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Genetics

Mutagenesis by the autoxidation of iron with isolated DNA

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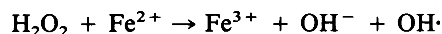
ABSTRACT Oxygen free radicals are highly reactive species generated by many cellular oxidation–reduction processes. These radicals damage cellular constituents and have been causally implicated in the pathogenesis of many human diseases. We report here that oxygen free radicals generated by Fe^{2+} in aqueous solution are mutagenic. Aerobic incubation of φX174 *am3* (amber 3 mutation) DNA with Fe^{2+} results in decreased phage survival when the treated DNA is transfected into *Escherichia coli* spheroplasts. Transfection of the treated DNA into SOS-induced spheroplasts results in an increase in mutagenesis as great as 50-fold. Both killing and mutagenesis can be prevented by binding of Fe^{2+} with deferoxamine or by the addition of catalase or mannitol. These results suggest that DNA damage and mutagenesis brought about by Fe^{2+} are likely to occur by a Fenton-type mechanism that involves the generation of (*i*) hydrogen peroxide by the autoxidation of iron and (*ii*) hydroxyl radicals by the interaction of the hydrogen peroxide with Fe^{2+}. DNA sequence analysis of the Fe^{2+}-induced mutants indicates that reversion of the phage phenotype to wild type occurs largely by a transversion type of mutation involving substitution of deoxyadenosine for thymidine opposite a template deoxyadenosine. Mutagenesis is not abolished by incubation of Fe^{2+}-treated φX174 *am3* DNA with an apurinic endonuclease and only partially abolished by incubation with alkali, suggesting that a large fraction of the mutagenesis by oxygen free radicals is not caused by formation of apurinic sites but instead involves an as-yet-to-be-defined alteration in deoxyadenosine. These findings raise the possibility that free iron localized in cellular DNA may cause mutations by the generation of oxygen free radicals.

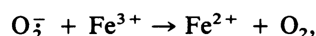
A portion of the total cellular oxygen metabolism proceeds by a sequence of one-electron reductions that result in oxygen free-radical intermediates. Processes reported to yield oxygen free radicals include phagocytosis, ischemic cell injury, and drug toxicity (1–4). The resultant free radicals have been hypothesized to be causative factors in aging (5), carcinogenesis (5, 6), and radiation injury (7) and to be a contributory factor in tumor promotion (8–10). Many of these effects could be mediated by interactions of oxygen free radicals with genomic DNA.

One of the simplest mechanisms for the generation of oxygen free radicals involves the reduction of H_2O_2 by divalent metal ions, particularly Fe^{2+}, with the formation of hydroxyl radicals (OH·) (Fenton reaction).



$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH\cdot$$

With limiting Fe^{2+} concentration, Fe^{3+} reduction is required for the continued formation of OH·. This can be accomplished by the superoxide anion:



$$O_2^{\bar{}} + Fe^{3+} \rightarrow Fe^{2+} + O_2,$$

with the overall reaction being the iron-catalyzed interaction between O_2 and H_2O_2 to form OH· or a similarly reactive species (Haber–Weiss reaction):

$$H_2O_2 + O_2^{\bar{}} \xrightarrow[Fe^{2+}]{} O_2 + OH^- + OH\cdot.$$

Some chelates—e.g., the Fe–EDTA complex—are active in the reaction, whereas others—e.g., the Fe–deferoxamine complex—are not. The ability of the iron–EDTA complex to cleave DNA *in vitro* (11) has been well documented. Tethering the Fe chelates to an intercalator enhances cleavage (12), and complexing the Fe with a specific hybridized oligonucleotide can be used to cleave specific sites on DNA (13). DNA cleavage by enzymatically generated oxygen radicals requires an additional metal catalyst, presumably Fe (14). DNA cleavage by H_2O_2 and chelated Fe has also been extensively studied (15, 16), and Cu-catalyzed degradation of DNA by H_2O_2 has been reported (17).

The extensive cleavage of biologically active DNA has made difficult the measurement and characterization of mutagenesis by oxygen radicals *in vitro*. A very sensitive assay using single-stranded φX174 *am3* (amber 3 mutation) DNA (18, 19) is used here for this purpose. In the *am3* region of the φX174 DNA genome, two proteins required for phage production are coded for by the same nucleotide sequence that is punctuated one reading frame apart. Only a limited number of nucleotide changes at the *am3* site are compatible with both proteins being active, and this could account for the low-background reversion frequency of mutants at the *am3* locus (18). As a result, use of φX174 *am3* DNA has made it possible to score for mutagenesis *in vitro*, even in the presence of extensive DNA cleavage and loss of biological activity. Using this assay, we have demonstrated the mutagenic effect of the autoxidation of Fe through the formation of oxygen free radicals. Mutagenesis is dependent on the induction of the SOS system and proceeds largely by T → A transversions.

MATERIALS AND METHODS

Chemicals. $FeSO_4$ (reagent grade) was obtained from Baker, and deferoxamine was from CIBA Pharmaceutical. HeLa apurinic endonuclease (fraction VII; specific activity, 200,000 units/mg) was purified by the method of Kane and Linn (20) and was supplied by D. Mosbaugh (Department of Chemistry, University of Texas, Austin). Catalase (bovine liver; 48,026 units/mg) was obtained from Cooper Biomedical (Malvern, PA), and superoxide dismutase (bovine erythrocytes; 12,300 units/mg) was obtained from Miles.

Bacteria and Bacteriophage. Bacterial strains, *Escherichia coli* HF4714 (su^+) and HF4704 (su^-) used for plating of φX174 phage and *E. coli* KT-1 for making spheroplasts, have been described (19, 21). Single-stranded φX174 *am3* DNA was obtained as before (21) and stored in 10 mM sodium phosphate buffer (pH 7.4) at −70°C.

Incubation of DNA with Fe^{2+}. φX174 *am3* DNA (29 μg/ml) was incubated in 10 mM sodium phosphate (pH 7.0) in the absence or presence of $FeSO_4$ in unsealed 1.5-ml Eppendorf

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plastic tubes. The final reaction mixture was either 0.05 or 0.15 ml, and incubation was for 30 min at 37°C with gentle agitation in a water bath. After addition of 1/10th volume of deferoxamine to yield a final concentration 10 times that of $FeSO_4$, 50 μl of each reaction mixture was immediately transfected into SOS-induced spheroplasts derived from *E. coli* strain KT-1. The resultant progeny phage were plated on HF4714 (su^+) and *E. coli* (su^-) to measure total and revertant phage, respectively. SOS-induced spheroplasts were prepared by irradiation of exponentially growing bacteria with UV light (254 nm) at 80 J/m^2 (22).

Preparation of Depurinated DNA. Incubation of φX174 single-stranded DNA was carried out in 30 mM KCl/10 mM Na citrate, pH 5.0, at 70°C for 15 min. After acid treatment, the reaction mixture containing the depurinated DNA was neutralized with NaOH. The DNA was precipitated with 0.3 M sodium acetate/70% ethanol at −70°C, and the precipitate was dissolved in 10 mM sodium phosphate (pH 7.4) and stored at 4°C. Approximately two apurinic sites per φX174 genome were formed as estimated from the sedimentation rate in alkaline sucrose gradients after hydrolysis of the apurinic sites in 0.1 M NaOH at 25°C for 2.5 hr (22).

Nucleotide Sequence Determination. Plaques containing revertant phage were picked and individually subcultured twice prior to phage growth and purification. The DNA from plaque-purified revertant phage was extracted by the procedure of Kunkel *et al.* (19) as recently modified (23). The nucleotide sequences were determined by the method of Sanger *et al.* with a 5′-^{32}P-labeled 15-mer primer that hybridizes to nucleotides 597–611 of the φX174 genome (24).

RESULTS

The lethal and mutagenic effects of Fe^{2+} were determined by incubating φX174 *am3* DNA with $FeSO_4$ in the presence of air and then measuring loss of biologic activity and frequency of revertants at the *am3* site after transfecting the treated DNA into *E. coli* spheroplasts. Incubation of single-stranded bacteriophage φX174 DNA with increasing concentrations of Fe^{2+} results in a progressive loss of biological activity, as measured by phage production after transfection of the DNA into *E. coli* spheroplasts (Fig. 1*A*). From an analysis of the loss of survival of phage as a function of Fe^{2+} concentration, we calculate that 2.7 μM Fe^{2+} introduces one lethal hit per phage genome in 30 min at 37°C. From the size distribution of the Fe^{2+}-treated φX174 DNA as determined by polyacrylamide gel electrophoresis, we estimate that approximately one break in the phosphodiester DNA backbone corresponds to one lethal event (results not shown). Mutagenesis was determined by measuring the frequency of reversions at the *am3* locus (Fig. 1*B*). With normal spheroplasts, the frequency of mutagenesis with 5 μM Fe^{2+} increased 2.3 ± 0.8-fold above the level of controls without Fe. With spheroplasts obtained from *E. coli* previously exposed to UV-irradiation (SOS-induced), the average increase in mutation frequency with 5 μM Fe^{2+} was 15-fold (nine separate experiments). A comparison of Fig. 1 *A* and *B* indicates that the reversion frequency per lethal event is approximately 15×10^{-6}. Thus, the majority of lesions are lethal because of the fragmentation of DNA, with the surviving DNA producing a high rate of mutation.

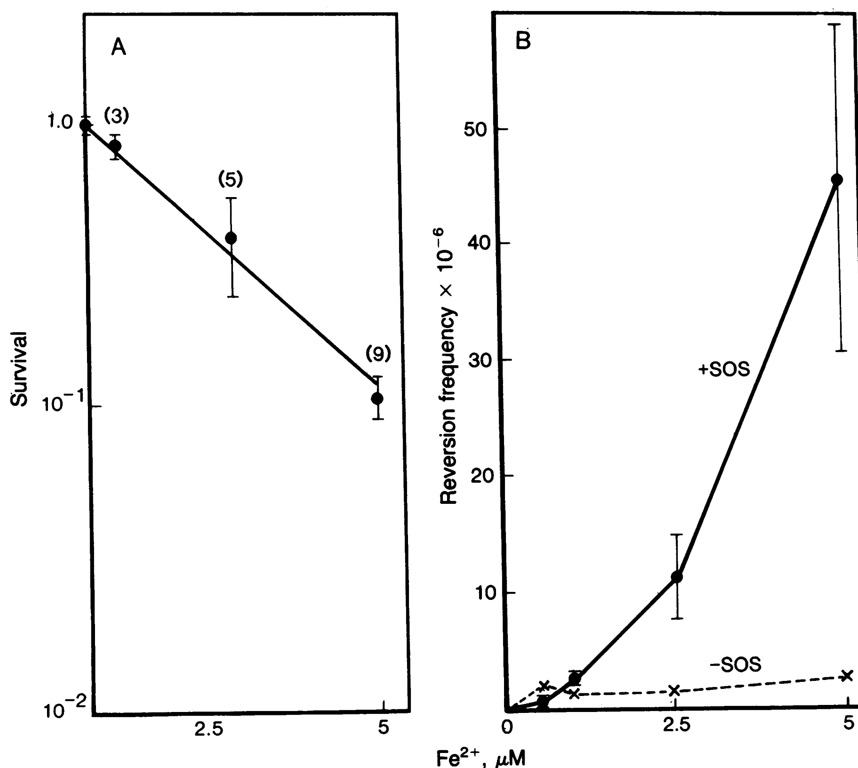


FIG. 1. Survival of single-stranded *am3* DNA and mutagenesis after incubation with Fe^{2+}. φX174 *am3* DNA (4 μg) was incubated in 0.15 ml of 10 mM sodium phosphate (pH 7.0) in the absence or presence of $FeSO_4$ at the concentrations indicated for 30 min at 37°C. After addition of deferoxamine at 10 times the Fe concentration to stop the reaction, 50 μl of each reaction mixture was immediately transfected into 2.5 ml of *E. coli* spheroplasts. (*A*) Plot of average number of plaques on HF4714 as a measure of survival after transfection in SOS-induced spheroplasts. The vertical bar is ±1 SD, and the number of experiments is in parentheses. (*B*) Plot of change in mutagenesis in a typical experiment with normal and SOS-induced spheroplasts. SOS induction was carried out before transfection by exposing the cells to UV light (80 J/m^2) prior to isolating spheroplasts. The reversion frequency in the absence of Fe^{2+} was 2.0×10^{-6} and 3.2×10^{-6} in the experiments with normal and SOS-induced spheroplasts, respectively; these were subtracted from each of the values given. Each assay was carried out in triplicate, and the vertical bar is ±1 SD.

To gain insight into the mechanisms of mutagenesis *in vitro* by Fe^{2+}, we studied the effects of adding different chelators and free radical or H_2O_2 scavengers to the reaction mixture (Table 1). Aerobic incubation with Fe^{2+}, by itself, reduced survival and enhanced mutagenesis. The single-stranded ϕX174 DNA had been extensively dialyzed to remove any chelators that may have copurified with the DNA. In a series of experiments, the addition of EDTA to the Fe^{2+} reaction had little or no effect on lethality and mutagenesis (Table 1 legend). In contrast, killing and mutagenesis by Fe^{2+} were essentially abolished by the prior addition of 50 μM deferoxamine, a chelator that sequesters Fe^{2+} in a form incapable of oxidation or reduction. The abolition of both the lethal and mutagenic effects of Fe^{2+} by catalase but not heated catalase indicates a requirement for hydrogen peroxide. Both the lethal and mutagenic effects of Fe^{2+} were markedly diminished by replacing air with argon during the incubation. The marked inhibitory effect of mannitol is compatible with the involvement of hydroxyl radicals (or a closely related species). In a number of experiments in which graded amounts of superoxide dismutase were added to the reaction, there was only a minimal reduction in either lethality or mutagenesis, arguing against a major direct involvement of superoxide radicals. The parallel lethal and mutagenic effects of Fe^{2+} under the different conditions tested suggest that both these processes are likely to be mediated by the same reactive species.

The nucleotide sequence changes observed in revertants obtained from treatment of ϕX174 *am3* DNA with Fe^{2+} and transfection into SOS-induced spheroplasts are given in Table 2. In 10 of 14 independently isolated revertant plaques obtained from controls in which single-stranded ϕX174 *am3* DNA was incubated in the absence of Fe^{2+}, the mutations resulted from the incorporation of deoxycytidine opposite the adenine residue at position 587 in the TAG amber codon. The presence of 5 μM Fe^{2+} increased mutagenesis 11.9-fold in 25 determinations (Table 2). Thus, 93% of the mutants sequenced were induced by Fe^{2+}, and 72% of these revertants were transversions involving substitution of deoxyadenosine for thymidine opposite deoxyadenosine on the (+)-strand template.

Table 1. Inhibition of Fe^{2+}-induced mutagenesis by chelators and free-radical scavengers

Exp.	Additions	Survival, % of control	Reversion frequency × 10^6
I	None	100	2
	Fe^{2+} (5 μM)	9	46
	Fe^{2+} (5 μM) + EDTA (10 μM)	6	30
	Fe^{2+} (5 μM) + deferoxamine (50 μM)	64	7
	Fe^{2+} (5 μM) + mannitol (0.1 M)	64	7
	Fe^{2+} (5 μM) + SOD (5.0 μg/ml)	16	23
	Fe^{2+} (5 μM) + catalase (5.8 μg/ml)	100	3
	Fe^{2+} (5 μM) + heated catalase (5.8 μg/ml)	22	40
II	None (incubated in air)	100	9
	None (incubated in argon)	62	8
	Fe^{2+} (15 μM) incubated in air	14	45
	Fe^{2+} (15 μM) incubated in argon	69	17

ϕX174 *am3* DNA (4 μg) was incubated in 0.15 ml of 10 mM sodium phosphate (pH 7.0) with the indicated added components for 30 min at 37°C. Transfection assays were carried out in SOS-induced spheroplasts; survival and reversion frequency were determined as described. The values given are the average of triplicate determinations. Catalase was inactivated by heating at 100°C for 20 min. In the control tubes without Fe^{2+}, the survival and the reversion frequency for deferoxamine, mannitol, superoxide dismutase (SOD), and catalase were, respectively, 100%, 100%, 100%, and 96% and 2.8, 2.3, 1.0, and 2.2 × 10^{-6}. In three other experiments with 15 μM Fe^{2+}, the addition of 30 μM EDTA increased the reversion frequency maximally about 2-fold.

Table 2. Frequency and types of base substitutions induced by exposure of ϕX174 DNA to Fe^{2+}

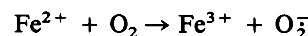
Additions	Reversion frequency × 10^6	Substitution at position 587		
		T → A	T → G	T → C
None	2.4 ± 2	4	0	10
Fe^{2+} (5 μM)	28.3 ± 14	23	7	2

ϕX174 *am3* DNA (4 μg) was incubated in 0.15 ml of 10 mM sodium phosphate (pH 7.0) in the absence or presence of 5 μM $FeSO_4$ for 30 min at 37°C. The reversion frequency is the average of 25 individual determinations in four different experiments. Plaques containing revertant phage from five different experiments were individually subcultured twice, and DNA was isolated and subjected to sequence analysis. Only changes at position 587 produce revertants because of the overlapping reading frames of genes *D* and *E* (18). An average of 108 nucleotides of sequence information was determined for each revertant, and no other changes in nucleotide sequence were observed.

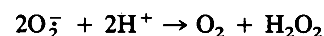
The SOS dependency and preference for misincorporation of dATP are characteristic for copying past apurinic sites (25). To determine whether or not Fe^{2+}-induced mutagenesis results from depurination, we exposed Fe-treated DNA to a HeLa cell apurinic endonuclease prior to transfection. As a control, we also examined ϕX174 *am3* DNA that had been depurinated by acid under conditions that are known to induce two apurinic sites per molecule (Table 3). Incubation of the acid-treated DNA with the HeLa apurinic endonucleases (18) entirely abolished mutagenesis. In contrast, only 26% of the Fe^{2+}-induced mutations were abolished by incubation with the same apurinic endonuclease prior to transfection. Mixing experiments in which graded amounts of acid- and Fe^{2+}-treated DNA were combined indicated that the resistance of the Fe^{2+}-treated DNA was not due to any inhibition of apurinic endonuclease activity by residual Fe^{2+} or by a product generated during the incubation with Fe^{2+}. Mutagenesis by Fe^{2+} is only partially abolished by alkali. Exposure of acid-treated DNA to alkali reduced mutagenesis by 93%, while mutagenesis by Fe^{2+}-treated DNA was decreased only 17% (Table 3) or 46% (legend to Table 3) upon incubation with alkali. These combined results suggest that mutagenesis resulting from treatment of ϕX174 *am3* DNA with Fe^{2+} is not primarily mediated by apurinic sites nor is it mediated by alkaline-labile lesions.

DISCUSSION

The studies presented suggest that the autooxidation of Fe^{2+} results in the production of oxygen radicals that are both lethal and mutagenic to DNA. The data from experiments with specific chelators and scavengers of reactive oxygen species (Table 1) suggest the following sequence of reactions that has been postulated in other systems (26–28):



$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bar{}}$$



$$2O_2^{\bar{}} + 2H^+ \rightarrow O_2 + H_2O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot.$$

In this scheme, Fe^{2+} reacts with molecular oxygen to generate Fe^{3+} and $O_2^{\bar{}}$, with $O_2^{\bar{}}$ undergoing spontaneous dismutation to H_2O_2. The interaction of Fe^{2+} + H_2O_2 proceeds by a classical Fenton reaction, with the production

Table 3. Effect of apurinic endonuclease and alkali on Fe^{2+}-induced mutagenesis

	Reversion frequency × 10^6			% reduction in mutagenesis	
ϕX174 *am3* DNA	Control	AP endo	NaOH	AP endo	NaOH
Control	4 ± 1				
Acid-treated	18 ± 3	3 ± 0.4	5 ± 2	>100	93
75% acid-treated + 25% Fe^{2+}-treated	25 ± 7	9 ± 1		76	
50% acid-treated + 50% Fe^{2+}-treated	22 ± 5	12 ± 5		56	
25% acid-treated + 75% Fe^{2+}-treated	28 ± 2	17 ± 1		46	
Fe^{2+}-treated	33 ± 5	25 ± 7	28 ± 5	26	17

Acid-treated ϕX174 *am3* DNA was made by incubation at pH 5.0 for 15 min at 70°C, yielding approximately two apurinic sites per molecule. Fe^{2+}-treated DNA was obtained by incubating aerobically ϕX174 *am3* DNA (240 ng) in 50 μl of 0.01 M sodium phosphate (pH 7.0)/60 μM $FeSO_4$ for 45 min at 37°C. After addition of 10 μl of 300 μM deferoxamine, groups of four identical reaction mixtures were combined and dialyzed for 3 hr in drops upon Millipore type V filter discs that were floated over 200 ml of 25 mM Tris·HCl (pH 8.0). Thereafter, the DNA was precipitated with 2 ml of 0.3 M sodium acetate/70% ethanol, and the pellet was redissolved in 25 mM Tris·HCl (pH 8.0) and transfected into *E. coli* spheroplasts. Each transfection reaction contained a total of 240 ng of either acid- or Fe^{2+}-treated ϕX174 *am3* DNA or a mixture of both in the indicated proportions. The DNA samples were incubated (*i*) with and without 16 units of HeLa apurinic endonuclease (AP endo) in 0.05 ml of 25 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/0.1 mM EDTA/0.005% Triton X-100 for 2 hr at 37°C or (*ii*) with and without 0.1 M NaOH for 2.5 hr at 25°C. Reactions and transfections were carried out in triplicate, and the results are given as the average ±1 SD. The percentage reduction in mutagenesis caused by treatment with apurinic endonuclease or alkali was obtained by subtracting the frequency of revertants with untreated DNA (controls) from that obtained after the indicated treatments. In a separate experiment, the reversion frequency after incubation of ϕX174 *am3* DNA in the absence (control) or presence of 30 μM Fe^{2+} was 1.0×10^{-6} and 24×10^{-6}, respectively. Subsequent treatment with NaOH reduced Fe-induced mutagens by 46%.

of OH· or a similarly reactive species, which is likely to be responsible for both the lethal and mutagenic effects of Fe^{2+}. The small decrease in mutagenesis upon the addition of superoxide dismutase suggests that O_2^- is not a major direct mutagen and that the Haber–Weiss reaction is not critical for the generation of the OH· required for mutagenesis. However, a requirement for O_2^- for oxygen radical-dependent DNA damage has been described in other systems (14, 15, 29, 30) and the partial inhibition by superoxide dismutase in our system may reflect a contribution by the Haber–Weiss reaction to the mutagenesis. The lack of an enhancement of mutagenesis by superoxide dismutase suggests that the spontaneous dismutation reaction is not a rate-limiting step in this pathway.

A chelator may be required to maintain the iron in solution in a catalytically active form. In contrast to other studies (31), we observe only a small enhancement in the inactivation of DNA by Fe^{2+} upon the addition of EDTA. However, we cannot eliminate the possibility that EDTA or a cellular chelator is present in our preparations of DNA; this may account for differences in the stoichiometry between the amount of added Fe^{2+} and the extent of mutagenesis observed with different preparations of ϕX174 *am3* DNA (compare Fig. 1 and Table 3). EDTA used in the preparation of DNA (19, 21) could copurify with the DNA despite the extensive dialysis. Alternatively, the DNA itself (32) or phosphate (26, 33) could chelate Fe^{2+}. Transition metal ions have been shown to be sequestered by nucleotides (34), and these complexes can induce the formation of short-lived free radicals that can damage surrounding DNA.

It seems highly probable that nucleotide damage by oxygen free radicals occurs at many sites on the ϕX174 genome, even though the ϕX174 *am3* DNA used in this study scores for only base substitution at nucleotide position 587. At this position, reversion to the wild-type phage occurs by substitution of deoxycytidine, deoxyguanosine, or deoxyadenosine for thymidine opposite deoxyadenosine in the template (+)-strand (18, 21). Damage to DNA by free radicals has been documented to produce single-stranded breaks as well as modified thymidines (35, 36) and deoxyguanosines (37, 38). Even though we cannot eliminate mutations resulting from alterations in thymidine or deoxyguanine at the neighboring nucleotide template positions, it seems more likely that the promutagenic alteration occurred on deoxyadenosine. Damage to adenine nucleosides by ionizing radiation and UV-irradiation has been demonstrated (39, 40) in processes that may involve free-radical intermediates.

The mutagenic potential of iron-dependent oxygen radical formation per lethal event is substantial and is equal to or greater than that observed by treatment of ϕX174 *am3* DNA with other agents, including those that produce predominantly apurinic sites (25). The ϕX174 DNA used in these studies is a single-stranded circle, and our assay is particularly sensitive to breaks in the phosphodiester backbone of DNA because one break per molecule is lethal. Mutagenesis is scored in only those molecules lacking breaks in DNA. Assuming breaks occur randomly, then 13% of damaging events at position 587 results in base-substitution mutations. Our finding that 23 of the 32 sequenced mutants contain deoxyadenosine at position 587 indicates that the majority of Fe^{2+}-induced mutations at this site involve a T → A transversion mutation. In contrast, spontaneous errors in DNA replication involve predominately transitions (41) as exemplified by our controls, which show primarily T → C transitions at this position. Since transversions involving deoxyadenosine substitution in SOS-induced cells are characteristic of mutagenesis via depurination (25), it seemed reasonable that the mutagenesis by oxygen free radicals might proceed by this pathway. Depurination of DNA has been shown to be enhanced by exposure of DNA to Cu^{2+}, Cr^{3+}, and Ni^{2+} (42), and apurinic sites have been shown to be a product of radiation damage to DNA (39), a closely related process. However, our inability to abolish mutagenesis by incubation of Fe^{2+}-treated DNA with an apurinic endonuclease or with alkali argues strongly against requirement of an apurinic site or a closely related lesion for mutagenesis. Identified structural analogues of abasic sites are alkaline labile (43–45), and evidence from studies with structural analogues of apurinic sites indicates that apurinic endonucleases detect the absence of a base rather than the presence of a deoxyribose moiety (46). Even though bulky adducts have also been shown to result in deoxyadenosine

substitution during DNA synthesis *in vitro* (47), there is no obvious source of these adducts in our purified system. Alteration of deoxyadenosine by oxygen free radicals has been demonstrated (J. Cadet, personal communication), with the formation of 8-hydroxyadenosine. The analogous guanine adduct, 8-hydroxyguanosine, is produced as a result of oxygen free-radical damage to DNA in a variety of systems (37, 38), but *in vitro* studies indicate that this analogue causes a general increase in the frequency of misincorporation (48) and does not specify the misinsertion of deoxyadenosine.

Mutagenesis by agents, including Fe (6, 49–51), that induce oxygen free radicals has been demonstrated both in procaryotic and eucaryotic cells. Since these studies utilize intact cells, molecular studies of the active species has been limited. The establishment of an *in vitro* system for measuring mutagenesis by oxygen radicals should permit us to identify the active mutagenic species and the lesions in DNA responsible for mutagenesis. Our finding that mutagenesis involves $T \rightarrow A$ base substitutions should be considered only within the limited context of mutations that can be scored by the ϕX174 system. It seems likely that many other mutations result from free radical damage to DNA and that some of these are even more frequent. As a result of the high mutagenic potential of oxygen free radicals, it should be feasible to establish a complete spectrum of mutations using a forward mutation assay. A comparison of such a spectrum with that exhibited by spontaneous mutations should allow one to assess the contributions of free-radical damage to both spontaneous and induced mutagenesis.

The effects of the autoxidation of Fe on DNA raises the possibility that in animal cells intracellular Fe could be a source of DNA damage and spontaneous mutation and, in this way, could be a contributor to pathologic processes. In this regard, a causal relationship between increased *in vivo* Fe stores and the risk of certain human cancers has been proposed based on epidemiologic studies (52, 53). Free Fe is present in low concentration in biological fluids, as it is predominately associated with Fe-binding proteins, heme proteins, and Fe–S centers. Although biologic Fe complexes may cause chromosomal abnormalities (54), DNA strand breaks (55), and the production of oxygen free radicals (56), the trace amounts of Fe bound to DNA (57) or in close proximity to it may be more significant with respect to DNA damage and mutagenesis.

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