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Determination of Genotoxicity Using a Chloroperoxidase-Mediated Model of PAH-DNA Adduct Formation

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In the past few decades, the oil industry has grown considerably and many sites near refineries and oil pipelines have become contaminated with hydrocarbons. Polycyclic aromatic hydrocarbons (PAH's) are a major class of polluting compounds that pose a potential health risk because of their possible mutagenic and carcinogenic activities (Thakker *et al* 1985).

Enzymes from cytochrome P450 superfamily, which are components of monooxygenase systems, are thought to play a major role in carcinogenic activation. The physicochemical properties of PAH's and the catalytic properties of cytochrome P450 suggest that PAH's are generally activated by two major mechanisms: One-electron oxidation with formation of radical cations and monooxygenation (two-electron oxidation) to yield bay-region diol epoxides (Devanesan *et al.* 1992; Wellemans *et al* 1994). Hemoproteins such as peroxidases, cytochromes, and hemoglobin are able to catalyze PAH's oxidation *in vitro* (Vazquez-Duhalt *et al.* 1994; Torres *et al.* 1995; Ortiz-Leon *et al.* 1995*).* The reaction products are mainly quinones and the mechanism involves free radical formation. Chloroperoxidase (CPO) from *Caldaryomyces fumago,* an enzyme with three different catalytic activities (halogenase, peroxidase and catalase), catalyzes phenol oxidation (Carmichael *et al.* 1985). On the other hand, chloroperoxidase shows close structural (Dawson 1988) and catalytic similarities with the cytochrome P450 (Sundaramoorthy *et al.* 1995). The similarities of both proteins could help to provide a better understanding of the biological activities and structure of cytochrome P450 *in vivo*. The primary activation of PAH's via one-electron by cytochrome P450 could be similar to the PAH-DNA binding mediated by chloroperoxidase.

Biomonitoring of chemical xenobiotics in the environment is an imperative activity; however, no conclusive methods exist to evaluate the genotoxicity of specific carcinogens or mutagens. DNA-adduction detected by postlabelling radiotechnique has been used for human biomonitoring (Hemminki 1995) and for environmental samples using microsomal preparations (Wang *et al.* 1990). The postlabelling assay that uses radiolabeled products is laborious and is not applicable to measurements in the field or modestly-equipped laboratories. Peroxidase activity on polluting compounds, such as PAH's, can be used to detect genotoxicity in environmental samples. Because of structural and catalytic similarities between cytochrome P450 and chloroperoxidase from *Caldariomyces fumago,* we propose to use chloroperoxidase activity as an *in vitro* model to determine the genotoxic potential of PAH's in environmental samples.

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MATERIALS AND METHODS

Pure PAH's were purchased from Aldrich Chemical Co (Milwaukee, WI). HPLC-grade solvents, dichloromethane and dimethyl sulfoxide were obteined from Merck (Darmstadt, Germany). Purified chloroperoxidase from *Caldariomyces fumago,* was a gift from Prof. Michael A. Pickard of the University of Alberta, Canada. Horseradish peroxidase and cytochrome C were purchased from Sigma Chemical Co. (St. Louis, MO). Lignin peroxidase, partially purified, was obtained from Tienzyme, Inc. (State College, PA).

PAH-DNA binding was performed in a 1-mL reaction mixture containing 0.5 mg of DNA (calf thymus), 50 µg of PAH's, 4 nmole enzyme, and 0.4 mM H_2O_2, 5% dimethyl sulfoxide in the respective buffer for each enzyme. PAH's were dissolved in dimethyl sulfoxide and added to the above reaction mixture and incubated for 2 min at room temperature. For the chloroperoxidase assay a 60 mM acetate buffer (pH 3) was used. Cytochrome C and horseradish peroxidase reactions were carried out in a 60 mM phosphate buffer (pH 6.1), and for lignin peroxidase a 40 mM sodium succinate buffer (pH 4.5) was used (Torres *et al.* 1995). The reaction was stopped by the addition of 100 µL NaCl (24 g / L) and 50 µL of sodium dodecyl sulfate (10 %), followed by three times extraction with 1 mL of ethyl acetate in order to eliminate unreacted hydrocarbons. DNA was precipitated with isopropanol and then washed once with ethyl alcohol. DNA-PAH adducts were measured by fluorometry (Luminescence Spectrometer, Perkin-Elmer, model LS 50). Adduct formation was estimated by flourescence intensity at 417 nm using an excitation wavelength of 300 nm and the extent of adduction was calculated as the relative intensity obtained with an equivalent amount of benzo(a)pyrene. [^{14}C]Benzo[a]pyrene was used to determine the amount of B[a]P binding to DNA in the *in vitro* reaction. Each datum from PAH-DNA adduct assay (including airborne particulate) is the mean from five replicates and the standard deviation was calculated. Linear regression analysis was used to evaluate the relationship of B[a]P concentration and the B[a]P-DNA adduct formation.

Airborne particulate matter was collected from different locations of Cuernavaca City by the Environmental Chemistry Laboratory, State University of Morelos. Glass fiber filters (Whatman GS-A) were removed every 24 h and stored in the dark. The filters were extracted three times with dichloromethane. Organic extracts were pooled, dried under vacuum, and weighed. The organic extract (125 µg) dissolved in dimethylsulfoxide was used for PAH-DNA adduction assay. This extract was analyzed by gas chromatography-mass spectrometry (GC-MS). GC-MS was performed on a Hewlett-Packard GCMS model 6890 equipped with a mass spectrometry detector (HP5972).

Ames mutagenicity assay (Maron and Ames 1983) was carried out by incubating 100 µL of *Salmonella typhimurium* TA100 cultured 16 hr at 37°C, 100 µL of S9 mix (10 % of microsomal protein S9 fraction) and 100 µL of a PAH dissolved in dimethyl sulfoxide for 30 min at 37°C. The incubated mixture was added to the agar and the revertant colonies were scored after 48 hr incubation at 37°C. Microsomal enzymes for the metabolic activation (S9) were obtained from rats (Sprague-Dawley) induced with Aroclor 1254. Positive mutagenicity was defined as having at least a twofold increase in reversion frequency of the negative control in a dose-response relationship and each datum is the mean ± standard deviation (SD) of the values of 8 replicate plates with statistical significance for Student-t test ($p<0.05$) with respect to the negative control.

Microtox tests assess acute toxicity by measuring the reduction of light emission produced by *Photobacterium phosphoreum,* in response to exposure to toxicants. PAH's were dissolved in dimethyl sulfoxide and 20 µL of these solutions were added to the total assay volume of 2.75 mL. EC_{50} values were obtained by testing successive dilutions of the initial concentration of each PAH's (170 µg/mL). The assays were performed with a Microtox Model 2055 instrument according to the procedure described in the manual (Microbics, Corp., Carlsbad, CA). Data were statistically analyzed according to the Microbics manual. The mean and the SD were calculated from 10 replicates.

RESULTS AND DISCUSSION

Four hemoproteins were assayed as biocatalyst for DNA adduction with B[a]P. We have detected B[a]P-DNA adduct formation with the activity of all the hemoproteins tested; chloroperoxidase, cytochrome C, horseradish peroxidase, and lignin peroxidase (Table 1). B[a]P-DNA adduct produced with all biocatalysts showed the same excitation (λ max 300 nm) and emission (λ max 417 nm) spectra, with a shoulder at 438 nm. Chloroperoxidase (CPO) was more active in binding PAH's to DNA than the other hemoproteins. This could be attributed to its catalytic and structural similarities with cytochrome P450 (Dawson 1988; Sundaramoorthy *et al.* 1995). Cytochrome P450 has at least three enzymatic activities (Sundaramoorthy *et* al. 1995) and it has been implicated in one-electron oxidation of several compounds (Cavalieri *et al.* 1983).

Strong evidence suggest that cytochrome P45Os catalyze *in vivo* PAH-DNA adduct formation through radical cation as intermediate (Devanesan *et al.* 1992). The one-electron oxidation with formation of radical cations is highly reactive (Cavalieri and Rogan 1983) and it seems to be the oxidation mechanism of chloroperoxidase (Okazaki and Guengerich 1993). Similar to cytochrome P450 (Devanessan *et al.* 1992), chloroperoxidase catalyzes the binding of B[a]P to DNA (Fig. la). In the enzymatic assay with chloroperoxidase no B[a]P-DNA adduct formation was detected when either enzyme or hydrogen peroxide were absent in the reaction (Fig. la). All the PAH-DNA adducts formed have identical fluorescence emission spectra, with a maximum emission wavelength at 417 nm, except perylene whose maximum emission was at 460 nm (Fig. lb). PAH-DNA adduct formation canalized by CPO is favored at low KCl concentrations (5 mM; Fig. 2). At high KCl concentrations (> 5 mM) the reaction was strongly inhibited. High levels of DNA adduction catalyzed by CPO were found without chlorine ions in the reaction mixture, suggesting that only the peroxidase activity of CPO is involved in the adduction.

Table 1 B[a]P-DNA adducts formed by different hemoproteins.[a]

Enzyme	Relative Intensity*
Chloroperoxidase	1.00 ± 0.10
Horseradish peroxidase	0.68 ± 0.05
Lignin peroxidase	0.14 ± 0.02
Cytochrome C	0.05 ± 0.02

[a]4 nmole of protein are used in all cases and emission intensity is reported relative to the chloroperoxidase signal. * (mean ± SD)

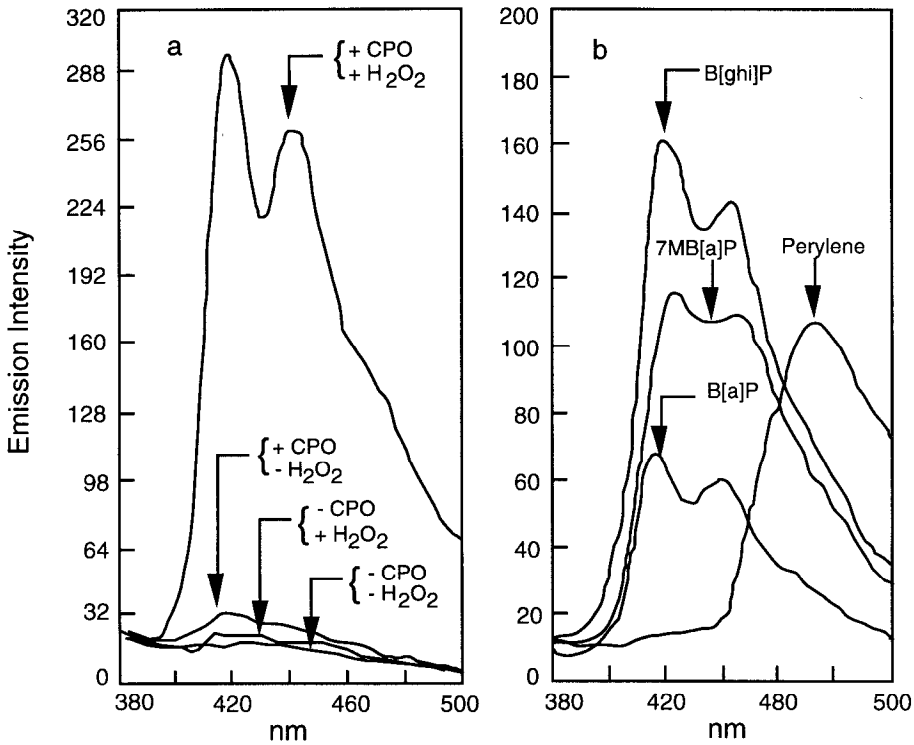

Figure 1. a) Emission spectra of B[a]P-DNA adducts formed: with both chloroperoxidae (CPO) and H_2O_2 { +, +}, without H_2O_2 { +, -}, without CPO {- , +} and without both {-, -}; b) Emission spectra of the PAH-DNA adducts formed; B[a]P (benzo[a]pyrene), 7MBP (7-methylbenzo[a]pyrene), B[ghi]P (benz[ghi]perylene).

B[a]P concentrations from 0 to 50 µg/ml in the reaction mixture showed a linear correlation (r = 0.97) with degree of adduct formation (Fig. 3). From quantitative data obtained by using [^{14}C]benzo[a]pyrene, we calculated that, 66.6 pmol B[a]P / nmol DNA (which corresponded to 1 adduct each 0.56 x 10^6 bases) were adducted under our reaction conditions. In agreement, data from Fig. 3 shows that 57.3 pmol B[a]P / nmol DNA were adducted when 50 µg/ml of non-labeled B[a]P were used. At concentrations higher than 50 µg/mL of non-labeled B[a]P, adduct formation was no longer linear.

Eight of 12 PAH's tested formed adducts (Table 2). PAH's with an ionization potential below 7.5 eV have formed adducts as in the case of 9,10-dimethylanthracene, benzo[a]pyrene, perylene, 1,2:5,6-dibenzanthracene, 7-methylbenzo[a]pyrene, 7,12-dimethylbenz[a]anthracene, and benz[ghi]perylene. These results provide evidence that chloroperoxidase-mediated binding of PAH to DNA occurs by one-electron oxidation.

The Microtox assay was used to evaluate the toxicity of PAH's with 3 to 5 aromatic rings and compared with the *in vitro* PAH-DNA adduct formation assay (Table 3). We found PAH's with 2 - 3 aromatic rings to be acutely toxic and poor toxicity was detected for PAH's with 4- 5 aromatic rings. These results contrast with those

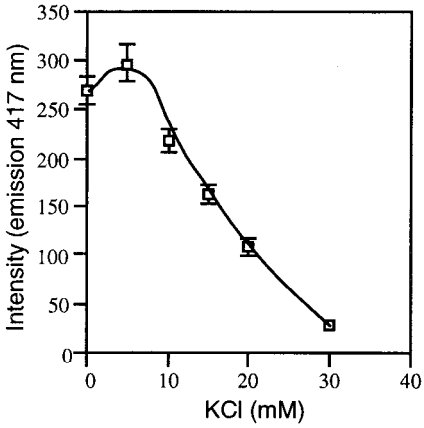

Figure 2. Effect of KCl concentration on B[a]P-DNA binding catalyzed by chloroperoxidase (mean ± SD).

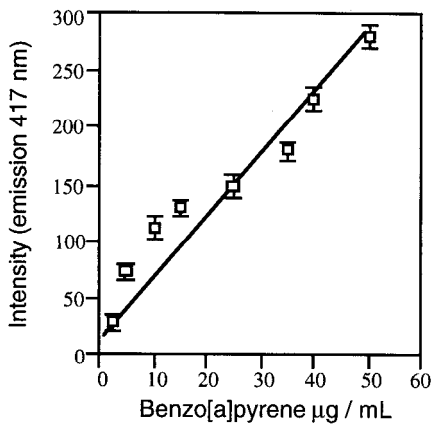

Figure 3. Effect of B[a]P concentration on B[a]P-DNA adduct formation mediated by chloroperoxidase (mean ± SD).

observed with the PAH-DNA adduction test, in which non-adduct formation was detected in PAH with 2 and 3 aromatic rings, while PAH's with 4 and 5 aromatic rings resulted in adduct formation (Table 2). One of the main factors for determining toxicity in a Microtox assay seems to be compound solubility or bioavailability. The Microtox assay is a microbial assay used for assessing the toxicity of complex wastes, the effectiveness of treatment of hazardous wastes, and it has recently extended to assessment of treatability of hazardous organic compounds in soil (Symons and Sims 1988). However, no single bioassay procedure can provide a comprehensive toxicity evaluation of a complex mixture such as oil. Due to mass transfer limitations and the lack of metabolic activation Microtox toxicity assay should be used cautiously to evaluate the toxicity of the PAH's.

Table 2. Relationship between Ionization Potential (IP) and DNA -Adduct Formed by chloroperoxidase.

PAH	IP (eV)[A]	Enzymatic genotoxicity (pmol BP / nmol DNA)[B]
Naphthalene	8.25[a]	NA[C]
Chrysene	8.01[a]	NA
Anthracene	7.55[a]	NA
2-Methylanthracene	7.42[a]	NA
Pyrene	7.72[a]	14.3 ± 1.6
9, 10- Dimethylanthracene	7.14[c]	34.4 ± 2.0
1,2:5,6-Dibenzanthracene	7.59[a]	60.0 ± 5.0
Benzo[a]pyrene	7.12[a]	57.3 ± 4.0
Perylene	7.10[a]	91.7 ± 6.0
7-Methylbenz[a]pyrene	7.17[c]	105.9 ± 5.0
7, 12- Dimethylbenz[a]anthracene	7.22[c]	114.6 ± 5.3
Benz[ghi]perylene	7.15[b]	149.0 ± 7.0

[A] Ionization potential (IP) from: [a] Rosenstock *et al* 1977; [b] Rhoda, Lias 1982; [c] Cavalieri *et al* 1983. [B] Adducts formed relative to B[a]P units. [C] (NA) No adduction detected, no significant difference with respect to the control (without PAH's)

Table 3 also shows the mutagenic activity of PAH's tested in *Salmonella typhimurium* TA100 in the presence of S9 fraction (Ames test). No mutagenic response was detected with 2-3 ringed aromatic hydrocarbons at concentrations as high as 333.3 µg/mL. On the opposite, 7,12-dimethylbenz[a]anthracene showed a mutagenic response at 1 µg/mL. With the aim of generating comparative data, results shown in table 3 were obtained by using a PAH's concentration of 3.3 µg/mL, in all the cases. These results of specific mutagenic activity are consistents with those obtained from chloroperoxidase-catalyzed PAH-DNA adduction (Table 2).

Enzymatic genotoxicity assay may be used for different mixtures of PAH's and crude oil fractions as well. PAH-DNA adducts were formed with a hydrocarbon mixture enriched with 20 µg/mL of B[a]P and a fluorescent intensity of 143 ± 5 was observed, in comparison of an intensity of 137 obtained from B[a]P alone in the reaction. Thus, there is no interaction with a hydrocarbon complex mixture, if it has at least one genotoxic compound such as B[a]P. Airborne particulates from urban aerosols were tested for PAH-DNA adduct formation (Table 4). Five of ten samples were able to form PAH-DNA adducts (in which the presence of at least one PAH-adduct forming compound was detected by mass spectometry). The PAH-DNA adduct formation reaction catalyzed by chloroperoxidase is specific and is not affected by mixture of hydrocarbons and has a resolution as low as 2.5 µg/mL of B[a]P compared to 3.3 µg/mL of B[a]P in the Ames assay.

Table 3. Microtox and Ames assay results for different polycyclic aromatic hydrocarbons

PAH	Aromatic rings	Microtox EC_{50} (µg / ml)*	Ames test [a] (revertants colonies)*
Naphthalene	2	0.71 ± 0.03	- [c]
Anthracene	3	7.39 ± 0.61	-
1-Methylanthracene	3	4.65 ± 0.39	-
9-Methylanthracene	3	5.86 ± 0.89	-
Pyrene	4	47.91 ± 3.13	ND[b]
7,12-Dimethylbenz[a]anthracene	4	65.33 ± 6.50[d]	689 ± 24.3
Benzo[a]pyrene	5	58.49 ± 7.04	618 ± 29.8
Perylene	5	32.78 ± 3.75	ND[b]
7-Methylbenz[a]pyrene	5	42.90 ± 4.51	608 ± 20.6

a) The reaction mixture contained 3.3 µg/mL of PAH; b) ND: no determined; c) negative sign means no significative response with respect to the control. * (all data are the mean ± SD); d) data of two replicates.

In conclusion, the chloroperoxidase-mediated genotoxicity assay is a rapid and accurate technique for determining the carcinogenic potential of environmental samples. Because the detection method is by fluorescent intensity and not by radiotechniques it could easily be applied in the field or non-sophisticated laboratories. The genotoxic potential of enviromental samples can be expressed as equivalents (pmol B[a]P / nmol DNA ; pmol B [a]P / mM DNA; or more specifically No. Adduct formed / No. bases of DNA) of B[a]P.

Table 4. DNA-adducts obtained with airborne particulate samples

Sample No.	Adduct (pmol B[a]P / nmol DNA)[a]
102	N A[b]
109	NA
232	9.2
234	NA
235	NA
236	NA
237	7.8
244	6.2
248	6.2
251	4.2

[a)] Adducts formed relative to B[a]P units;

[b)] no adduction detected

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