Disposition and Biological Activity of Benzo[*a*]pyrene-7,8-dione. A Genotoxic Metabolite Generated by Dihydrodiol Dehydrogenase[†,‡]

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ABSTRACT: A novel pathway of polycyclic aromatic hydrocarbon metabolism involves the oxidation of non-K-region *trans*-dihydrodiols to yield *o*-quinones, a reaction catalyzed by dihydrodiol dehydrogenase (DD). We have recently shown that in isolated rat hepatocytes (±)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene (BP-diol) was oxidized by this route to yield benzo[*a*]pyrene-7,8-dione (BPQ). We now report the disposition of BPQ and its mutagenic and genotoxic properties. Using [^{3}H]BPQ it was found that 30% of the radioactivity was sequestered by rat hepatocytes into the cell pellet. Isolation of hepatocyte DNA provided evidence for a low level of covalent incorporation of BPQ into DNA (30 ± 17 adducts/10^6 base pairs). Examination of the hepatocellular DNA by agarose gel electrophoresis following treatment with BPQ indicated that extensive fragmentation had occurred. DNA fragmentation was also observed when hepatocytes were treated with BP-diol and this effect was attenuated by indomethacin, a DD inhibitor. Hepatocytes treated with either BP-diol or BPQ were found to produce large quantities of superoxide anion radical ($O_2^{\bullet-}$). The amount of $O_2^{\bullet-}$ generated by BP-diol was blocked by DD inhibitors. These data suggest that by diverting BP-diol to BPQ reactive oxygen species (ROS) were generated which caused DNA fragmentation. The ability of BPQ to cause DNA strand scission was further studied using supercoiled ϕX174 DNA. It was found that BPQ caused concentration-dependent (0.05−10 μM) strand scission in the presence of 1 mM NADPH (which promoted redox-cycling) provided $CuCl_2$ (10 μM) was present. Complete destruction of the DNA was observed using 10 μM BPQ. This strand scission was prevented by catalase and hydroxyl radical scavengers but not by superoxide dismutase. These data indicate that ROS were responsible for the destruction of the DNA. Using 20 μM (±)-*anti*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [(±)-*anti*-BPDE] only single nicks in the DNA were observed indicating that BPQ was the more potent chemical nuclease. BPQ was also found to be a direct-acting mutagen in the Ames test using *Salmonella typhimurium* tester strains TA97a, TA98, TA100, TA102, and TA104, but was 10−5500-fold less efficient as a mutagen than (±)-*anti*-BPDE. Our data indicate that DD suppresses the mutagenicity of (±)-*anti*-BPDE by producing BPQ, but in doing so a potent chemical nuclease is produced which causes extensive DNA fragmentation via the generation of ROS.

Polycyclic aromatic hydrocarbons (PAH)[1] are ubiquitous environmental pollutants and potential human carcinogens. They require activation by host metabolism to exert their mutagenic and carcinogenic effects (Dipple, 1985). Benzo[*a*]pyrene (BP) is a representative PAH and several routes of activation have been documented. One route proceeds through the intermediate non-K-region *trans*-dihydrodiol [(−)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene] to yield predominantly the (+)-*anti*-diol epoxide [(+)-*anti*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; (+)-*anti*-BPDE] which alkylates DNA (Jeffrey et al., 1976) and is one of the most mutagenic, tumorigenic, and carcinogenic PAH metabolites known (Dipple, 1985; Newbold & Brookes, 1976). Similar pathways of activation exist for other PAH (Figure 1).

Dihydrodiol dehydrogenase (DD) represents one enzyme that can catalyze an alternative route of *trans*-dihydrodiol (proximate carcinogen) metabolism. Homogeneous rat liver DD has been shown to oxidize a variety of non-K-region *trans*-dihydrodiols, including those derived from BP, chrysene, 5-methylchrysene, benz[*a*]anthracene, and 7,12-dimethylbenz[*a*]anthracene (Smithgall et al., 1986, 1988a). The enzymatic oxidation of *trans*-dihydrodiols resulted in the

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[1] Abbreviations and trivial names: BP, benzo[*a*]pyrene; (±)-*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; DD, dihydrodiol dehydrogenase (*trans*-1,2-dihydrobenzene-1,2-diol dehydrogenase; EC 1.3.1.20); dG, deoxyguanosine; indomethacin, 1-[*p*-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid; LIN, linear DNA; 6MPA, 6-medroxyprogesterone acetate (6α-methyl-17α-hydroxyprogesterone acetate); OC, open circular DNA; $O_2^{\bullet-}$, superoxide anion radical; PAH, polycyclic aromatic hydrocarbon; ROS, reactive oxygen species; RP-HPLC, reverse-phase high-performance liquid chromatography; SC, supercoiled DNA; SOD, superoxide dismutase; Tiron, 4,5-dihydroxy-1,3-benzenedisulfonic acid.

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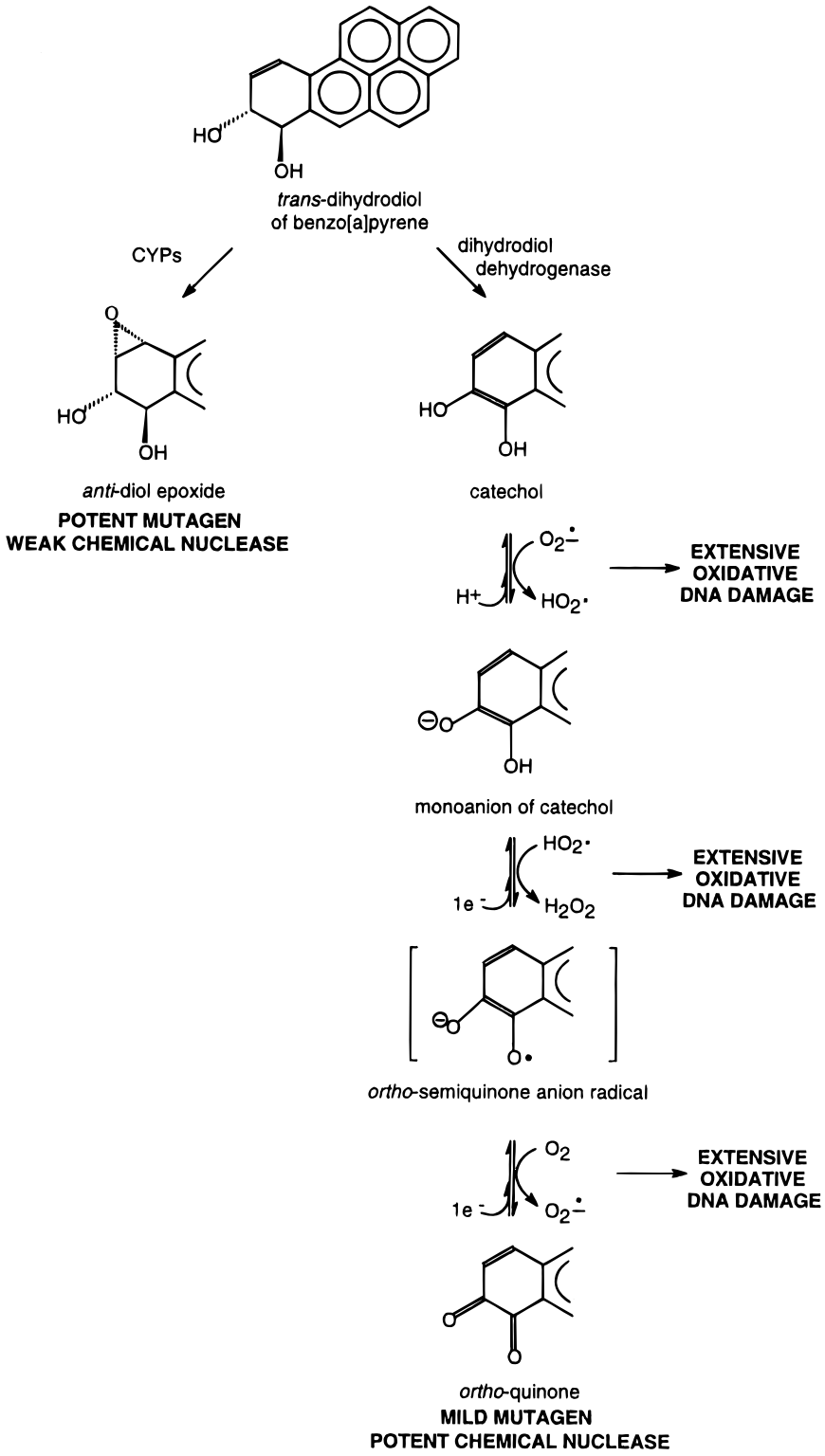

FIGURE 1: Two pathways for the activation of *trans*-dihydrodiols of PAH.

formation of the corresponding PAH *o*-quinones (Smithgall et al., 1988b). Using fortified rat liver subcellular fractions or isolated rat hepatocytes we have shown that (±)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene [BP-diol] was metabolized to benzo[*a*]pyrene-7,8-dione [BPQ] and that this transformation was blocked by DD inhibitors (Shou et al., 1992; Flowers-Geary et al., 1995a). Our studies in isolated rat hepatocytes indicated that BP-diol was converted to either BPQ or diol epoxides to an equal extent implying that DD and CYPs contributed equally to the metabolism of BP-diol (Flowers-Geary et al., 1995a). Although the formation of *o*-quinones may reduce the accumulation of the *anti*-diol epoxides, quinones, in general, are considered to be highly reactive and may represent products of an alternative pathway of PAH activation.

PAH *o*-quinones are formed by DD *via* the autoxidation of intermediate catechols, a process which generates *o*-semiquinone anion radicals and reactive oxygen species [ROS, superoxide anion radical, hydrogen peroxide, and hydroxyl radical] (Penning et al., 1996). Once formed the

PAH *o*-quinones can act as Michael acceptors and undergo addition reactions with cellular nucleophiles. Mercapturic acid-, glutathionyl-, and deoxyguanosine-BPQ adducts have all been characterized (Murty & Penning, 1992; Shou et al., 1993). Apart from the capacity to form covalent adducts, PAH *o*-quinones also undergo successive one-electron enzymatic reductions to form *o*-semiquinone anion radicals and catechols. Subsequent air oxidation regenerates the *o*-quinone. By entering these futile one-electron redox-cycles, *o*-semiquinone anion radicals and ROS are produced multiple times (Flowers-Geary et al., 1992a, 1993). This mechanism of free radical amplification may endow additional cytotoxic and genotoxic properties to the PAH *o*-quinones.

In this study the disposition and genotoxic properties of BPQ were examined in isolated rat hepatocytes. Although BPQ formed covalent DNA adducts, its most pronounced effect was to cause extensive fragmentation of hepatocellular DNA *via* the generation of ROS. BPQ was also shown to be a mild mutagen in the Ames test, but its mutagenicity was dwarfed by that observed with (±)-*anti*-BPDE. The ability of DD to reduce the accumulation of the mutagenic *anti*-diol epoxide and at the same time produce a potent chemical nuclease that causes pronounced strand scission of DNA is discussed.

MATERIALS AND METHODS

Materials. Leibovitz's L-15 media was obtained from Gibco BRL Life Technologies, Inc. (Gaithersburg, MD). Gentamycin was purchased from JRH Biosciences (Lenexa, KS). Glucose, indomethacin, 6-medroxyprogesterone acetate, glucose-6-phosphate dehydrogenase (type IX from Baker's yeast), superoxide dismutase (SOD; type I from bovine erythrocytes), catalase (from bovine liver), cytochrome *c* (type VI from horse heart), nuclease P1 (from *Penicillium citrinum*), alkaline phosphatase (type III from *Escherichia coli*), sodium benzoate, formic acid, mannitol, Tiron, ferrous sulfate, hydrogen peroxide, and *tert*-butyl hydroperoxide were obtained from Sigma Chemical Co. (St. Louis, MO). Cupric chloride, 4-nitro-*o*-phenylenediamine, sodium azide, and cumene hydroperoxide were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Methyl glyoxal was obtained from Fluka Chemical Corp. (Ronkonkoma, NY). L-Histidine and D-biotin were obtained from ICN Biochemicals (Cleveland, OH). Collagenase (Type 2) was purchased from Worthington Biochemical Corp. (Freehold, NJ). Acetylated cytochrome *c* was prepared by the published method (Azzi et al., 1975). NADPH, $NADP^+$, proteinase K, and RNase A (from bovine pancreas) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). C18 Sep-Pak cartridges were from Waters Associates (Milford, MA). Supercoiled ϕX174 DNA (RF I; SC) and open circular ϕX174 DNA (RF II; OC) were from New England Biolabs (Beverly, MA). The linearized (LIN) form of ϕX174 DNA was obtained by *Pst*I digestion of SC ϕX174 DNA. *Salmonella typhimurium* tester strains were provided by Dr. Bruce Ames (University of California, Berkeley, CA). Oxoid nutrient broth No. 2 was obtained from Unipath Co. (Ogdensburg, NY). Induced (Aroclor 1254) rat liver S9 (Sprague-Dawley) was obtained from Molecular Toxicology, Inc. (Annapolis, MD). All other chemicals were of the highest grade available. All solvents were HPLC grade.

Purity of PAH Metabolites. BP-diol and BPQ were synthesized according to their respective published methods (Fu & Harvey, 1977; Sukumaran & Harvey, 1980). The purity of BP-diol and BPQ was confirmed by reverse-phase high-performance liquid chromatography [RP-HPLC; purity > 95%] (Flowers-Geary et al., 1995a). [1,3-^{3}H]BP-diol (456 mCi/mmol; >98% purity) and [1,3-^{3}H]BPQ (383 mCi/mmol; >95% purity) were obtained from Chemsyn Science Laboratories (Lenexa, KS). (±)-*anti*-BPDE was purchased from Midwest Research Institute (Kansas City, MO), and its purity was checked prior to use by measuring the formation of the thiol−ether adduct with 2-mercaptoethanol (final concentration, 2 mM) in 50 mM glycine buffer (pH 9.0) at 25 °C as described (Flowers-Geary et al., 1992b). Analysis by RP-HPLC showed the presence of a single peak indicating that the diol epoxide was greater than 95% pure. *CAUTION: All PAH are potentially hazardous and should be handled in accordance with "NIH Guidelines for the Laboratory Use of Chemical Carcinogens".*

Isolation of Hepatocytes and Disposition Studies. Hepatocytes were prepared from adult male Sprague-Dawley rats according to the collagenase perfusion method (Seglen, 1976) with modifications (Failla & Cousins, 1978). Generally, 300 $\times$ 10^6 cells were obtained per rat liver. Cell viability was determined by trypan blue exclusion, and the viability of cells used was 95−99%. Freshly isolated hepatocytes (1.5 $\times$ 10^6 cells/mL) were incubated at 37 °C with shaking with 20 μM [^{3}H]BPQ (100 000 cpm/nmol) in Me_2SO [2% (v/v) final] in Leibovitz's L-15 media (pH 7.4) supplemented with gentamycin (0.05 mg/mL) and glucose (1 mg/mL). Reaction flasks were gassed with 95% O_2 for 1 min before incubation. After incubation, extracellular media and cells were separated by centrifugation. Cells were lysed in water, and the cell lysate and cell pellet were separated. Cell pellets were solubilized in 10 mM Tris-HCl (pH 7.8) containing 0.5% (w/v) sodium dodecyl sulfate for 30 min at 37 °C. Extracellular media, cell lysates, and solubilized cell pellets were extracted with 2 $\times$ 2 vol of ethyl acetate saturated with water, and in each instance the ethyl acetate layers were combined and dried. The distribution of radioactivity into the aqueous and organic phases of the extracellular media, cell lysate, and cell pellet was determined by scintillation counting.

Determination of DNA Adduct Formation in Hepatocytes. DNA was isolated from hepatocytes that were treated with either [^{3}H]BP-diol or [^{3}H]BPQ. Cells were solubilized in TNE buffer [10 mM Tris-HCl, 150 mM NaCl, and 10 mM EDTA (pH 8.0)] containing 0.5% (v/v) Triton X-100. Cell pellets were treated with proteinase K, and RNA was digested with RNase A. DNA was isolated by successive phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Purity and concentration of the DNA were determined by measuring the A_{260}/A_{280} ratio (>1.7). DNA was repetitively extracted and precipitated until radioactivity per amount of DNA (cpm/μg) remained constant. The number of stable adducts per 10^6 base pairs was determined assuming that 1 μg of DNA contains 3240 pmol of dNp (Reddy & Randerath, 1986) and that the specific activity of BP-diol or BPQ was unchanged at 100 000 cpm/nmol. PAH-adducted DNA was further digested to obtain the constituent deoxyribonucleosides by treating the purified DNA successively with nuclease P1 and alkaline phosphatase according to previously published methods (Fiala et al., 1989). The resulting hydrolysates were applied to C18 Sep-Pak car-

tridges, and unmodified deoxyribonucleosides were eluted with water. PAH−deoxyribonucleoside adducts were eluted with 100% methanol and analyzed by RP-HPLC as described (Shou et al., 1993).

Fragmentation of DNA in Hepatocytes. DNA fragmentation was determined in hepatocytes (1.5×10^6 cells/mL) following treatment with either 20 μM BP-diol or 20 μM BPQ for 30 min at 37 °C. Incubations with BP-diol were also performed in the presence of indomethacin (30 μM). Genomic DNA was isolated from intact hepatocyte nuclei using a kit to isolate cellular DNA (Qiagen, Inc., Chatsworth, CA). DNA was separated by agarose gel electrophoresis. Bands were visualized by staining with ethidium bromide, and gels were irradiated with UV light at 254 nm and photographed.

Determination of Superoxide Anion Radical Formation in Cell Suspensions. Superoxide anion radical ($O_2^{\bullet-}$) formation was followed from time zero in hepatocyte suspensions (1×10^6 cells/mL) treated with either 20 μM BP-diol or 20 μM BPQ in Me_2SO [8% (v/v) final] by measuring the rate of reduction of acetylated cytochrome *c* (60 μM) that was inhibited by SOD (500 units/mL). Incubations were also performed in the presence of known DD inhibitors, i.e., indomethacin, 6-medroxyprogesterone acetate, and 1-(4′-nitrophenyl)-2-propyn-1-ol (Ivins & Penning, 1987; Penning et al., 1984; Ricigliano & Penning, 1989). The rate of acetylated cytochrome *c* reduction was measured at 550 nm ($\epsilon = 19\,600$ M^{-1} cm^{-1}) in an Aminco DW-2A UV/vis spectrophotometer (Silver Spring, MD) equipped with a magnetic stirrer at 25 °C. For all determinations control incubations were performed in the absence of PAH metabolites to determine the basal rate of $O_2^{\bullet-}$ production. Conditions were employed which ensured linearity of the rate of acetylated cytochrome *c* reduction with respect to cell number.

DNA Strand Scission Experiments. Strand scission of SC ϕX174 DNA was measured following treatment with either BPQ under conditions of redox-cycling or (±)-*anti*-BPDE alone. Briefly, reaction mixtures (20 μL) containing 0.2 μg of SC ϕX174 DNA, 0−10 μM BPQ or 0−20 μM (±)-*anti*-BPDE in Me_2SO [5% (v/v) final], and 10 mM Tris-HCl (pH 8.0) were incubated for 2 h at 37 °C. In experiments with BPQ reactions were as above with the addition of 1 mM NADPH and 10 μM $CuCl_2$. Strand scission was monitored by measuring the conversion of SC to OC and LIN forms. No strand scission occurred in the presence of BPQ only. The three forms of DNA were separated by agarose gel electrophoresis. 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 0.1% (w/v) sodium thiosulfate, and the bands were quantified using a video densitometer and UNISCAN software (Analtech, Newark, DE).

Mutagenicity Methods. The mutagenicity of BPQ and (±)-*anti*-BPDE was compared in *S. typhimurium* tester strains TA97a, TA98, TA100, TA102, and TA104 using the preincubation procedure (Maron & Ames, 1983). Briefly, master plates of frozen permanents were prepared and tested for genotype, frequency of spontaneous reversion, and characteristic reversion frequencies to test mutagens (Levin et al., 1982; Marnett et al., 1985; Maron & Ames, 1983). In each experiment spontaneous reversion of the tester strains was measured and was within the published limits (Levin et al., 1982; Marnett et al., 1985; Maron & Ames, 1983). The mutagens used as positive controls were 4-nitro-*o*-phenylenediamine [TA97a and TA98]; sodium azide [TA100]; methyl glyoxal [TA102 and TA104]; *tert*-butyl hydroperoxide [TA102]; and cumene hydroperoxide [TA102 and TA104]. Control mutagens and PAH metabolites were freshly prepared and were tested over a range of concentrations.

To determine whether PAH metabolites were direct-acting mutagens, assays were performed in reaction mixtures (0.7 mL) containing 50 mM sodium phosphate buffer (pH 7.4), 0−100 μM PAH metabolites in Me_2SO [8% (v/v) final], and 0.1 mL of an overnight culture of the tester strain grown in Oxoid nutrient broth No. 2. Reaction mixtures were incubated in the dark for 30 min at 37 °C. Molten top agar (2 mL) containing minimal amounts of histidine and biotin was added and the mixture was plated on agar plates containing minimal glucose. The histidine revertants were scored after 48 h at 37 °C. To ensure that PAH metabolites were not bacteriocidal, the presence of a background lawn of bacterial growth was confirmed before scoring the plates. Results were expressed as the total histidine revertants per plate ± SE at the concentration of mutagen which gave the highest response.

Scintillation Counting. Samples were counted in 5 mL of Ecolite (ICN Biomedicals, Inc., Irvine, CA) in a Tracor Analytical scintillation counter with a machine efficiency of 53% for tritium. Radioactivity was reported as corrected cpm.

Table 1: Disposition of [^{3}H]BPQ in Isolated Rat Hepatocytes[a]

	distribution of radioactivity (% of total)[b]				
	media		lysate		
conditions	organic	aqueous	organic	aqueous	pellet
no incubation	40 ± 3	17 ± 2	2 ± 1	1 ± 1	8 ± 1
incubation (30 min)	14 ± 2	14 ± 1	5 ± 1	1 ± 1	30 ± 2

[a] Isolated hepatocytes (1.5×10^6 cells/mL) were incubated with 20 μM [^{3}H]BPQ in supplemented Leibovitz's L-15 media (pH 7.4) as described in Materials and Methods. After incubation, the distribution of radioactivity in the organic and aqueous phases of the fractions indicated was determined. Values are expressed as the mean ± SE of three experiments. [b] 100% = 2×10^6 cpm/20 nmol of BPQ.

RESULTS

Formation of Covalent BPQ−DNA Adducts in Isolated Rat Hepatocytes. In the present study [^{3}H]BPQ was incubated with isolated hepatocytes to determine its disposition. It was found that a significant portion of the radioactivity (30%) was sequestered into the cell pellet [RNA, DNA, and protein] (Table 1). DNA was isolated from hepatocytes incubated with either [^{3}H]BPQ or [^{3}H]BP-diol. The radioactivity bound to the DNA was then taken as a measure of covalent adduct formation (Table 2). The number of stable adducts observed with BPQ was 30 ± 17 per 10^6 base pairs and was far fewer than that observed with BP-diol (204 ± 30 per 10^6 base pairs). These data suggest that the adducts attributed to BP-diol were unlikely to have arisen from BPQ. This conclusion was supported by digestion of the radiolabeled DNA to its constituent deoxyribonucleosides and subsequent RP-HPLC analysis of the fraction containing PAH−deoxyribonucleoside adducts (data not shown). Radiolabeled adducts obtained from BP-diol-treated cells had retention times similar to those reported for (±)-*anti*-BPDE-

Table 2: Incorporation of [^{3}H]BP-Diol and [^{3}H]BPQ into the DNA Fraction of Isolated Rat Hepatocytes[a]

PAH metabolite	radioactivity (cpm × 10^3/10^6 cells)			covalent adducts (adducts/10^6 base pairs)
	cell pellet	isolated DNA	deoxyribonucleosides	
BP-diol	130 ± 8	4.0 ± 0.2	3.5 ± 0.9	204 ± 54
BPQ	390 ± 26	0.6 ± 0.04	0.5 ± 0.2	30 ± 17

[a] Isolated rat hepatocytes were incubated with either 20 μM [^{3}H]BP-diol or [^{3}H]BPQ and the distribution of radioactivity into the cell pellet, isolated DNA, and the constituent deoxyribonucleosides was determined as described in Materials and Methods. The concentration of the DNA was determined by measuring the A_{260}/A_{280} ratio. The DNA was subjected to repetitive phenol/chloroform/isoamyl alcohol extractions and ethanol precipitations until the cpm/μg of DNA remained constant. The number of stable adducts per 10^6 base pairs was calculated using the specific activity of the PAH metabolite and the conversion factor 1 μg of DNA = 3240 pmol dNp (Reddy & Randerath, 1986). Values are the means ± SE of three determinations.

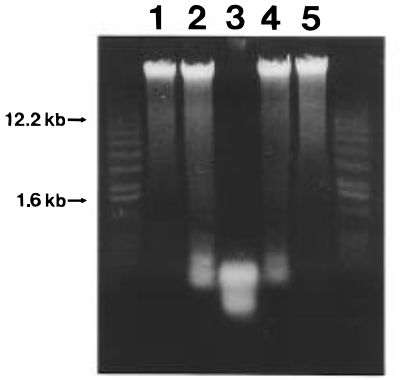

FIGURE 2: Fragmentation of rat hepatocyte genomic DNA by PAH metabolites. Genomic DNA was isolated from intact hepatocyte (1.5×10^6 cells/mL) nuclei after incubation with either BP-diol or BPQ for 30 min at 37 °C. Following isolation the DNA was subjected to agarose gel electrophoresis. Me_2SO-treated cells (lane 1); cells treated with 20 μM BP-diol (lane 2); cells treated with 20 μM BPQ (lane 3); cells treated with BP-diol plus 30 μM indomethacin (lane 4); and cells treated with indomethacin (lane 5). The outside lanes contain a 1 kb DNA ladder.

deoxyguanosine (dG) adducts (Shou et al., 1993). By contrast, adducts obtained from BPQ-treated cells did not survive the digestion and subsequent chromatographic steps. The ability of BPQ and (±)-*anti*-BPDE to form covalent DNA adducts with calf thymus DNA, plasmid DNA, and oligonucleotides has been previously compared (Shou et al., 1993). With the exception of plasmid DNA, the recovery of BPQ-dG adducts was consistently less, suggesting that BPQ-dG adducts are more unstable. In the present study the low incorporation of BPQ into hepatocyte DNA may also be related to the sequestration of the quinone by other macromolecules as less than 1% of the radioactivity in the cell pellet was bound to the DNA.

BPQ Promotes Extensive Fragmentation of Hepatocyte DNA. In order to determine whether fragmentation of hepatocyte DNA occurred upon exposure to BPQ, isolated rat hepatocytes were incubated with this *o*-quinone, and the genomic DNA was isolated, purified, and analyzed by agarose gel electrophoresis (Figure 2). DNA from untreated cells was of a high molecular mass (>23.0 kb). DNA obtained from hepatocytes treated with 20 μM BPQ for 30 min had undergone extensive fragmentation, yielding a range of species with an average size of 0.5 kb. When hepatocytes were incubated with 20 μM BP-diol, about one-third of the DNA was fragmented to the lower molecular weight species, and this effect could be attenuated by indomethacin, a DD inhibitor. These experiments were performed under essentially the same conditions in which hepatocytes were shown to convert BP-diol to BPQ (Flowers-Geary et al., 1995a). It is concluded that in hepatocytes DD converts BP-diol into BPQ, and as a consequence extensive DNA fragmentation can result.

Table 3: Production of Superoxide Anion Radical in Isolated Hepatocytes Treated with BP-diol or BPQ[a]

treatment	superoxide anion radical (pmol/min/10^6 cells)
cells	28 ± 5
cells + BP-diol (20 μM)[b]	191 ± 21
cells + BPQ (20 μM)[b]	620 ± 74
cells + BP-diol + indomethacin (30 μM)[c]	14 ± 2
cells + BP-diol + 6MPA (10 μM)[c]	19 ± 3
cells + BP-diol + 1-(4′-nitrophenyl)-2-propyn-1-o1 (500 μM)[c]	9 ± 1

[a] Superoxide anion radical formation was measured as the rate of cytochrome *c* reduction that was inhibited by SOD. Isolated hepatocytes in suspension (1×10^6 cells/mL) were treated with either BP-diol (20 μM) or BPQ (20 μM), and superoxide anion radical production was measured from time zero in the presence and absence of the inhibitors indicated. Values are expressed as the mean ± SE of three to four determinations. [b] $p < 0.005$. The statistical significance was determined using the *t*-test by comparison to the corresponding Me_2SO control. [c] $p < 0.005$. The statistical significance was determined using the *t*-test by comparison to treatment of cells with BP-diol alone.

ROS Are Involved in the DNA Fragmentation Mediated by BPQ. To determine whether ROS are responsible for the fragmentation of hepatocellular DNA, superoxide anion radical ($O_2^{\bullet -}$) formation was measured in isolated rat hepatocytes. Hepatocytes were found to produce a basal level of $O_2^{\bullet -}$ (Table 3). Incubation of hepatocytes with BP-diol resulted in a robust production of $O_2^{\bullet -}$ which could be obliterated with a series of DD inhibitors, i.e., indomethacin [a potent competitive inhibitor], 6-medroxyprogesterone acetate [6MPA, a potent mixed inhibitor], and 1-(4′-nitrophenyl)-2-propyn-1-ol [a suicide substrate] (Ivins & Penning, 1987; Penning et al., 1984; Ricigliano & Penning, 1989). Treatment of hepatocytes with BPQ increased the amount of $O_2^{\bullet -}$ produced over that seen with BP-diol by 3-fold. This increase correlated with the increase in DNA fragmentation observed with BPQ over that observed with BP-diol in isolated hepatocytes.

BPQ Causes Strand Scission of ϕX174 DNA Under Redox-Cycling Conditions. To understand the mechanisms by which BPQ caused extensive fragmentation of hepatocyte DNA, the ability of BPQ to cause DNA strand scission in a model system was examined. For these studies, double-stranded supercoiled (SC) ϕX174 DNA was used. A single nick in the DNA is sufficient to cause the transition into open circular (OC) DNA, two nicks are sufficient to form linearized (LIN) DNA, and multiple nicks on both strands would lead to fragmentation to a low molecular weight species. Upon incubation of ϕX174 DNA with low concentrations of BPQ (0.01 μM) in the presence of NADPH

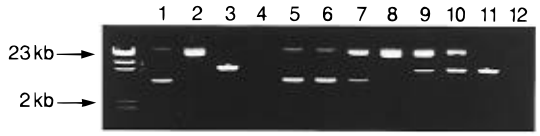

FIGURE 3: Strand scission of ϕX174 DNA under redox-cycling conditions. Strand scission of 0.2 μg of SC ϕX174 DNA was measured following treatment with BPQ in the presence of NADPH (1 mM) and $CuCl_2$ (10 μM) [complete system]. Strand scission was monitored by separating SC from OC and LIN forms by agarose gel electrophoresis. 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 BPQ (lane 5); and complete system plus 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, and 10.0 μM BPQ (lanes 6−12, respectively). The outside lane contains a *Hin*dIII digest of λ phage.

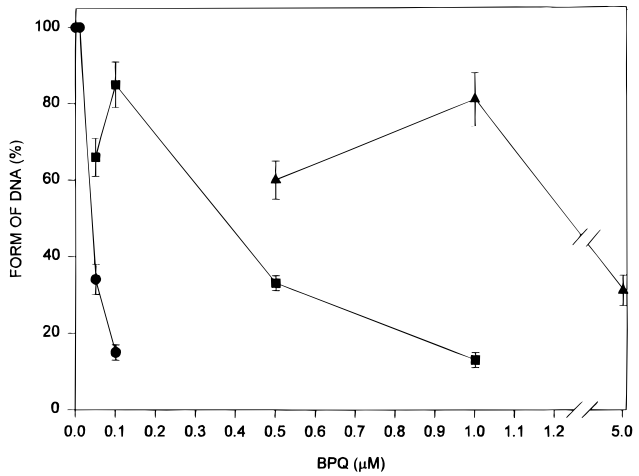

FIGURE 4: Concentration-dependent strand scission of ϕX174 DNA mediated by BPQ under redox-cycling conditions. The quantity of SC (●), OC (■), and LIN (▲) forms of ϕX174 DNA observed following treatment with BPQ (0.01−10 μM), NADPH (1 mM), and $CuCl_2$ (10 μM) was determined by densitometry and plotted against quinone concentration.

and $CuCl_2$ no effect on the SC DNA was observed (Figure 3). However, at concentrations between 0.05 μM and 10 μM BPQ there was a concentration-dependent destruction of the SC DNA that progressed through OC and LIN forms until the DNA was completely destroyed (Figure 4). At the highest concentration of BPQ (10 μM) the DNA was no longer detectable on the gel. This result was identical to that observed for a positive control in which ROS were generated by the Fenton reaction ($FeSO_4$ and H_2O_2).

To identify the ROS responsible for BPQ-mediated strand scission, attempts were made to block DNA fragmentation with free radical scavengers (Table 4). Catalase and hydroxyl radical scavengers (sodium benzoate, formic acid, and mannitol) were all able to block the strand scission seen with BPQ. By contrast, superoxide dismutase was unable to protect the ϕX174 DNA from fragmentation which may be explained by the fact that dismutation of $O_2^{\bullet-}$ results in the formation of H_2O_2. Tiron, a chemical trap for $O_2^{\bullet-}$, was able to prevent the DNA strand scission. Of the radical scavengers that provided protection, none completely ameliorated the effects of BPQ. These data suggest that NADPH reduces BPQ to the catechol and that during the subsequent autoxidation back to the *o*-quinone, $O_2^{\bullet-}$ is produced which dismutates to H_2O_2. In the presence of $CuCl_2$, hydroxyl radical is generated which in turn causes strand scission of the DNA. It is proposed that a similar sequence may be occurring in isolated hepatocytes treated with BPQ.

Table 4: Effects of Radical Scavengers on BPQ-Induced Strand Scission of ϕX174 DNA[a]

conditions	form of DNA: supercoiled	open circular	linear	complete scission
CS[b]	−	−	−	100[c]
CS + SOD (210 units/mL)	−	−	−	100
CS + Tiron (10 mM)	14 ± 2	69 ± 5	−	−
CS + catalase (880 units/mL)	29 ± 2	53 ± 4	−	−
CS + sodium benzoate (5 mM)	27 ± 3	53 ± 5	−	−
CS + formic acid (5 mM)	27 ± 3	67 ± 5	−	−
CS + mannitol (5 mM)	28 ± 3	55 ± 5	−	−

[a] The quantities of SC, OC, and LIN forms of ϕX174 DNA were determined by densitometry. [b] Complete systems (CS) contained BPQ (10 μM), NADPH (1 mM), and $CuCl_2$ (10 μM). [c] Values are expressed as a percent of untreated DNA and are the mean ± SE of three determinations.

FIGURE 5: Comparison of ϕX174 DNA strand scission by BPQ under redox-cycling conditions versus (±)-*anti*-BPDE. Strand scission of 0.2 μg of SC ϕX174 DNA was measured following treatment with either BPQ in the presence of NADPH (1 mM) and $CuCl_2$ (10 μM) [complete system] or (±)-*anti*-BPDE. Strand scission was monitored by separating SC from OC and LIN forms by agarose gel electrophoresis. SC ϕX174 DNA (lane 1); OC ϕX174 DNA (lane 2); LIN ϕX174 DNA (lane 3); complete system minus BPQ (lane 4); complete system plus 1.0, 5.0, and 10.0 μM BPQ (lanes 5−7, respectively); and 0.0, 1.0, 5.0, 10.0, and 20.0 μM (±)-*anti*-BPDE (lanes 8−12, respectively). The outside lane contains a *Hin*dIII digest of λ DNA.

The strand scission induced by BPQ under redox-cycling conditions was compared to that caused by (±)-*anti*-BPDE alone (Figure 5). At the concentration of BPQ (10 μM) that caused complete fragmentation of the DNA, (±)-*anti*-BPDE caused only single nicks in the DNA to produce OC DNA. A direct comparison of the strand scission experiments (Figure 3 versus Figure 5) showed that 0.1 μM BPQ was capable of converting SC DNA to the OC form, whereas 20 μM (±)-*anti*-BPDE was required for the same effect. The results indicate that BPQ is approximately 200-fold more potent as a chemical nuclease than (±)-*anti*-BPDE.

BPQ as a Direct-Acting Mutagen in the Ames Test. Using the preincubation method (Maron & Ames, 1983), BPQ was found to be a direct-acting mutagen in five tester strains [TA97a, TA98, TA100, TA102, and TA104] (Table 5). In the absence of an activation system BPQ caused predominantly frameshift mutations. Modest mutation was observed in strains sensitive to oxidative mutagens. Addition of an activation system that would promote redox-cycling of BPQ (rat liver S9 plus glucose-6-phosphate dehydrogenase and $NADP^+$) failed to increase the number of revertants (data not shown) and may be related to the sequestration of the *o*-quinone by the added protein. It should be emphasized that in each tester strain the mutagenic efficiency observed with BPQ was equal to or greater than that observed with the test mutagens. However, the most important result was that the mutagenic efficiency of BPQ was dwarfed by that observed with (±)-*anti*-BPDE. Thus, the mutagenic efficiency of (±)-*anti*-BPDE exceeded that observed for BPQ by 10−5500-fold depending on the tester strain.

Table 5: Comparison of the Mutagenic Efficiency of BPQ *versus* (±)-*anti*-BPDE in the Ames Test[a]

tester strain	compound	concn (nmol/plate)[b]	revertants (rev/plate)[c]	*n*-fold increase[d] (*n*)	mutagenic efficiency[e]	mutation type[f]
TA97a	BPQ[g]	70	463 ± 57	4.1	5.9	frameshift
TA98	BPQ[h]	70	52 ± 5	1.8	2.6	frameshift
TA100	BPQ[g]	70	192 ± 6	1.6	2.3	point
TA102	BPQ[i]	35	368 ± 12	1.5	4.3	oxidative
TA104	BPQ[j]	35	553 ± 15	1.6	4.6	oxidative
TA97a	(±)-*anti*-BPDE[j]	0.1	444 ± 27	4.0	4 000	frameshift
TA98	(±)-*anti*-BPDE[j]	0.1	426 ± 33	14.7	14 700	frameshift
TA100	(±)-*anti*-BPDE[j]	0.1	845 ± 48	7.0	7 000	point
TA102	(±)-*anti*-BPDE[j]	16.5	2200 ± 120	9.1	55	oxidative
TA104	(±)-*anti*-BPDE[j]	3.3	1620 ± 85	4.6	140	oxidative

[a] Preincubation assays were performed as described in Materials and Methods in reaction mixtures (0.7 mL) containing 50 mM sodium phosphate buffer (pH 7.4), 0.1 mL bacterial strain, and either BPQ or (±)-*anti*-BPDE at 37 °C for 30 min. Top agar was added, and the mixture was poured onto minimal agar plates. Assay plates were incubated for 48 h at 37 °C and then scored for revertants. [b] The concentration indicated gave the greatest number of revertants and was on the linear portion of the dose−response curve. [c] Histidine revertants are represented as total revertants per plate and are the mean values ± SE calculated from three determinations. Each determination was performed in triplicate. Statistical significance was determined using the *t*-test by comparing the spontaneous revertants/plate with the revertants/plate following treatment with either BPQ or (±)-*anti*-BPDE within the same experimental group. Spontaneous reversions ($n = 26$) were 112 ± 6 (TA97a), 29 ± 1 (TA98), 120 ± 8 (TA100), 242 ± 16 (TA102), and 354 ± 15 (TA104). [d] The *n*-fold increase is the number of histidine revertants over the spontaneous revertants. [e] The mutagenic efficiency was calculated as the [(number of revertants over the number of spontaneous revertants)/nmol of mutagen] × 100. The mutagenic efficiency of test mutagens was 1.6 (TA97a) and 3.5 (TA98) for 4-nitro-*o*-phenylenediamine (1300 nmol/plate), 0.6 (TA100) and 1.2 (TA104) for methyl glyoxal (560 nmol/plate), 0.4 (TA102) for *tert*-butyl hydroperoxide (1110 nmol/plate), and 0.8 (TA102) and 0.3 (TA104) for cumene hydroperoxide (660 nmol/plate). [f] The mutation type represents the most likely mutation for each tester strain according to Levin et al. (1982), Marnett et al. (1985), and Maron and Ames (1983). [g] $p < 0.005$. [h] $p < 0.02$. [i] $p < 0.01$. [j] $p < 0.001$.

DISCUSSION

It was originally proposed that DD oxidized BP-diol to the corresponding catechol and, thereby, suppressed the formation of (±)-*anti*-BPDE and its inherent mutagenicity (Glatt et al., 1979; Vogel et al., 1980). We have previously shown that in isolated rat hepatocytes DD converted BP-diol into BPQ. Under the conditions employed BPQ and BP-tetraols were formed to an equal extent, implying that in hepatocytes from uninduced rats, DD and CYPs contributed equally to the metabolism of BP-diol (Flowers-Geary et al., 1995a). This study addresses the genotoxicity of BPQ.

A major finding of the present work was that BPQ was a mild mutagen when compared to (±)-*anti*-BPDE which would support a role for this enzyme in the detoxication of PAH *trans*-dihydrodiol proximate carcinogens. However, there was a disadvantage in diverting BP-diol into BPQ. Our studies show that rather than acting as a direct mutagen, BPQ acted as a potent chemical nuclease by causing extensive fragmentation of hepatocyte DNA. When hepatocytes were treated with either BP-diol or BPQ, significant amounts of $O_2^{\bullet -}$ were produced. However, when hepatocytes were treated with DD inhibitors, the conversion of BP-diol into BPQ was blocked (Flowers-Geary, et al., 1995a) and the generation of ROS and DNA fragmentation was attenuated. The residual DNA fragmentation observed may be attributed to the formation of (±)-*anti*-BPDE in hepatocytes. Our findings indicate that the DD-catalyzed oxidation of BP-diol generated sufficient ROS to cause DNA damage *ex vivo* and that the reactions depicted in Figure 1 were responsible for this damage.

Using ϕX174 DNA as a model system to investigate the mechanisms by which BPQ mediated strand scission, we provide evidence that the responsible agents were, at least in part, hydroxyl radicals derived from H_2O_2. Thus, strand scission was observed only in complete systems which contained BPQ, reducing equivalents (NADPH), and $CuCl_2$. The observed strand scission was blocked by Tiron, catalase, and hydroxyl radical scavengers, but not by superoxide dismutase which would generate H_2O_2. Using similar concentrations of (±)-*anti*-BPDE only single nicks in the SC ϕX174 DNA occurred. We estimate that BPQ is 200-fold more potent than (±)-*anti*-BPDE as a chemical nuclease. The weak nuclease property of (±)-*anti*-BPDE agrees with previously published results (Gamper et al., 1977).

The nature of the strand breaks observed with BPQ in both hepatocytes and ϕX174 DNA indicated that extensive fragmentation occurred and that this, in turn, must result from multiple nicks in the DNA. Furthermore, these nicks must occur on both strands in close proximity to one another to produce the low molecular weight fragments observed. These multiple nicks would occur under conditions of pronounced oxidative insult. Since there was no characteristic laddering of the DNA, such an insult might lead to necrotic rather than apoptotic cell death (Wyllie, 1980). Interestingly, when hepatocytes were exposed to the same concentrations of BPQ that caused DNA fragmentation, cell viability was reduced by 30% over a 2 h period (Flowers-Geary, et al., 1995b). There may be a number of explanations for the discrepancy between the degree of DNA fragmentation and late cell death. First, hepatocytes might contain appropriate DNA repair enzymes that would catalyze recombination events and double-strand break repair (Birkenbihl & Subramani, 1992; Finnie et al, 1995). Second, DNA fragmentation may not lead to immediate cell death. Third, cell viability was measured by trypan blue exclusion which indicated only whether the cells contained an intact plasma membrane.

Oxidative damage of DNA is not only associated with cell death but is also involved in mutagenesis and carcinogenesis. For example, ionizing radiation produces ROS, and the production of hydroxyl radicals may be responsible for radiation-induced carcinogenesis (Frenkel, 1992; Wood et al., 1990). Two types of oxidative damage that have been correlated with mutagenesis and carcinogenesis are the production of oxidatively damaged bases and DNA strand breaks (Breimer, 1990; Frenkel, 1992). 8-Oxo-2′-deoxygua-

nosine represents an oxidatively damaged form of guanosine (Breimer, 1990; Frenkel, 1992) which has been implicated in G → T transversion mutagenesis (Kuchino et al., 1987). This type of mutation has been observed in the *ras* proto-oncogene (Colapietro et al., 1993; Mass et al., 1993) and the p53 tumor suppressor gene (Puisieux et al., 1991). Further, levels of 8-oxo-2′-deoxyguanosine have been correlated with the incidence of end-organ carcinogenicity in animals and humans (Kasai et al., 1987, 1989). It has been found that G → T transversions in the *ras* proto-oncogene are a common occurrence in mouse skin papillomas induced by a variety of PAH. Although the transversions have been attributed to the formation of depurinating PAH−DNA adducts (Chakravarti et al., 1995), the formation of oxidatively damaged bases via the redox-cycling of PAH *o*-quinones could also contribute to this outcome. DNA strand breaks and subsequent illegitimate recombination may also play a role in mutagenesis and carcinogenesis (Emerit et al., 1982). It should be emphasized that the extensive DNA fragmentation observed with BPQ represents a biological end point that may never be reached under *in vivo* conditions. In the studies described, conditions were optimized to achieve a measurable index of oxidative DNA damage. Less extensive oxidative DNA damage may result from exposure to BPQ *in vivo*, and the mutagenic and carcinogenic events associated with the formation of oxidatively damaged DNA bases and recombination events may occur.

The substantial production of ROS observed when hepatocytes were treated with BP-diol is believed to result from the redox-cycling of the BPQ produced by DD. We have previously ascribed this redox-cycling to the one-electron reduction of BPQ mediated by NADPH-cytochrome P450 reductase, NADH-cytochrome b_5 reductase, and NADH: ubiquinone oxidoreductase (Flowers-Geary et al., 1992a, 1993). BPQ is not a substrate for DT-diaphorase (Flowers-Geary et al., 1992a, 1993). The ability to detect ROS in hepatocyte suspensions treated with BP-diol suggests that DD generates more ROS than can be eliminated by endogenous protective enzymes (e.g., catalase, SOD, and glutathione peroxidase). Therefore, PAH *trans*-dihydrodiols are anticipated to generate significant oxidative insult by this pathway. This pathway may provide a mechanism by which PAH can act as initiators and promoters (i.e., act as complete carcinogens). It is known that ROS can act as selective mitogens to cause initiated cells to divide (Ames & Gold, 1990; Cohen & Ellwein, 1990). Furthermore, ROS will activate protein kinase C and induce the transcription of proto-oncogenes (Amstad et al., 1992; Larsson & Cerutti, 1989).

From the studies described, a clearer picture of the biological profile of BPQ has emerged. Once formed, BPQ can act as a substrate for enzymes that catalyze one-electron redox-cycling, and as a consequence *o*-semiquinone anion radicals and ROS can be produced multiple times (Flowers-Geary et al., 1992a, 1993). The ROS that are generated can be measured in hepatocyte suspensions, indicating that neighboring cells are exposed to oxidative insult. In addition, the *o*-quinone is sequestered into the macromolecule fraction of the cell. Although covalent *o*-quinone−DNA adducts are formed to a limited extent, they do not survive the procedures required for chromatographic analysis, suggesting that they may be unstable. A large portion of BPQ that is associated with the cell pellet is unaccounted for and may be adducted with other macromolecules (RNA or protein). Rather than form DNA adducts, BPQ can cause extensive oxidative damage of DNA leading to fragmentation.

In summary, when rat hepatocytes are treated with PAH *trans*-dihydrodiols they can either form *anti*-diol epoxides which are highly mutagenic or they can form *o*-quinones which act as potent chemical nucleases via the production of ROS. In the latter case the resultant genotoxicity and the effects of ROS on tumor promotion may be important contributors to PAH carcinogenesis.

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