Fe^{2+}, Fe^{3+}, and Oxygen React with DNA-Derived Radicals Formed during Iron-Mediated Fenton Reactions†

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Received May 24, 1996; Revised Manuscript Received July 17, 1996⊗

ABSTRACT: Oxidative DNA damage is decreased by the presence of O_2 during Fe^{2+}-mediated Fenton reactions when H_2O_2 is in excess. During these reactions, the presence of DNA increases H_2O_2 consumption relative to Fe^{2+} consumption under anaerobic conditions, but decreases H_2O_2 consumption relative to Fe^{2+} consumption under aerobic conditions. The pseudobimolecular rate constant of H_2O_2 consumption is the same under both conditions, however, indicating that the presence of DNA affects the oxidation and/or reduction of the iron pool. To understand the basis of these effects, DNA was replaced with ethanol as a model compound. Computer simulations of Fe^{2+} and H_2O_2 consumption were experimentally verified and allowed identification of the predominant reactions leading to the changes in stoichiometry. Based upon these results and upon qualitative and quantitative differences in DNA damages between aerobic and anaerobic conditions, it was concluded that, in the presence of DNA, Fe^{3+} is reduced by some DNA radicals. However, if O_2 is present, these radicals react instead with O_2 and the product of these reactions can then oxidize Fe^{2+}. Mechanisms proposed for the alteration by O_2 of products from dC- and dG-containing substrates after exposure to Fe and H_2O_2 fit these general schemes. These results provide another distinction between DNA damage caused by ionizing radiation and that caused by Fenton reactions.

H_2O_2 is a byproduct of aerobic metabolism and is implicated in cell death, aging, and a variety of pathologies (Imlay & Linn, 1988; Lindahl, 1993; Halliwell & Gutteridge, 1990). The toxic effects of H_2O_2 are attributable to DNA oxidation *via* Fenton-like reactions. Although in its simplest form the Fenton reaction generates $^{\bullet}OH$,

$$Fe^{2+} + H^+ + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + H_2O$$

it is clear that when DNA is present, more complex radicals may be generated (Imlay & Linn, 1988; Luo *et al.*, 1994a). $^{\bullet}OH$ can attack DNA at near diffusion limited rates, either forming an OH adduct or abstracting a hydrogen. In either case, a DNA radical is formed (von Sonntag, 1987). The strongly oxidizing agent generated by the Fenton reaction might be an iron−oxo species such as the ferryl radical, possibly depending upon the nature of the iron chelation (Walling, 1975; Yamazaki & Piette, 1991; Bielski, 1992; Halliwell & Gutteridge, 1992; Goldstein *et al.*, 1993; Koppenol, 1994; Wink *et al.*, 1994, Winterbourn, 1995; Wardman & Candeias, 1996).

$$Fe^{2+} + H_2O_2 \rightarrow FeO^{2+} + H_2O$$

Imlay and Linn (1988) found that killing of *Escherichia coli* by H_2O_2 is due to DNA damage, requires iron, and can be subdivided into two kinetically distinct modes. Mode I has a maximal effect between 0.5 and 2.5 mM H_2O_2, and mode II predominates above 5 mM H_2O_2. These kinetics also apply to temperate phage induction and mutagenesis by H_2O_2 and are observed for *in vitro* DNA strand breakage (Imlay & Linn, 1988; Luo *et al.*, 1994a). It appears that the existence of several modes is due to differences in iron/DNA associations (Luo *et al.*, 1994a) which cause a change in the reactivity of the iron and/or nascent oxidant with H_2O_2, alcohols, and other agents. In further studies of these reactions, we were surprised to find that the 1:1 stoichiometry of Fe^{2+}:H_2O_2 consumption was severely perturbed by the presence of DNA and that these perturbations were themselves affected by the presence of O_2. This report describes studies of the chemistry of these changes and how they relate to DNA damage.

EXPERIMENTAL PROCEDURES

Materials. Horseradish peroxidase (HRP)[1] from Sigma was assayed according to Pütter and Becker (1983). PM2 DNA was prepared as described (Luo *et al.*, 1994b). For reactions in which only Fe^{2+} and H_2O_2 were monitored, salmon testes DNA (Sigma) was used. It had been treated with RNase, extracted consecutively with phenol, diethyl ether, and chloroform, and finally extensively dialyzed in a 50-kDa cutoff membrane against 10 mM Tris-HCl, 100 mM sodium EDTA, pH 8.0, and 1 M NaCl, followed by 50 mM NaCl or 50 mM $NaClO_4$. The resulting DNA had an ϵ_{260} of 6950 ± 100, an A_{250}/A_{260} of 0.856 ± 0.010, an A_{280}/A_{260} of 0.540 ± 0.010, and a pK_a of 5.97 ± 0.04. Other materials are described elsewhere (Luo *et al.*, 1994b).

Degradation of DNA and DNA Constituents by Iron/H_2O_2. Reactions containing iron, H_2O_2, and DNA and the subse-

† Supported by NIH Grants R29GM19020 and T32ES070075.

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⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1996.

[1] Abbreviations: HRP, horseradish peroxidase; ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); DNPH, (2,4-dinitrophenyl)-hydrazine.

S0006-2960(96)01235-4 CCC: $12.00

quent enzymatic conversion of DNA products to nucleosides, product identification, and product quantitation were described previously (Luo *et al.*, 1994b).

Stoichiometry Determinations. Reactions were carried out in borosilicate tubes at 1 atm and 22 °C for 10 min with vigorous bubbling of either N_2 or air. The gases had been prewashed successively in 0.1 M NaOH and a solution containing components of the reaction except for iron or DNA. The sequence of addition to reaction mixtures was NaCl, organic substrate (DNA, *tert*-butyl alcohol, or ethanol), $FeSO_4$, and H_2O_2. NaCl (25−50 mM) was added when DNA was present. Fenton reactions containing DNA were adjusted to pH 6.2−6.4 with NaOH. So long as Fe^{2+} and H_2O_2 were not simultaneously present, Fe^{2+}, H_2O_2, DNA, ethanol, and *tert*-butyl alcohol were stable under both aerobic and anaerobic conditions for the times employed for the experiments. Reactions without DNA started at pH 5.7 and dropped to pH 3.7−4.2, depending upon the amount of Fe^{2+} remaining. Exogenous buffers were avoided as they could have changed the iron redox couple, interfered with the intrinsic iron binding abilities of DNA, and/or scavenged radicals.

Determination of Fe^{2+} and H_2O_2 Concentrations. Quantitation of stock solutions of Fe^{2+} and H_2O_2 were described previously (Luo *et al.*, 1994b). Consumption of Fe^{2+} and H_2O_2 were halted by adding an equal volume of 4 mM 3-(2-pyridyl)-5,6-bis(4-phenylsulfonyl)-1,2,4-triazine (ferrozine)/200 mM sodium acetate, pH 5.0. Within 1 min the ferric hydrate precipitate and/or insoluble Fe^{3+}/DNA complexes were removed by filtration through a 3-kDa centrifree Millipore filter. (Fe^{2+} determinations with ferrozine in control studies showed that the presence of DNA did not affect the amount of assayed Fe^{2+}/ferrozine complex. At this point, the Fe^{2+}/ferrozine and the H_2O_2 concentrations remained unchanged for at least 2 h at 0 °C). With one-half of the sample, the A_{562} was determined to estimate the Fe^{2+} concentration (Luo *et al.*, 1994b), and the A_{420} was determined as a blank for H_2O_2 determinations. A 4-fold volume excess of 2.38 mM 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1.25 U/mL HRP, and 100 mM sodium acetate, pH 5.0 was added to the other half of the sample. After at least 30 min at 22 °C, the A_{420} of the solution was measured. The increase in A_{420} per μM H_2O_2 present was approximately 0.05 depending upon the stock solutions of ABTS and HRP. The increase was linear up to 40 μM H_2O_2, and the colorimetric response of ABTS oxidation per H_2O_2 was reproducible to within 2% for a given batch of ABTS/HRP but varied from batch to batch. When the A_{420} or A_{562} was above 1.5, the sample was diluted with 100 mM sodium acetate, pH 5.0.

Acetaldehyde Determination. Fifty microliters of 2.5 mg/mL (2,4-dinitrophenyl)hydrazine (DNPH) in 2 M HCl was added to 200 μL of a sample containing up to 500 μM acetaldehyde in a gas tight vessel and incubated at 40 °C for 1 h. Acetonitrile (450 μL) and 300 μL of 0.67 M potassium phosphate, pH 7, were then added at 0 °C with mixing. One hundred microliters of the final mixture was finally injected onto an RP-C_{18} high performance liquid chromatography column (25 cm × 4.6 mm), and the column was eluted with 45% acetonitrile at 1 mL/min. DNPH eluted around 7 min, and acetaldehyde (2,4-dinitrophenyl)hydrazone eluted around 18 min. The acetaldehyde (2,4-dinitrophenyl)-hydrazone A_{367} peak area was proportional to initial acetaldehyde concentration.

Computer Simulation of Reaction Kinetics. The FORTRAN program which was devised to simulate the reaction kinetics of Fenton reactions in the presence of ethanol considered 32 reactions (Table 1) which could have affected the kinetics of H_2O_2 and Fe^{2+} consumption in the presence of ethanol under aerobic and anaerobic conditions. The combined set of reactions were reformulated as differential equations for individual yield changes with respect to time. Additionally, an empirically-determined relationship was used to estimate the pH from the amount of Fe^{3+} produced. The set of stiff coupled ordinary differential equations was solved for the change in yield of each species assuming a steady state for all other species during an appropriately small time interval (Edelson, 1981). The time intervals for integration were reduced until the differences in the results were negligible (<1%). The ratio of H_2O_2 consumption to Fe^{2+} oxidation of a completed reaction was assessed when 98% of either H_2O_2 or Fe^{2+} was consumed. Program statements also recorded the contribution of a given reaction to the simulation. The FORTRAN program is available from the authors.

Rate Constant Determinations. The pseudobimolecular rate constants (k_S) for the consumption of H_2O_2 [$k_{(H_2O_2)}$] or the oxidation of Fe^{2+} [$k_{(Fe^{2+})}$] are defined as

$$k_S = (d[S]/dt)/([H_2O_2][Fe^{2+}])$$

where [S] is either $[H_2O_2]$ or $[Fe^{2+}]$.

RESULTS

Stoichiometry of H_2O_2 and Fe^{2+} Consumption in the Presence of DNA and/or O_2. Damage to the 4 nucleosides in DNA due to Fe^{2+}/H_2O_2 was assessed when H_2O_2 was in excess over Fe^{2+}. It was found that less damage occurred to each of the DNA nucleosides under aerobic conditions than under anaerobic conditions (Table 2). Similar results were seen with the individual 3′- or 5′-mononucleotides and with small oligonucleotides of dG (Henle *et al.*, 1996), dC (Luo *et al.*, 1996), dT, and dA (R. Jin, R. Chattopadhyaya, E. S. Henle, Y. Luo, and S. Linn, unpublished experiments). This effect is not observed during γ-irradiation (LaFleur & Retel, 1993) and therefore was subject to further study. For these studies, the initial H_2O_2 concentration was chosen to be 0.5−2 mM as these may be biologically relevant concentrations (Imlay & Linn, 1988).

In the absence of DNA or other organic radical scavengers, O_2 had no discernible effect upon the ratio of H_2O_2 consumption to Fe^{2+} consumption (Figure 1). (Although O_2 can oxidize Fe^{2+}, this rate is negligible compared to that of the Fenton reaction under these conditions.) In the presence of DNA, however, with H_2O_2 initially in excess of Fe^{2+}, H_2O_2 consumption relative to Fe^{2+} consumption was increased under anaerobic conditions, but was decreased under aerobic conditions (Figure 1). Representative time courses verify that these changes brought about by the presence of DNA and O_2 were due to changes in the rates of Fe^{2+} consumption throughout the reaction and changes in H_2O_2 consumption in the later phases of the reaction (Figure 2A).

Assuming that the Fenton reaction is rate limiting (see Table 1), the pseudobimolecular rate constants for H_2O_2 consumption and Fe^{2+} oxidation during the reaction were

Table 1: Reactions Considered in This Study

reaction no.	reaction	rate const (M^{-1} s^{-1})	ref
	Reactions without Ethanol or Oxygen		
1	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$	7.6×10^1	Walling, 1975
2	$Fe^{2+} + HO_2^{\bullet} \rightarrow Fe^{3+} + HO_2^-$	1.2×10^6	Bielski *et al.*, 1985
3	$Fe^{3+} + HO_2^{\bullet} \rightarrow Fe^{2+} + H^+ + O_2$	3.1×10^5	Bielski *et al.*, 1985[a]
4	$H_2O_2 + {}^{\bullet}OH \rightarrow HO_2^{\bullet} + H_2O$	2.7×10^7	Buxton *et al.*, 1988
5	$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + H^+ + HO_2^{\bullet}$	2.7×10^{-1}	*b*
6	$Fe^{2+} + {}^{\bullet}OH \rightarrow Fe^{3+} + OH^-$	3.5×10^8	Buxton *et al.*, 1988
7	$^{\bullet}OH + HO_2^{\bullet} \rightarrow H_2O + O_2$	7×10^9	Buxton *et al.*, 1988
8	$2HO_2^{\bullet} \rightarrow H_2O_2 + O_2$	1.7×10^7	Bielski *et al.*, 1985[c]
9	$HO_2^{\bullet} + H_2O_2 \rightarrow {}^{\bullet}OH + O_2 + H_2O$	5×10^{-1}	Bielski *et al.*, 1985
10	$2{}^{\bullet}OH \rightarrow H_2O_2$	5.5×10^9	Buxton *et al.*, 1988
	Reactions Involving Ethanol and Its Products but Not Oxygen		
11	$CH_3CH(OH)^{\bullet} + H_2O_2 \rightarrow CH_3CHO + H_2O + {}^{\bullet}OH$	1.5×10^5	Sedon & Allen, 1967
12	$Fe^{3+} + CH_3CH(OH)^{\bullet} \rightarrow CH_3CHO + Fe^{2+} + H^+$	2.7×10^8	Berdnikov *et al.*, 1977
13	$^{\bullet}CH_2CH_2OH + Fe^{2+} \rightarrow [CH_2CH_2OH]^- + Fe^{3+}$	1.0×10^5	Walling & El-Taliawi, 1973[d]
14	$2CH_3CH(OH)^{\bullet} \rightarrow CH_3CHO + CH_3CH_2OH$	1.1×10^9	Burchill & Ginns, 1970[e]
15	$^{\bullet}OH + CH_3CH_2OH \rightarrow CH_3CH(OH)^{\bullet} + H_2O$	1.6×10^9	Buxton *et al.*, 1988[f]
16	$^{\bullet}OH + CH_3CH_2OH \rightarrow {}^{\bullet}CH_2CH_2OH + H_2O$	2.8×10^8	Buxton *et al.*, 1988[f]
17	$^{\bullet}CH_2CH_2OH + CH_3CH_2OH \rightarrow CH_3CH(OH)^{\bullet} + CH_3CH_2OH$	2×10^2	Burchill & Ginns, 1970
18	$2{}^{\bullet}CH_2CH_2OH \rightarrow CH_3CHO + CH_3CH_2OH$	1.1×10^9	Burchill & Ginns, 1970[e]
19	$CH_3CH(OH)^{\bullet} + {}^{\bullet}OH \rightarrow CH_3CHO + H_2O$	1.0×10^{10}	*g*
20	$^{\bullet}CH_2CH_2OH + {}^{\bullet}OH \rightarrow HOCH_2CH_2OH$	1.0×10^{10}	*g*
21	$CH_3CH(OH)^{\bullet} + {}^{\bullet}CH_2CH_2OH \rightarrow CH_3CHO + CH_3CH_2OH$	1.1×10^9	*e*, *h*
	Reactions Involving Ethanol and Its Products That Can Proceed if Oxygen Is Present		
22	$CH_3CH(OH)O_2^{\bullet} \rightarrow CH_3CHO + HO_2^{\bullet}$	5.0×10^1	Bothe *et al.*, 1983[i]
23	$CH_3CH(OH)^{\bullet} + O_2 \rightarrow CH_3CH(OH)O_2^{\bullet}$	4.6×10^9	Neta *et al.*, 1990
24	$^{\bullet}CH_2CH_2OH + O_2 \rightarrow {}^{\bullet}O_2CH_2CH_2OH$	6.6×10^9	Neta *et al.*, 1990
25	$2CH_3CH(OH)O_2^{\bullet} \rightarrow H_2O_2 + O_2 + 2CH_3CHO$	3.5×10^8	Neta *et al.*, 1990
26	$CH_3CH(OH)O_2^{\bullet} + Fe^{2+} \rightarrow [CH_3CH(OH)O_2{-}Fe]^{2+}$	1.7×10^6	Butler *et al.*, 1974[j]
27	$^{\bullet}O_2CH_2CH_2OH + Fe^{2+} \rightarrow [Fe{-}O_2CH_2CH_2OH]^{2+}$	1.7×10^6	*k*
28	$2{}^{\bullet}O_2CH_2CH_2OH \rightarrow O_2 + OCHCH_2OH + HOCH_2CH_2OH$	1.0×10^8	Neta *et al.*, 1990[l]
29	$CH_3CH(OH)O_2H \rightarrow CH_3CO(OH) + H_2O$	2.1×10^4	Butler *et al.*, 1974[i,j]
30	$CH_3CH(OH)O_2^{\bullet} + {}^{\bullet}O_2CH_2CH_2OH \rightarrow O_2 + CH_3CO(OH) + OCHCH_2OH$	3.5×10^8	*m*
31	$CH_3CH(OH)O_2H + Fe^{2+} \rightarrow CH_3CH_2O^{\bullet} + OH^- + Fe^{3+}$	1×10^3	Garnier-Suillert *et al.*, 1984
32	$HO_2CH_2CH_2OH + Fe^{2+} \rightarrow [HOCH_2CH_2O{-}Fe]^{2+}$	1×10^3	Garnier-Suillert *et al.*, 1984

[a] The same rate is assumed for the reaction of Fe^{3+} with $O_2^{\bullet -}$. [b] E. S. Henle, and S. Linn, unpublished experiments; see also Walling and Goosen (1973). [c] The rate is pH-dependent and pH 5 is assumed. [d] The reaction is also described by Walling (1975) and Walling and El-Taliawi (1973), and similar reactions are reviewed by Fischer (1990). [e] The products of eqs 14, 18, and 21 may also contain 2,3-, 1,4-, or 1,3-butanediol, respectively. [f] The proportion of α-hydroxy radical to β-hydroxy radical is between 85% and 97% during these reactions. Rate constants are based on an overall $^{\bullet}OH$ scavenging rate constant of 1.9×10^9 and upon the assumption that 85% α-hydroxyethanol radical is formed. [g] These values are estimated from similar diffusion controlled reactions. Even at these rates, the reactions have negligible effects on the simulations. [h] The reaction rate is estimated to be equal to those of reactions 14 and 18. [i] These are first order reactions (s^{-1}). [j] The estimate is based upon the rate constants for the α-hydroxy peroxyl radical of 2-propanol and also upon rates of comparable reactions in Neta *et al.* (1990). [k] The reaction rate is estimated to be equal to that of reaction 24. [l] The products are assumed. [m] The reaction rate is estimated to be equal to those of reactions 25 and 26, and the products are assumed.

Table 2: Percent of Each Nucleoside Damaged during Fenton Reaction with DNA Present[a]

DNA nucleoside	% damaged	
	air	N_2
dG	8	14
dC	9	14
dA	6	9
dT	10	12

[a] Reactions contained 1 mM PM2 DNA (nucleotide residues), 50 mM NaCl, 1 mM $FeSO_4$, and 2 mM H_2O_2. Reactions and analyses were carried out as described by Luo *et al.* (1994b).

estimated from the time courses of Figure 2A (Figure 2B). Throughout most of the reaction, the rate of H_2O_2 consumption was proportional to $[H_2O_2][Fe^{2+}]$ and the proportionality factors—i.e., the pseudobimolecular rate constants for H_2O_2 consumption, $k_{(H_2O_2)}$, were the same under anaerobic and aerobic conditions (110 ± 7 M^{-1} s^{-1}) (Figure 2C). (Note that the equivalence of the rate constants under aerobic and anaerobic conditions is consistent with H_2O_2 consumption being due to the same reactions under both conditions (see below).) It is significant that $k_{(H_2O_2)}$ in the presence of DNA is larger than that expected from the simple Fenton reaction (76 M^{-1} s^{-1}; Walling, 1975), possibly reflecting an increase in the rate of the Fenton reaction when Fe^{2+} is associated with DNA.

In contrast to the situation for H_2O_2 consumption, the pseudobimolecular rate constant for Fe^{2+} consumption, $k_{(Fe^{2+})}$, differed between anaerobic and aerobic conditions in the presence of DNA. Under anaerobic conditions and in the presence of 2 mM DNA nucleotide, $k_{(Fe^{2+})}$ was 70% of $k_{(H_2O_2)}$ (Figure 2B,C). The lesser value of $k_{(Fe^{2+})}$ as compared to $k_{(H_2O_2)}$ could be explained by replenishment of Fe^{2+} due to the presence of DNA during the anaerobic Fenton reaction. In contrast to the anaerobic observations under aerobic conditions in the presence of 2 mM DNA, $k_{(Fe^{2+})}$ *exceeded* $k_{(H_2O_2)}$ by roughly 2-fold (Figure 2B,C). This increase in $k_{(Fe^{2+})}$ could be explained by enhanced oxidation of Fe^{2+} due to the presence of DNA during the Fenton reaction.

In summary, the rate of H_2O_2 consumption was increased roughly 30% by the presence of DNA irrespective of

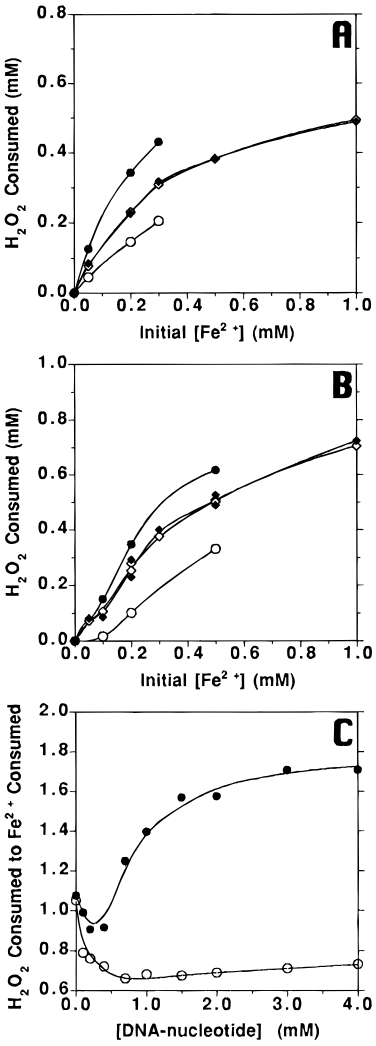

FIGURE 1: Effect of DNA and oxygen upon H_2O_2 consumption *vs* Fe^{2+} consumption. In panel A, reactions contained 0.5 mM H_2O_2, $FeSO_4$ as indicated, and 4 mM DNA-nucleotide as indicated. In panel B, reactions were as in panel A except that 1 mM H_2O_2 was utilized. In panel C, reactions contained 0.5 mM H_2O_2, 0.2 mM $FeSO_4$, and DNA as indicated. The H_2O_2 remaining after 12−15 min was determined as described in Experimental Procedures. ♦, purged with N_2, DNA absent; ●, purged with N_2, DNA present; ◊, aerated, DNA absent; ○, aerated, DNA present.

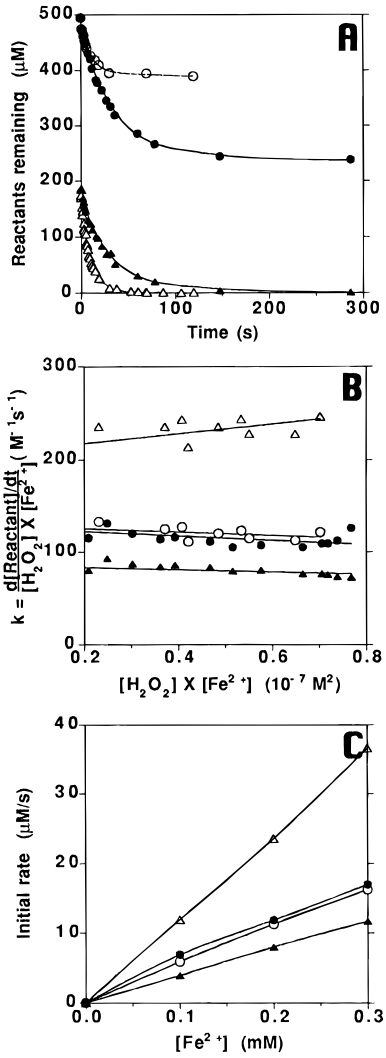

FIGURE 2: Kinetics of H_2O_2 consumption and Fe^{2+} oxidation in the presence and absence of DNA. Reactions contained 2 mM DNA-nucleotide, 0.5 mM H_2O_2, and 0.2 mM $FeSO_4$ except that initial $FeSO_4$ concentrations were as indicated in panel C. H_2O_2 and Fe^{2+} concentrations were determined as described in Experimental Procedures. Panel B shows the pseudobimolecular rate constants as defined in Experimental Procedures calculated from the data of panel A. Panel C shows initial rates obtained from curves such as those in panel A. ●, purged with N_2, H_2O_2 consumption; ○, aerated, H_2O_2 consumption; ▲, purged with N_2, Fe^{2+} consumption; △, aerated, Fe^{2+} consumption.

aeration. Conversely, Fe^{2+} consumption was enhanced roughly 2-fold by the presence of DNA under aerobic conditions such that it exceeded that of H_2O_2 consumption. On the other hand, Fe^{2+} consumption under anaerobic conditions was somewhat lower than H_2O_2 consumption.

Comparison of the Effects of DNA to Those of Ethanol and tert-Butyl Alcohol upon Fe^{2+} and H_2O_2 Consumption. To attempt to better understand the basis of the effects of DNA upon the stoichiometry of Fe^{2+} and H_2O_2 consumption during aerobic and anaerobic Fenton reactions, the effects of simple model compounds, ethanol, and to a lesser extent, *tert*-butyl alcohol, were studied. These alcohols were chosen because they had previously been studied in reactions with oxygen radicals. In addition, since the majority of radicals formed by •OH attack on ethanol, but not *tert*-butyl alcohol, are reducing radicals (Walling, 1975), the effects of these radicals could be distinguished. Therefore, H_2O_2 consumption was measured in reactions containing 0.5 mM H_2O_2 and 0.2 mM Fe^{2+} in the presence of various concentrations of DNA up to 4 mM nucleotide residues, ethanol up to 3 mM, and *tert*-butyl alcohol up to 10 mM (Figure 3). For purposes of comparison, the concentrations of organic substrates have been normalized in this figure according to the rate constants of their reaction with •OH. In all cases, when the reaction was completed, some H_2O_2 remained, but Fe^{2+} was totally consumed.

Under aerobic conditions, H_2O_2 consumption decreased with increasing concentration of each of the compounds (open symbols, Figure 3). At the highest concentrations of the alcohols, the ratio of H_2O_2 consumed to Fe^{2+} consumed was approximately 0.5. In the case of DNA this ratio was 0.68. Under anaerobic conditions (closed symbols, Figure 3), the results were quite different. The presence of DNA or ethanol increased the rate of H_2O_2 consumption by up to 75% and 240%, respectively. *tert*-Butyl alcohol, however, did not effect a change in H_2O_2 consumption.

The decrease in H_2O_2 consumption due to the presence of the organic compounds under aerobic conditions could

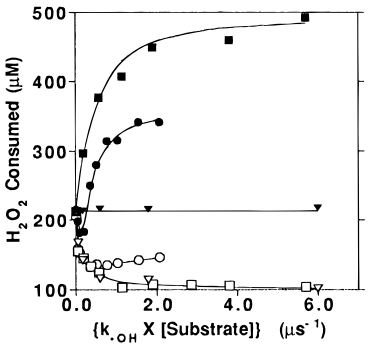

FIGURE 3: H_2O_2 consumption during the Fe^{2+}-mediated Fenton reaction in the presence of DNA, ethanol, or *tert*-butyl alcohol. Reactions contained 0.2 mM $FeSO_4$, 0.5 mM H_2O_2, and 0−4 mM DNA-nucleotide, 0−3 mM ethanol, or 0−10 mM *tert*-butyl alcohol, as indicated. The H_2O_2 remaining after 12−15 min was determined as described in Experimental Procedures. The data for DNA are from the same experiment as those in Figure 1C. The abscissa is the concentration of the organic substrate (DNA, ethanol, or *tert*-butyl alcohol) multiplied by its rate constant in reacting with •OH, as reviewed by Buxton *et al.* (1988): 5.2×10^8 M^{-1} s^{-1}, 1.9×10^9 M^{-1} s^{-1}, and 6.0×10^8 M^{-1} s^{-1} for DNA, ethanol, and *tert*-butyl alcohol, respectively. ●, purged with N_2, DNA present; ○, aerated, DNA present; ■, purged with N_2, ethanol present; □, aerated, ethanol present; ▼, purged with N_2, *tert*-butyl alcohol present; ▽, aerated, *tert*-butyl alcohol present.

be explained if carbon-centered radicals were formed on the substrates which would react with O_2 to form peroxyl radicals (von Sonntag, 1987). These peroxyl radicals would then in turn react with Fe^{2+}. The resulting effect of these reactions would be an apparent diminishment of H_2O_2 consumption relative to Fe^{2+} consumption.

The increase in H_2O_2 consumption due to the presence of the compounds under anaerobic conditions could be explained if carbon-centered radicals were to reduce the iron atoms which had been oxidized by H_2O_2 in the Fenton reaction. The varying effects of the added compounds would then be explained by the different radicals which each can form. With ethanol, 85−97% of the radicals would have a reducing nature (Buxton *et al.*, 1988; Asmus *et al.*, 1973; Burchill & Ginns, 1970; Berdnikov *et al.*, 1977). (See footnote *f* of Table 1 for the definition of the radical character of ethanol radicals.) With DNA, however, the radicals are more heterogeneous and many of them are redox-ambivalent or oxidizing (Steenken, 1989). Hence ethanol would be better at re-forming Fe^{2+} from Fe^{3+} than would DNA. Radicals formed from *tert*-butyl alcohol are not reducing, however, so that, in the presence of *tert*-butyl alcohol, Fe^{2+} should not be replenished. This is consistent with the observation (Figure 3) that, under anaerobic conditions, the presence of *tert*-butyl alcohol had no effect upon the extent of H_2O_2 consumption.

Modeling of H_2O_2 and Fe^{2+} Consumption by Fenton Reactions in the Presence of Ethanol. Although it would be desirable to validate the above concepts by quantitatively modeling the reaction kinetics of the iron-mediated Fenton reaction in the presence of DNA, the complexity and heterogeneity of DNA reactions with oxygen radicals do not allow this. Thus ethanol was chosen as a model system from which proposals could be derived which could apply to DNA. With ethanol the pertinent free radical reactions and their rate constants are known or can be adequately estimated, so that 32 reactions (Table 1) were considered in a computer simulation of the kinetics of H_2O_2 and Fe^{2+} consumption

Sequence I

Reaction	1	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$
	15	$\cdot OH + CH_3CH_2OH \rightarrow CH_3CH(OH)\cdot + H_2O$
	12	$CH_3CH(OH)\cdot + Fe^{3+} \rightarrow Fe^{2+} + CH_3CHO + H^+$

Sequence II

Reaction	1	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$
	15, 16	$\cdot OH + EtOH \rightarrow \cdot(EtOH) + H_2O$
	23, 24	$\cdot(EtOH) + O_2 \rightarrow \cdot(O_2EtOH)$
	26, 27	$Fe^{2+} + H^+ + \cdot(O_2EtOH) \rightarrow Fe^{3+} + HO_2EtOH$

Sequence III

Reaction	1	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$
	15	$\cdot OH + CH_3CH_2OH \rightarrow CH_3CH(OH)\cdot + H_2O$
	23	$CH_3CH(OH)\cdot + O_2 \rightarrow CH_3 - CH(OH)O_2\cdot$
	22	$CH_3 - CH(OH)O_2\cdot \rightarrow CH_3CHO + HO_2\cdot$
	2	$Fe^{2+} + HO_2\cdot \rightarrow Fe^{3+} + HO_2^-$
	3	$Fe^{3+} + HO_2\cdot \rightarrow Fe^{2+} + H^+ + O_2$

Sequence IV

Reaction	1	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$
	33	$DNA + \cdot OH \rightarrow DNA\cdot$
	34	$DNA\cdot + Fe^{3+} \rightarrow DNAox + Fe^{2+} + H^+$

Sequence V

Reaction	1	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$
	33	$DNA + \cdot OH \rightarrow DNA\cdot$
	35	$DNA\cdot + O_2 \rightarrow DNAO_2\cdot$
	36	$DNAO_2\cdot + Fe^{2+} + H^+ \rightarrow DNAO_2H + Fe^{3+}$

Sequence VI

Reaction	1	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$
	33	$DNA + \cdot OH \rightarrow DNA\cdot$
	35	$DNA\cdot + O_2 \rightarrow DNAO_2\cdot$
	37	$DNAO_2\cdot \rightarrow DNAox + HO_2\cdot$
	2	$Fe^{2+} + HO_2\cdot \rightarrow Fe^{3+} + HO_2^-$
	3	$Fe^{3+} + HO_2\cdot \rightarrow Fe^{2+} + H^+ + O_2$

FIGURE 4: Reaction sequences for explaining altered stoichiometries of the Fe^{2+}-mediated Fenton reaction in the presence of ethanol or DNA. Reaction numbers correspond to those in Table 1. In sequence II, •(EtOH) is either the α- or the *β*-hydroxy radical at a ratio of about 20:1 (see Table 1). Thus, •(O_2EtOH) can be the α- or *β*-peroxyl radical, which are reduced by Fe^{2+} to form $CH_3CH(OH)$-O_2H or $HO_2CH_2CH_2OH$, respectively.

under both aerobic and anaerobic conditions. By monitoring the contribution of each reaction to the overall reactant consumptions during the simulation, one can identify the predominant reactions for such a simulation. In this way it became apparent that while many of the reactions listed in Table 1 could contribute to the stoichiometry of Fe^{2+} and H_2O_2 utilization, only a few reactions predominate. These predominant reactions can be grouped into three reaction sequences (sequences I−III, Figure 4), one for anaerobic conditions and two for aerobic conditions.

The rate limiting step in all three sequences is the Fenton reaction which leads to an ethanol radical. Under anaerobic conditions (sequence I, Figure 4), the •OH formed by the Fenton reaction reacts with ethanol to form the α-hydroxy radical. Fe^{2+} can be replenished from Fe^{3+} by the α-hydroxy radical. Thus several equivalents of H_2O_2 might be consumed for each Fe^{2+} initially present.

Under aerobic conditions (sequences II and III, Figure 4) •OH forms ethanol hydroxy radicals which in turn react with O_2 to form the corresponding peroxyl radicals. The nascent peroxyl radicals can then oxidize Fe^{2+} to Fe^{3+} (sequence II). Alternatively, the α-peroxy radical could decay to form acetaldehyde and superoxide, and the superoxide could react with iron ions to change the Fenton reaction stoichiometry (sequence III, Figure 4).

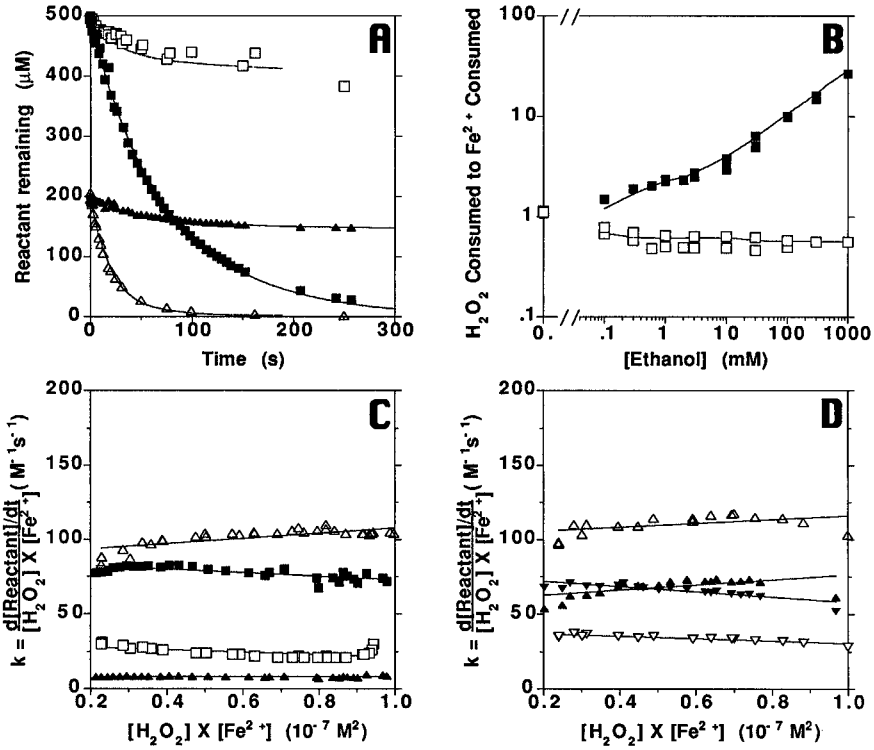

FIGURE 5: H_2O_2 and Fe^{2+} consumption during Fenton reactions in the presence of ethanol or *tert*-butyl alcohol. All reactions contained 0.2 mM $FeSO_4$ and 0.5 mM H_2O_2. Reactions of panels A and C contained 100 mM ethanol, those of panel B contained ethanol as indicated, and those of panel D contained 200 mM *tert*-butyl alcohol. The symbols represent individual data points. In panels A and B the solid lines are interpolated fits from the results of computer simulations which considered the relevant reactions listed in Table 1, but which showed that those of sequences I−III of Figure 4 represented the predominant reactions. In panel C the pseudobimolecular rate constants for H_2O_2 and Fe^{2+} consumption as defined in Experimental Procedures were calculated for the time course shown in panel A. In panel D under anaerobic conditions, the pseudobimolecular rate constants for Fe^{2+} and H_2O_2 consumption were 72 ± 7 M^{-1} s^{-1} and 67 ± 7 M^{-1} s^{-1}, respectively. △, aerated, Fe^{2+} consumption; ▲, purged with N_2, Fe^{2+} consumption; ■, purged with N_2, ethanol present, H_2O_2 consumption; □, aerated, ethanol present, H_2O_2 consumption; ▼, purged with N_2, *tert*-butyl alcohol present, H_2O_2 consumption; ▽, aerated, *tert*-butyl alcohol present, H_2O_2 consumption.

The assignment of the reactions of sequences I, II, and III as being predominant was tested by comparing the computer-calculated predictions of Fe^{2+} and H_2O_2 consumption to experimental results. Time courses for H_2O_2 and Fe^{2+} remaining in the presence of 100 mM ethanol (Figure 5A) and the ratio of H_2O_2 to Fe^{2+} consumed in the presence of varying ethanol concentrations (Figure 5B) were measured and compared to the values predicted by the computer simulations. The fits between the data and the simulations under both aerobic (open symbols) and anaerobic conditions (closed symbols) are within experimental error.

The experimental results under anaerobic conditions demonstrate also that, in the presence of 100 mM ethanol, H_2O_2 is consumed with a pseudobimolecular rate constant expected for the Fenton reaction, 76 M^{-1} s^{-1} (Walling, 1975) (Figure 5C). On the other hand, $k_{(Fe^{2+})}$ was only one-eighth that rate (Figure 5C). Moreover, between 3 and 1000 mM ethanol, the amount of acetaldehyde formed equaled the amount of H_2O_2 consumed within 5%, as expected for sequence I of Figure 4 (Figure 6). When similar time courses were done with 200 mM *tert*-butyl alcohol present under anaerobic conditions, $k_{(H_2O_2)}$ was again approximately that of the Fenton reaction, and this was also the case for $k_{(Fe^{2+})}$ (Figure 5D). These results are consistent with the reducing nature of the α-hydroxyethanol radical which can reduce Fe^{3+} to Fe^{2+} and with the nonreducing nature of the β-hydroxy *tert*-butyl alcohol radical, which does not react with Fe^{3+}.

As expected from sequences II and III of Figure 4, the aerobic situation is more complex. In the presence of 100 mM ethanol, H_2O_2 is depleted less rapidly than is Fe^{2+} (Figure 5A). Throughout the reaction, $k_{(H_2O_2)}$ is roughly one-third that of the Fenton reaction, whereas $k_{(Fe^{2+})}$ is somewhat greater than that of the Fenton reaction (Figure 5C). The ratio of H_2O_2 consumed per Fe^{2+} consumed in an aerobic reaction with 0.1−1000 mM ethanol present was 0.55 (Figure 5B). If only the reactions of sequence II had occurred, the ratio would have been 0.5. Should only the reactions of sequence III have occurred, the ratio could also have been 0.5, but only if reactions 2 and 3 had occurred at equal frequency—i.e., if superoxide had reduced Fe^{3+} and oxidized Fe^{2+} to equal extents. $k_{(H_2O_2)}$ in the presence of 100 mM ethanol and O_2 was 22 M^{-1} s^{-1}, whereas that for the

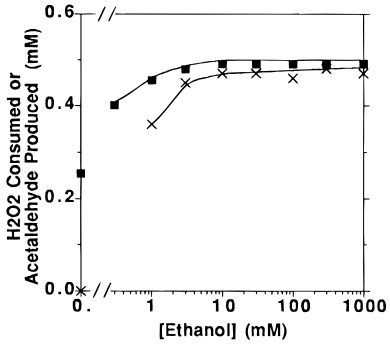

FIGURE 6: Acetaldehyde production during Fenton reactions in the presence of ethanol. Reactions were purged with N_2 and contained 0.2 mM $FeSO_4$, 0.5 mM H_2O_2, and ethanol as indicated. After 20 min, acetaldehyde was measured as described in Experimental Procedures. ■, H_2O_2 consumed; ×, acetaldehyde produced.

anaerobic reaction was 73 ± 4 M^{-1} s^{-1} (Figure 5C). Sequence II would predict a rate constant of 76 M^{-1} s^{-1}—i.e., the Fenton reaction alone would determine the rate of H_2O_2 consumption. Hence, replenishment of H_2O_2 would appear to be significant in the aerobic reactions. Since only reaction 2 in sequence III replenishes H_2O_2, sequence III must be occurring to a significant extent, utilizing both reactions 2 and 3 to roughly equal extents.

Application of the Ethanol Studies to DNA. The studies of Fenton reactions in the presence of ethanol can serve as a model by which to begin to understand the complexity of the reactions in the presence of DNA which were observed in Figures 1−3. Based upon the results with ethanol, reaction sequences might be proposed by which iron reacts with DNA-derived radicals (Figure 4, sequences IV−VI). In all cases, the Fenton reaction would generate a reactive oxygen species which in turn would react with DNA, thereby generating DNA radicals (DNA•). For purposes of stoichiometry, the reactive oxygen species is simply given as •OH, though it could be an alternative radical such as the ferryl radical. If the DNA radical were to have a reducing nature and if O_2 were unavailable, then, by analogy to sequence I, the DNA radical could reduce Fe^{3+} back to Fe^{2+}, thereby generating an oxidized DNA product, DNAox (reaction 34, sequence IV, Figure 4). The regenerated Fe^{2+} could react again with H_2O_2, more DNA damage could ensue, and multiple equivalents of H_2O_2 would be consumed for each Fe^{2+} initially entering into this sequence, as was observed under anaerobic conditions in the presence of DNA (Figures 1−3 and Table 2). Since O_2 reacts at diffusion limited rates with most carbon-centered radicals, this sequence is likely only to proceed under conditions where O_2 concentrations remain very low.

Under aerobic conditions, DNA peroxyl radicals are assumed to be formed by reaction of the DNA radicals with O_2 (sequences V and VI, Figure 4), analogously to the case for ethanol (sequences II and III). In one case, sequence V, the peroxyl radicals could oxidize additional Fe^{2+} ions (reaction 36), thereby diminishing the pool of Fe^{2+}. As a consequence, the overall generation of the Fenton oxidant would be diminished and hence the amount of DNA damage would be reduced. On the other hand, the DNA peroxides which are generated by this sequence would be predicted to give rise to unique damage products, and products consistent with this sequence of reactions have been observed (Luo *et al.*, 1996; Henle *et al.*, 1996).

An alternative sequence of reactions under aerobic conditions, sequence VI, would be the analog of sequence III. The DNA peroxyl radicals would decompose *via* reaction 37 into superoxide and oxidized DNA (DNAox). The superoxide radicals could then react with either Fe^{2+} or Fe^{3+} (reactions 2 or 3), respectively.

Depending upon the relative rates of reactions 36 and 37, either sequence V or sequence VI would predominate. However, $k_{(H_2O_2)}$ observed in the presence of DNA was approximately the same under aerobic and anaerobic conditions and somewhat greater than that of the Fenton reaction in a pure system (110 M^{-1} s^{-1} *vs* 76 M^{-1} s^{-1}) (Figure 2B). This observation would imply that the reactions of sequence VI utilizing reaction 2 would not occur to an appreciable extent as $k_{(H_2O_2)}$ would have been diminished by this reaction. In addition, if sequence VI proceeded *via* reaction 3, Fe^{2+} would have been replenished, contrary to the data of Figure 2 in which DNA increased the consumption of Fe^{2+}. Therefore, sequence V would appear to be dominant over sequence VI.

DISCUSSION

The data presented in this paper and the proposals that it has generated may be summarized as follows. During a Fenton reaction between Fe^{2+} and H_2O_2 with no additional reactants, the amounts of H_2O_2 consumed per Fe^{2+} consumed are equal under aerobic or anaerobic conditions. However, when DNA is present, an increase in the ratio of H_2O_2 consumed to Fe^{2+} consumed occurs under anaerobic conditions. This increase is ascribed to replenishment of Fe^{2+} by a DNA radical which is produced in the presence of iron and H_2O_2. Conversely, under aerobic conditions a decrease in the ratio of H_2O_2 consumed to Fe^{2+} consumed occurs in the presence of DNA. This decrease is ascribed to Fe^{2+} oxidation by a DNA radical which was formed by reaction of O_2 with a precursor DNA radical formed in the presence of iron and H_2O_2.

As noted in Table 2, there were substantial quantitative differences in the degree of damage to each of the four DNA nucleosides in aerobic *vs* anaerobic reactions. We have begun to characterize the specific base damages to DNA and its derivatives, and we have observed some qualitative differences between aerobic and anaerobic conditions (Luo *et al.*, 1996; Henle *et al.*, 1996; R. Jin, R. Chattopadhyaya, E. S. Henle, Y. Luo, and S. Linn, unpublished experiments). 2′-Deoxyguanosine 5′-aldehyde was observed to be formed preferentially under aerobic conditions whereas 5′,8-cyclo-2′-deoxyguanosine was observed preferentially under anaerobic conditions (Henle *et al.*, 1996). A possible mechanism for the formation of these products, based upon studies by Dizdaroglu (1986) and Langfinger and von Sonntag (1985) for ionizing radiation, is that a 5′-carbon-centered radical would be formed by reaction with a Fenton oxidant. Under anaerobic conditions, the 5′-radical could attack the guanine base, and oxidation of the resulting guanine radical by a ferric ion would yield 5′,8-cyclo-2′-deoxyguanosine analogously to sequence IV of Figure 4. Under aerobic conditions, on the other hand, the 5′-radical could also react with O_2 to produce a peroxyl radical which would ultimately be converted to the 5′-aldehyde.

In a similar study with d-CpC (Luo *et al.*, 1996), it was found that, among the major degradation products, 1-carbamoyl-1-carboxy-4-(2-deoxyribosyl)glycinamide was observed to be formed preferentially under aerobic conditions whereas 5-hydroxy-2′-deoxycytidine was observed only under anaerobic conditions. Based on the general mechanisms for the generation of these products by Cadet *et al.* (1991) by ionizing radiation in the absence of iron and H_2O_2, a possible explanation was proposed for these differences (Luo *et al.*, 1996). The major site of reaction of the Fenton oxidant is C5 of cytosine whereby a 5-hydroxy-6-yl radical is formed. Under anaerobic conditions this radical might be oxidized by Fe^{3+} so as to form 5-hydroxy-2′-deoxycytidine analogously to sequence IV of Figure 4. Under aerobic conditions, on the other hand, the 5-hydroxy-6-yl radical would instead react with O_2 leading to a peroxyl radical which could oxidize Fe^{2+}, forming 1-carbamoyl-1-carboxy-4-(2-deoxyribosyl)glycinamide (Luo *et al.*, 1996). These reactions would be analogous to sequence V, Figure 4.

In future studies we hope to utilize defined DNA oligonucleotides. The goal would be to be able to monitor reactions leading to particular DNA damages so as to ultimately understand them to the extent to which we understand the reactions with ethanol. In so doing, we would hope to validate the sequences of Figure 4 and to define exactly the intermediates and products.

ACKNOWLEDGMENT

We are indebted to Dr. Rajagopal Chattopadhyaya for the early observations which led to the initiation of this study.

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BI961235J