DNA Strand Scission by Polycyclic Aromatic Hydrocarbon *o*-Quinones: Role of Reactive Oxygen Species, Cu(II)/Cu(I) Redox Cycling, and *o*-Semiquinone Anion Radicals[†,‡]

Lynn Flowers,[§] S. Tsuyoshi Ohnishi,[||] and Trevor M. Penning*[,§]

Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6084, and Philadelphia Biomedical Research Institute, King of Prussia, Pennsylvania 19406

Received February 18, 1997; Revised Manuscript Received May 8, 1997[⊗]

ABSTRACT: In previous studies, benzo[a]pyrene-7,8-dione (BPQ), a polycyclic aromatic hydrocarbon (PAH) *o*-quinone, was found to be 200-fold more potent as a nuclease than (±)-*anti*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene, a suspect human carcinogen. The mechanism of strand scission mediated by naphthalene-1,2-dione (NPQ) and BPQ was further characterized using either ϕX174 DNA or poly(dG)·poly(dC) as the target DNA. Strand scission was extensive, dependent on the concentration of *o*-quinone (0−10 μM), and required the presence of NADPH (1 mM) and $CuCl_2$ (10 μM). The production of reactive species, i.e., superoxide anion radical, *o*-semiquinone anion (SQ) radical, hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and Cu(I), was measured in the incubation mixtures. The formation of SQ radicals was measured by EPR spectroscopy under anaerobic conditions in the presence of NADPH. A Cu(II)/Cu(I) redox cycle was found to be critical for DNA cleavage. No strand scission occurred in the absence of Cu(II) or when Cu(I) was substituted, yet Cu(I) was required for $OH^{\bullet}$ production. Both DNA strand scisson and $OH^{\bullet}$ formation were decreased to an equal extent, albeit not completely, by the inclusion of $OH^{\bullet}$ scavengers (mannitol, soduim benzoate, and formic acid) or Cu(I) chelators (bathocuproine and neocuproine). In contrast, although the SQ radical signals of NPQ and BPQ were quenched by DNA, no strand scission was observed. When calf thymus DNA was treated with PAH *o*-quinones, malondialdehyde (MDA) was released by acid hydrolysis. The formation of MDA was inhibited by $OH^{\bullet}$ scavengers suggesting that $OH^{\bullet}$ cleaved the 2′-deoxyribose moiety in the DNA to produce base propenals. These studies indicate that for PAH *o*-quinones to act as nucleases, NADPH, Cu(II), Cu(I), H_2O_2, and $OH^{\bullet}$, were necessary and that the primary species responsible for DNA fragmentation was $OH^{\bullet}$, generated by a Cu(I)-catalyzed Fenton reaction. The genotoxicity of PAH *o*-quinones may play a role in the carcinogenicity and mutagenicity of the parent hydrocarbons.

Polycyclic aromatic hydrocarbons (PAHs)[1] are ubiquitous environmental pollutants found in tobacco smoke and car exhaust (Dipple, 1985) and are believed to be causative agents of human lung cancer (Denissenko et al., 1996). PAHs are by themselves innocuous compounds, but through metabolic activation to electrophilic species, e.g., *anti*-diol epoxides (Dipple, 1985), radical cations (Cavalieri & Rogan, 1992), and *o*-quinones (Smithgall et al., 1988) (Scheme 1), they can alkylate DNA (Cavalieri & Rogan, 1992; Dipple, 1985; Jeffrey et al., 1976; Shou et al., 1993) and ultimately cause proto-oncogenes (Colapietro et al., 1993; Mass et al., 1993) and tumor suppressor genes (Denissenko et al., 1996; Puisieux et al., 1991) to be mutated.

Enzyme systems that generate *anti*-diol epoxides, radical cations, and *o*-quinones include enzymes of the P450 gene superfamily (primarily CYP1A1) (Dipple, 1985), cytochrome peroxidase (Cavalieri & Rogan, 1992), and dihydrodiol dehydrogenase (DD) (Smithgall et al., 1986, 1988), respectively. Our laboratory has focused on the role of *o*-quinones as activated PAH metabolites produced by DD. The homogeneous rat liver DD will oxidize a variety of PAH *trans*-dihydrodiols to yield the corresponding *o*-quinones (Smithgall et al., 1986, 1988). Thus, (±)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene (BP-diol) is oxidized to benzo[a]pyrene-7,8-dione (BPQ) (Scheme 1). Currently, there are four human DDs that are presumed to catalyze the same reaction (Hara et al., 1990, 1996). The rat liver enzyme is sufficiently abundant that the conversion of BP-diol to BPQ can be measured in isolated normal rat hepatocytes (Flowers-Geary et al., 1995). Furthermore, in rat hepato-

[†] This work was supported by NCI Grant CA39504 awarded to T.M.P.

[‡] Preliminary accounts of this work were presented at the eighty-seventh annual meeting of the American Association for Cancer Research, Washington, DC, April 20−24, 1996.

* Address for correspondence: Department of Pharmacology, University of Pennsylvania School of Medicine, 3620 Hamilton Walk, Philadelphia, PA 19104-6084. Tel: (215) 898-9445. Fax: (215) 573-2236. E-mail: penning@pharm.med.upenn.edu.

[§] University of Pennsylvania.

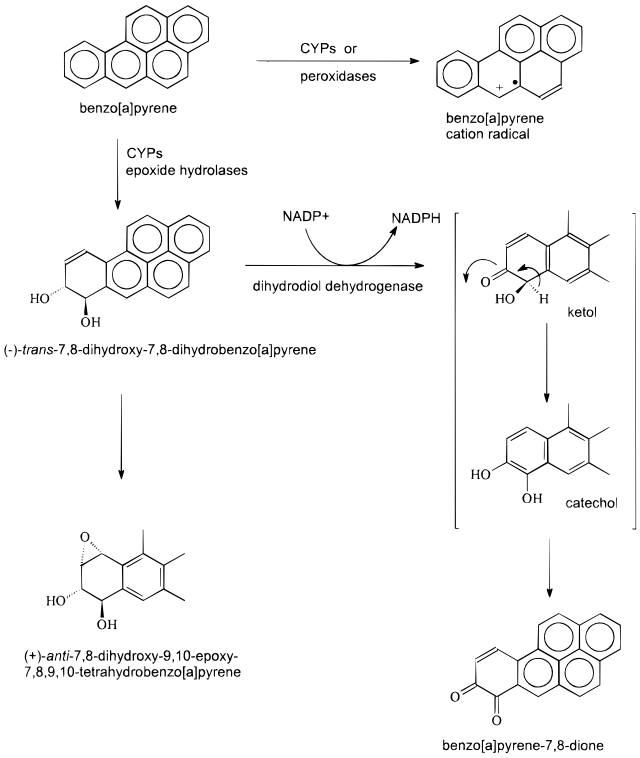
[||] Philadelphia Biomedical Research Institute.

[⊗] Abstract published in *Advance ACS Abstracts,* June 15, 1997.

[1] Abbreviations: bathocuproine, 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline; (±)-*anti*-BPDE, (±)-*anti*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; BP-diol, (±)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene; BPQ, benzo[a]pyrene-7,8-dione; CYP, protein members of the P450 gene superfamily; cyt *c*, cytochrome *c*; DD, dihydrodiol dehydrogenase (*trans*-1,2-dihydrobenzene-1,2-diol: dehydrogenase; EC 1.3.1.20); EPR, electron paramagnetic resonance; H_2O_2, hydrogen peroxide; LIN, linear; MDA, malondialdehyde; neocuproine, 2,9-dimethyl-1,10-phenanthroline; NPQ, naphthalene-1,2-dione; $O_2^{\bullet-}$, superoxide anion radical; OC, open circular; $OH^{\bullet}$, hydroxyl radical; PAH, polycyclic aromatic hydrocarbon; ROS, reactive oxygen species; SC, supercoiled; SOD, superoxide dismutase; SQ, *o*-semiquinone anion; TBA, thiobarbituric acid; Tiron, 4,5-dihydroxy-1,3-benzene disulfonic acid.

S0006-2960(97)00367-X CCC: $14.00

Scheme 1: Pathways Responsible for the Metabolic Activation of Benzo[a]pyrene and the Reaction Sequence Catalyzed by Dihydrodiol Dehydrogenase

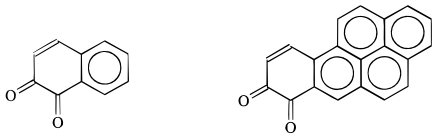


cytes, the conversion of BP-diol to form either the corresponding *anti*-diol epoxide [(±)-*anti*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene, *anti*-BPDE] or BPQ occurs to an equal extent. Both *anti*-BPDE and BPQ can form stable DNA adducts by alkylation of 2′-deoxyguanosine (Jeffrey et al., 1976; Shou et al., 1993). Examination of the mutagenicity of *anti*-BPDE and BPQ has revealed the former to be 10−5500-fold more mutagenic than BPQ in the Ames test (Flowers et al., 1996a).

One deleterious aspect of the generation of PAH *o*-quinones by DD is the production of reactive oxygen species (ROS) that can oxidatively damage DNA. During the enzymatic oxidation of *trans*-dihydrodiols, a catechol is formed which undergoes two successive one-electron oxidations (Smithgall et al., 1988) resulting in the formation of superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and *o*-semiquinone anion (SQ) radical. In the presence of trace Fe(II), H_2O_2 can undergo the Fenton reaction to produce hydroxyl radical ($OH^{\bullet}$). The production of $O_2^{\bullet -}$, H_2O_2, and $OH^{\bullet}$ during the enzymatic conversion of *trans*-dihydrodiols to *o*-quinones has been documented (Penning et al., 1996). Once formed, the *o*-quinone can enter futile redox cycles so that ROS are produced multiple times (Flowers-Geary et al., 1992, 1993, 1996a,b). This mechanism of free-radical amplification may generate sufficient ROS to cause oxidative damage of bases (e.g., formation of 8′-oxo-2-deoxyguanosine) within DNA.

8′-Oxo-2-deoxyguanosine can give rise to G to T transversions, which is one of the most common mutations observed in select codons in the H-*ras* proto-oncogene and the *p53* tumor suppressor gene (Denissenko et al., 1996; Colapietro et al., 1993; Mass et al., 1993; Puisieux et al., 1991). During replication, 8′-oxo-2-deoxyguanosine mispairs preferentially with adenine, giving rise to G·C to T·A transversions (Grollman & Moriya, 1993). Thus, modification of DNA by ROS provides an alternate route to these oncogenic mutations.

Scheme 2: Structures of PAH *o*-Quinones Produced by Dihydrodiol Dehydrogenase[a]



[a] Legend: NPQ, naphthalene-1,2-dione; BPQ, benzo[a]pyrene-7,8-dione.

ROS can also cause DNA strand scission and subsequent illegitimate recombination may play a role in mutagenesis and carcinogenesis (DeMarini et al., 1989; Emerit et al., 1982; Povirk & Austin, 1991). Incubation of isolated rat hepatocytes with BP-diol results in substantial generation of ROS, which is accompanied by DNA strand scission. Both effects can be attenuated by DD inhibitors implying that redox cycling of enzymatically generated BPQ was responsible (Flowers et al., 1996a). Using ϕX174 DNA, it was shown that BPQ was at least 200-fold more potent as a nuclease than *anti*-BPDE.

We now report studies on the mechanism of DNA strand scission by two representative PAH *o*-quinones (naphthalene-1,2-dione [NPQ] and BPQ) (Scheme 2) that are produced by DD. Evidence is provided that there is a requirement for redox cycling and that Cu(II), Cu(I), and $OH^{\bullet}$ are participants in the strand scission. By contrast, SQ radicals detected by EPR spectroscopy do not act as nucleases by themselves.

MATERIALS AND METHODS

Materials. NPQ and BPQ were synthesized according to published methods (Fieser, 1943; Sukumaran & Harvey, 1980). Superoxide dismutase (SOD; type I from bovine erythrocytes), catalase (from bovine liver), cytochrome *c* (cyt *c*; type VI from horse heart), calf thymus DNA (type I), ferrous sulfate, ferric chloride, hydrogen peroxide, Tiron, sodium benzoate, formic acid, mannitol, bathocuproine, neocuproine, sodium thiosulfate, ferrous ammonium sulfate, and potassium thiocyanate were obtained from Sigma Chemical Co. (St. Louis, MO). Cupric chloride, cuprous chloride, 2′-deoxy-D-ribose, thiobarbituric acid (TBA), trichloroacetic acid, and malondialdehyde bis(dimethyl) acetal were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). NADPH was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Supercoiled (RF I; SC) ϕX174 DNA and open circular (RF II; OC) ϕX174 DNA were from New England Biolabs (Beverly, MA). The linearized (LIN) form of ϕX174 DNA was obtained by *Pst* I (Boehringer Mannheim Biochemicals) digestion of SC ϕX174 DNA. Poly(dG)·poly(dC) was purchased from Pharmacia Biotech, Inc. (Piscataway, NJ). All other chemicals were of the highest grade available. ***Caution:*** *All PAHs are potentially hazardous and should be handled in accordance with "NIH Guidelines for the Laboratory Use of Chemical Carcinogens".*

Strand Scission of ϕX174 DNA and Fragmentation of Poly(dG)·Poly(dC) by Either PAH o-Quinones or SQ

Radicals. Strand scission of SC ϕX174 DNA or fragmentation of poly(dG)·poly(dC) was measured following treatment with PAH *o*-quinones under conditions of redox cycling. Briefly, reaction mixtures (20 μL) containing either 0.2 μg of SC ϕX174 DNA or 2 μg of poly(dG)·poly(dC), 0−10 μM NPQ or BPQ in Me_2SO [5% (v/v) final], 1 mM NADPH, 10 μM $CuCl_2$, and 10 mM Tris-HCl (pH 8.0) buffer were incubated for 2 h at 37 °C. Control incubations contained NPQ or BPQ alone, NPQ or BPQ in the presence of NADPH, or NADPH and $CuCl_2$ alone. Under these conditions no strand scission occurred. Incubations with ϕX174 DNA were also performed using 10 μM Cu(I), Fe(II), or Fe(III) in place of Cu(II). The effects of free radical scavenging agents or transition metal chelators on ϕX174 DNA strand scission were determined under conditions where the phage DNA was damaged to the point of complete scission. Strand scission due to the SQ radicals of NPQ and BPQ, and the NPQ-derived radical with the $g = 2.004$ signal was determined in reaction mixtures containing either 0.2 μg ϕX174 DNA or 2.0 μg poly(dG)·poly(dC). In this instance, radical species were generated prior to treatment of the DNA. The Fenton reaction was used to cause either complete scission of the SC ϕX174 DNA or complete fragmentation of poly(dG)·poly(dC) as a positive control. Reagents were prepared fresh daily. Strand scission of SC ϕX174 DNA was determined by separation of the SC, OC, and LIN forms of the DNA by electrophoresis through 0.8% agarose gels. Fragmentation of poly(dG)·poly(dC) was monitored by electrophoresis through 1.5% agarose gels. In each case, bands were visualized by staining with ethidium bromide, and gels were irradiated with UV light at 254 nm and photographed. Negatives were fixed by soaking the film backing in a solution of 0.1% (w/v) sodium thiosulfate, and the bands were quantified using a video densitometer and UNISCAN software (Analtech, Newark, DE).

Measurement of $O_2^{\bullet-}$ and SQ Radical Formation during the Redox Cycling of PAH o-Quinones. Rates of $O_2^{\cdot-}$ formation were determined from time zero in reaction mixtures (1 mL final volume) containing 0−10 μM NPQ or BPQ in Me_2SO [5% (v/v) final], NADPH (1 mM), $CuCl_2$ (10 μM), and 10 mM Tris-HCl (pH 8.0) buffer at 37 °C by following the rate of reduction of cyt *c* (50 μM) that was inhibited by SOD (500 units/mL). SQ radical formation was taken as the rate of reduction of cyt *c* that was not inhibited by SOD. The rate of cyt *c* reduction was measured at 550 nm [$\epsilon = 19\,600\ M^{-1}\ cm^{-1}$] (Azzi et al., 1975) in a Beckman DU640 spectrophotometer. Conditions were employed which ensured linearity of the rate of cyt *c* reduction with respect to time.

Measurement of H_2O_2 Formation during the Redox Cycling of PAH o-Quinones. Hydrogen peroxide formation was determined by the method of Thurman et al. (1972). Reaction mixtures (500 μL) containing 0−10 μM NPQ or BPQ in Me_2SO [5% (v/v) final], 1 mM NADPH, 10 μM $CuCl_2$, and 10 mM Tris-HCl (pH 8.0) buffer were incubated over a 2 h time period at 37 °C. At various time points, 0.1 mL of 10 mM ferrous ammonium sulfate (1.6 mM final concn) and 0.05 mL of 2.5 M potassium thiocyanate (200 mM final concn) were added to reaction mixtures. Ferrithiocyanate complex formation was measured at 480 nm ($\epsilon = 34\,300\ M^{-1}\ cm^{-1}$). Nanomoles of H_2O_2 produced were calculated from a standard curve which was linear over the range of 0−45 nmol.

Measurement of OH• Formation and Degradation of Calf Thymus DNA during the Redox Cycling of PAH o-Quinones. Hydroxyl radical formation was determined by the method of Gutteridge (1987). Reaction mixtures (100 μL) containing 0−10 μM NPQ or BPQ in Me_2SO [5% (v/v) fina1], 1 mM NADPH, 10 μM $CuCl_2$, and 10 mM Tris-HCl (pH 8.0) buffer were incubated over a 2 h time period at 37 °C in the presence of 2′-deoxy-D-ribose (2 mM), and the formation of TBA-reactive substances was determined. To measure the degradation of calf thymus DNA by PAH *o*-quinones, the same reaction conditions were used but calf thymus DNA (1 mg/mL) was substituted for 2′-deoxy-D-ribose. In the subsequent assay, 0.5 mL of TBA (1% [w/v]; in 50 mM NaOH) and 0.5 mL of 2.8% (w/v) trichloroacetic acid were added to the reaction mixtures at various times, and the mixtures were heated at 90 °C for 20 min. Reactions were cooled to room temperature, and the absorbance was measured at 532 nm [$\epsilon = 15\,800\ M^{-1}\ cm^{-1}$] (Giloni et al., 1981). Since the TBA-reactive substance produced from either the reaction of OH• with 2′-deoxy-D-ribose or the reaction of PAH *o*-quinones with calf thymus DNA is thought to be malondialdehyde (MDA) (Cheeseman et al., 1988; Giloni et al., 1981; Gutteridge, 1987), a standard curve for MDA formation was generated as follows: a 10 mM stock solution of MDA was prepared by the reaction of malondialdehyde bis(dimethyl) acetal in 1% (v/v) H_2SO_4 for 2 h at 25 °C (Cheeseman et al., 1988) and 0−100 nmol of MDA was then reacted with TBA as described.

Measurement of Cu(I)−Bathocuproine Complex Formation during the Redox Cycling of PAH o-Quinones. Reaction mixtures (500 μL) containing either 100 μM NPQ or 20 μM BPQ in EtOH (100%), 100 mM NADPH, 100 μM $CuCl_2$, and 100 mM bathocuproine were monitored spectrally at 480 nm for 30 min at 25 °C for evidence of Cu(I)−bathocuproine complex formation ($\epsilon = 13\,500\ M^{-1}\ cm^{-1}$) (Nesbar, 1964).

EPR Spectroscopy Measurements. EPR spectra of the SQ radicals of NPQ (0.78 mM) or BPQ (50 μM) were determined in the presence of NADPH (10 mM) under anaerobic conditions using Me_2SO [20% (v/v) for NPQ and 50% for BPQ] as cosolvent and Chelex-100 treated sodium phosphate (5 mM; pH 7.2) buffer containing NaCl (100 mM). The radical species derived from NPQ (2.0 mM) with the characteristic $g = 2.004$ signal was obtained under aerobic conditions in the presence of EDTA (5 mM). The effect of DNA on the radical signals was determined by the addition of either ϕX174 DNA (20 μg/mL) or poly(dG)·poly(dC) (200 μg/mL). EPR spectra were recorded on a Varian E109 EPR spectrometer operating in the X-band (9.25 GHz), employing 0.5 modulation amplitude and 20 mW microwave power with the receiver gain set between 1×10^4 and 1×10^5 at ambient temperature.

RESULTS

Strand Scission of DNA by PAH o-Quinones Requires NADPH and Cupric Chloride. Previous studies have shown that BPQ will cause extensive DNA strand scission in isolated rat hepatocytes (Flowers et al., 1996a). Model studies with BPQ or the more soluble NPQ showed that both of these PAH *o*-quinones caused a concentration-dependent destruction of ϕX174 DNA provided 1 mM NADPH and 10 μM $CuCl_2$ were present (Figure 1, panels A and B). Complete destruction of the DNA occurred at a concentration

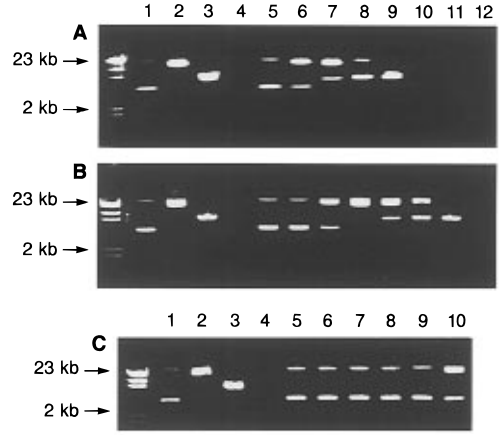


FIGURE 1: Strand scission of ϕX174 DNA by PAH *o*-quinones in the presence of NADPH and Cu(II) or by *o*-semiquinone anion radicals. Strand scission of 0.2 μg of ϕX174 DNA was measured following treatment with (A) NPQ and (B) BPQ in the presence of NADPH (1 mM) and $CuCl_2$ (10 μM) [complete system], or (C) the SQ radicals of NPQ and BPQ, and the NPQ-derived radical with the $g = 2.004$ signal. Strand scission was monitored by separating SC from OC and LIN forms by agarose gel electrophoresis. (Panels A and B) SC ϕX174 DNA (lane 1); OC ϕX174 DNA (lane 2); LIN ϕX174 DNA (lane 3); SC DNA plus 100 μM $FeSO_4$ and 0.1% H_2O_2 (lane 4); complete system minus PAH *o*-quinone (lane 5); and complete system plus 0.01 μM, 0.05 μM, 0.1 μM, 0.5 μM, 1.0 μM, 5.0 μM and 10.0 μM PAH *o*-quinone (lanes 6−12, respectively). (Panel C) SC ϕX174 DNA (lane 1); OC ϕX174 DNA (lane 2); LIN ϕX174 DNA (lane 3); SC DNA plus 100 μM $FeSO_4$ and 0.1% H_2O_2 (lane 4); SC DNA plus EDTA (5 mM) under aerobic conditions (lane 5); SC DNA plus NPQ (2 mM) and EDTA (5 mM) under aerobic conditions, i.e., the NPQ-derived radical with the $g = 2.004$ signal (lane 6); SC DNA plus NADPH (10 mM) under anaerobic conditions (lanes 7 and 9); SC DNA plus NPQ (0.78 mM) and NADPH (10 mM) under anaerobic conditions, i.e., the SQ radical of NPQ (lane 8); SC DNA plus BPQ (50 μM) and NADPH (10 mM) under anaerobic conditions, i.e., the SQ radical of BPQ (lane 10). The outside lanes contain a HindIII digest of λ phage.

of 1 μM for NPQ and 10 μM for BPQ, indicating that NPQ was 10-fold more potent as a nuclease than BPQ. Similar results were obtained when a ds-oligonucleotide [poly(dG)·poly(dC)] (Figure 2, panels A and B) was substituted for ϕX174 DNA. No significant strand scission occurred when Cu(I), Fe(II), or Fe(III) were substituted for Cu(II) (data not shown), indicating that *o*-quinone-mediated strand scission had a strict requirement for Cu(II).

Reactive Species Produced during the Redox cycling of PAH o-Quinones. To identify the species that could account for the DNA strand scission, the formation of reactive species [i.e., $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$, Cu(I), and/or SQ radical] was measured during the redox cycling of *o*-quinones in the presence of NADPH and $CuCl_2$. First, the rate of $O_2^{\bullet-}$ production was measured as the rate of cyt *c* reduction that was inhibited by SOD. The rate of reduction that was not inhibited by SOD was taken as a measure of SQ radical formation. With NPQ plus NADPH, it was found that there was robust $O_2^{\bullet-}$ production and SQ radical formation (Table 1). The inclusion of Cu(II) accelerated the rate of $O_2^{\bullet-}$ production and decreased the formation of SQ radical. In contrast, BPQ plus NADPH produced 10-fold less $O_2^{\bullet-}$ but SQ radical formation was high. The inclusion of Cu(II) decreased both $O_2^{\bullet-}$ and SQ radical formation. The ability of BPQ to produce less $O_2^{\bullet-}$ than NPQ was in accord with previous studies which showed that the SQ radical of BPQ was quite stable and that $O_2^{\bullet-}$ was produced slowly in the

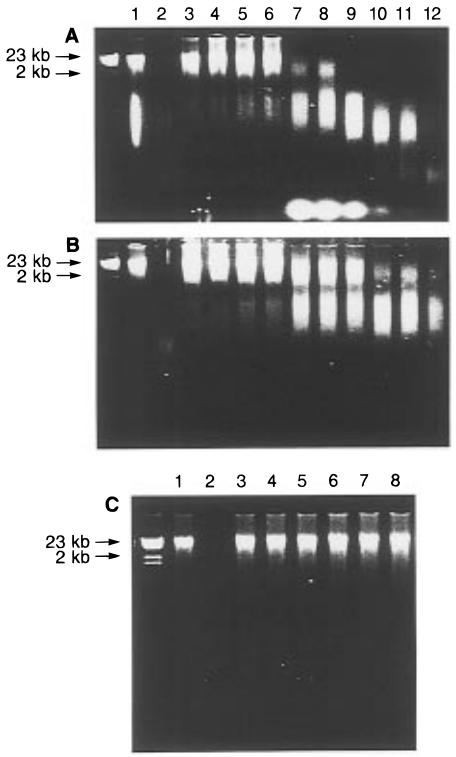


FIGURE 2: Fragmentation of poly(dG)·poly(dC) DNA by PAH *o*-quinones in the presence of NADPH and Cu(II) or by *o*-semiquinone anion radicals. Strand scission of 2.0 μg poly(dG)·poly(dC) DNA was measured following treatment with (A) NPQ and (B) BPQ in the presence of NADPH (1 mM) and $CuCl_2$ (10 μM) [complete system], or (C) the SQ radicals of NPQ and BPQ, and the NPQ-derived radical with the $g = 2.004$ signal. Fragmentation was monitored by agarose gel electrophoresis. (Panels A and B) Poly(dG)·poly(dC) DNA (lane 1); poly(dG)·poly(dC) DNA plus 100 μM $FeSO_4$ and 0.1% H_2O_2 (lane 2); complete system minus NADPH and $CuCl_2$ (lane 3); complete system minus $CuCl_2$ (lane 4); complete system minus NADPH (lane 5); complete system minus PAH *o*-quinone (lane 6); and complete system plus 0.1 μM, 0.5 μM, 1.0 μM, 5.0 μM, 10.0 μM, and 20 μM PAH *o*-quinone (lanes 7−12, respectively). (Panel C) Poly(dG)·poly(dC) DNA (lane 1); poly(dG)·poly(dC) DNA plus 100 μM $FeSO_4$ and 0.1% H_2O_2 (lane 2); poly(dG)·poly(dC) DNA plus EDTA (5 mM) under aerobic conditions (lane 3); poly(dG)·poly(dC) DNA plus NPQ (2 mM) and EDTA (5 mM) under aerobic conditions, i.e., the NPQ-derived radical with the $g = 2.004$ signal (lane 4); poly(dG)·poly(dC) DNA plus NADPH (10 mM) under anaerobic conditions (lanes 5 and 7); poly(dG)·poly(dC) DNA plus NPQ (0.78 mM) and NADPH (10 mM) under anaerobic conditions, i.e., the SQ radical of NPQ (lane 6); poly(dG)·poly(dC) DNA plus BPQ (50 μM) and NADPH (10 mM) under anaerobic conditions, i.e., the SQ radical of BPQ (lane 8). The outside lanes contain a HindIII digest of λ phage.

subsequent one-electron oxidation of the SQ radical to form BPQ (Penning et al., 1996). The differential effects of Cu(II) on $O_2^{\bullet-}$ production may be explained by differences in the reactions of 1,2-dihydroxynaphthalene and 7,8-dihydroxybenzo[a]pyrene with Cu(II) to produce the intermediate SQ radical. Cu(II) has been shown to oxidize hydroquinone and catechol estrogens with the concomitant production of ROS (Li & Trush, 1993; Li et al., 1994). Direct comparison of the reactions of the PAH catechols with Cu(II) was not feasible due to the instability of 7,8-dihydroxybenzo[a]pyrene. Differences in the potency of NPQ and BPQ as nucleases may reflect differences in the stability of the SQ radicals and, therefore, their ability to generate ROS.

Second, the formation of SQ radicals from NPQ (Figure 3) and BPQ (Figure 4) was verified by EPR spectroscopy. Small amounts of SQ radicals could be detected in the

Table 1: Formation of Reactive Species during the Redox Cycling of PAH *o*-Quinones in the Presence of NADPH and Cu(II)[a]

conditions	$O_2^{\bullet-}$ (nmol/mL × min)[b]	*o*-semiquinone anion radical (nmol/mL × min)[b]	H_2O_2 (nmol/mL)[c]	$OH^{\bullet}$ (nmol/mL)[d]	Cu(I) (nmol/mL)[e]
$FeSO_4$ (100 μM) + H_2O_2 (0.1%)				97.1 ± 7.5	
NADPH + $CuCl_2$	ND[f]	ND	ND	11.2 ± 0.7	65.80
NPQ	ND	ND	ND	11.0 ± 0.8	
NPQ + NADPH	27.1 ± 3.0	44.4 ± 5.1	53.2 ± 0.6	26.1 ± 1.7	
NPQ + NADPH + $CuCl_2$	98.4 ± 3.1	18.9 ± 2.5	37.3 ± 0.5	84.3 ± 6.1	74.80
+ SOD (210 units/mL)			37.5 ± 0.5	77.0 ± 7.4	
+ Tiron (10 mM)			22.1 ± 0.4	36.6 ± 0.9	
+ catalase (880 units/mL)			ND	26.0 ± 0.3	
+ mannitol (5 mM)				20.4 ± 0.7	
+ Na-benzoate (5 mM)				21.0 ± 17.0	
+ formic acid (5 mM)				17.0 ± 36.2	
+ bathocuproine (200 μM)			21.1 ± 0.3	25.3 ± 0.9	
BPQ	ND	ND	ND	4.4 ± 0.2	
BPQ + NADPH	2.6 ± 0.1	11.7 ± 0.5	51.7 ± 0.4	23.2 ± 2.4	
BPQ + NADPH + $CuCl_2$	0.5 ± 0.1	6.1 ± 0.5	31.8 ± 0.5	50.7 ± 3.6	94.20
+ SOD (210 units/mL)			31.5 ± 0.5	41.1 ± 0.7	
+ Tiron (10 mM)			24.0 ± 0.8	34.9 ± 0.3	
+ catalase (880 units/mL)			ND	21.9 ± 0.7	
+ mannitol (5 mM)				13.4 ± 0.7	
+ Na-benzoate (5 mM)				10.4 ± 0.7	
+ formic acid (5 mM)				9.8 ± 0.2	
+ bathocuproine (200 μM)			21.1 ± 0.6	18.3 ± 0.8	

[a] The formation of reactive species was determined in reaction mixtures containing NPQ (10 μM) or BPQ (10 μM), NADPH (1 mM), and $CuCl_2$ (10 μM) in the absence and presence of free radical scavenging agents and metal chelators according to Materials and Methods unless otherwise indicated. All reactions were performed in triplicate. [b] The rate of $O_2^{\bullet-}$ formation was determined by the rate of reduction of cyt *c* that was inhibited by SOD. The rate of SQ radical formation was taken as the rate of reduction of cyt *c* that was not inhibited by SOD. [c] The amount of H_2O_2 formed was determined by the formation of ferrithiocyanate as described in Materials and Methods. [d] The amount of $OH^{\bullet}$ formed was determined in the presence of 2′-deoxy-D-ribose (2 mM) as described in Materials and Methods. [e] The amount of Cu(I) formed was determined as the Cu(I)−bathocuproine complex in mixtures containing either NPQ (100 μM) or BPQ (20 μM) in EtOH (100%), 100 μM NADPH, 100 μM $CuCl_2$, and 100 μM bathocuproine. [f] ND = not detectable.

presence of cosolvent (Me_2SO) only (Figures 3a and 4a). The addition of NADPH under anaerobic conditions significantly enhanced the SQ radical signal (Figures 3b and 4b). The top panels of Figures 3 and 4 indicate simulated EPR spectra for the SQ radicals of NPQ and BPQ, respectively. The spectrum of the SQ radical of NPQ showed the presence of a 12-line hyperfine splitting pattern with apparent splitting constants of $a = 4.55$, 1.4, 1.4, and 0.7 G. The spectrum indicated that the unpaired electron was, for the most part, delocalized around both rings of the *o*-seminaphthoquinone radical. An EPR spectrum for the SQ radical of NPQ obtained under aprotic conditions has been published previously and differs from that shown here only in the degree of hyperfine splitting (Pederson, 1985). In contrast, the spectrum for the SQ radical of BPQ was characterized by a 4-line splitting pattern with splitting constants of $a = 4.4$ and 1.1 G. This implies that either the unpaired electron was not delocalized over the entire pyrene ring system or the broad 1:1:1:1 splitting pattern represented an envelope of unresolved hyperfine splitting that could indicate delocalization over the entire ring system. It should be noted that, in the presence of EDTA and air, NPQ had a radical signal characterized by $g = 2.004$. No evidence of this signal existed under conditions in which the corresponding SQ radical was generated.

Third, both NPQ and BPQ produced large amounts of H_2O_2 during redox cycling catalyzed by NADPH (Table 1). The addition of $CuCl_2$ decreased the detection of H_2O_2. The production of H_2O_2 was blocked by catalase. In contrast, Tiron (a chemical scavenger for $O_2^{\bullet-}$) only attenuated the production of H_2O_2, whereas SOD had no effect. The inclusion of bathocuproine, a Cu(I) chelator, caused a decrease in the final concentration of H_2O_2 indicating that Cu(I) was involved in its removal.

Fourth, incubations containing NPQ or BPQ in the presence of NADPH produced significant amounts of $OH^{\bullet}$ (Table 1). The addition of $CuCl_2$ significantly increased the production of $OH^{\bullet}$ to a level that was comparable to that produced by the Fenton reaction. The production of $OH^{\bullet}$ was significantly decreased by the addition of Tiron or catalase, whereas SOD was without effect. Hydroxyl radical scavengers (mannitol, sodium benzoate, and formic acid) significantly inhibited the detection of $OH^{\bullet}$ but did not completely abolish its formation. The inclusion of bathocuproine decreased the formation of $OH^{\bullet}$, indicating a dependency on Cu(I), consistent with a Cu(I)-mediated Fenton reaction.

Fifth, formation of Cu(I) during the NADPH/$CuCl_2$-catalyzed redox cycling of NPQ and BPQ was verified by complex formation of Cu(I) with bathocuproine (Table 1). Significant amounts of this complex were formed when NPQ or BPQ was incubated with NADPH and $CuCl_2$. However, a major portion of the Cu(I)−bathocuproine complex arose from the reduction of $CuCl_2$ by NADPH in the absence of *o*-quinones. This reaction, which has been described previously for NADH, was an indicator for $NAD^{\bullet}$ formation and resulted in DNA strand scission (Oikawa & Kawanishi, 1996). Here, the formation of Cu(I) during the reaction of $CuCl_2$ with NADPH was presumed to be accompanied by $NADP^{\bullet}$ formation, but no strand scission was observed unless an *o*-quinone was included. These data provided evidence for the formation of $O_2^{\bullet-}$, SQ radical, H_2O_2, $OH^{\bullet}$, and Cu(I) as reactive species that may play a role in PAH *o*-quinone-mediated DNA strand scission.

Role of Reactive Species in DNA Strand Scission Caused by PAH o-Quinones. To identify which of the reactive species were responsible for *o*-quinone-mediated strand scission, attempts were made to block DNA fragmentation

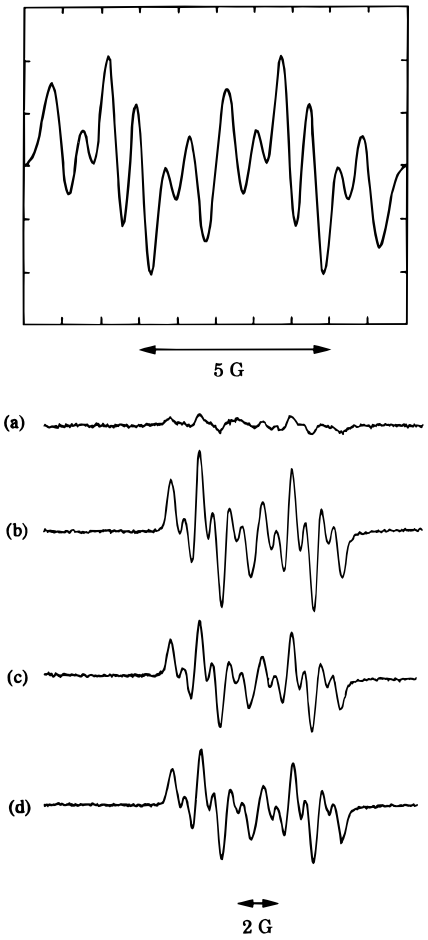


FIGURE 3: EPR spectra of the *o*-semiquinone anion radical (SQ) generated from NPQ in the absence and presence of DNA. The top panel is a simulated spectrum of the SQ radical of NPQ (0.78 mM) generated in the presence of NADPH (10 mM) under anaerobic conditions. Spectra of the SQ radical of NPQ generated in the presence of cosolvent [20% (v/v) Me_2SO] (curve a); in the presence of NADPH under anaerobic conditions (curve b); in the presence of NADPH and poly(dG)·poly(dC) DNA (200 μg/mL) under anaerobic conditions (curve c); in the presence of NADPH and ϕX174 DNA (20 μg/mL) under anaerobic conditions (curve d). SQ radicals were generated prior to the addition of DNA. EPR conditions were described in Materials and Methods.

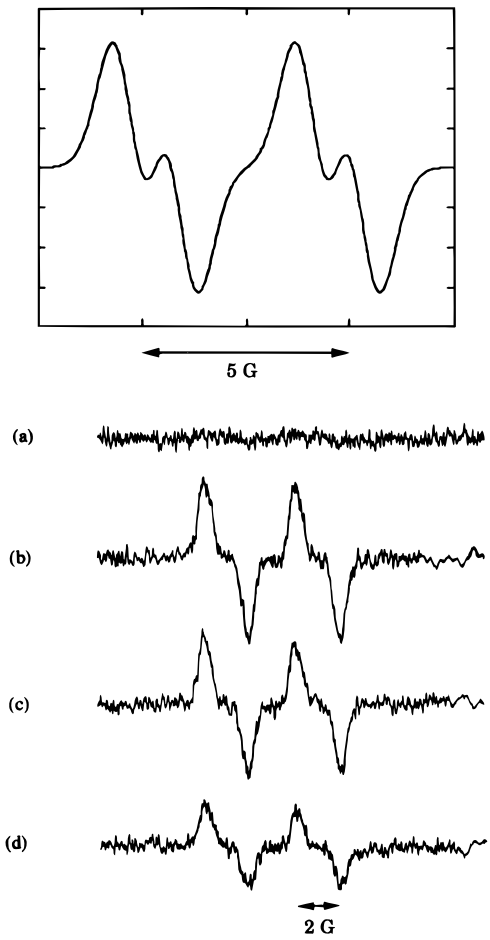


FIGURE 4: EPR spectra of the *o*-semiquinone anion radical (SQ) generated from BPQ in the absence and presence of DNA. The top panel is a simulated spectrum of the SQ radical of BPQ (50 μM) generated in the presence of NADPH (10 mM) under anaerobic conditions. Spectra of the SQ radical of BPQ generated in the presence of cosolvent [50% (v/v) Me_2SO] (curve a); in the presence of NADPH under anaerobic conditions (curve b); in the presence of NADPH and poly(dG)·poly(dC) DNA (200 μg/mL) under anaerobic conditions (curve c); in the presence of NADPH and ϕX174 DNA (20 μg/mL) under anaerobic conditions (curve d). SQ radicals were generated prior to the addition of DNA. EPR conditions were described in Materials and Methods.

with free radical scavenging agents and transition metal chelators (Table 2). Catalase and OH• scavengers were able to block the strand scission observed with *o*-quinone treatment. By contrast, SOD provided no protection. Tiron was able to prevent the DNA strand scission. Metal chelators (bathocuproine and neocuproine) also protected the DNA from fragmentation. A striking correlation existed between the ability of free radical scavenging agents and metal chelators to reduce OH• production (Table 1) and their ability to attenuate *o*-quinone-mediated DNA strand scission (Table 2). It should be noted that none of the free radical scavenging agents and metal chelators used completely ameliorated strand scission.

Role of SQ Radicals in DNA Strand Scission. To access the contribution of SQ radicals to strand scission, the SQ radicals of NPQ or BPQ were incubated with either ϕX174 DNA or poly(dG)·poly(dC) and the effects on EPR amplitude and strand scission were measured. It was found that the signal of the SQ radical of NPQ was quenched by both ϕX174 DNA and poly(dG)·poly(dC) (Figure 3d and c) without significant strand scission (Figure 1C). In systems containing BPQ, the EPR signal of the SQ radical was quenched only by ϕX174 DNA (Figure 4d and c), and this was accompanied by single nicks in the DNA (Figure 1C). When the NPQ-derived radical with the characteristic $g = 2.004$ signal was incubated with either phage DNA or poly(dG)·poly(dC) (Figures 1C and 2C) no strand scission was observed. Thus, SQ radicals produced during the redox cycling of PAH *o*-quinones are not directly involved in DNA strand scission.

Role of OH• in DNA Degradation Caused by PAH o-Quinones. When calf thymus DNA was treated with PAH *o*-quinones under redox-cycling conditions, MDA was released by acid hydrolysis. Detection of MDA was taken as evidence for the presence of base propenals that result from OH• attack on the 2′-deoxyribose moiety in DNA (Cheeseman, 1988; Giloni et al., 1981; Gutteridge, 1987). In incubations containing either NPQ or BPQ in the presence of NADPH, $CuCl_2$, and calf thymus DNA, there was significant production of MDA (Table 3), which was reduced by the addition of SOD, Tiron, and catalase. When OH• scavengers were included in the incubation medium, MDA

Table 2: Effects of Free Radical Scavenging Agents and Transition Metal-Chelators on PAH *o*-Quinone-Induced Strand Scission of ϕX174 DNA[a]

conditions	form of DNA[b] SC	OC	LIN	complete scission
CS[c] [NPQ]				100[d]
CS + SOD (210 units/mL)				100
CS + Tiron (10 mM)		28 ± 4	42 ± 3	
CS + catalase (880 U/mL)		25 ± 4	42 ± 5	
CS + mannitol (5 mM)			79 ± 8	
CS + Na-benzoate (5 mM)		9 ± 2	69 ± 3	
CS + formic acid (5 mM)			79 ± 7	
CS + bathocuproine (200 μM)			51 ± 4	
CS + neocuproine (200 μM)			50 ± 4	
CS [BPQ]				
CS + SOD (210 units/mL)				100
CS + Tiron (10 mM)	14 ± 2	69 ± 5		
CS + catalase (880 units/mL)	29 ± 2	53 ± 4		
CS + mannitol (5 mM)	28 ± 3	55 ± 5		
CS + Na-benzoate (5 mM)	27 ± 3	53 ± 5		
CS + formic acid (5 mM)	27 ± 3	67 ± 5		
CS + bathocuproine (200 μM)			51 ± 4	
CS + neocuproine (200 μM)			53 ± 4	

[a] Strand scission of 0.2 μg SC ϕX174 DNA was measured following treatment with NPQ (1 μM) or BPQ (10 μM), NADPH (1 mM), and $CuCl_2$ (10 μM) in the absence and presence of scavengers. [b] Strand scission was monitored by separating SC from OC and LIN forms by agarose gel electrophoresis and was quantitated by densitometry. [c] Complete systems (CS) contained the *o*-quinone, NADPH (1 mM), and $CuCl_2$ (10 μM). [d] Values are expressed as a percent of untreated DNA. The ϕX174 DNA used in these studies was >83% SC DNA with OC DNA as the contaminant.

Table 3: Degradation of Calf Thymus DNA by PAH *o*-Quinones in the Presence of NADPH and Cu(II)[a]

conditions	malondialdehyde (nmol/mL)	% inhibition
NPQ + NADPH + $CuCl_2$	78.8 ± 5.7	
+ SOD (210 units/mL)	74.8 ± 5.7	5.1
+ Tiron (10 mM)	42.4 ± 2.2	46.2
+ catalase (880 units/mL)	34.4 ± 2.2	56.3
+ mannitol (5 mM)	18.9 ± 0.5	76.1
+ Na-benzoate (5 mM)	21.0 ± 0.5	73.3
+ formic acid (5 mM)	16.8 ± 0.5	78.7
+ bathocuproine (200 μM)	25.4 ± 0.5	67.7
BPQ + NADPH + $CuCl_2$	53.5 ± 2.7	
+ SOD (210 units/mL)	39.6 ± 2.1	25.9
+ Tiron (10 mM)	33.8 ± 2.0	36.8
+ catalase (880 units/mL)	22.3 ± 0.5	58.3
+ mannitol (5 mM)	15.4 ± 0.5	71.2
+ Na-benzoate (5 mM)	14.0 ± 0.5	73.8
+ formic acid (5 mM)	14.4 ± 0.5	73.1
+ bathocuproine (200 μM)	15.4 ± 0.6	71.2

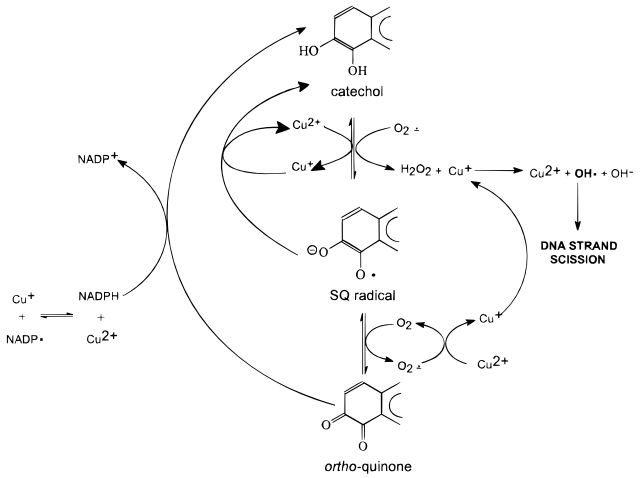
[a] The degradation of calf thymus DNA (1 mg/mL) was determined in reaction mixtures containing NPQ (10 μM) or BPQ (10 μM), NADPH (1 mM), and $CuCl_2$ (10 μM) in the absence and presence of free radical scavenging agents and a metal chelator as described in Materials and Methods.

levels were again decreased suggesting that these scavengers protected the DNA (70−80%) from degradation. Bathocuproine prevented MDA formation, indicating that Cu(I) plays an important role in the degradation of the calf thymus DNA presumably by the formation of $OH^{\bullet}$.

DISCUSSION

We have previously shown that in normal rat hepatocytes BP-diol was oxidized by DD to form BPQ (Flowers-Geary et al., 1995) and that this event was accompanied by $O_2^{\bullet-}$ production and DNA fragmentation (Flowers-Geary et al., 1996a). In model studies, BPQ was found to be 200-fold more potent as a nuclease than *anti*-BPDE, a suspect human carcinogen (Flowers-Geary et al., 1996a), yet both were produced in equal amounts in rat hepatocytes (Flowers-Geary et al., 1995). In this study, the mechanism of PAH *o*-quinone-induced DNA strand scission was elucidated using either ϕX174 DNA or poly(dG)·poly(dC) as the target DNA. NADPH was utilized to promote redox cycling of the *o*-quinones. Cu(II) was included since it is present in chromosomes (Bryan et al., 1981) and is closely associated with DNA bases, particularly guanine (Geierstanger et al., 1991). It is presumed that endogenous copper bound to DNA was a requirement for the BPQ-induced strand scission in isolated hepatocytes.

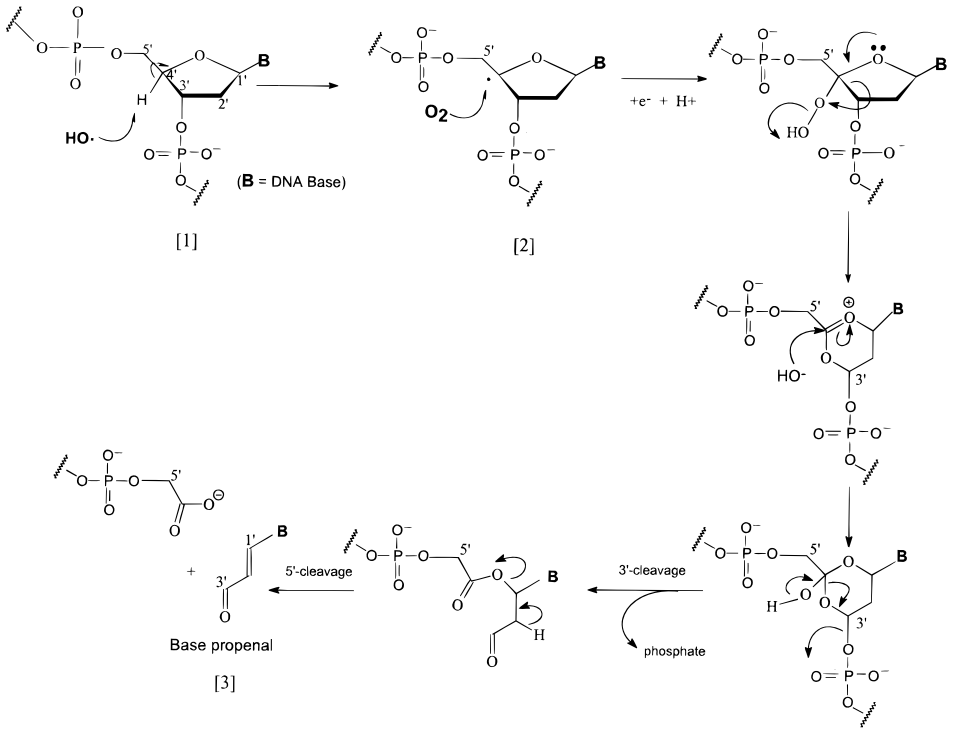
Scheme 3: Redox Cycling of PAH *o*-Quinones in the Presence of NADPH and Cupric Chloride and the Formation of Reactive Species



We found that PAH *o*-quinones in the presence of NADPH and Cu(II) induced extensive fragmentation of both phage DNA and ds-oligonucleotide DNA. DNA strand breaks were dependent on the presence of both NADPH and Cu(II). Other metals, including Cu(I), Fe(II), and Fe(III) would not suffice. A Cu(II)/Cu(I) redox cycle was critical to PAH *o*-quinone-induced DNA damage. Indicative of such a redox mechanism was the significant inhibitory effect of bathocuproine and neocuproine on the observed strand scission. These compounds are primarily Cu(I)-specific chelators that can strongly bind Cu(I) forming a stable complex, thereby removing Cu(I) from the reaction mixture. Although the Cu(I) chelator, 1,10-phenanthroline, forms a complex that is redox active which can induce DNA strand breaks (Byrnes et al., 1992), the Cu(I) chelators used here contain bulky substituents which may prevent redox cycling of Cu(I) due to steric hindrance (Sigman, 1986).

The reaction of PAH *o*-quinones with NADPH and Cu(II) led to the production of ROS, including $O_2^{\bullet-}$, H_2O_2, and $OH^{\bullet}$ (Scheme 3). NADPH promoted redox-cycling of the *o*-quinones by providing the reducing equivalents to form the catechol which autoxidized to yield the SQ radical and then the *o*-quinone. During this process, H_2O_2 and $O_2^{\bullet-}$ were produced. Cu(II) may be reduced by $O_2^{\bullet-}$ to form Cu(I) [$Cu(II) + O_2^{\bullet-} \rightarrow Cu(I) + O_2$]. The formation of both Cu(I) and H_2O_2 leads to a Fenton-type reaction in which H_2O_2 is consumed to yield $OH^{\bullet}$ [$Cu(I) + H_2O_2 \rightarrow Cu(II) + OH^-$

Scheme 4: Proposed Reaction Mechanism of DNA Strand Scission by Hydroxyl Radical Produced during the Reaction of PAH *o*-Quinones, NADPH, and Cupric Chloride[a]



[a] Only hydroxyl attack at C-4′ is indicated. Although other mechanisms of strand scission are possible, aerobic DNA cleavage via a C-4′ Criegee rearrangement is preferred (Breen & Murphy, 1995; Giloni et al., 1981).

+ OH•]. This sequence explains why Cu(II) decreases the detection of H_2O_2. It also predicts that Cu(I) should act as a substitute for Cu(II) to produce OH• provided H_2O_2 is present. Although H_2O_2 is produced during the redox cycling of the *o*-quinones, Cu(I) was unable to mediate strand scission. This suggested an additional function for Cu(II), namely the establishment of a Cu(II)/Cu(I) redox cycle that accelerates the conversion of catechols to SQ radicals. This would lead to an increase in ROS, notably OH• formation, and this is observed in systems containing PAH *o*-quinones, NADPH and $CuCl_2$ (Table 1). A similar role has been proposed for Cu(II) in strand scission mediated by hydroquinone and the catechol estrogens (Li & Trush, 1993; Li et al., 1994). In our studies, Tiron attenuated the production of H_2O_2 but SOD did not. The Tiron result may be due to its competing properties to act as a trap for $O_2^{\bullet-}$ and metal-ion chelator (Greenstock & Miller, 1975). The SOD result was expected since it catalyzes the dismutation of $O_2^{\bullet-}$ to form H_2O_2. In these studies, both OH• formation and strand scission of phage DNA were significantly decreased by the inclusion of catalase, OH• scavengers, and Cu(I) chelators, indicating a major role for the Cu(I)-catalyzed Fenton reaction in DNA cleavage.

Studies on the degradation of calf thymus DNA by PAH *o*-quinones in the presence of NADPH and $CuCl_2$ pointed to the mechanism of DNA strand scission by OH• radical. The release of MDA by acid hydrolysis was taken as evidence for the formation of base propenals. In the proposed sequence (Scheme 4), strand scission is initiated by abstraction of the C-4′ proton of 2′-deoxyribose by OH• [1]. A peroxyl radical is formed at this position by the addition of molecular oxygen [2] followed by oxidative cleavage of the C-3′−C-4′ bond yielding a base propenal [3] (Breen & Murphy, 1995; Giloni et al., 1981). Acid hydrolysis of the base propenal yields MDA (Giloni et al., 1981). It is presumed that the mechanism of degradation of calf thymus DNA by PAH *o*-quinones parallels that for phage DNA strand scission. A similar mechanism has been proposed for the DNA strand scission and chromosomal aberrations induced by bleomycin (Giloni et al., 1981; Povirk & Austin, 1991).

Although OH• scavengers decreased both strand scission of phage DNA and the formation of MDA from calf thymus DNA, these effects were not completely ameliorated. Hydroxyl radical production and subsequent DNA damage may be site specific due to the binding affinity of metal ions to select DNA sequences (Gutteridge, 1987; Samuni et al., 1983). Therefore, OH• scavengers must compete with metal−DNA complexes for OH• making complete scavenging of the radical virtually impossible. Strand scission not inhibited by OH• scavengers may also occur via a Cu(I)−peroxide [e.g., Cu(I)-OOH] complex (Masarwa et al., 1988; Oikawa & Kawanishi, 1996; Yamamoto & Kawanishi, 1989), which would react with DNA but would not be trapped by OH• scavengers. However, peroxy and alkoxyl radicals formed from iron chelates and *tert*-butyl hydroperoxide do not release MDA from 2′-deoxy-D-ribose (Gutteridge, 1987). Since MDA was detected in these studies, it was concluded that *o*-quinone-induced strand scission in the presence of NADPH and Cu(II) was due to OH• production and not Cu(I)-peroxide.

Semiquinone anion radicals of several quinone-containing anticancer drugs can covalently modify DNA, RNA, and protein with deleterious effects (Lusthoff et al., 1990; Sinha & Chignell, 1979). The SQ radical signals of NPQ and BPQ were quenched by DNA with little influence on strand scission, indicating that they are not directly involved in DNA strand breaks. The nature of the SQ radical−DNA adducts

is unknown and their formation may be influenced by aerobic conditions, which would lead to the regeneration of the SQ radical by redox cycling.

We have shown that strand scission is a measurable index of oxidative DNA damage caused by PAH *o*-quinones. The extensive fragmentation of phage DNA represents a toxicological endpoint that may never be reached *in vivo*. A milder insult may be the formation of oxidatively damaged bases, e.g., 8-oxo-2′-deoxyguanosine. Du et al. (1994) have shown that low concentrations of ROS do not cause strand scission of plasmids containing the H-*ras* proto-oncogene, but rather cause oncogene activation through G to T transversions. Thus, DNA damage resulting from exposure to PAH *o*-quinones *in vivo* may lead to the mutagenic events associated with cellular transformation.

In summary, our data provide evidence for the formation of $O_2^{\bullet -}$, SQ radical, H_2O_2, $OH^{\bullet}$, and Cu(I) as reactive species that may play a role in PAH *o*-quinone-induced DNA strand scission. The reactive species responsible for the degradation of DNA was found to be $OH^{\bullet}$, generated by the Cu(I)-catalyzed Fenton reaction with H_2O_2. These studies suggest that the PAH *o*-quinones produced by DD are genotoxic species and may play a role in PAH-induced carcinogenesis and mutagenesis.

REFERENCES

Azzi, A., Montecucco, C., & Richter, C. (1975) *Biochem. Biophys. Res. Commun. 65*, 597−603.

Breen, A. P., & Murphy, J. A. (1995) *Free Rad. Biol. Med. 18*, 1033−1077.

Bryan, S. E., Vizard, D. L., Beary, D. A., LaBiche, R. A., & Hardy, K. J. (1981) *Nucleic Acids Res. 9*, 5811−5823.

Byrnes, R. W., Antholine, W. E., & Petering, D. H. (1992) *Free Rad. Biol. Med. 12*, 457−469.

Cavalieri, E. L., & Rogan, E. C. (1992) *Pharmac. Ther. 55*, 183−199.

Cheeseman, K. H., Beavis, A., & Esterbauer, H. (1988) *Biochem. J. 252*, 649−653.

Colapietro, A.-M., Goodell, A. L., & Smart, R. C. (1993) *Carcinogenesis 14*, 2289−2295.

DeMarini, D. M., Brockman, H. E., deSerres, F. J., Evans, H. H., Stankowski, L. F., Jr., & Hsie, A. W. (1989) *Mutat. Res. 220*, 11−29.

Denissenko, M. F., Pao, A., Tang, M.-S., & Pfeifer, G. P. (1996) *Science 274*, 430−432.

Dipple, A. (1985) in *Polycyclic Hydrocarbons and Carcinogenesis* (Harvey, R. G., Ed.) pp 1−17, ACS Symposium Series 283, American Chemical Society, Washington, DC.

Du, M.-Q., Carmichael, P. L., & Phillips, D. H. (1994) *Mol. Carcinog. 11*, 170−175.

Emerit, I., Keck, M., Levy, A., Feingold, J., & Michelson, A. M. (1982) *Mutation Res. 103*, 165−172.

Fieser, L. F. (1943) *Org. Synth. 2*, 430−435.

Flowers-Geary, L., Harvey, R. G., & Penning, T. M. (1992) *Biochem. (Life Sci. Adv.) 11*, 49−58.

Flowers-Geary, L., Harvey, R. G., & Penning, T. M. (1993) *Chem. Res. Toxicol. 6*, 252−260

Flowers-Geary, L., Harvey, R. G., & Penning, T. M. (1995) *Carcinogenesis 16*, 2707−2715.

Flowers, L., Bleczinski, W. F., Burczynski, M. E., Harvey, R. G., & Penning, T. M. (1996a) *Biochemistry 35*, 13664−13672.

Flowers-Geary, L., Bleczinski, W., Harvey, R. G., & Penning, T. M. (1996b) *Chem. Biol. Interact. 99*, 55−72.

Geierstanger, B. H., Kagawa, T. F., Chen, S.-L., Quigley, G. J., & Ho, P. S. (1991) *J. Biol. Chem. 266*, 20185−20191.

Giloni, L., Takeshita, M., Johnson, F., Iden, C., & Grollman, A. P. (1981) *J. Biol. Chem. 256*, 8608−8615.

Greenstock, C. L., & Miller, R. W. (1975) *Biochim. Biophys. Acta 396*, 11−16.

Grollman, A. P., & Moriya, M. (1993) *Trends Genet. 9*, 246−249.

Gutteridge, J. M. C. (1987) *Biochem. J. 243*, 709−714.

Hara, A., Taniguchi, H., Nakayama, T., & Sawada, H. (1990) *J. Biochem. 108*, 250−254.

Hara, A., Matsuura, K., Tamada, Y., Sato, K., Miyabe, Y., Deyashiki, Y., & Ishida, M. (1996) *Biochem. J. 313*, 373−376.

Jeffrey, A. M., Jennette, K. W., Blobstein, S. H., Weinstein, I. B., Beland, F. A., Harvey, R. G., Kasai, H., Miura, I., & Nakanishi, K. (1976) *J. Amer. Chem. Soc. 98*, 5714−5715.

Li, Y., & Trush, M. A. (1993) *Arch. Biochem. Biophys. 300*, 346−355.

Li, Y., Trush, M. A., & Yager, J. D. (1994) *Carcinogenesis 15*, 1421−1427.

Lusthof, K. J., De Mol, N. J., Janssen, L. H. M., Egberink, R. J. M., Verboom, W., & Reinhoudt, D. N. (1990) *Chem. Biol. Interact. 76*, 193−209.

Masarwa, M., Cohen, H., Meyerstein, D., Hickman, D. L., Bakac, A., & Espenson, J. H. (1988) *J. Am. Chem. Soc. 110*, 4293−4297.

Mass, M. J., Jeffers, A. J., Ross, J. A., Nelson, G., Galati, A. J., Stoner, G. D., & Nesnow, S. (1993) *Mol. Carcinog. 8*, 186−192.

Nesbar, B. (1964) *Anal. Chem. 36*, 1961−1965.

Oikawa, S., & Kawanishi, S. (1996) *Biochemistry 35*, 4584−4590.

Pederson, J. A., Ed. (1985) *CRC Handbook of EPR Spectra from Quinones and Quinols*, p 39, CRC Press, Inc., Boca Raton, FL.

Penning, T. M., Ohnishi, S. T., Ohnishi, T., & Harvey, R. G. (1996) *Chem. Res. Toxicol. 9*, 84−92.

Povirk, L. F., & Austin, M. J. F. (1991) *Mutat. Res. 257*, 127−143.

Puisieux, A., Lim, S., Groopman, J., & Ozturk, M. (1991) *Cancer Res. 51*, 6185−6189.

Samuni, A., Aronovitch, J., Godinger, D., Chevion, M., Czapski, G. (1983) *Eur. J. Biochem. 137*, 119−124.

Shou, M., Harvey, R. G., & Penning, T. M. (1993) *Carcinogenesis 14*, 475−482.

Sigman, D. S. (1986) *Acc. Chem. Res. 19*, 180−183.

Sinha, B. K., & Chignell, C. F. (1979) *Chem. Biol. Interact. 28*, 301−308.

Smithgall, T. E., Harvey, R. G., & Penning, T. M. (1986) *J. Biol. Chem. 261*, 6184−6191.

Smithgall, T. E., Harvey, R. G., & Penning, T. M. (1988) *J. Biol. Chem. 263*, 1814−1820.

Sukumaran, K. B., & Harvey, R. G. (1980) *J. Org. Chem. 45*, 4407−4413.

Thurman, R. G., Ley, H. G., & Scholz, R. (1972) *Eur. J. Biochem. 25*, 420−430.

Yamamoto, K., & Kawanishi, S. (1989) *J. Biol. Chem. 264*, 15435−15440.

BI970367P