Strand Cleavage of Supercoiled DNA by Water-Soluble Peroxyl Radicals. The Overlooked Importance of Peroxyl Radical Charge†

Thomas Paul,*,‡ Mary Jane Young,‡ Irene E. Hill,§ and K. U. Ingold*,‡

Steacie Institute for Molecular Sciences and Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6

Received June 25, 1999; Revised Manuscript Received September 16, 1999

ABSTRACT: It is well established that the peroxyl radicals formed during the thermal decomposition of 2,2′-azobis(amidinopropane), ABAP, in oxygenated water can cleave double-stranded DNA, from which fact it has been concluded that peroxyl radicals, as a general class, can induce DNA strand scission. However, the ABAP-derived radicals are positively charged, and DNA is a negatively charged polyanion. Moreover, the relatively small and, therefore, free to diffuse peroxyl radicals likely to be formed in vivo will generally be negatively charged or neutral. Plasmid supercoiled DNA [pBR 322, 4361 base pairs (bp)] was reacted with known, equal fluxes of two positively charged peroxyl radicals, a negatively charged peroxyl radical, and a neutral peroxyl radical. The two positively charged peroxyl radicals degraded ≥80% of the supercoiled pBR 322 at a flux of 4 radicals/bp, but the negatively charged and neutral peroxyl radicals had no significant effect even at a flux as high as 24 radicals/bp. The same lack of effect on the DNA was also observed with high fluxes of superoxide/hydroperoxyl radicals. Similar results were obtained with another supercoiled DNA, pUC 19, except that pUC 19 is somewhat more sensitive to strand scission by positively charged peroxyl radicals than pBR 322. We conclude that most of the peroxyl radicals likely to be formed in vivo have little or no ability to induce DNA strand scission and that the potential role of electrostatics in radical/DNA reactions should always be considered.

Quantitative studies on free radical reactions require reproducible and precisely known rates of radical formation. Much of our present understanding of free radical chemistry in homogeneous systems has been acquired by utilizing the thermal decomposition of azo compounds to provide specific radicals at known and reproducible rates, eq 1. [In this equation, e[1] is the efficiency of escape of the geminate radicals from

$$\mathrm{R{-}N{=}N{-}R} \rightarrow 2e\ \mathrm{R^{\bullet}} + \mathrm{N_2} \qquad (1)$$

the solvent cage in which they are produced; typically $e \approx 0.5$ (*1*−*3*)].

To undertake quantitative studies of lipid peroxidation on biomimetic aqueous lipid dispersions, 2,2′-azobis(amidinopropane) dihydrochloride (ABAP or AAPH) was introduced in 1984 by one of us (*3*) and, independently, by Yamamoto et al. (*4*). This water-soluble azo compound decomposes slowly at 37 °C and, in air- or oxygen-saturated buffer solutions at physiological pHs (∼7), yields a steady flux of the water-soluble, positively charged peroxyl radical ($^{+}$AOO$^{\bullet}$) for many hours (eq 2).

$$\underset{\text{ABAP}}{[(\mathrm{H_2N})_2^{+}\mathrm{CC(CH_3)_2N{=}}]_2} \rightarrow 2e\ (\mathrm{H_2N})_2^{+}\mathrm{CC^{\bullet}(CH_3)_2} \xrightarrow{\mathrm{O_2}} 2e\ (\mathrm{H_2N})_2^{+}\mathrm{CC(CH_3)_2OO^{\bullet}} \quad {}^{+}\mathrm{AOO^{\bullet}} \qquad (2)$$

Since 1984, ABAP has been used extensively, and largely uncritically, in "biomimetic" studies involving water-soluble peroxyl radicals. However, $^{+}$AOO$^{\bullet}$ is clearly not a biomimetic peroxyl radical because it is *positively* charged whereas most of the relatively small and, therefore, free to diffuse peroxyl radicals likely to be formed in vivo will be *negatively* charged, e.g., those formed during the peroxidation of free fatty acids ($\mathrm{RCO_2^-} \rightarrow {}^{-}\mathrm{O_2CR_{-H}OO^{\bullet}}$) and the superoxide anion radical ($^{-}$OO$^{\bullet}$), or neutral, e.g., those formed during the peroxidation of phospholipids and the conjugate acid of superoxide (HOO$^{\bullet}$). [Although radical attack on free amino acids may yield peroxyl radicals, the positively charged arginine, histidine, and lysine are more resistant to such attack than the neutral amino acids leucine, proline, and valine (*5*).]

† This work was supported by a grant from the National Foundation for Cancer Research.

* To either of whom correspondence should be addressed at the Steacie Institute for Molecular Sciences. Phone: 613-990-0938; Fax: 613-941-8447; e-mail: Thomas.Paul@nrc.ca, Keith.Ingold@nrc.ca.

‡ Steacie Institute for Molecular Sciences.

§ Institute for Biological Sciences.

[1] Abbreviations: ABAP, 2,2′-azobis(amidinopropane) dihydrochloride; $^{+}$AOO$^{\bullet}$, 2-amidinoprop-2-peroxyl radical; ABCBS, 3,3′-azobis-(3-cyano-1-butanesulfonic acid) disodium salt; $^{-}$BOO$^{\bullet}$, 1-butanesulfonate-3-cyano-3-peroxyl radical; ABDBA, 2,2′-azobis(*N*,*N*′-dimethyleneisobutyramidine) dihydrochloride; $^{+}$DOO$^{\bullet}$, *N*,*N*′-dimethyleneisobutyramidine-2-peroxyl radical; ABMHP, 2,2′-azobis[2-methyl-*N*-(2-hydroxyethyl)propionamide]; COO$^{\bullet}$, 2-methyl-*N*-(2-hydroxyethyl)propionamide-2-peroxyl radical; SOTS-1, di(4-carboxybenzyl)hyponitrite; $^{-}\mathrm{O_2}^{\bullet}$, superoxide radical anion; HOO$^{\bullet}$, hydroperoxyl radical; *e*, efficiency of escape from the solvent cage of geminate pairs of radicals derived from the azo compounds; AA, acetaldehyde; XO, xanthine oxidase; CAT, catalase; SC DNA, supercoiled DNA; R DNA, relaxed DNA; L DNA, linear DNA; desferal, deferoxamine mesylate.

10.1021/bi991463o
Published on Web 03/14/2000

We started to consider the potential importance of charge on "biomimetic" reactions of water-soluble peroxyl radicals shortly after we invented the first Superoxide Thermal Source (SOTS-1) (*6*).

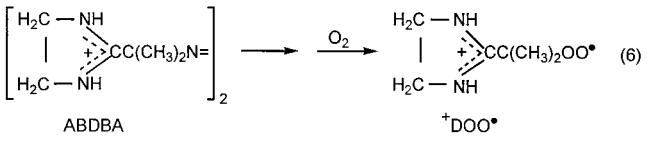
$$[^{-}O_2CC_6H_4CH_2ON{=}]_2 \xrightarrow[37\ ^\circ C]{H_2O,\ pH\ 7} \xrightarrow{O_2} 0.40\ O_2^{\bullet -} \quad (3)$$
SOTS-1

One matter of concern was the extensive and sole use of ABAP to investigate the ability of water-soluble alkylperoxyl radicals to cleave DNA (*7*−*12*). In this earlier work, there is uniform agreement that the radicals derived from ABAP can cleave DNA. However, DNA is a polyanion and although the negatively charged phosphate groups will be partially screened in vivo by mono- and divalent cations which may overcome its polyanionic character at the local level it appeared possible, even likely, that electrostatic attraction between DNA and ABAP/$^{+}$AOO$^{\bullet}$ could yield results, i.e., DNA strand cleavage by $^{+}$AOO$^{\bullet}$, of little or no relevance to the in vivo situation. The results reported herein show that this is, indeed, the case both for "naked" DNA and for DNA "protected" by physiological concentrations of NaCl, KCl, and $MgCl_2$. That is, two plasmid DNAs have been found to be very, very much more resistant to strand scission by a water-soluble, negatively charged alkylperoxyl radical, $^{-}$BOO$^{\bullet}$ (eq 4), and by a water-soluble, neutral alkylperoxyl radical, COO$^{\bullet}$ (eq 5)

$$[^{-}O_3SC_2H_4C(CH_3)(CN)N{=}]_2 \xrightarrow{O_2} {}^{-}O_3SC_2H_4C(CH_3)(CN)OO^{\bullet} \quad (4)$$
ABCBS
$^{-}$BOO$^{\bullet}$

$$[HOC_2H_4NHC(O)C(CH_3)_2N{=}]_2 \xrightarrow{O_2} HOC_2H_4NHC(O)C(CH_3)_2OO^{\bullet} \quad (5)$$
ABMHP
COO$^{\bullet}$

than by two water-soluble, positively charged alkylperoxyl radicals, $^{+}$AOO$^{\bullet}$ (eq 2) and $^{+}$DOO$^{\bullet}$ (eq 6).



(6)

At physiological concentrations, the salts provided only slight protection against strand cleavage by the positively charged peroxyl radicals. In agreement with earlier work (*13*−*16*), plasmid DNA has also been shown to be highly resistant to strand scission by the $^{-}O_2^{\bullet}$/HOO$^{\bullet}$ couple.

MATERIALS AND METHODS

Materials. 2,2′-Azobis(amidinopropane) dihydrochloride (ABAP), 2,2′-azobis(*N*,*N*′-dimethyleneisobutyramidine) dihydrochloride (ABDBA), and azobis[2-methyl-*N*-(2-hydroxyethyl)propionamide] (ABMHP) were purchased from Wako Chemicals USA, Inc. (Richmond, VA). 3,3′-Azobis(3-cyano-1-butanesulfonic acid) disodium salt (ABCBS) was synthesized as described by Phelisse and Quiby (*17*). The synthesis of SOTS-1 has been reported earlier (*6*). Acetaldehyde (Aldrich) was distilled immediately prior to use. Xanthine oxidase (EC 1.1.3.22, XO) was purchased from Boehringer Mannheim. Tris buffer, catalase (EC 1.11.1.6, CAT), cytochrome *c*, desferal, and Chelex 100 were purchased from Sigma. Plasmid supercoiled DNAs (pBR 322 and pUC 19) were purchased from MBI Fermentas Inc., ON, and salmon sperm DNA was purchased from GIBCO.

Table 1: Kinetic Data for the Decomposition of Some Azo Radical Sources at 37 °C in D_2O, pD ∼7

azo compound (peroxyl radical)	rate constant (s^{-1})	half-life (h)	*e*	pK_a
ABAP ($^{+}$AOO$^{\bullet}$)	$k_2 = 1.3 \times 10^{-6}$	148	0.5[a]	∼9.5[b]
ABDBA ($^{+}$DOO$^{\bullet}$)	$k_6 = 5.2 \times 10^{-6}$	37	0.5[a]	∼10.5[b]
ABMHP (COO$^{\bullet}$)	$k_5 = 1.8 \times 10^{-7}$	1070	0.5[a]	
ABCBS ($^{-}$BOO$^{\bullet}$)	$k_4 = 3.5 \times 10^{-7}$	550	0.5[a]	∼−6[c]
SOTS-1 ($^{-}$OO$^{\bullet}$)	$k_3 = 1.5 \times 10^{-4}$	1.3	0.2	

[a] Assumed, see text. [b] From titration curve. [c] Value for methane sulfonic acid.

Methods. (i) Rates of Peroxyl Radical Formation. For the planned, quantitative investigation of the abilities of the four water-soluble alkylperoxyl radicals mentioned above (viz., $^{+}$AOO$^{\bullet}$, $^{-}$BOO$^{\bullet}$, COO$^{\bullet}$, and $^{+}$DOO$^{\bullet}$) to induce strand scission in plasmid DNA, it was essential to be able to treat the DNA with known and approximately equal fluxes of these peroxyl radicals. There are data on the rates of thermal decomposition in water of ABAP at 37 °C (*3*) and of ABAP, ABDBA, and ABMHP at temperatures of 40 °C and higher (*18*), but there are no data for ABCBS. We therefore decided to measure the decomposition rates for all four azo compounds. These decomposition rates were measured at 37 °C in D_2O by monitoring the decrease in intensity of the signal due to the CH_3 groups in the starting azo compounds using ^{1}H NMR. Decay of these azo compounds followed clean first-order kinetics in all cases. The derived first-order rate constants and half-lives are summarized in Table 1. The efficiencies of escape of the tertiary alkyl radicals from the solvent cage, *e*, were assumed to be 0.5 (*1*−*3*). The rate constant for decay of SOTS-1 at 37 °C and pH 6.5−8.0 has been reported previously together with its remarkably small *e* value of 0.2 (*6*). These data allow the rates of generation of positively charged, negatively charged, and neutral tertiary alkylperoxyl radicals and superoxide radical anions to be matched using simple chemical sources for each of these various radicals. The positively and negatively charged azo compounds and the peroxyl radicals derived from them will be essentially fully ionized at the experimental pH of 7.4.

Two positively charged peroxyl radicals were used in these studies because it is known (*18*, *19*), though usually ignored or overlooked, that ABAP can undergo a base-catalyzed hydrolysis to the thermally much more stable amide, eq 7:

$$[(H_2N)_2^{+}CC(CH_3)_2N{=}]_2 + 2H_2O \rightarrow [H_2NC(O)C(CH_3)_2N{=}]_2 + 2NH_4^{+} \quad (7)$$

We have estimated that under our conditions ca. 90% of the ABAP decays to radicals (eq 2), and we have therefore ignored the small contribution hydrolysis makes to our

measured rate of decomposition of ABAP in calculating the rate of formation of ($^{+}$AOO$^{\bullet}$) peroxyl radicals from this azo compound. The hydrolysis of ABDBA would appear to be even slower than the hydrolysis of ABAP (*20*).

Superoxide was also generated by the well-established aerobic xanthine oxidase/acetaldehyde (XO/AA) system [acetaldehyde being chosen as the substrate because it is more water-soluble than xanthine (*8*, *21*)]. By monitoring superoxide formation using the cytochrome *c* assay (*22*), conditions were discovered where the total flux of superoxide from XO/AA could be matched with its production from SOTS-1. However, it should be borne in mind that SOTS-1 is the "cleaner" source of superoxide because commercial XO generally contains "excess" iron, i.e., more iron than can be accounted for by the enzyme's two iron−sulfur clusters. This "contaminating" iron is not easy to remove, and it may generate the highly reactive hydroxyl radical by reaction with the H_2O_2 (*23*, *24*) (which is both produced directly in the XO/AA reaction and by dismutation of the "side-product", superoxide, eqs 8 and 9).

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (8)$$

$$H_2O_2 + Fe^{2+} \rightarrow \rightarrow HO^{\bullet} + HO^- + Fe^{3+} \quad (9)$$

The HO$^{\bullet}$ radical is capable of cleaving DNA, and its potential production together with superoxide in the XO/AA system makes the assignment of any observed DNA damage to superoxide problematical (*25*). Indeed, we consider it likely that different levels of iron contamination and HO$^{\bullet}$ formation are the reason for there being reports that superoxide is very inefficient at cleaving double-stranded DNA (*13*−*16*, *25*) and that it can be fairly efficient in this regard (*8*, *9*).

(ii) Interaction of Double-Stranded DNA with Peroxyl Radicals. The effects of the peroxyl radicals produced from ABAP, ABDBA, ABCBS, ABMHP, SOTS-1, and XO/AA on plasmid DNA (pBR 322 and pUC 19) derived from *E. coli* were examined. Buffer [Tris/HCl (50 mM), pH 7.4] was treated with Chelex 100 for at least 24 h before use. Immediately prior to its use, this buffer was filter-sterilized, and the oxygen content was increased by bubbling with dioxygen for 15 min. Samples prepared in 15 μL of this buffer contained 320 ng of pBR 322 DNA [4361 base pairs (bp) ≡ 32 μM bp] or 320 ng of pUC 19 (2686 bp), 15 units of catalase (CAT), 50 μM desferal, and an appropriate concentration of one of the azo compounds listed in Table 1. The CAT was added to eliminate hydrogen peroxide (eq 10):

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2 \quad (10)$$

and the desferal to chelate any adventitious soluble iron. (It should be noted that DNA may be contaminated with adventitious transition metal ions and that these cations may be rather tightly bound to polyanionic DNA.) Together these two additives should minimize any contribution to DNA strand scission by hydroxyl radicals derived from H_2O_2 via Fenton chemistry and by alkoxyl radicals derived from any tertiary alkyl hydroperoxides formed by reaction of the alkylperoxyl radicals with hydrogen atom donors, SH, eqs 11 and 12:

$$ROO^{\bullet} + SH \rightarrow ROOH + S^{\bullet} \quad (11)$$

$$ROOH + Fe^{2+} \rightarrow \rightarrow RO^{\bullet} + HO^- + Fe^{3+} \quad (12)$$

Both HO$^{\bullet}$ and RO$^{\bullet}$ radicals are, of course, very much more reactive than ROO$^{\bullet}$ radicals. To investigate the possible effects of mono- and divalent cations on peroxyl radical-induced DNA cleavage, different salts were added to the Tris buffer at physiological concentrations: NaCl (145 mM, extracellular), KCl (140 mM, intracellular), and $MgCl_2$ (30 mM, intracellular).

In matched experiments, samples of pBR 322 or pUC 19 DNA containing an appropriate concentration of one of the azo compounds were incubated at 37 °C for 4 or 24 h in order to produce a total quantity of ca. 0.13 and 0.78 mM, respectively, of each of the four tertiary alkylperoxyl radicals. SOTS-1 was used at two different concentrations to generate total superoxide quantities of ca. 0.13 and 0.78 mM after 4 h incubations at 37 °C. We have found that XO is deactivated during turnover and, for this reason, the incubation time for the XO/AA system was reduced to 1 h using 20 mM AA and XO concentrations adjusted to generate total superoxide concentrations of 0.13 and 0.78 mM. At the end of the incubation period, 4 μL of loading buffer (0.2 M EDTA, 0.1% w/w bromophenol blue, 50% w/w glycerol) was added to each sample, followed by heating to 65 °C for 10 min. Samples were then loaded onto a 1% or 2% agarose slab gel (the latter being found to give a better separation of the DNA bands). Electrophoresis in a Tris/acetic acid buffer (5 mM; EDTA, 1 mM; pH 8) at 24 V until the dye reached the end of the gel was followed by a soak in an aqueous solution of ethidium bromide (1 μg/mL). The gel was photographed with UV transillumination.

The incubations described above were started at such times that they were all completed at the same time.

(iii) Association of SC DNA with the Azo Compounds. The relative abilities of each of the four azo compounds to aggregate with DNA were determined by examining their ability to inhibit the fluorescence due to intercalation of ethidium bromide into salmon sperm DNA (*26*). The fluorescence was measured after incubating the DNA at room temperature with each azo compound and ethidium bromide, their order of addition to the DNA having no effect on the fluorescence. Peroxyl radical formation from the azo compounds was negligible under these conditions. Spermine served as a positive control since this compound is known to bind to DNA and to inhibit the fluorescence of DNA-intercalated ethidium bromide (*26*). The effects of physiological concentrations of NaCl, KCl, and $MgCl_2$ on the binding of the four azo compounds and spermine to salmon sperm DNA were also examined (Supporting Information, Figure S4B and Table S1).

RESULTS

The results of our peroxyl radical/pBR 322 DNA experiments are shown in Figure 1. In each lane, the lower band is due to supercoiled (SC) DNA, the upper band to relaxed (R) DNA (sometimes called open-circular DNA) and between is a band which sometime appears and is due to linear (L) DNA. It is normally assumed that it takes only a single strand scission event to convert SC DNA into R DNA (*27*,

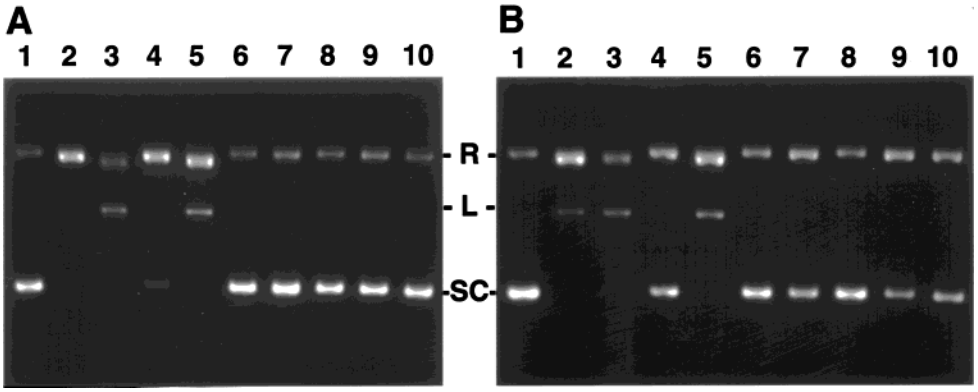


FIGURE 1: Agarose gel 2% electrophoresis of supercoiled pBR 322 DNA treated with peroxyl radicals. All solutions contained 320 ng of pBR 322, 15 units of CAT, 50 μM desferal and were prepared in oxygenated 50 mM Tris buffer (pH 7.4). Reaction mixtures (15 μL) were incubated at 37 °C for 4 and 24 h to generate a total of 0.13 and 0.78 mM peroxyl radicals, respectively. Experiments were as follows: untreated DNA (lane 1), DNA with 6.9 mM (4 h) and 7.2 mM (24 h) ABAP (lanes 2 and 3), DNA with 1.8 mM (4 h) and 2.2 mM (24 h) ABDBA (lanes 4 and 5), DNA with 50 mM ABMHP for 4 and 24 h (lanes 6 and 7), DNA with 26 mM ABCBS for 4 and 24 h (lanes 8 and 9), DNA alone for 24 h (lane 10). Panel A, no added salts. Panel B, with added KCl (140 mM) plus $MgCl_2$ (30 mM).

28) and the latter seems always to be present (in variable amounts depending on the batch) in SC DNA. A second strand scission event converts R DNA into L DNA provided this event occurs on the other (i.e., the uncut) strand and probably within about 5 bp of the break in the first strand. If the second strand scission event and all subsequent strand scission events occur randomly, the conversion of 50% of R pBR 322 DNA into L DNA will then, on average, require about 28 separate strand scission events (see Supporting Information). The L DNA never becomes a major product because it is continuously degraded to small DNA fragments.

Figure 1A shows the effect of incubating pBR 322 DNA with the four azo compounds in Tris buffer without added salts, and Figure 1B shows matched experiments (also lane-matched) carried out in Tris buffer containing intracellular concentrations of KCl (140 mM) and $MgCl_2$ (30 mM). In both of these figures, lane 1 shows untreated pBR 322 DNA and lane 10 the DNA incubated with no peroxyl radical source for 24 h. The incubations with a total of 0.13 mM radicals correspond to the formation of about 4 radicals/bp over the duration of the experiment. Neutral (lane 6) and negatively charged (lane 8) tertiary alkylperoxyl radicals generated in this amount produced no observable strand scission. However, generation of this quantity of the two positively charged tertiary alkylperoxyl radicals degraded all (ABAP, lane 2) or almost all (ABDBA, lane 4) of the SC DNA to R DNA, which in the case of ABAP was further degraded to give traces of L DNA in the salt-free experiments. The added KCl and $MgCl_2$ provided a small protective effect against *only* the positively charged peroxyl radicals from ABDBA (cf. lane 4 in Figure 1A,B). Increasing the total quantity of radicals to 0.78 mM, which corresponds to the formation of about 24 radicals/bp, still produced no sign of significant strand scission for the neutral (lane 7) and negatively charged (lane 9) alkylperoxyl radicals. However, this quantity of the two positively charged peroxyl radicals (lanes 3 and 5) produced far more damage with all of the SC DNA destroyed and much of the R DNA converted into L DNA, there being no obvious protection by the added salts. The, at best, occasional and minor protective effect against positively charged peroxyl radical induced strand cleavage of pBR 322 by $MgCl_2$ (30 mM), by NaCl (145 mM), and by KCl (140 mM) is shown in Figures S1A,B,C in the Supporting Information (compare with Figure 1A).

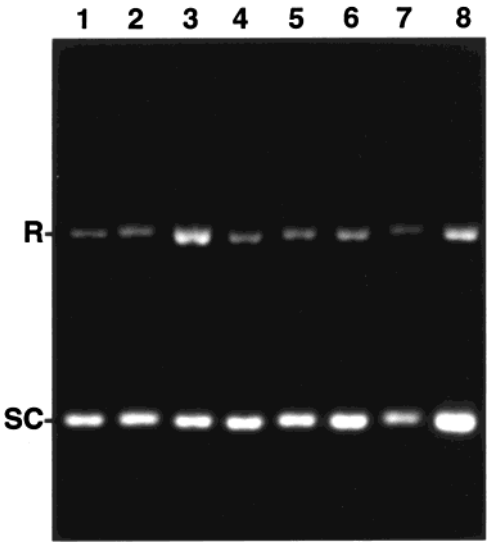


FIGURE 2: Agarose gel 1% electrophoresis of supercoiled pBR 322 DNA treated with superoxide. All solutions contained 320 ng of pBR 322, 15 units of CAT, 50 μM desferal and were prepared in oxygenated 50 mM Tris buffer (pH 7.4). Reaction mixtures (15 μL) were incubated at 37 °C to generate a total of 0.13 and 0.78 mM superoxide. Incubations were as follows: DNA alone for 4 h (lane 1), 20 mM AA with 0.024 and 0.16 unit/mL XO incubated for 1 h (lanes 2 and 3), 0.4 and 2.2 mM SOTS-1 incubated for 4 h (lanes 4 and 5), 0.4 and 2.2 mM decomposed SOTS-1 incubated for 4 h (lanes 6 and 7), and untreated pBR 322 (lane 8).

The above-described results with pBR 322 were closely reflected by the results of similar experiments on SC pUC 19. The pUC 19 was, however, somewhat more susceptible to strand cleavage by the positively charged peroxyl radicals. Specifically, all SC pUC 19 was destroyed by treatment with both ABAP and ABDBA at a dose of 4 radicals/bp (see Supporting Information Figure S2A and compare lane 4 with lane 4 in Figure 1A). A high, nonphysiological, concentration of salts (145 mM NaCl plus 140 mM KCl) provided some protection to the pUC 19 against cleavage by the two positively charged peroxyl radicals (Figure S2B).

The results of experiments with pBR 322 DNA and the superoxide anion radical are shown in Figure 2. Generation of 0.13 and 0.78 mM superoxide from the aerobic XO/AA couple (lanes 2 and 3, respectively) and from SOTS-1 (lanes

4 and 5, respectively) caused observable strand scission only with the larger quantity of superoxide derived from XO/AA. Similarly, SC pUC 19 was not visibly degraded by superoxide derived from XO/AA (even with the larger quantity of superoxide) or from SOTS-1 (Supporting Information, Figure S2C). Interestingly, on repeating the XO/AA experiments on SC pBR 322 with a quite different sample of XO, there was no detectable strand scission even at the higher level of superoxide (0.78 mM, results not shown). The ability of the aerobic XO/AA couple to effect DNA strand scission is clearly dependent on the purity of the XO. (Control experiments showed no sign of strand cleavage by XO alone, nor by AA alone, nor by either in the presence of SOTS-1; results not shown.) As was mentioned above, we consider it likely that different levels of XO contamination by adventitious iron and consequently different levels of $HO^{\bullet}$ radical formation (reaction 9) are probably responsible for there being reports that superoxide is very inefficient at cleaving double-stranded DNA (*13*−*16*, *25*) and that it can be fairly efficient in this regard (*8*, *9*).

The effect on pBR 322 of a wider range of radical/bp ratios was also examined for the positively charged peroxyl radicals from ABAP. Roughly 50% of the SC DNA was degraded to R DNA at a radical/bp ratio of ca. 0.2 (Supporting Information, Figure S3A).

Measurements were made of the reduction in ethidium bromide/DNA fluorescence over a range of concentrations of each of the four azo compounds relative to fluorescence in the absence of any azo compound (*26*). Fluorescence was reduced 40% by ca. 1 mM ABAP and 2 mM ABDBA, the two positively charged azo compounds, by 10 mM negatively charged ABCBS (which, however, interacts directly with the ethidium bromide), and by 50 mM neutral ABMHP (see Supporting Information, Figure S4A and Table S1). For comparison, fluorescence was decreased 40% by ca. 3 μM spermine, which has a DNA binding constant of 5×10^6 M^{-1} (*26*). These results indicate that the two positively charged azo compounds are more strongly absorbed onto the DNA than the neutral and negatively charged azo compounds, a phenomenon we attribute to attractive electrostatic forces. In the presence of physiological concentrations of salts, the association with the DNA of two positively charged azo compounds is reduced by roughly 1 order of magnitude (Supporting Information, Table S1). These salts reduce the binding of (positively charged) spermine to the DNA by probably 2 orders of magnitude (Figure S4B).

DISCUSSION

Tertiary alkylperoxyl radicals are expected to have very similar reactivities *in the absence of* "*special factors*". In their reaction with the polyanion DNA, it is quite clear that electrostatic charge is a special factor which perturbs reactivity. That is, DNA is very resistant to strand scission by neutral and negatively charged alkylperoxyl radicals. These (non) results are fully consistent with the very low rate constants which have been found for the reactions of peroxyl radicals with compounds having the same functional groups as those present in DNA's deoxyribose backbone [ethers, esters, etc. (*29*)] which must be attacked for strand scission to occur. The main reaction of these peroxyl radicals is therefore most likely to be their bimolecular self-reaction, eq 13:

$$2ROO^{\bullet} \rightarrow \text{nonradical products} \qquad (13)$$

together, possibly, with some oxidative modifications of the bases which do not lead to strand scission. The steady-state concentration of tertiary alkylperoxyl radicals, $[ROO^{\bullet}]_{ss}$, will, of course, depend on the rate of their formation, $2ek_1[RNNR]$, and the rate of their destruction, $2k_{13}[ROO^{\bullet}]^2$, and is given by

$$[ROO^{\bullet}]_{ss} = (2ek_1[RNNR]/2k_{13})^{1/2} \qquad (14)$$

It is possible that $2k_{13}$ will be somewhat smaller for charged tertiary alkylperoxyl radicals than for neutral tertiary alkylperoxyl radicals because of their mutual electrostatic repulsion. This would lead to a somewhat higher $[ROO^{\bullet}]_{ss}$ for charged than for neutral tertiary alkylperoxyls. However, the absence of any observable DNA damage by the negatively charged peroxyls implies that this factor is not responsible for DNA damage by the two positively charged peroxyl radicals.

The two positively charged tertiary alkylperoxyl radicals and their precursors were expected to be electrostatically attracted to the DNA, and, indeed, it was demonstrated by their suppression of ethidium bromide/DNA fluorescence that the two positively charged precursors were associated with the DNA. It therefore appears likely that some fraction of the positively charged peroxyl radicals will actually be formed right on the DNA rather than in the aqueous phase. This would certainly encourage their attack on the DNA. ABAP appears to cause somewhat more damage to SC DNA than ABDBA, which is consistent with ABAP's slightly stronger binding to salmon sperm DNA, as measured with ethidium bromide. Nevertheless, attack on SC DNA leading to strand scission by the ABAP-derived peroxyls is not very efficient. That is, it takes 1 single strand scission to convert SC DNA into R DNA (*27*, *28*), yet it requires ca. 0.2 positively charged peroxyl radical from ABAP per base pair to degrade about 50% of SC pBR 322 DNA into its R form, vide supra. This means that approximately 900 (i.e., 0.2×4361) of these particular positively charged peroxyl radicals must be generated per SC DNA molecule to have a 50% probability of causing one single strand scission event. (Presumably somewhat fewer than 900 of these radicals would be required to achieve the same level of strand scission in SC pUC 19, which appears to be more sensitive to positively charged peroxyls than pBR 322.) The generation of roughly 120 (24 radicals/bp ÷ 0.2 radical/bp) times as many neutral or negatively charged *tert*-alkylperoxyl radicals (lanes 7 and 9, respectively, in Figure 1A) induced no discernible strand scission of pBR 322. That is, *if* these peroxyl radicals *can* induce strand scission, more than 110 000 (900×120) are required per DNA molecule to achieve an observable level of scission. (Occasionally, and quite irreproducibly, the negatively charged peroxyl radicals at the high dose of 24 radicals/bp produced an obvious but only partial degradation of SC to R DNA; see Supporting Information, lanes 9 in Figures S1B, S1C, and S2A, but contrast with lanes 9 in Figures 1A,B, S1A, and S2B. The reasons for this irreproducibility were not explored.) What

is clear, is that *positively charged peroxyl radicals can cleave SC DNA but this process has an efficiency per radical generated of only ca. 0.1%. Nevertheless, the positively charged peroxyl radicals are at least 2 orders of magnitude more effective at DNA strand scission than the neutral or negatively charged peroxyl radicals*. [The importance of Coulombic attraction between DNA and radical precursors has recently been reported for the reactions of triplet benzophenones with DNA (*30*).]

We conclude that the extensive and uncritical use of ABAP to investigate the ability of peroxyl radicals to cleave DNA (*7*−*12*) has led to the misleading conclusion that peroxyl radicals *as a class* readily induce DNA strand scission. Most relatively small and, therefore, free to diffuse peroxyl radicals formed in vivo will be negatively charged or neutral. Our results show that *such radicals have little or no ability to induce DNA strand scission.*

The inability of the superoxide radical anion to induce strand scission in pBR 322 DNA (see Figure 2) and pUC 19 (Supporting Information, Figure S2C) arises from a number of causes. The thermodynamics of hydrogen atom abstraction by hydroperoxyl and alkylperoxyl radicals are very similar. [The O−H bond dissociation enthalpies, BDE, in H_2O_2 and in various ROOH are both ca. 88−89 kcal mol^{-1} (*31*).] Furthermore, there is evidence that the reactivities of HOO$^\bullet$ and ROO$^\bullet$ in hydrogen atom abstractions are similar (*29*, *32*, *33*). Since there would appear to be little or no reaction between neutral alkylperoxyl radicals and SC DNA, the failure of superoxide to induce DNA strand scission via its conjugate acid, HOO$^\bullet$ (present, in any case, at only ca. 0.2% of the $^-O_2^\bullet$ concentration at pH 7.4), is, therefore, readily explained. Hydrogen atom abstraction by the superoxide radical anion is even less favorable thermodynamically than by the HOO$^\bullet$ radical (O−H BDE for $^-$OOH is ca. 60 kcal mol^{-1}), so this radical should not be capable of inducing, nor expected to induce, DNA strand scission.

CONCLUSION

Electrostatic effects must be considered in any study of biomimetic free radical reactions. Failure to do so can lead to incorrect conclusions.

ACKNOWLEDGMENT

We thank Brenda Neely, Leanne Bedard, Jake Majewski, and Ginette Dubuc for superb technical help, John MacManus, Willem Siebrand, and Janusz Lusztyk for valuable discussions, and two anonymous referees for their extremely helpful comments and criticisms.

SUPPORTING INFORMATION AVAILABLE

Derivation by Dr. W. Siebrand of the equation used to calculate the average number of random strand scissions required to convert 50% of the R DNA into L DNA. Figure S1: effect of added salts on peroxyl radical-induced cleavage of SC pBR 322: (A) 30 mM $MgCl_2$; (B) 140 mM KCl; (C) 145 mM NaCl. Figure S2: reactions of peroxyl radicals with SC pUC 19: (A) effects of alkylperoxyls in the absence of added salts; (B) their effects in the presence of 145 mM NaCl plus 140 mM KCl; (C) effect of superoxide. Figure S3: effect of different quantities of peroxyl radicals on pBR 322: (A) $^+$AOO$^\bullet$ radicals from ABAP; (B) COO$^\bullet$ from ABMHP. Figure S4: effects of various concentrations of additives on ethidium bromide/DNA fluorescence: (A) ABAP, ABDBA, ABCBS, and ABMHP; (B) spermine and spermine with added salts. Table S1: effect of salts on the inhibition of ethidium bromide/DNA fluorescence by azo compounds and spermine (7 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Hammond, G. S., Sen, J. N., and Boozer, C. E. (1955) *J. Am. Chem. Soc*. *77*, 3244−3248.
2. Howard, J. A., and Ingold, K. U. (1962) *Can. J. Chem. 40*, 1851−1864.
3. Barclay, L. R. C., Locke, S. J., MacNeil, J. M., VanKessel, J., Burton, G. W., and Ingold, K. U. (1984) *J. Am. Chem. Soc. 106*, 2479−2481.
4. Yamamoto, Y., Haga, S., Niki, E., and Kamiya, Y. (1984) *Bull. Chem. Soc. Jpn. 57*, 1260−1264.
5. Gebicki, S., and Gebicki, J. M. (1993) *Biochem. J. 289*, 743−749.
6. Ingold, K. U., Paul, T., Young, M. J., and Doiron, L. (1997) *J. Am. Chem. Soc. 119*, 12364−12365.
7. Hiramoto, K., Johkoh, H., Sako, K.-I., and Kikugawa, K. (1993) *Free Radical Res. Commun. 19*, 323−332.
8. Dix, T. A., Hess, K. M., Medina, M. A., Tilly, S. L., and Sullivan, R. W. (1994) in *Biological Oxidation and Antioxidants* (Packer, L., and Cadenas, E., Eds.) pp 13−23, Hippokratus, Stuttgart.
9. Dix, T. A., Hess, K. M., Medina, M. A., Sullivan, R. W., Tilly, S. L., and Webb, T. L. L. (1996*) Biochemistry 35*, 4578−4583.
10. Harkin, L. A., and Burcham, P. C. (1997) *Biochem. Biophys. Res. Commun. 237*, 1−5.
11. Martini, M., and Termini, J. (1997) *Chem. Res. Toxicol. 10*, 234−241.
12. Valentine, M. R., Rodriguez, H., and Termini, J. (1998) *Biochemistry 37*, 7030−7038.
13. Van Hemmen, J. J., and Meuling, W. J. A. (1975) *Biochim. Biophys. Acta 402*, 133−141.
14. Lesko, S. A., Lorentzen, R. J., and Ts'o, P. O. P. (1980) *Biochemistry 19*, 3023−3033.
15. Brawn, K., and Fridovich, I. (1981) *Arch. Biochem. Biophys. 206*, 414−419.
16. Ward, B., Skorobogaty, A., and Dabrowiak, J. C. (1986) *Biochemistry 25*, 6875−6883.
17. Phelisse, J. A., and Quiby, C. A., (1960) *Deutsche Auslageschrift 1111395*, Société des Usines Chimiques Rhône-Polenc, Germany. *Chem. Abstr*. (1962) *56*, 3681g.
18. Wako Technical Information Bulletins.
19. Ito, K. (1973) *J. Polym. Sci. II 11*, 1673−1681.
20. Rojas Wahl, R. U., Zeng, L., Madison, S. A., DePinto, R. L., and Shay, B. J. (1998) *J. Chem. Soc., Perkin Trans. 2*, 2009−2017.
21. Thomas, M. J., Mehl, K. S., and Pryor, W. A. (1982) *J. Biol. Chem. 257*, 8343−8347.
22. Bielski, B. H. J., and Richter, H. W. (1977) *J. Am. Chem. Soc. 99*, 3019−3023.
23. Lloyd, R. V., and Mason, R. P. (1990) *J. Biol. Chem. 265*, 16733−16736.
24. Britigan, B. E., Pou, S., Rosen, G. M., Lilleg, D. M., and Buettner, G. B. *J. Biol. Chem. 265*, 17533−17538.
25. Denq, R.-Y., and Fridovich, I. (1989) *Free Radical Biol. Med. 6*, 123−129.
26. Morgan, A. R., Lee, J. S., Pulleyblank, D. E., Murray, N. L., and Evans, D. H. (1979) *Nucleic Acids Res. 7*, 547−569.
27. Inouye, S. (1984) *FEBS Lett. 172*, 231−234.
28. Häring, M., Rüdiger, H., Demple, B., Boiteux, S., and Epe, B. (1994) *Nucleic Acids Res. 22*, 2010−2015.

29. Howard, J. A., and Scaiano, J. C. (1984) in *Landolt-Börnstein, New Series, Vol. 13, Part d* (Fischer, H., Ed.) Springer, Berlin. Howard, J. A. (1997) in *Landolt-Börnstein, New Series, Vol. 18, Part d2* (Fischer, H., Ed.) Springer, Berlin.
30. Mohtat, N., Cozens, F. L., Hancock-Chen, T., Scaiano, J. C., McLean, J., and Kim, J. (1998) *Photochem. Photobiol. 67*, 111−118.
31. Cremer, D. (1983) in *The Chemistry of Peroxides* (Patai, S., Ed.) pp 1−84, John Wiley & Sons, Chichester.
32. von Sonntag, C., and Schuchmann, H.-P. (1991) *Angew. Chem., Int. Ed. Engl. 30*, 1229−1253.
33. Bielski, B. H. J., Cabelli, D. E., Arudi, R. L., and Ross, A. B. (1985) *J. Phys. Chem. Ref. Data 14*, 1041−1100. Neta, P., Huie, R. E., and Ross, A. B. (1990) *J. Phys. Chem. Ref. Data 19*, 413−513.

BI991463O