Binding of Benzo[a]pyrene to DNA by Cytochrome P-450 Catalyzed One-Electron Oxidation in Rat Liver Microsomes and Nuclei†

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ABSTRACT: To investigate whether cytochrome P-450 catalyzes the covalent binding of substrates to DNA by one-electron oxidation, the ability of both uninduced and 3-methylcholanthrene (MC) induced rat liver microsomes and nuclei to catalyze covalent binding of benzo[a]pyrene (BP) to DNA and formation of the labile adduct 7-(benzo[a]pyren-6-yl)guanine (BP–N7Gua) was investigated. This adduct arises from the reaction of the BP radical cation at C-6 with the nucleophilic N-7 of the guanine moiety. In the various systems studied, 1–9 times more BP–N7Gua adduct was isolated than the total amount of stable BP adducts in the DNA. The specific cytochrome P-450 inhibitor 2-[(4,6-dichloro-*o*-biphenyl)oxy]ethylamine hydrobromide (DPEA) reduced or eliminated BP metabolism, binding of BP to DNA, and formation of BP–N7Gua by cytochrome P-450 in both microsomes and nuclei. The effects of the antioxidants cysteine, glutathione, and *p*-methoxythiophenol were also investigated. Although cysteine had no effect on the microsome-catalyzed processes, glutathione and *p*-methoxythiophenol inhibited BP metabolism, binding of BP to DNA, and formation of BP–N7Gua by cytochrome P-450 in both microsomes and nuclei. The decreased levels of binding of BP to DNA in the presence of glutathione or *p*-methoxythiophenol are matched by decreased amounts of BP–N7Gua adduct and of stable BP–DNA adducts detected by the ^{32}P-postlabeling technique. This study represents the first demonstration of cytochrome P-450 mediating covalent binding of substrates to DNA via one-electron oxidation and suggests that this enzyme can catalyze peroxidase-type electron-transfer reactions.

Covalent binding of chemical carcinogens to DNA is thought to be the first critical step in the initiation of the tumor formation process (Miller, 1970; Miller & Miller, 1981). The ultimate goals of most research in chemical carcinogenesis are to elucidate the structure of various DNA–carcinogen adducts and to establish their biological significance. Two immediate objectives that can be realized by elucidating adduct structures are the determination of the mechanism of carcinogenic activation and the nature of the enzymes involved in this catalytic process.

Cytochrome P-450 isozymes, which are components of the monooxygenase enzyme system, are thought to play a major role in carcinogenic activation. This process has been thought to occur via formation of oxygenated metabolites (Miller, 1970; Miller & Miller, 1981). More specifically, in the case of polycyclic aromatic hydrocarbons (PAH),[1] diol epoxides have been proposed as the almost exclusive ultimate carcinogenic forms that react with DNA to initiate cancer (Sims & Grover, 1981; Conney, 1982). Several lines of evidence, however, suggest that some PAH can be enzymically activated by one-electron oxidation to form radical cation intermediates that covalently bind to DNA (Cavalieri & Rogan, 1985a,b).

One-electron oxidation has been implicated as the initial step in the cytochrome P-450 catalyzed oxidation of alkylamines (Burka et al., 1985; Macdonald et al., 1989), sulfides (Watanabe et al., 1980), sulfoxides (Watanabe et al., 1982), quadricyclane (Stearns & Ortiz de Montellano, 1985), and benzo[a]pyrene (BP) to generate BP diones (Cavalieri et al., 1988). Dihydropyridine (Augusto et al., 1982) and cycloalkylamines (Hanzlik & Tullman, 1982; Macdonald et al., 1982; Bondon et al., 1989) induce suicide inactivation of cytochrome P-450 via an initial one-electron oxidation of the substrate.

These findings suggest that cytochrome P-450 can catalyze binding of BP to DNA by a one-electron oxidation mechanism analogously to horseradish peroxidase (HRP), which catalyzes formation of the labile 7-(benzo[a]pyren-6-yl)guanine (BP–N7Gua) and the stable adduct containing BP bound at C-6 to the C-8 of deoxyguanosine (Rogan et al., 1988). HRP also catalyzes the one-electron oxidation of many other substrates (Bartsch & Hecker, 1971; Bartsch et al., 1972; Griffin & Ting, 1978; Metzler & McLachlan, 1978; Galliani & Rindone, 1981; Josephy et al., 1982a,b; Kalyanaraman & Mason, 1982). In the one-electron oxidation mechanism, binding of BP radical cation to nucleophiles occurs at the C-6 position, in which the charge is mainly localized (Rochlitz, 1967; Jeftic & Adams, 1970; Wilk & Girke, 1972; Johnson & Calvin, 1973; Cavalieri

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[1] Abbreviations: BP, benzo[a]pyrene; BP–NC, 6-isocyanobenzo[a]pyrene; BP–N7Gua, 7-(benzo[a]pyren-6-yl)guanine; CuOOH, cumene hydroperoxide; DNA-P, DNA phosphate; DPEA, 2-[(4,6-dichloro-*o*-biphenyl)oxy]ethylamine hydrobromide; FAB MS/MS, fast atom bombardment tandem mass spectrometry; HRP, horseradish peroxidase; MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbon(s).

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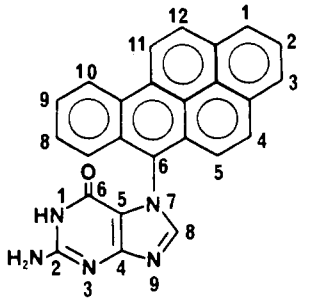
& Auerbach, 1974; Blackburn et al., 1974; Rogan et al., 1988; Cremonesi et al., 1989). One of the adducts produced by anodic oxidation of BP in the presence of deoxyguanosine contains BP bound at C-6 to the N-7 position of the guanine moiety, i.e., BP–N7Gua (Rogan et al., 1988). Formation of



this adduct results in complete loss of deoxyribose from the deoxyribonucleoside due to destabilization of the glycosidic link. The possibility that the adduct BP–N7Gua, which results from the HRP-catalyzed binding of BP to DNA, can also be formed by cytochrome P-450 prompted the study of the binding of BP to calf thymus DNA with rat liver microsomes and to endogenous DNA in rat liver nuclei.

Experimental Procedures

Metabolism and Binding of BP to DNA Catalyzed by Rat Liver Microsomes and Nuclei. Liver microsomes were prepared from both uninduced or 3-methylcholanthrene (MC) induced (100 μmol/kg of body weight administered on two consecutive days) 8-week-old male Wistar rats (Eppley Colony) as has been previously described (Wong et al., 1986). Liver nuclei were prepared from MC-induced (100 μmol/kg of body weight administered on two consecutive days) 3-week-old male Wistar rats as was previously described (Rogan & Cavalieri, 1974). The nuclei were structurally intact and free of significant contamination, as determined by electron microscopy.

Microsome-catalyzed reactions of [^{3}H]BP (550 Ci/mol) were performed in 5-mL incubation mixtures. The cumene hydroperoxide (CuOOH) supported metabolism was started by preincubation of 5 mg of microsomal protein, calf thymus DNA (2.8 mM DNA-P), 150 mM KCl, and 5 mM $MgCl_2$ in 50 mM Tris-HCl, pH 7.5, for 3 min at 37 °C. The enzymic reactions were initiated by the addition of 400 nmol of the substrate, followed immediately by addition of 2 mM CuOOH and incubation for 10 min. In the NADPH-supported metabolism, 5 mg of microsomal protein, calf thymus DNA (2.8 mM DNA-P), NADPH-generating system (0.43 mM NADP, 1.29 mM glucose 6-phosphate, 0.43 unit/mL glucose-6-phosphate dehydrogenase), 150 mM KCl, and 5 mM $MgCl_2$ in 50 mM Tris-HCl, pH 7.5, were preincubated for 5 min at 37 °C to generate NADPH; the incubation was continued for 30 min after addition of the substrate. With both cofactors, a 1-mL aliquot was removed at the end of the incubation to determine the level of binding of BP to DNA. The reaction was terminated by addition of phenol (see below). The remaining 4-mL portion was analyzed for metabolites and the BP–N7Gua adduct. The reaction was terminated by addition of ethanol (see below).

To measure the binding of BP to DNA, the DNA was purified from the 1-mL aliquot of the reaction mixture by extraction with water-saturated phenol, pH 8.0, and $CHCl_3$, followed by two precipitations with ethanol (Rogan & Cavalieri, 1974). The level of binding of BP to DNA was determined by counting [^{3}H]BP and measuring DNA by absorbance at 260 nm. This allows determination of the amount of stable BP adducts in the DNA.

To identify the BP metabolites and the BP–N7Gua adduct, 8 mL of absolute ethanol was added to the remaining 4-mL portion of incubation mixture to precipitate the DNA and to obtain a supernatant that contained the metabolites and the BP–N7Gua adduct. The supernatant was then evaporated to dryness under argon and analyzed by HPLC.

Nuclei-catalyzed reactions of [^{3}H]BP were performed in 4-mL incubation mixtures. In the NADPH-supported metabolism, 0.6 mM NADPH, nuclei (4 mg of protein), and 50 μM [^{3}H]BP in 50 mM Tris-HCl, pH 7.5, were incubated for 20 min at 37 °C. Preincubation was omitted because NADPH itself was used. The reactions were terminated by addition of 1% sodium dodecyl sulfate. A 1-mL aliquot was removed for the determination of BP binding to DNA, and the remaining nuclear DNA was then precipitated with 2 volumes of absolute ethanol. The resulting supernatant was analyzed for BP metabolites and for BP–N7Gua. The values reported here for metabolism, binding of BP to DNA, and BP–N7Gua are representative of two to three replicates.

Analysis of Metabolites and BP–N7Gua by HPLC. The residue from evaporation of the supernatant fraction was dissolved in a minimum volume (0.5 mL) of DMSO, followed by addition of an equal volume of CH_3OH. The undissolved material was removed by centrifugation. An aliquot of the supernatant was first analyzed by using HPLC on an Altex Ultrasphere 5-μm reverse-phase column (Spectra Physics SP8700 solvent delivery system). The column was eluted for 5 min with 30% CH_3OH in H_2O, followed by a 70-min linear gradient to 100% CH_3OH at a flow rate of 0.8 mL/min. Peaks were detected via UV absorbance (at 302 nm) and by radioactivity (RAMONA radiometric detector, IN/US, Fairfield, NJ). HPLC was conducted in the presence of the electrochemically synthesized reference adduct BP–N7Gua (Rogan et al., 1988). The metabolite BP 4,5-dihydrodiol and the BP–N7Gua adduct eluted together and were collected. The solution was evaporated to dryness, and the residue was redissolved in DMSO/CH_3OH (1:1) and further analyzed by HPLC by using a CH_3CN/H_2O gradient. The column was first eluted for 5 min with 20% CH_3CN in H_2O, followed by an 80-min linear gradient to 100% CH_3CN at a flow rate of 0.8 mL/min. The radioactive BP–N7Gua coeluted with the BP–N7Gua standard, whereas the BP 4,5-dihydrodiol eluted later.

To confirm further the presence of BP–N7Gua, the fraction containing the presumed BP–N7Gua from the CH_3CN/H_2O gradient was collected and evaporated. The residue was redissolved in DMSO/CH_3OH (1:1) and reanalyzed by HPLC using the CH_3OH/H_2O gradient described above. Once again, the radioactive compound coeluted with the BP–N7Gua standard.

Structure Analysis. Four 25-mL microsomal incubations were carried out for both the NADPH and 2 mM CuOOH supported reactions, as described above, and the supernatants combined. The BP–N7Gua adduct was isolated and purified by HPLC using the CH_3OH/H_2O gradient, followed by the CH_3CN/H_2O gradient. A total of 1.7–2 μg of BP–N7Gua was obtained from both microsomal preparations.

The structure of the BP–N7Gua adduct obtained from incubation of BP and DNA with MC-induced rat liver microsomes supported by either NADPH or CHP was determined by using fast atom bombardment combined with tandem mass spectrometry (FAB MS/MS) (Rogan et al., 1988).

Because rat liver nuclei produce much smaller amounts of metabolites and adducts than do microsomes, the structure

Table I: Inhibition of BP Metabolism, DNA Binding, and BP–N7Gua Formation by DPEA[a]

sample	uninduced			MC-induced		
	total metabolites (nmol)	binding to DNA (μmol of BP/mol of DNA-P)	BP–N7Gua (μmol/mol of DNA-P)	total metabolites (nmol)	binding to DNA (μmol of BP/mol of DNA-P)	BP–N7Gua (μmol/mol of DNA-P)
microsomes						
NADPH						
control (no cofactor)	16	2.1	0	20	8	0
complete	77	10.0	32	163	79	107
1× DPEA	15 (81)[b]	4.0 (60)	7.1 (78)	150 (8)	50 (37)	64 (40)
10× DPEA	12 (84)	3.3 (67)	0 (100)	22 (87)	9 (89)	0 (100)
CuOOH						
control (no cofactor)	16	3.1	0	20	2.5	0
complete	40	6.6	7.1	48	6.4	14.3
1× DPEA	16 (60)	6.4 (3)	3.6 (50)	18 (62)	5.6 (12)	5 (65)
10× DPEA	4 (90)	4.0 (39)	0 (100)	12 (75)	4.8 (25)	0 (100)
nuclei						
NADPH						
complete				7.9	9	83
1× DPEA				6.7 (15)	6 (33)	72 (13)
10× DPEA				1.4 (82)	0 (100)	0 (100)

[a] Amounts are calculated on the basis of 5-mL reaction mixtures (400 nmol of BP) for microsomes and 2-mL reaction mixtures (100 nmol of BP) for nuclei. The amounts of DPEA are presented relative to BP. [b] Numbers in parentheses indicate percent inhibition.

of the BP–N7Gua adduct obtained from incubation of BP with rat liver nuclei and NADPH was determined by fluorescence line-narrowed spectrometry (Zamzow et al., 1989). Only 20 pg of BP–N7Gua, representing a small portion isolated from one preparation, was required for this determination.

Effect of the Cytochrome P-450 Inhibitor DPEA on BP Metabolism and Binding of BP to DNA. The cytochrome P-450 inhibitor 2-[(4,6-dichloro-*o*-biphenyl)oxy]ethylamine hydrobromide (DPEA) was added to 5-mL (microsomes) or 4-mL (nuclei) reaction mixtures at a concentration of 80 (molar ratio of 1:1 with respect to BP) or 800 μM (molar ratio of 10:1 with respect to BP) in ≤0.1 mL of DMSO prior to preincubation. The reactions were carried out as described above. A 1-mL aliquot of the mixture was used to determine the level of binding of BP to DNA. The remaining mixture was analyzed by using HPLC to determine BP metabolites and the presence of the BP–N7Gua adduct.

Effect of Cysteine on BP Metabolism and Binding of BP to DNA. Cysteine was added to 5-mL microsomal reaction mixtures at a concentration of 1 (molar ratio of 12:1 with respect to BP) or 5 mM (60:1 vs BP) prior to preincubation. The reactions were carried out and analyzed as described above for the experiments with DPEA.

Effect of Glutathione or p-Methoxythiophenol on BP Metabolism and Binding of BP to DNA. Glutathione or *p*-methoxythiophenol was added to 5- (microsomes) or 4-mL (nuclei) incubation mixtures at a concentration of 0.08 (molar ratio of 1:1 with respect to BP), 0.5 (6:1 vs BP), 1 (12:1 vs BP), or 5 mM (60:1 vs BP) prior to preincubation (microsomes) or incubation (nuclei). The reactions were carried out and analyzed as described above for the experiments with DPEA.

^{32}P-Postlabeling Analysis of Stable BP–DNA Adducts. The nuclease P1 ^{32}P-postlabeling method was used as previously described (Bodell et al., 1989) to analyze the stable BP–DNA adducts formed by microsomes and nuclei both with and without addition of 5 mM (60×) glutathione or *p*-methoxythiophenol.

Results

Identification of the BP–N7Gua Adduct Formed by Microsomes and Nuclei. [^{3}H]BP was bound to DNA with uninduced and MC-induced microsomes supported by either NADPH or CuOOH as the cofactor. The DNA was then precipitated and the supernatant analyzed by using HPLC. The supernatant fraction from the NADPH-supported binding of BP to DNA by MC-induced microsomes was first analyzed by using HPLC with the CH_3OH/H_2O gradient. BP–N7Gua was added to the supernatant as a UV marker (Figure 1A). Metabolites and possible adducts observed were, in order of elution, BP 9,10-dihydrodiol, BP 4,5-dihydrodiol plus BP–N7Gua, BP 7,8-dihydrodiol, BP quinones, and BP phenols. The fraction containing both BP 4,5-dihydrodiol and BP–N7Gua was collected and rechromatographed by using the CH_3CN/H_2O gradient (Figure 1B). In this case, two radiolabeled peaks were observed, one coeluting with the BP–N7Gua marker and a second, of 10-fold greater abundance, was identified as BP 4,5-dihydrodiol. The BP–N7Gua peak in Figure 1B was collected and rechromatographed with the CH_3OH/H_2O gradient (Figure 1C). The adduct again coeluted with the BP–N7Gua marker.

Similar results were obtained with uninduced microsomes and NADPH, except that lower levels of metabolites and less BP–N7Gua (0.45 nmol) were found. This level of adduct was much less than that obtained with MC-induced microsomes (1.5 nmol) (Table I). With CuOOH as the cofactor, very similar results were obtained for both uninduced and MC-induced microsomes (Figure 2 and Table I). This is as expected because CuOOH utilizes only a specific constitutive isozyme (Cavalieri et al., 1987). At the concentration of CuOOH used (2 mM), no phenols were formed, but the level of quinones was high, as previously reported (Wong et al., 1986; Cavalieri et al., 1987). The amount of BP–N7Gua formed with CuOOH was less than the amount formed with NADPH (Table I).

The BP–N7Gua adduct obtained from the supernatant fraction when BP and DNA were incubated in the presence of MC-induced microsomes with either NADPH or CuOOH was isolated from large preparations by using HPLC. The adduct-containing fractions were analyzed by using FAB and FAB MS/MS. FAB of a portion of the adduct-containing fraction produced $(M + H)^+$ ions of m/z 402 (observed 402.1335, theoretical for $C_{25}H_{16}N_5O$ 402.1355, relative error 4.9 ppm). Upon collisional activation (see Figure 4B), the $(M + H)^+$ ions decompose predominantly to form ions of m/z 277 (BP–NC$^{\bullet+}$) and of m/z 251 ($C_{20}H_{11}^+$ of the BP moiety). The

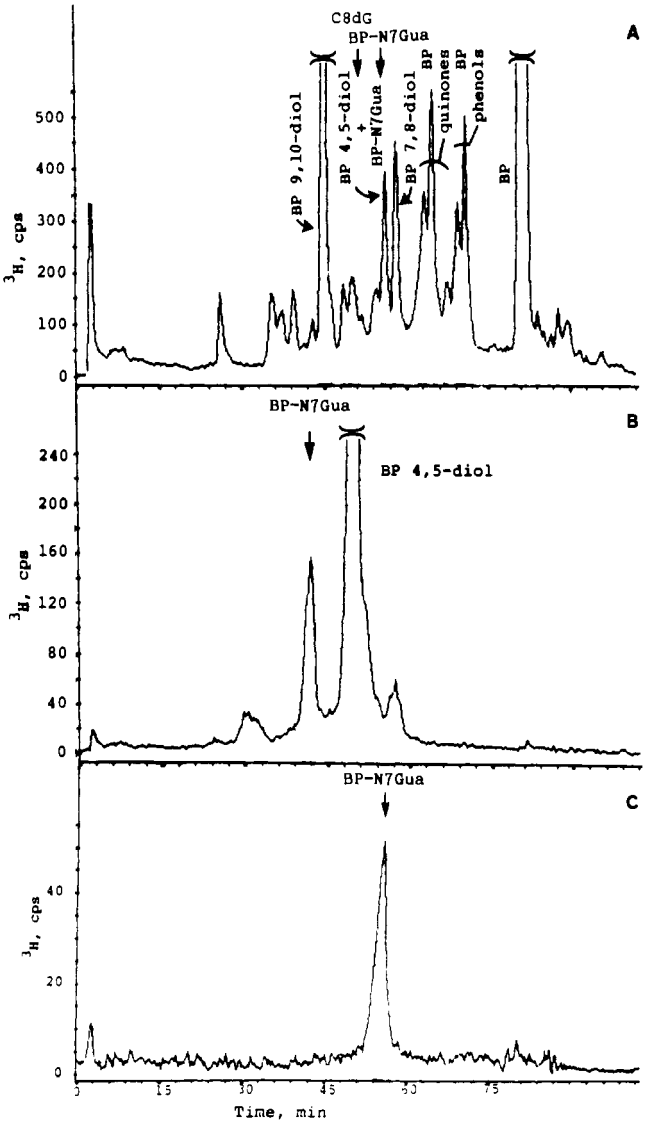


FIGURE 1: (A) HPLC profile of the supernatant from NADPH-supported binding of BP to DNA by MC-induced microsomes. The supernatant was cochromatographed with BP–N7Gua as a UV marker by using the CH_3OH/H_2O gradient. (B) Rechromatography of the BP 4,5-dihydrodiol peak in (A) using the CH_3CN/H_2O gradient. (C) Rechromatography of the BP–N7Gua peak in (B) using the CH_3OH/H_2O gradient.

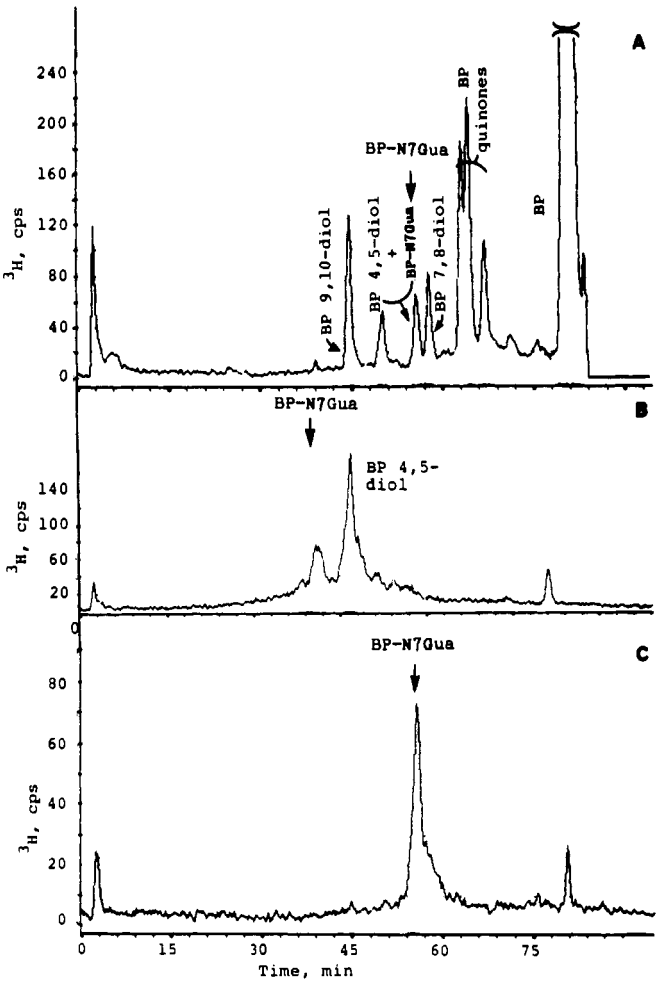


FIGURE 2: (A) HPLC profile of the supernatant from CuOOH-supported binding of BP to DNA by uninduced microsomes. The supernatant was cochromatographed with BP–N7Gua as a UV marker by using the CH_3OH/H_2O gradient. (B) Rechromatography of the BP 4,5-dihydrodiol peak in (A) using the CH_3CN/H_2O gradient. (C) Rechromatography of the BP–N7Gua peak in (B) using the CH_3OH/H_2O gradient.

fragment ion distribution observed in the MS/MS spectra of the adduct formed with either CuOOH (Figure 4B) or NADPH (not shown) compares favorably with the MS/MS spectrum obtained for authentic BP–N7Gua (Figure 4A). The MS/MS spectrum is also consistent with that of BP–N7Gua isolated from the binding of BP to DNA catalyzed by HRP (Rogan et al., 1988).

[^{3}H]BP was bound to the DNA of MC-induced nuclei in the NADPH-supported reaction (Table I). Insignificant binding of BP to DNA was observed for uninduced nuclei plus NADPH and for uninduced or MC-induced nuclei with CuOOH. When the supernatant fraction from MC-induced nuclei and NADPH was analyzed by using HPLC, it was found that the amounts of BP 4,5-dihydrodiol and BP 7,8-dihydrodiol formed by the nuclei were relatively small compared to the amounts formed when microsomes were used. Conversely, the amount of phenols detected for the nuclei experiments was relatively large (Figure 3A). BP–N7Gua was isolated by further HPLC in a manner analogous to the procedure described for the microsomal incubations (Figure 3B,C). Because of the relatively small amount of BP–N7Gua obtained from nuclei, an aliquot of the isolated fraction containing approximately 20 pg of BP–N7Gua was analyzed by using fluorescence line-narrowed spectrometry. With this technique the fluorescence spectrum is characteristic of the adduct. Therefore, comparison with the authentic sample allows unequivocal identification. The BP–N7Gua adduct was detected, thus confirming formation of this adduct by nuclei (Zamzow et al., 1989).

Table II: Relative Amounts of Stable BP–DNA Adducts and BP–N7Gua[a]

binding system	ratio of BP-N7Gua/ stable adducts
microsomes + NADPH	1.4:1
microsomes + 2 mM CuOOH	2:1
nuclei + NADPH	9:1

[a] Calculated from values presented in Table I for complete incubations with MC-induced microsomes and nuclei.

Since the depurinated BP–N7Gua adduct is obtained in both microsomal and nuclear preparations, it is of interest to compare the relative amounts of this labile BP–DNA adduct to the amounts of stable adducts. In such a comparison (Table II), formation of BP–N7Gua is clearly seen to be the predominant process in the binding of BP to DNA by nuclei with NADPH and by microsomes with either NADPH or CuOOH.

Table III: Effect of Cysteine on BP Metabolism, DNA Binding, and BP–N7Gua Formation[a]

	total metabolites (nmol)	binding to DNA (μmol of BP/mol of DNA-P)	BP–N7Gua (μmol/mol of DNA-P)
complete	153	75	114
12× cysteine	125 (18)[b]	71 (5)	114 (0)
60× cysteine	98 (36)	67 (11)	86 (25)

[a] Incubations were conducted with MC-induced microsomes and NADPH. Amounts are calculated on the basis of 5-mL reaction mixtures (400 nmol of BP). The amounts of cysteine are presented relative to BP. [b] Numbers in parentheses indicate percent inhibition.

Inhibitory Effect of DPEA on Metabolism and Binding of BP to DNA. To demonstrate that the formation of the BP–N7Gua adduct was catalyzed by cytochrome P-450, incubations were carried out in the presence of DPEA, a specific inhibitor of cytochrome P-450 (Table I). With NADPH-supported uninduced microsomes, equimolar amounts of DPEA and BP resulted in an 81% inhibition of total metabolism, a 60% inhibition of BP binding to DNA, and a 78% inhibition of the formation of BP–N7Gua. With levels of DPEA 10 times greater than those of BP, the metabolism and binding of BP to DNA were inhibited even more, and BP–N7Gua could not be detected. With MC-induced microsomes and equimolar amounts of DPEA and BP, metabolism was only slightly inhibited (8%), and the levels of binding of BP to DNA and BP–N7Gua adduct formation were inhibited by approximately 40%. When a 10-fold excess of DPEA to BP was used, metabolism was inhibited by approximately 85%, and no BP–N7Gua was detected. With CuOOH as the cofactor and equimolar amounts of DPEA and BP, similar reductions in total metabolism and BP–N7Gua were observed with both uninduced and MC-induced microsomes. This is expected because CuOOH selects a constitutive isozyme of cytochrome P-450 (Cavalieri et al., 1987). Addition of a 10-fold excess of DPEA to BP resulted in almost quantitative inhibition of both metabolism and formation of the labile adduct. The binding of BP to DNA in the CuOOH-supported reactions appeared to be only slightly reduced by DPEA, although the low levels of binding make comparison to the uninhibited reactions more difficult.

With MC-induced nuclei, only slight inhibitions of BP metabolism, binding of BP to DNA, and formation of BP–N7Gua were seen at 1:1 levels of DPEA and BP. Almost total inhibition of all three parameters was observed at 10:1 levels of DPEA to BP.

Effect of Cysteine, Glutathione, and p-Methoxythiophenol on BP Metabolism and Binding of BP to DNA. The effects of antioxidants on one-electron oxidation were investigated in the cytochrome P-450 catalyzed BP metabolism and binding of BP to DNA by using two natural thiols and one synthetic

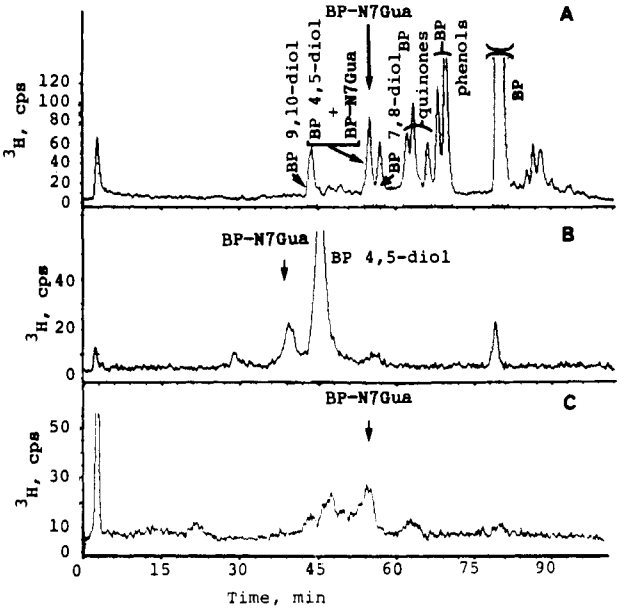


FIGURE 3: (A) HPLC profile of the supernatant from NADPH-supported binding of BP to DNA in MC-induced nuclei. The supernatant was cochromatographed with BP–N7Gua as a UV marker by using the CH_3OH/H_2O gradient. (B) Rechromatography of the BP 4,5-dihydrodiol peak in (A) using the CH_3CN/H_2O gradient. (C) Rechromatography of the BP–N7Gua peak in (B) using the CH_3OH/H_2O gradient.

thiol. When incubations were conducted with MC-induced microsomes, NADPH, and 12- or 60-fold excess amounts of cysteine, insignificant inhibition of BP metabolism and binding was observed (Table III). When a similar experiment was conducted with MC-induced microsomes and four levels of glutathione (Table IV), however, a dose response in the level of inhibition of total metabolism and binding of BP to DNA was observed, although the relative inhibition of binding exceeded the inhibition of metabolism. Maximum inhibition of formation of BP–N7Gua appeared to occur with only 6-fold excess glutathione, but the significance of this result is questionable because the amount of isolated BP–N7Gua is small. With MC-induced nuclei, reductions in metabolism and the levels of binding of BP are insignificant. Again, the dose–response relationship of the inhibition of formation of BP–N7Gua is questionable.

When the effect of 60-fold excess of glutathione on formation of stable BP–DNA adducts by MC-induced microsomes (corresponding to the measurement of binding of BP to DNA) was analyzed by using ^{32}P postlabeling, the level of various adducts detected was inhibited about 60% (data not shown). This result parallels the value reported in Table IV with 60-fold excess glutathione for overall binding (58% inhibition). When the same experiment was conducted with MC-induced nuclei, no inhibition of adduct formation was

Table IV: Inhibition of BP Metabolism, DNA Binding, and BP–N7Gua Formation by Glutathione[a]

	MC-induced microsomes			MC-induced nuclei		
sample	total metabolites (nmol)	binding to DNA (μmol of BP/mol of DNA-P)	BP-N7Gua (μmol/mol of DNA-P)	total metabolites (nmol)	binding to DNA (μmol of BP/mol of DNA-P)	BP-N7Gua (μmol/mol of DNA-P)
no inhibitor	23	60	50	8	9	128
1× glutathione	23 (0)[b]	61 (0)	46 (9)	7 (12)	7 (22)	106 (17)
6× glutathione	22 (4)	47 (22)	20 (60)	8 (0)	7 (22)	72 (44)
12× glutathione	18 (22)	39 (35)	18 (64)	7 (12)	7 (22)	61 (52)
60× glutathione	15 (35)	25 (58)	16 (67)	7 (12)	6 (33)	44 (65)

[a] Amounts are calculated on the basis of 5-mL reaction mixtures (400 nmol of BP) for microsomes and 2-mL reaction mixtures (100 nmol of BP) for nuclei. The amounts of glutathione are presented relative to BP. [b] Numbers in parentheses indicate percent inhibition.

Table V: Inhibition of BP Metabolism, DNA Binding, and BP–N7Gua Formation by *p*-Methoxythiophenol[a]

	MC-induced microsomes			MC-induced nuclei		
sample	total metabolites (nmol)	binding to DNA (μmol of BP/mol of DNA-P)	BP-N7Gua (μmol/mol of DNA-P)	total metabolites (nmol)	binding to DNA (μmol of BP/mol of DNA-P)	BP-N7Gua (μmol/mol of DNA-P)
no inhibitor	20	60	22	9	12	122
1× MTP	19 (5)[b]	44 (27)	21 (6)	9 (0)	7 (42)	106 (14)
6× MTP	19 (5)	20 (67)	15 (32)	8 (12)	6 (50)	72 (41)
12× MTP	16 (20)	9 (85)	11 (52)	7 (22)	4 (67)	56 (55)
60× MTP	12 (40)	2 (97)	9 (61)	6 (33)	2 (83)	39 (68)

[a] Amounts are calculated on the basis of 5-mL reaction mixtures (400 nmol of BP) for microsomes and 2-mL reaction mixtures (100 nmol of BP) for nuclei. The amounts of *p*-methoxythiophenol are presented relative to BP. [b] Numbers in parentheses indicate percent inhibition.

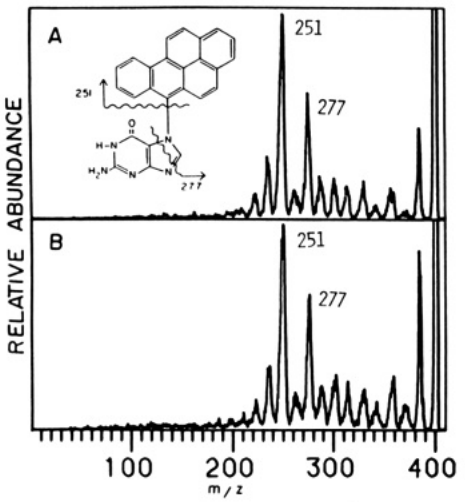


FIGURE 4: FAB MS/MS spectra of $(M + H)^+$ ion of m/z 402 for (A) an authentic sample of 2 μg of BP–N7Gua and (B) BP–N7Gua (1.5 μg) isolated from the CHP-supported binding of BP to DNA by uninduced microsomes.

observed by using ^{32}P postlabeling (data not shown).

When the antioxidant *p*-methoxythiophenol was used (Table V), a dose–response inhibition was observed with MC-induced microsomes and in MC-induced nuclei, although inhibition of metabolism was less effective than inhibition of binding. The dramatic inhibition of binding by 60× *p*-methoxythiophenol was confirmed by ^{32}P postlabeling, results that revealed about 85% inhibition of all stable adducts detected in nuclei (Figure 5) and about 95% inhibition of all stable adducts with microsomes (data not shown).

Discussion

When BP is bound to DNA by rat liver microsomes supported by NADPH or CuOOH, the labile adduct BP–N7Gua is formed (Figures 1, 2, and 4). With NADPH-supported uninduced and MC-induced microsomes, the levels of binding of BP to DNA and the amount of BP–N7Gua adduct formed are greater than with uninduced and MC-induced microsomal preparations supported by CuOOH (Table I). Furthermore, with NADPH, the level of binding and amount of BP–N7Gua formed are larger with MC-induced microsomes than with uninduced microsomes. These results contrast with those using CuOOH in which the levels of metabolism, the binding of BP to DNA, and the extent of BP–N7Gua adduct formation are similar with both uninduced and MC-induced microsomes. This is a consequence of the selection by CuOOH of a constitutive isozyme that catalyzes metabolism (Cavalieri et al., 1987) and binding of BP to DNA.

With nuclei, the binding of BP to DNA and formation of BP–N7Gua could be observed only with MC-induced nuclei supported by NADPH (Figure 3).

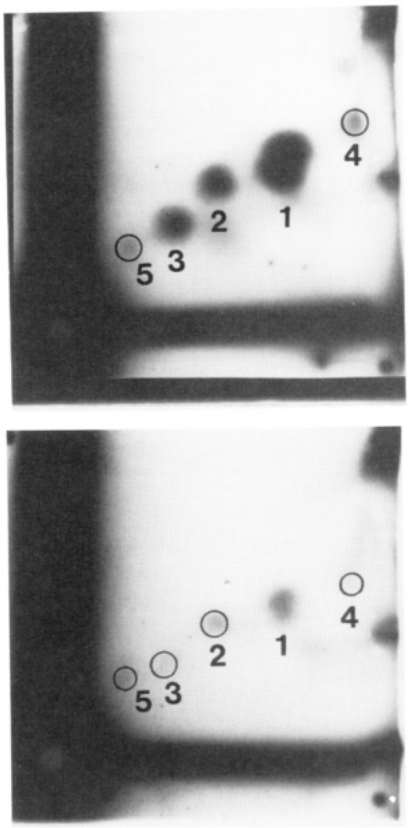


FIGURE 5: Autoradiogram of ^{32}P-postlabeled BP–DNA from MC-induced nuclei. The DNA was chromatographed as previously described (Bodell et al., 1989), and the film was exposed at room temperature for 18 h. (Top) Control incubation of BP, nuclei, and NADPH. (Bottom) Incubation of BP, nuclei, NADPH, and 50 mM (60×) *p*-methoxythiophenol.

In both rat liver microsomes and nuclei, the large ratio of the isolated BP–N7Gua to stable BP–DNA adducts (Table II) suggests that BP is primarily bound to DNA by the one-electron oxidation mechanism. The ratios presented in Table II are conservative because the value for BP–N7Gua represents the amount of isolated adduct, which is considerably less than the amount formed. This is because two consecutive HPLCs are required and recovery of BP–N7Gua from HPLC is particularly poor (Rogan et al., 1988).

The inhibitory effect of DPEA, a specific cytochrome P-450 inhibitor, on BP metabolism and binding of BP to DNA as well as in formation of the BP–N7Gua adduct provides evidence that cytochrome P-450 is involved in these three processes (Table I). Cysteine was ineffective in preventing BP metabolism and binding of BP to DNA by microsomes (Table III). In dose–response studies, glutathione effectively inhibited binding of BP to DNA, formation of BP–N7Gua, and, to a lesser extent, metabolism of BP (Table IV). No inhibitory

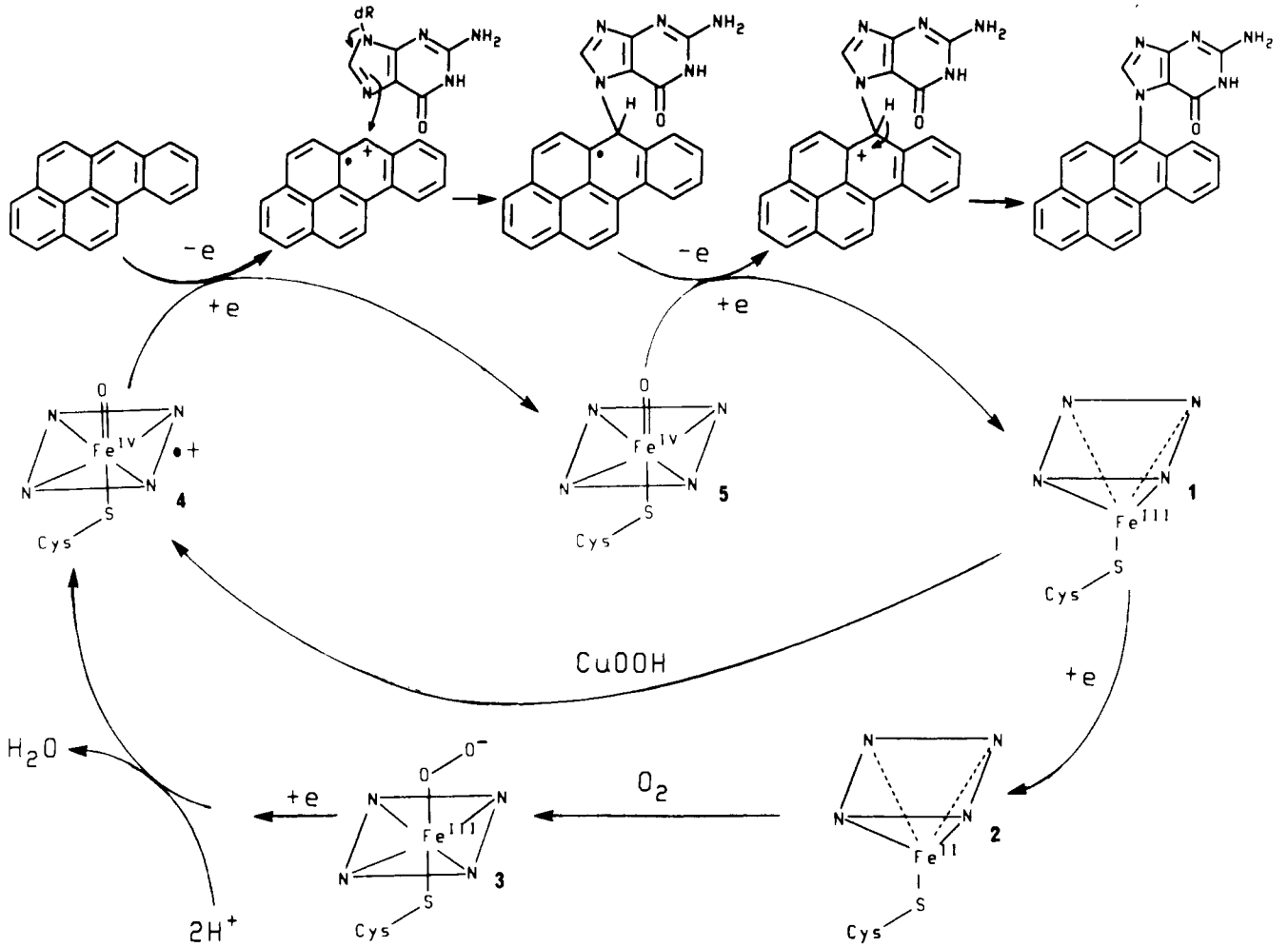


FIGURE 6: Catalytic cycles of cytochrome P-450 via the NADPH-supported reaction (**1** → **2** → **3** → **4**) and the CuOOH-supported reaction (**1** → **4**) with concomitant formation of BP–N7Gua via an electron-transfer reaction.

effect of glutathione on BP metabolism or binding was observed in the nuclei experiments (Table IV).

With *p*-methoxythiophenol, the inhibition of BP metabolism and binding was efficient for both rat liver microsomes and nuclei (Table V). The extent of inhibition of BP binding was greater than the inhibition of metabolism. Although inhibitory effects on metabolism and binding were observed with both glutathione and *p*-methoxythiophenol, these results have not advanced our understanding of cytochrome P-450 catalyzed metabolism and binding of BP to DNA.

Formation of BP quinone metabolites occurs via an initial electron transfer from BP to cytochrome P-450 (Cavalieri et al., 1988). Therefore, the reaction of the BP radical cation, obtained by one-electron oxidation of BP, with DNA may be preceded by diffusion of this reactive intermediate from the substrate binding pocket deep within the enzyme (Poulos et al., 1985; Poulos, 1986). This process seems rather unlikely for binding of BP to DNA for two reasons: (1) the rate of the oxygen rebound mechanism to form metabolites (Ortiz de Montellano & Stearns, 1987) could compete successfully with the rate of diffusion and, more importantly, (2) the BP radical cation would react with other nucleophiles before reaching DNA.

A more plausible hypothesis is the formation of a preliminary physical complex between BP and DNA, followed by one-electron oxidation of BP catalyzed by cytochrome P-450 and covalent binding of the BP radical cation to DNA. In this case, electron transfer from BP would be more likely to occur at an exposed heme edge of the cytochrome P-450. This is consistent with the hypothesis that the active form of cytochrome P-450 is an Fe^{IV} radical cation (Lowe et al., 1986; Dawson, 1988; Champion, 1989) with the charge delocalized on the periphery of the heme (**4**, Figure 6). The hypothetical intermediate **4** is formed via two pathways, one supported by NADPH and one by CuOOH. In the NADPH-supported pathway, the high-spin five-coordinate Fe^{III} derivative (**1**, Figure 6) is reduced to the ferrous state **2**, which, by addition of dioxygen, produces an oxycytochrome P-450 intermediate **3**. Two further unidentified steps, which involve cleavage of the O–O bond with formation of H_2O, lead to the hypothetical intermediate **4**. This intermediate can also be obtained directly from **1** in one step by using CuOOH instead of NADPH. This is the alternative peroxide shunt pathway.

Formation of the BP–N7Gua adduct would occur by transfer of one electron from BP to intermediate **4**, resulting in the formation of BP radical cation and, concomitantly, the Fe^{IV}–oxo group **5**. Nucleophilic attack of the N-7 of the guanine moiety in DNA at the charge-localized C-6 position of the BP radical cation follows (see the introduction). The N-7–C-6 bond would destabilize the glycosidic link; as a result the BP–N7Gua adduct depurinates. Further reduction of **5** to high-spin Fe^{III} **1** produces an arenium ion which, by loss of a proton, would yield the BP–N7Gua adduct.

This study represents the first demonstration that cytochrome P-450 catalyzes covalent binding of substrates to DNA by one-electron oxidation. On the basis of these results, we think that cytochrome P-450 catalyzes not only oxygen-transfer reactions, as commonly recognized, but also electron transfers

characteristic of peroxidase-type reactions.

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