

$$DNA{\equiv}CuL_2^{2+} + O_2^- \xrightarrow{k_3} DNA{\equiv}CuL_2^{+} + O_2 \quad (3)$$

$$-\frac{d[O_2^-]}{dt} = (k_1[CuL_2^{2+}] + k_3[DNA{\equiv}CuL_2^{2+}])[O_2^-] = k_{obsd}[O_2^-]$$

$$[O_2^-]_t = [O_2^-]_0 e^{-k_{obsd}t}$$

$$-\frac{d[CuL_2^{+}]}{dt} = k_1[CuL_2^{2+}][O_2^-] = k_1[CuL_2^{2+}][O_2^-]_0 e^{-k_{obsd}t}$$

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$$[CuL_2^+]_t = \frac{k_1[CuL_2^{2+}][O_2^-]_0}{k_{obsd}}(1 - e^{-k_{obsd}t})$$

and

$$[DNA{\equiv}CuL_2^+]_t = \frac{k_3[DNA{\equiv}CuL_2^{2+}][O_2^-]_0}{k_{obsd}}(1 - e^{-k_{obsd}t})$$

$$OD_0 = 0$$

$$OD_t = l\epsilon_{DNA{\equiv}CuL_2^+}[DNA{\equiv}CuL_2^+]_t + l\epsilon_{CuL_2^+}[CuL_2^+]_t$$

$$OD_\infty = \frac{l[O_2]_0}{k_{obsd}}(k_1\epsilon_{CuL_2^+}[CuL_2^{2+}] + k_3\epsilon_{DNA{\equiv}CuL_2^+}[DNA{\equiv}CuL_2^{2+}])$$

$$OD_t/OD_\infty = 1 - e^{-k_{obsd}t}$$

$$\frac{OD_\infty - OD_t}{OD_\infty} = e^{-k_{obsd}t}$$

therefore

$$\ln \frac{OD_\infty - OD_t}{OD_\infty} = -k_{obsd}t$$

and

$$dOD/dt = k_{obsd}OD$$

Appendix B

The Kinetics of the Absorbance Decay Due to the Reoxidation of the Cuprous Complexes by Oxygen in the Presence of DNA and SOD:

$$CuL_2^+ + O_2 \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} CuL_2^{2+} + O_2^- \quad (1)$$

$$DNA + CuL_2^+ \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} DNA{\equiv}CuL_2^+ \quad (4)$$

$$DNA{\equiv}CuL_2^+ \underset{k_{-9}}{\overset{k_9}{\rightleftharpoons}} DNA{\equiv}CuL^+ + L \quad (9)$$

$$O_2^- + O_2^- \xrightarrow[2H^+]{SOD} O_2 + H_2O_2$$

$$d[O_2^-]/dt = k_{-1}[CuL_2^+][O_2] - k_1[CuL_2^{2+}][O_2^-] - k_{cat}[SOD][O_2^-] = 0$$

$$[O_2^-]_{s.s} = \frac{k_{-1}[CuL_2^+][O_2]}{k_1[CuL_2^{2+}] + k_{cat}[SOD]}$$

In the absence of DNA:

$$-\frac{d[CuL_2^+]}{dt} = k_{-1}[CuL_2^+][O_2] - k_1[CuL_2^{2+}][O_2^-] = \frac{k_{-1}k_{cat}[O_2][SOD]}{k_1[CuL_2^{2+}] + k_{cat}[SOD]}[CuL_2^+] = k_{obsd}[CuL_2^+]$$

In the presence of DNA, where rapid equilibria 4 and 9 are reached:

$$[CuL_2^+]_0 = \frac{[O_2^-]_0}{1 + K_4[DNA]_0n_4(1 + K_9/[L])} = \frac{[O_2^-]_0}{\alpha}$$

$$[DNA{\equiv}CuL_2^+]_0 = \frac{[O_2^-]_0K_4[DNA]_0n_4}{\alpha}$$

$$[DNA{\equiv}CuL^+]_0 = \frac{[O_2^-]_0K_4[DNA]_0n_4K_9/[L]}{\alpha}$$

and

$$-\frac{d[CuL_2^+]}{dt} = \frac{k_{obsd}}{\alpha}[CuL_2^+] = k_{obsd}'[CuL_2^+]$$

$$[CuL_2^+]_t = [CuL_2^+]_0e^{-k_{obsd}'t} = \frac{[O_2^-]_0}{\alpha}e^{-k_{obsd}'t}$$

$$[DNA{\equiv}CuL_2^+]_t = K_4[DNA]_0n_4[CuL_2^+]_t$$

$$[DNA{\equiv}CuL^+]_t = (K_4[DNA]_0n_4K_9/[L])[CuL_2^+]_t$$

$$OD_t = l\epsilon_{CuL_2^+}[CuL_2^+]_t + l\epsilon_{DNA{\equiv}CuL_2^+}[DNA{\equiv}CuL_2^+]_t + l\epsilon_{DNA{\equiv}CuL^+}[DNA{\equiv}CuL^+]_t = (\epsilon_{CuL_2^+} + \epsilon_{DNA{\equiv}CuL_2^+}K_4[DNA]_0n_4 + \epsilon_{DNA{\equiv}CuL^+}K_4[DNA]_0n_4K_9/[L])\frac{[O_2^-]_0}{\alpha}e^{-k_{obsd}'t} = \beta e^{-k_{obsd}'t}$$

$$OD_0 = \beta$$

$$OD_\infty = 0$$

therefore

$$\frac{OD_t - OD_\infty}{OD_0 - OD_\infty} = e^{-k_{obsd}'t}$$

and

$$-\frac{dOD}{dt} = k_{obsd}'OD$$

In the case of OP where reaction 10 also occurs, assuming $[DNA{\equiv}CuL^+] \ll [DNA{\equiv}CuL^{+*}]$, $[DNA{\equiv}CuL_2^+]$:

$$k_{obsd}' = \frac{k_{obsd}}{1 + K_4[DNA]_0n_4(1 + K_9K_{10}/[L][DNA])}$$

Registry No. Cu^I-bpy, 100994-53-4; Cu^{II}-bpy, 16482-45-4; Cu^I-OP, 100994-51-2; Cu^{II}-OP, 15891-89-1; Cu^I-5-NO_2-OP, 100994-52-3; Cu^{II}-5-NO_2-OP, 100994-50-1; H_2O_2, 7722-84-1; O_2, 7782-44-7; $O_2^{\cdot-}$, 11062-77-4.