Role of Superoxide in Deoxyribonucleic Acid Strand Scission†

Stephen A. Lesko,* Ronald J. Lorentzen, and Paul O. P. Ts'o

ABSTRACT: Single-strand scissions were produced in T7 DNA upon incubation with potassium superoxide or hydrogen peroxide in aqueous solution at neutral pH. The number of strand scissions induced per intact single strand of DNA was calculated from the molecular weight determined by band centrifugation through alkaline sodium chloride. The degradation was completely inhibited by EDTA and catalase, suggesting that metal ions and hydrogen peroxide (produced by dismutation of superoxide) are involved. Moreover, inhibition of strand scission by very small amounts of the chelator diethylenetriaminepentaacetic acid indicates that superoxide and hydrogen peroxide by themselves are not effective agents for breaking DNA. Inhibition of strand scission by hydroxyl radical scavengers indicates the intermediacy of hydroxyl radicals in strand breakage. The hydroxyl radicals are most likely produced via a Fenton reaction in which hydrogen peroxide reacts with adventitious metal ions. The rate of strand breakage is higher, compared to a preincubated sample, when DNA is treated with potassium superoxide. This is probably the result of a metal-catalyzed Haber–Weiss reaction in which adventitious metal ions are cycled by first being reduced by superoxide and then oxidized by hydrogen peroxide with the concomitant production of hydroxyl radicals. In the presence of superoxide dismutase, the rates of strand scission observed with potassium superoxide and a preincubated sample are similar. This investigation indicates that DNA strand scission induced by superoxide involves an intermediate in common with ionizing radiation, i.e., the hydroxyl radical. The carcinogenicity of ionizing radiation and the involvement of the hydroxyl radical in DNA damage suggest that the hydroxyl radical may be the most simple carcinogen known. Moreover, since there are pathways for the spontaneous and chemical formation of superoxide in living systems, the reactive hydroxyl radical may be indeed ubiquitous. The implications of the possible carcinogenic effect on mammalian cells of reactive hydroxyl radicals generated from superoxide deserve careful evaluation.

In reduction of molecular oxygen, spin restriction hinders the divalent pathway and favors the univalent pathway (Taube, 1965). The univalent pathway for reduction of ground-state oxygen results in the formation of initially the superoxide radical anion (O_2^-),[1] then H_2O_2, then OH·, and finally H_2O in an obligatory stepwise fashion. The OH· is now considered to be responsible for about 90% of the damage induced in DNA in vitro by ionizing radiation (Armel et al., 1977). H_2O_2 has been shown to cause DNA strand breakage, liberation of DNA bases, and alteration of DNA bases (Rhaese & Freese, 1968; Massie et al., 1972). There is increasing evidence that reduced oxygen species produced in chemical autoxidative processes may be responsible for the toxic or carcinogenic properties of certain antitumor agents, carcinogens, or catecholamine analogues (Lorentzen et al., 1979; Lorentzen & Ts'o, 1977; Cohen & Heikkila, 1974; Cone et al., 1976; Biaglow et al., 1977; Goodman & Hochstein, 1977).

This investigation was initiated to determine if O_2^-, the first reduced oxygen species in the univalent pathway, can cause DNA strand scission directly in vitro. The role of O_2^- in DNA damage has not been adequately examined because of the great tendency of O_2^- toward dismutation in protic solvents. Special procedures are required to generate a "flux" of O_2^- in aqueous solutions. The availability of KO_2 has facilitated research on the chemical reactions of O_2^- since it can be manipulated quite easily in aprotic solvents such as Me_2SO. In this study, we have examined DNA strand scission by using a very sensitive method. This method involves incubation of homogeneous T7 DNA with KO_2 in 10 mM sodium phosphate buffer, pH 7, with subsequent size determination of DNA by band sedimentation through alkaline sodium chloride as described by Studier (1965).

Experimental Rationale

Table I shows some of the relevant reactions that would be expected to occur when KO_2 is introduced to a solution of DNA in 10 mM sodium phosphate buffer, pH 7. As a result of reactions 1–4, all reactive reduced oxygen species can be expected to be present: O_2^-, H_2O_2, and OH·. However, in relatively pure systems, reaction 4 does not proceed at an appreciable rate ($k = 0.13\ M^{-1}\ s^{-1}$). On the other hand, there is evidence for a metal-catalyzed two-step reaction shown by eq 7 and 8 in which OH· is generated from O_2^- (McCord & Day, 1978; Halliwell, 1978). Reactions 7 and 8 would be expected to occur through the mediation of adventitious metal ions. Because of the fast dismutation (reaction 2), relatively large amounts of KO_2 were added to the DNA solutions.

In this investigation, we have used the specificity and high catalytic efficiency of SOD (reaction 2a) and catalase (reaction 9) to probe for the involvement of O_2^- and H_2O_2 in DNA strand scission. Metal chelators, exogenous metal ions, and OH· scavengers have been used to determine if a Haber–Weiss cycle (reactions 7 and 8) is operational and responsible for strand scissions in our KO_2–DNA system.

Experimental Procedures

Materials

KO_2 (purity 96.5%) was purchased from Ventron Corp., Alfa Division, Danvers, MA. SOD (EC 1.15.1.1) from bovine blood, nitro blue tetrazolium, cytochrome *c* from horse heart (type III), and DETAPA were purchased from Sigma Chemical Co., St. Louis, MO. SOD (2900 units/mg of protein) did not show any catalase activity when measured at 10

† From the Division of Biophysics, The Johns Hopkins University, Baltimore, Maryland 21205. *Received September 7, 1979; revised manuscript received March 6, 1980.* Part of this work was presented at the 70th annual meeting of the Americal Association for Cancer Research, New Orleans, LA, 1979. This work was supported in part by Grant R01 ES 01659-06 from NIEHS. This is paper 5 in a series concerned with the involvement of polycyclic aromatic hydrocarbons and reduced oxygen radicals in carcinogenesis.

[1] Abbreviations used: OH·, hydroxyl radical; O_2^-, superoxide radical anion; DETAPA, diethylenetriaminepentaacetic acid; Me_2SO, dimethyl sulfoxide; SOD, superoxide dismutase; $s^0_{20,w}$, sedimentation coefficient at 20 °C, the density and viscosity of water and zero concentration; KO_2, potassium superoxide.

0006-2960/80/0419-3023$01.00/0

Table I: Relevant Reactions of Reduced Oxygen Species and Their Rate Constants at Neutral pH

reaction no.	reaction	k (M^{-1} s^{-1})	ref
1	$HO_2\cdot \rightleftharpoons O_2^- + H^+$ ($pK_a = 4.8$)		*a*
2	$HO_2\cdot + O_2^- + H^+ \longrightarrow H_2O_2 + O_2$	1×10^5	*b*
2a	$HO_2\cdot + O_2^- + H^+ \xrightarrow{SOD} H_2O_2 + O_2$	2×10^9	*b*
3	$H_2O_2 + Fe^{2+} \longrightarrow OH\cdot + OH^- + Fe^{3+}$	76	*c*
4	$H_2O_2 + O_2^- \longrightarrow OH\cdot + OH^- + O_2$	0.13	*d*
5	$OH\cdot + HCOO^- \longrightarrow CO_2^- + H_2O$	2.5×10^9	*e*
6	$CO_2^- + O_2 \longrightarrow O_2^- + CO_2$	4.5×10^9	*f*
7	$O_2^- + M^{n+} \longrightarrow O_2 + M^{(n-1)+}$		*g*
8	$M^{(n-1)+} + H_2O_2 \longrightarrow OH\cdot + OH^- + M^{n+}$		*g*
9	$2H_2O_2 \xrightarrow{catalase} 2H_2O + O_2$	4×10^7	*h*

[a] Rabani & Nielsen (1969). [b] McCord et al. (1977). [c] Wilshire & Sawyer (1979). [d] Weinstein & Bielski (1979). [e] Thomas (1965). [f] Adams et al. (1968). [g] McCord & Day (1978). [h] Schonbaum & Chance (1976).

or 100 μg of protein per mL by the procedure of Beers & Sizer (1952). Catalase (EC 1.11.1.6) was purchased from Worthington Biochemical Corp., Freehold, NJ, as a sterile aqueous solution containing 40 000 units/mg of protein. Catalase at 10 μg/mL did not show SOD activity as determined by its inability to inhibit the reduction of nitro blue tetrazolium by O_2^- (see assay for O_2^-). Dianisidine (3,3'-dimethoxybenzidine) was purchased from Aldrich Chemical Co., Milwaukee, WI.

Me_2SO was dried as described by Johnson et al. (1966) and stored over 4A molecular sieves. The distilled water was obtained from the house supply which is prepared with a Barnstead still. It was passed through a Barnstead bantam standard bed deionizing cartridge and then redistilled in an all-glass distilling apparatus. NaH_2PO_4, Na_2HPO_4, and sodium formate were recrystallized two times from this purified water, the first time in the presence of 0.1 mM DETAPA. The DETAPA was recrystallized twice before use. All glassware was cleaned with acidic chromium trioxide and rinsed with purified, glass-distilled water.

T7 Bacteriophage DNA. T7 DNA was extracted from purified phage particles by the sodium dodecyl sulfate procedure described by Murphy et al. (1974). The procedures for growth and purification of the bacteriophage were those described by Englund (1972). The DNA obtained was homogeneous and intact with an $s^0_{20,w}$ around 36. This corresponds to a single-strand molecular weight of 1.21×10^7.

Methods

Preparation of O_2^-. KO_2 was ground to a fine powder with a mortar and pestle in a glovebag under a dry nitrogen atmosphere. The powder was added to dry Me_2SO in a capped tube and vigorously vortexed for at least 5 min. The sample was then filtered through a medium sintered-glass filter or centrifuged for about 30 s at low speed. The resultant sample was a dilute solution or a fine colloidal suspension of KO_2. The suspension was always vortexed before use to ensure uniform sampling. These samples gave a positive test of O_2^- (blue solution or precipitate) when added to an aqueous solution of nitro blue tetrazolium (Beauchamp & Fridovich, 1971) and were capable of reducing cytochrome *c* (McCord & Fridovich, 1969).

Assay for O_2^-. The concentration of O_2^- prepared as described above was estimated by measuring the reduction of nitro blue tetrazolium (0.1 mM) dissolved in 50 mM carbonate buffer–0.1 mM EDTA, pH 10.2 (Beauchamp & Fridovich, 1971). KO_2 in Me_2SO (5 μL) was added slowly to 2.1 mL of nitro blue tetrazolium with rapid stirring, and the absorbance at 560 nm was measured. The quantity of O_2^- present was determined as follows. An absorbance change of 0.26 equals 50 nmol (Valentine & Curtis, 1975). Under the same conditions in the presence of SOD (10 μg/mL), the reduction of nitro blue tetrazolium was inhibited around 85%, indicating that the species responsible for reduction of nitro blue tetrazolium was O_2^-.

Assay for H_2O_2. A procedure similar to that previously described was used (Lorentzen et al., 1975). To 5.9 mL of 1.7 mM EDTA, pH 4.6, were added 50 μL of KO_2 in Me_2SO or 50 μL of H_2O_2 and 50 μL of 1% dianisidine in methanol. After allowing about 1 min for O_2^- to disproportionate to H_2O_2, 2.95 mL was transferred to a cuvette, and the remainder was used as a reference. Horseradish peroxidase (50 μL, 1 mg/mL in water) was added to the sample cuvette, and the absorbance at 460 nm was quickly measured. H_2O_2 concentration was determined from the absorbance at 460 nm by using a molar extinction coefficient of 1.13×10^4 cm^{-1} for oxidized dianisidine.

Incubation of T7 DNA with KO_2 or H_2O_2. Incubations were carried out at 37 °C in 150 μL of recrystallized sodium phosphate (10 mM, pH 7) with a DNA concentration of about 0.1 mM in nucleotide. The amount of KO_2 initially added was determined by measuring H_2O_2 concentration of stock KO_2 (concentration of KO_2 equals $2 \times H_2O_2$; reaction 2, Table I). Addition of KO_2 caused a slight rise in pH. At a O_2^-/DNA base ratio of 10, the condition most commonly used, the pH was 7.5. At the highest ratio used, a O_2^-/DNA base ratio equal to 15, the pH was 7.85. H_2O_2 (diluted from 30% Baker Analyzed reagent) was added in purified water. KO_2 preincubations were carried out at 23 °C in 10 mM sodium phosphate buffer, pH 7. The H_2O_2 concentration remained constant for at least 1 h under the conditions of preincubation. The strand scission reaction was stopped by placing the incubation mixture on ice after addition of 5 μL of 50 mM EDTA and 5 μL of catalase (770 μg/mL). EDTA (10 μM) or catalase (10 μg/mL) will completely inhibit strand scission induced by O_2^- (Figure 3, Table II).

Assay for DNA Single-Strand Scission. Sedimentation measurements were made in a Beckman Model E analytical ultracentrifuge equipped with UV optics as previously described (Lorentzen & Ts'o, 1977). The mean number of DNA single-strand scissions, *P*, in the treated samples was calculated by using the relationship (Charlesby, 1954)

$$\frac{M_r}{M_r(\text{control})} = \frac{2[e^{-P} + (P - 1)]}{P^2}$$

This assay measures the sum of single-strand bands plus alkali-labile bands.

Inactivation of SOD. SOD was irreversibly inactivated by incubation with H_2O_2 in 0.5 M sodium carbonate buffer, pH 10, for 1 h at 23 °C as described by Hodgson & Fridovich (1975). The ratio of H_2O_2 to SOD (18.8 μM) was 400.

Assay for SOD Activity. The method employed was essentially that of Valentine & Curtis (1975) and is based on the ability of SOD to inhibit the reduction of nitro blue tetrazolium by O_2^-.

Assay for OH· Formation in Fenton-Type Reactions. The decrease in thymine absorbance at 264.5 nm (λ_{max}) was followed after treatment of 50 mM H_2O_2 in the presence and absence of 10 μM metal salts. Incubations were at 37 °C in glass-distilled water with approximately 1 $A_{264.5}$/mL thymine. After 2 and 4 h of incubation, catalase was added (4 μg/mL) and spectra were taken in a Cary 14 spectrophotometer. Lown et al. (1978) have detected the *N-tert*-butyl α-phenyl nitroxide radical after mixing 1 mM $FeSO_4$ and 3% H_2O_2 with the spin

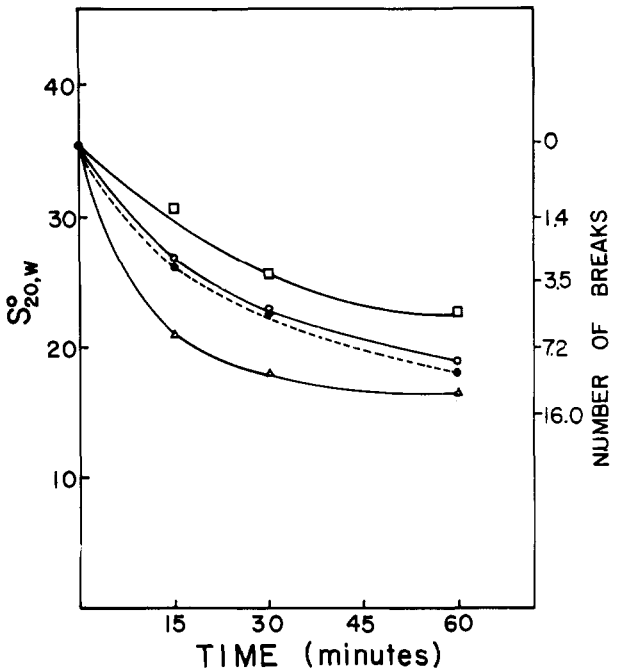

FIGURE 1: Kinetics of T7 DNA single-strand scission after treatment with reduced oxygen species. All incubations were at 37 °C and contained 0.11 mM DNA with the O_2^-/DNA base ratios being 10 and the H_2O_2/DNA base ratio being 5. Reactions were stopped by addition of EDTA and catalase as described under Experimental Procedures. Sedimentation coefficients were calculated after band sedimentation of the DNA through 0.1 N NaOH and 0.9 M NaCl. The number of single-strand scissions (nonlinear scale on the right) induced per intact DNA strand was calculated from the molecular weight (see Experimental Procedures). (□) Preincubated O_2^-; (○) O_2^-; (△) O_2^- plus 0.1 M sodium formate; (●) H_2O_2.

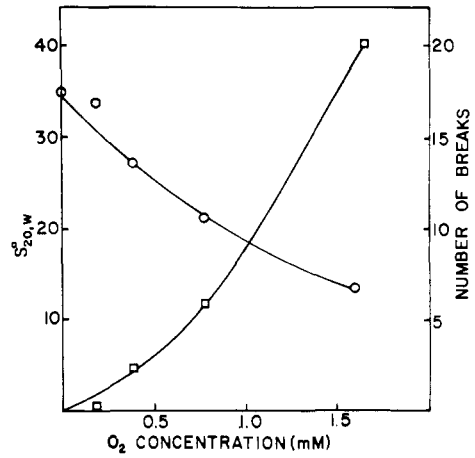

FIGURE 2: Dependence of T7 DNA single-strand scission on O_2^- concentration. Incubations were at 37 °C for 1 h and contained 0.11 mM DNA. See Figure 1 for other details. (○) Reduction of sedimentation coefficient of treated DNA; (□) mean number of strand breaks per intact single strand of DNA.

trap *N-tert*-butyl-α-phenylnitrone. In addition, non-UV-absorbing 5,6-dihydro-5,6-dihydroxythymidine has been determined to be the principal product formed when thymidine is oxidized in neutral aqueous solution by H_2O_2 + Fe^{2+} (Schellenberg, 1979). These data are compelling evidence for the formation of OH· with Fenton's reagent and indicate that measurement of loss of thymine absorbance is a valid assay for OH· generation.

Other Methods. Protein was determined by a modified Lowry procedure (Hartree, 1972) with bovine serum albumin as a standard. DNA concentration in moles of nucleotides was determined by using a molar extinction coefficient of 6.6 × 10^3 cm^{-1} at 258 nm.

Results

DNA Strand Scission by Reduced Oxygen Species. The kinetics of DNA strand scission obtained by using a O_2^-/DNA base ratio of 10 under various reaction conditions are shown in Figure 1. The number of strand scissions is greater when DNA is incubated with KO_2 than with preincubated KO_2. After 15 min of incubation, the number of strand scissions introduced into each intact T7 DNA strand by equivalent amounts of reduced oxygen was calculated to be 1.3 with preincubated KO_2 and 2.7 with KO_2. The dashed line in Figure 1 shows the kinetics obtained with an equimolar amount of H_2O_2 (H_2O_2/DNA base = 5). Addition of an equivalent amount of Me_2SO (the solvent used for addition of KO_2 to the H_2O_2 incubations) resulted in a decrease in the observed number of breaks to that obtained with preincubated KO_2 (Table IV). Thus, H_2O_2 and preincubated KO_2 produced the same amount of DNA strand scission when added as equivalent amounts of H_2O_2. The number of strand breaks induced under any of the conditions varied slightly with different DNA preparations; therefore, all the data shown in Figure 1 were obtained with one DNA preparation. The number of breaks induced by O_2^- was dose dependent as shown in Figure 2. Incubation of O_2^- with T7 DNA at 37 °C for 1 h at a O_2^-/DNA base ratio equal to 10 resulted in 7–9 single-strand scissions per intact DNA strand (Figure 2, Table II).

Table II: Effect of Superoxide Dismutase and Catalase on DNA Strand Scission

condition	reduced O_2/DNA base	no. of strand scissions per intact strand: no enzyme	SOD (10 μg/mL)	inactivated SOD (10 μg/mL)	catalase (10 μg/mL)
preincubated	5.2[a]	1.2 (30.8)[c]	1.3 (30.6)		
	7.4[b]	1.5 (30.8)	2.0 (29.2)		
	10.6[b]	4.2 (23.8)			0 (35.6)
O_2^-	5.4[b]	4.0 (24.8)	0.9 (32.4)		
	5.8[b]	4.0 (24.6)	2.4 (28.1)	3.9 (25.0)	
	10.0[b]	6.9 (20.6)			0 (35.9)
H_2O_2	2.6[a]	2.3 (27.6)	2.6 (26.9)		
	4.3[b]	6.6 (21.2)	7.5 (20.3)	6.4 (21.4)	
	5.4[b]	6.7 (20.8)			0 (35.9)

[a] Incubation was at 37 °C for 30 min. [b] Incubation was at 37 °C for 1 h. [c] Sedimentation coefficients are given in parentheses.

Effect of SOD and Catalase on DNA Strand Scission. DNA strand scission observed with KO_2 was sensitive to SOD at 10 μg/mL; however, complete protection was never observed at this enzyme concentration (Table II). Inactivated SOD (10 μg/mL) did not provide any protection against strand scission induced by O_2^- or H_2O_2 (Table II). There was no protection against strand scission by SOD when T7 DNA was treated with H_2O_2 or preincubated KO_2, conditions where little or no O_2^- should be present (Table II).

Increasing the SOD concentration to 20 μg/mL resulted in a small increase in protection. The sedimentation coefficient increased to 34.8 compared to 32.4 at 10 μg/mL and 36.4 for untreated control. A further increase in SOD concentration to 50 μg/mL did not result in any further protection. This increased protection with 20–50 μg/mL SOD may be due to a nonspecific effect since addition of inactivated SOD at 50 μg/mL resulted in an increase in the sedimentation coefficient of KO_2-treated DNA from 24.8 to 27.8.

DNA strand scission induced with KO_2, preincubated KO_2, and H_2O_2 was completely inhibited by catalase at 10 μg/mL (Table II).

Effect of Chelators and Metal Ions on DNA Strand Scission. DNA strand scission induced by O_2^- and H_2O_2 was completely inhibited by EDTA (Table III). The effect of

Table III: Effect of Metal Ions and Chelators on DNA Strand Scission Induced by Reduced Oxygen Species[a]

addition	concn (μM)	no. of strand scissions per intact strand: KO_2	H_2O_2
O		4.3	4.4
EDTA[b]	100	<0.25	<0.25
8-hydroxyquinoline	10	<0.25	
DETAPA	10		<0.25
$FeSO_4$	10	4.6	40.6
	50	10.2	
$FeCl_3$	10	4.2	5.4
	50	6.5	
$CuSO_4$	1	13.0	
	10	36.9	54.4
$MnCl_2$	10	11.4	4.7
$ZnCl_2$	10	7.8[c]	4.5

[a] Incubation was at 37 °C for 1 h; H_2O_2/DNA base 3.2:1; O_2^-/DNA base 6.4:1. [b] H_2O_2/DNA base 5:1; O_2^-/DNA base 10:1. [c] O_2^-/DNA base 8.8:1; control DNA had 5.1 breaks.

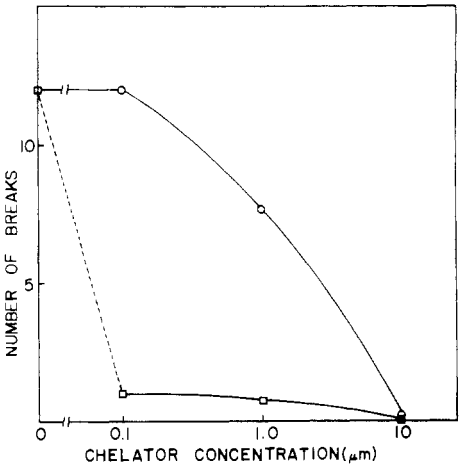

FIGURE 3: Effect of chelator concentration of T7 DNA strand scission induced by O_2^-. Incubations were at 37 °C for 1 h and contained 0.11 mM DNA with O_2^-/DNA base ratios of 10. See Figure 1 for other details. (□) DETAPA; (○) EDTA.

chelator concentration on DNA strand scission induced by O_2^- is shown in Figure 3; concentrations as low as 10 μM EDTA were effective in inhibiting DNA strand scission. The data also indicate that DETAPA is a more potent inhibitor of DNA strand scission at low concentration than EDTA. DNA strand scission induced by O_2^- was also effectively inhibited by 10 μM 8-hydroxyquinoline-5-sulfonic acid (Table III). The effectiveness of the three chelators in inhibiting O_2^--induced DNA strand scission is DETAPA > EDTA ≥ 8-hydroxyquinoline-5-sulfonic acid; this is correlated to their chelating ability (Martel & Calvin, 1952; Buettner et al., 1978).

The effect of addition of small amounts of metal ions on DNA strand scission induced by O_2^- is shown in Table III. Strand scission was greatly enhanced (8-fold) by 10 μM $CuSO_4$. Enhancement of strand scission (1.5–3-fold) was also observed with 10 μM $ZnCl_2$, 10 μM $MnCl_2$, and 1 μM $CuSO_4$. $FeSO_4$ and $FeCl_3$ did not produce any significant enhancement at 10 μM; however, some enhancement was seen at 50 μM.

The effect of exogenous metal ions on DNA strand scission induced by H_2O_2 is also shown in Table III. Strand scission was greatly enhanced by 10 μM $FeSO_4$ (9-fold) and by 10 μM $CuSO_4$ (12-fold). There was a slight enhancement with 10 μM $FeCl_3$ while 10 μM $MnCl_2$ and 10 μM $ZnCl_2$ had little, if any, effect.

Effect of OH· Scavengers on DNA Strand Scission. Table IV shows that OH· scavengers such as ethyl alcohol, *tert*-butyl alcohol, Me_2SO, and NaN_3 inhibit strand scission induced by

Table IV: Effect of Hydroxyl Radical Scavengers on DNA Strand Scission Induced by H_2O_2[a]

scavenger	concn (M)	H_2O_2/DNA base ratio	protection (%)
ethyl alcohol	1.14	3.2	50
tert-butyl alcohol	0.71	3.2	100
Me_2SO	0.94	3.2	91
	0.94	5	34
	0.47	5	13
NaN_3	0.02	4.2	87
KCl	0.1	3.2	5

[a] Incubation was at 37 °C for 1 h.

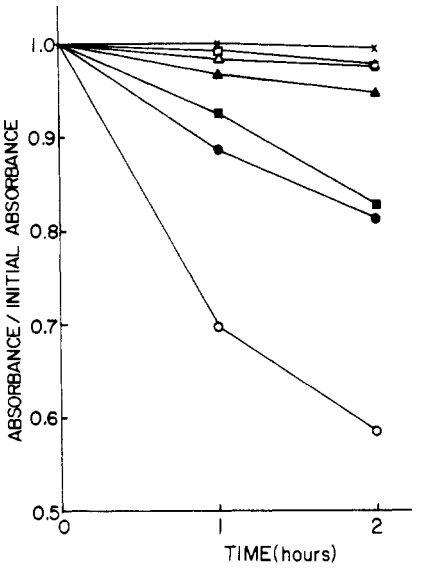

FIGURE 4: Decrease of thymine absorbance after treatment with 50 mM H_2O_2 and 10 μM metal ions. Incubations were at 37 °C and contained about 1 $A_{264.5}$/mL. Catalase (4 μg/mL) was added to stop reactions and then spectra were measured. (○) $FeSO_4$, (●) $FeCl_3$, (■) $CuSO_4$, (▲) $MnCl_2$, (△) H_2O_2 alone, (□) $ZnCl_2$, and (X) H_2O_2–$FeSO_4$–*tert*-butyl alcohol.

H_2O_2. With Me_2SO, the degree of protection observed was shown to be dependent on the scavenger/H_2O_2 ratio. KCl, which is not a very efficient scavenger, had very little effect on strand scission at a concentration of 100 mM. Control experiments showed that these compounds did not noticeably react with H_2O_2.

Figure 1 shows that incubation of DNA with KO_2 in the presence of 100 mM sodium formate, an efficient OH· scavenger (reaction 5, Table I), increased the number of strand breaks compared to that obtained with KO_2 alone. Mannitol was effective in inhibiting DNA strand scission in KO_2–sodium formate incubations (data not shown).

Effect of Various Metal Ions on OH· Formation in Fenton-Type Reactions. The loss of thymine absorbance that results from saturation of the 5,6 double bond upon treatment with Fenton's reagent (H_2O_2 + Fe^{2+}) can be used as a measure of OH· formation (see Experimental Procedures). Figure 4 shows the decrease in absorbance of thymine at 264.5 nm after incubation in glass-distilled water with 50 mM H_2O_2 in the presence and absence of 10 μM metal salts. There is very little decrease in absorbance after incubation for 2 h at 37 °C with H_2O_2 alone. Addition of $FeSO_4$, $FeCl_3$, and $CuSO_4$ enhances the reaction substantially, with $FeSO_4$ showing the greatest effect. Addition of $MnCl_2$ has very little effect on the decrease in thymine absorbance while $ZnCl_2$ shows no effect at all. The large decrease in thymine absorbance observed after incubation with H_2O_2 plus $FeSO_4$ could be completely eliminated by

addition of 100 mM *tert*-butyl alcohol, a OH· scavenger (Figure 4).

Discussion

In this investigation, DNA strand scission was observed after incubation of T7 DNA with KO_2, preincubated KO_2, and H_2O_2. Inhibition of strand scission by very small amounts of the chelator DETAPA (Figure 3) indicates that O_2^- and H_2O_2 by themselves are not effective agents for breaking DNA. The complete protection observed with catalase and metal chelators strongly suggests that H_2O_2 (produced by dismutation of O_2^-; reaction 2, Table I) and metal ions are directly involved in the strand scission observed with KO_2 and preincubated KO_2. The enhancement of H_2O_2-induced strand scission by the addition of exogenous $FeSO_4$ or $CuSO_4$ suggests that H_2O_2 manifests its activity by being converted to OH· in a Fenton-type reaction (reactions 3 and 8, Table I). This is supported by the data in Table IV which demonstrate the inhibition of H_2O_2-induced DNA strand scission by OH· scavengers such as ethyl alcohol, *tert*-butyl alcohol, Me_2SO, and NaN_3. The OH· is most likely produced near the site of cleavage on the DNA by reaction of adventitious metal ions with H_2O_2.

In the presence of both O_2^- and H_2O_2, additional OH· can be produced efficiently via a metal-catalyzed Haber–Weiss cycle (reactions 7 and 8). In these two reactions, O_2^- serves as a reducing agent to regenerate the reduced metal ion from the oxidized metal ion. The increased strand breakage observed with KO_2 as compared to preincubated KO_2 (Figure 1) is the result of additional OH· produced via reactions 7 and 8 (Table I). Strand breakage induced by KO_2 is inhibited by SOD because the enzyme removes the source of reducing power, i.e., the O_2^-, thereby removing the source of the reduced metal ion needed for formation of OH·. The existence of a metal-catalyzed Haber–Weiss cycle has been reported by other investigators (McCord & Day, 1978; Halliwell, 1978; Koppenol et al., 1978).

This system is not without its complexities since complications do arise in attempting to explain some of the data. First, the addition of exogenous $FeSO_4$ and $FeCl_3$ (10 μM) to KO_2–DNA incubations did not result in any significant enhancement of DNA strand scission (Table III). This is in contrast to the data obtained with H_2O_2 where a 9-fold increase was observed with exogenous $FeSO_4$. Massie et al. (1972) also reported an enhancement of DNA degradation by H_2O_2 with addition of exogenous $FeCl_2$, and Halliwell (1975) has reported that Fe^{2+} or Fe^{3+} does not affect the reduction of nitro blue tetrazolium by O_2^-. The lack of enhancement of DNA strand scission by Fe^{2+} and Fe^{3+} ions may be the result of complex formation between ligand, iron, and O_2^-. Caspary et al. (personal communication, 1979) have electron paramagnetic resonance data to indicate that the O_2^- signal is stabilized by ferric or ferrous iron and suggest that an outer-sphere ion-pair complex is formed between ligand-bound iron and O_2^-. Thus, exogenous iron may complex with O_2^- and render it nonavailable for reducing adventitious metal ions that are ultimately responsible for the enhancement of DNA strand scission. The enhancement of strand scission observed at 50 μM iron is most likely due to an increase in the rate of the Fenton reaction. A second problem that arises is that addition of exogenous $MnCl_2$ and $ZnCl_2$ does not result in an enhancement of DNA strand scission by H_2O_2 while there is a 1.5–2.5-fold enhancement with KO_2. This could occur if Mn^{2+} and Zn^{2+} do not produce OH· efficiently in the presence of H_2O_2. This appears to be the case since incubation of thymine with H_2O_2 in the presence of $MnCl_2$ or $ZnCl_2$ results in a very small or no decrease in thymine absorbance while $CuSO_4$, $FeCl_3$, or $FeSO_4$ decreases the absorbance quite substantially (Figure 4). Saturation of the 5,6 double bond in thymine upon incubation with Fenton's reagent has been shown to be due to reaction with OH· as determined by product analyses (Schellenberg, 1979; Cadet & Teoule, 1978). DNA strand scission induced with O_2^- is enhanced by $MnCl_2$ and $ZnCl_2$ because this system is capable of cycling the metals between oxidized and reduced forms.

Another set of data appears to refute the conclusion that OH· is responsible for DNA strand scission, viz., that addition of sodium formate, an efficient OH· scavenger, resulted in an increase in the rate and extent of strand scission (Figure 1). The increased rate most likely results from further reaction of the formate radical anion formed by reaction 5 (Table I). The lifetime of the OH· is probably very short compared to that of the formate radical anion. Subsequent reaction of this more stable radical with DNA could lead to some of the increase in strand scission observed. A similar mechanism has been proposed for increased membrane lysis (Michelson, 1977) and for the luminescence that accompanies xanthine oxidase oxidation of acetaldehyde (Hodgson & Fridovich, 1976). In addition, the formate radical anion could react with molecular oxygen to produce a small amount of O_2^- (reaction 6, Table I) which could have a measurable effect due to the sensitivity of the assay. Any O_2^- thus produced can reduce adventitious metal ions which then can serve to form OH· from H_2O_2 in juxtaposition for strand breakage. The increased strand scission observed with 0.1 M formate is most likely due to contributions by both mechanisms outlined above. However, this can only be resolved when the products of the reaction are isolated and analyzed.

Conclusions similar to those reached in this investigation have been reported by others using different systems. The inactivation of biologically active ϕX174 DNA in sodium formate solutions by γ irradiation has been reported to result from a combination of the protonated form of O_2^- and H_2O_2 (Van Hemmen & Meuling, 1975). It has been suggested that the degradation of DNA by streptonigrin (Cone et al., 1976) and by mitomycin C (Lown et al., 1976) results from OH· produced by interaction of O_2^- and H_2O_2.

This investigation indicates that DNA strand scission induced by O_2^- and ionizing radiation involves a common intermediate, i.e., the very reactive OH·. It is well established that ionizing radiation is carcinogenic and that the OH· is primarily responsible for DNA damage induced by the irradiation process (Roots & Okada, 1972; Achey & Duryea, 1974; Ward & Kuo, 1973; Ward, 1975, and references therein; Armel et al., 1977). Since there are indeed pathways for the formation of O_2^- in normal living systems, OH· may be the most simple and ubiquitous carcinogen known. The implication of the possible carcinogenic effect on mammalian cells of OH· generated from O_2^- deserves careful consideration.

Acknowledgments

The authors acknowledge the technical assistance of Shu-Uin Yang and Nathan Dreon.

References

Achey, P., & Duryea, H. (1974) *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 25*, 595.

Adams, G. E., Michael, B. D., & Willson, R. L. (1968) *Adv. Chem. Ser. No. 81*, 289.

Armel, P. R., Strniste, G. F., & Wallace, S. S. (1977) *Radiat. Res. 69*, 328.

Beauchamp, C., & Fridovich, I. (1971) *Anal. Biochem. 44*, 276.
Beers, R. F., & Sizer, I. W. (1952) *J. Biol. Chem. 195*, 133.
Biaglow, J. E., Jacobson, B. E., & Nygaard, O. F. (1977) *Cancer Res. 37*, 3306.
Buettner, G. R., Oberley, L. W., & Leuthauser, S. W. H. C. (1978) *Photochem. Photobiol. 28*, 693.
Cadet, J., & Teoule, R. (1978) *Photochem. Photobiol. 28*, 661.
Charlesby, A. (1954) *Proc. R. Soc. London, Ser. A 224*, 120.
Cohen, G., & Heikkila, R. (1974) *J. Biol. Chem. 249*, 2447.
Cone, R., Hasan, S. K., Lown, J. W., & Morgan, A. R. (1976) *Can. J. Biochem. 54*, 219.
Englund, P. (1972) *J. Mol. Biol. 66*, 209.
Goodman, J., & Hochstein, P. (1977) *Biochem. Biophys. Res. Commun. 77*, 797.
Halliwell, B. (1975) *FEBS Lett. 56*, 34.
Halliwell, B. (1978) *FEBS Lett. 92*, 321.
Hartree, E. (1972) *Anal. Biochem. 48*, 422.
Hodgson, E. K., & Fridovich, I. (1975) *Biochemistry 14*, 5294.
Hodgson, E. K., & Fridovich, I. (1976) *Arch. Biochem. Biophys. 172*, 202.
Johnson, E., Pool, K., & Hamm, R. (1966) *Anal. Chem. 38*, 183.
Koppenol, W. H., Butler, J., & Van Leeuwen, J. W. (1978) *Photochem. Photobiol. 28*, 655.
Lorentzen, R., & Ts'o, P. O. P. (1977) *Biochemistry 16*, 1467.
Lorentzen, R., Caspary, W., Lesko, S., & Ts'o, P. (1975) *Biochemistry 14*, 3970.
Lorentzen, R., Lesko, S., McDonald, K., & Ts'o, P. O. P. (1979) *Cancer Res. 39*, 3194.
Lown, J. W., Begleitter, A., Johnson, D., & Morgan, A. R. (1976) *Can. J. Biochem. 54*, 110.
Lown, J. W., Sim, S. K., & Chen, H. H. (1978) *Can. J. Biochem. 56*, 1042.
Martel, A., & Calvin, M. (1952) *Chemistry of Metal Chelate Compounds*, Prentice-Hall, Englewood Cliffs, NJ.
Massie, H., Samis, H., & Baird, M. (1972) *Biochim. Biophys. Acta 272*, 539.
McCord, J. M., & Fridovich, I. (1969) *J. Biol. Chem. 244*, 6049.
McCord, J. M., & Day, E. D. (1978) *FEBS Lett. 86*, 139.
McCord, J. M., Crapo, J. D., & Fridovich, I. (1977) in *Superoxide and Superoxide Dismutases* (Michelson, A. M., McCord, J. M., & Fridovich, I., Eds.) p 11, Academic Press, New York.
Michelson, A. (1977) in *Superoxide and Superoxide Dismutases* (Michelson, A. M., McCord, J. M., & Fridovich, I., Eds.) p 245, Academic Press, New York.
Murphy, J. R., Pappenheimer, A. M., & Tayart de Borms, S. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 11.
Rabani, J., & Nielson, S. (1969) *J. Phys. Chem. 72*, 3836.
Rhaese, H.-J., & Freese, E. (1968) *Biochim. Biophys. Acta 155*, 476.
Roots, R., & Okada, S. (1972) *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 21*, 329.
Schellenberg, K. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, Abstr. 1433, 501.
Schonbaum, G. R., & Chance, B. (1976) *Enzymes, 3rd Ed. 13*, 363.
Studier, W. F. (1965) *J. Mol. Biol. 11*, 373.
Taube, H. (1965) *J. Gen. Physiol. 49*, 29.
Thomas, J. K. (1965) *Trans. Faraday. Soc. 61*, 702.
Valentine, J. S., & Curtis, A. B. (1975) *J. Am. Chem. Soc. 97*, 224.
Van Hemmen, J. J., & Meuling, W. J. A. (1975) *Biochim. Biophys. Acta 402*, 133.
Ward, J. F. (1975) *Adv. Radiat. Biol. 5*, 81.
Ward, J. F., & Kuo, I. (1973) *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 23*, 543.
Weinstein, J., & Bielski, B. H. J. (1979) *J. Am. Chem. Soc. 101*, 58.
Wilshire, J., & Sawyer, D. (1979) *Acc. Chem. Res. 12*, 105.

Prothrombin–Membrane Interaction. Effects of Ionic Strength, pH, and Temperature†

Robert M. Resnick and Gary L. Nelsestuen*

ABSTRACT: The effects of ionic strength, pH, and temperature on three separate aspects of prothrombin–phospholipid membrane binding were studied. The three parameters include a calcium-dependent protein transition, a calcium–membrane interaction, and, finally, the binding of calcium-saturated protein to a calcium-saturated phospholipid membrane. The results are consistent with calcium binding to carbonyl groups in the protein and to phosphate in the phospholipids. These interactions show the expected pH profiles and sensitivity to ionic strength. Temperature effects indicate a small negative enthalpy change for each process. The binding of calcium-saturated protein to calcium-saturated membrane shows very little variation between pH 6 and pH 9, is accompanied by no detected enthalpy change, and is relatively insensitive to ionic strength. These latter results indicate that ionic calcium bridging between the protein and membrane is not important. A chelation model for prothrombin–membrane binding is proposed where the two interacting species have no net charge; ligands on the protein complete the coordination sphere of membrane-bound calcium and vice versa.

The binding of vitamin K dependent proteins to membranes is dependent on the presence of γ-carboxyglutamic acid residues. There are 10 of these residues in the amino-terminal region of prothrombin [see Stenflo & Suttie, (1977) and Suttie & Jackson (1977) for reviews]. Several lines of evidence have shown that native protein structure is required for tight calcium binding to prothrombin (Henriksen & Jackson, 1975; Nelsestuen et al., 1975) and for prothrombin–membrane interaction (Nelsestuen, 1976). In other words, the 10 γ-carboxyglutamic acid residues do not act independent of a larger

† From the Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108. *Received January 8, 1980.* Supported in part by Grant HL 15728 from the National Institutes of Health.

* Correspondence should be addressed to this author. He is an Established Investigator of the American Heart Association.

0006-2960/80/0419-3028$01.00/0