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Effects of Ozonation on Mutagenic Activity of Polycyclic Aromatic Hydrocarbons

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Polycyclic aromatic hydrocarbons (**PAH**) represent a broad class of pollutants which are widely distributed in the environment (Neff 1979; Nikolaou *et al.* 1984) .These aromatic hydrocarbons are generated by incomplete combustion processes: many of them have been shown to possess mutagenic and carcinogenic activities (I.A.R.C. 1983). Therefore studies were undertaken in order to test the efficacy of various techniques to get rid of PAH from water and laboratory wastes (Castegnaro et *al.* 1983).

However, as far as water is concerned, problems may arise during the decontamination process itself as, for instance, chlorination was shown to produce mutagenic halogenated hydrocarbons like trihalomethane and chloramine (Shih and Lederberg 1976) .Therefore Burleson *et al* (1979) have tried ozonation of water in order to eliminate PAH such as Benzo(a)pyrene, 3-methylcholanthrene and 7,12-dimethylbenzo(a)-anthracene and measured its mutagenic activity before and after the ozone treatment.They found that mutagenicity from the above three PAH was rapidly destroyed after a short period of ozonation.

In our study, four polycyclic aromatic hydrocarbons were tested. Benzo(a)pyrene (**B(a)P**), Chrysene (**CH**), 7,12-dimethylbenzo(a)-anthracene (**DMBA**) and 3-methylcholanthrene (**MCA**) in hexane were treated with ozone to determine the effectiveness of degradation and to evaluate the genetic properties of ozone byproducts. Two types of ozonation were carried out: partial ozonation and total ozonation. The disappearance of parent compounds and the appearance of ozone byproducts were measured by high performance liquid chromatography (**HPLC**) coupled with spectrofluorimetry and U.V. spectrophotometry. Plate incorporation mutagenicity assay, using a *Salmonella typhimurium* strain, was used to test the ozone byproducts with and without metabolic activation.

MATERIALS AND METHODS

Polycyclic aromatic hydrocarbons, B(a)P, MCA,(Fluka A.G. chem.), DMBA, CH (Eastman Kodak) were solubilized in hexane (Merck

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analytical grade) at a concentration of 50 mg/L. Methanol was HPLC grade and water was distilled in glass.

Analysis of polycyclic aromatic hydrocarbons was performed by HPLC (Kontron) using a reverse phase spherisorb ODS2 column (Chrompack) with particle size of 5µm (25 cm x 4.6 mm). The proper isocratic eluent system was developed for each PAH: methanol/water (98:2) was used for B(a)P, MCA, DMBA and (90:10) for CH. Elution was carried out at a flow rate of 1 mL/min. . Under these conditions, the retention times obtained were as follows: MCA (7.2 min.), DMBA (8.2 min.), CH (8.8 min.) and B(a)P (10.8 min.). The eluted PAH were detected with a U.V. spectrophotometer at 254 nm and a spectrofluorimeter at variable wavelengths (Table 1).

Table 1. Spectral data of PAH

PAH	Excitation wavelength nm	Emission wavelength nm
Benzo(a)pyrene	**382**	**405**
Chrysene	**260**	**385**
7,12-dimethyl-benzo(a)anthracene	**296**	**430**
3-methylcholanthrene	**360**	**420**

Ozone was obtained from a Trailigaz air-fed unit, Labo 76 type, with a production rate of 10 to 15 mg O_3/liter of air. A 100 mL sample of PAH solution was ozonized in a bubble chamber. For each PAH , two types of ozonation were tested : total ozonation (over 99%) and partial ozonation at about 50%. (Table 2)

After 99.9% ozonation, the remaining traces of PAH were shown not to give a positive test. The ozonated solutions were concentrated to about 1 mL under reduced pressure using a rotary evaporator, then evaporated to dryness under continuous nitrogen flow. The residue was dissolved into 1.25 mL DMSO.
After ozonation at about 50%, separation of ozone byproducts was achieved with one of the following methods according to the type of PAH:
1) Separation by elution on silica gel (Merck SI 60 , 65-70 mesh) column was used for DMBA, MCA and CH byproducts. Two successive elutions were carried out, one by cyclohexane and the other by methanol. The methanol fraction was concentrated to about 1 mL under reduced pressure using a rotary evaporator ,then evaporated to dryness under continuous nitrogen flow.The residue was dissolved into 1.25 mL of DMSO.

2) Separation on semi-preparative HPLC system using a reverse phase spherisorb ODS2 column with particle size of 5 µm (30 cm x 7.5 mm i.d.) was used for B(a)P byproducts . The isocratic eluent was methanol (100%). Elution was carried out at a flow rate of 3 mL/min.. Under these conditions, the polar fraction (ozone byproducts) was eluted in 12 min.. This fraction was concentrated to about 1 mL using a vacuum rotary evaporator and then evaporated to dryness under continuous nitrogen flow. The residue was dissolved into DMSO (1.25 mL).

Table 2. Ozonation of PAH

PAH	Ozone (mg)	% of degradation
Benzo(a)pyrene	**1**	**50.0**
	10	**99.95**
Chrysene	**5**	**57.8**
	50	**99.90**
7,12-dimethyl-benzo(a)anthracene	**2**	**53.6**
	10	**99.95**
3-methylcholanthrene	**2**	**49.0**
	10	**99.89**

Salmonella typhimurium TA 100 histidine auxotroph strains were kindly provided by Dr B.N. Ames, University of California, Berkeley, USA, for use in the plate incorporation test , which detects reverse mutations from histidine dependence to histidine independence (Ames *et al.* 1975). Cultures were grown overnight from frozen stocks (-80°C) in nutrient broth. The presence of R factor in this strain was checked by seeding bacteria on agar containing ampicillin.
Cultures were checked for crystal violet sensitivity and mutability using methyl methane sulfonate (**MMS**) and amino anthracene (**AA**) in the presence of S9 metabolic activating system.; 0.1 µL MMS per plate induced 200-300 revertants; 2 µg AA in the presence of 50 µL S9 per plate induced 800-1000 revertants.
Adult male Sprague Dawley rats (≃ 200 g) (Iffa Credo L'arbresle , France) pretreated with Aroclor 1254 (500 mg/Kg) were used to obtain liver for the S9 metabolic activation system.
Five days after intraperitoneal injection of Aroclor, rats were killed and S9 was prepared and stocked at - 80°C. The S9 mix was prepared daily by adding to 0.1 mL S9, 33 µM KCl, 8 µM $MgCl_2$, 4 µM glucose-6-phosphate, 5 µM NADP and 100 µM sodium phosphate buffer (pH 7.4) per mL.

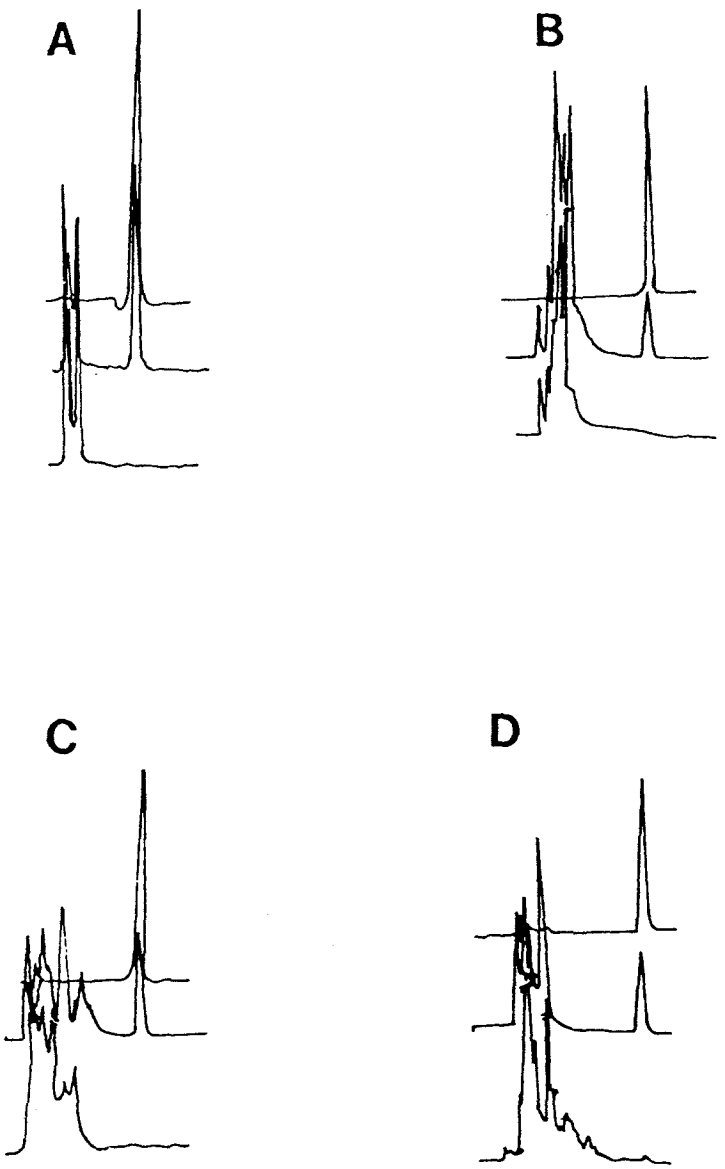

Figure.1. Top trace : HPLC-UV chromatogram of polycyclic aromatic hydrocarbon standard **(A) MCA, (B) CH, (C) DMBA, (D) B(a)P** . Middle trace : HPLC-UV chromatogram of reaction mixture after partial ozonation of corresponding PAH. Bottom trace : HPLC-UV chromatogram of reaction mixture after ozonation at about 99.9% of corresponding PAH.

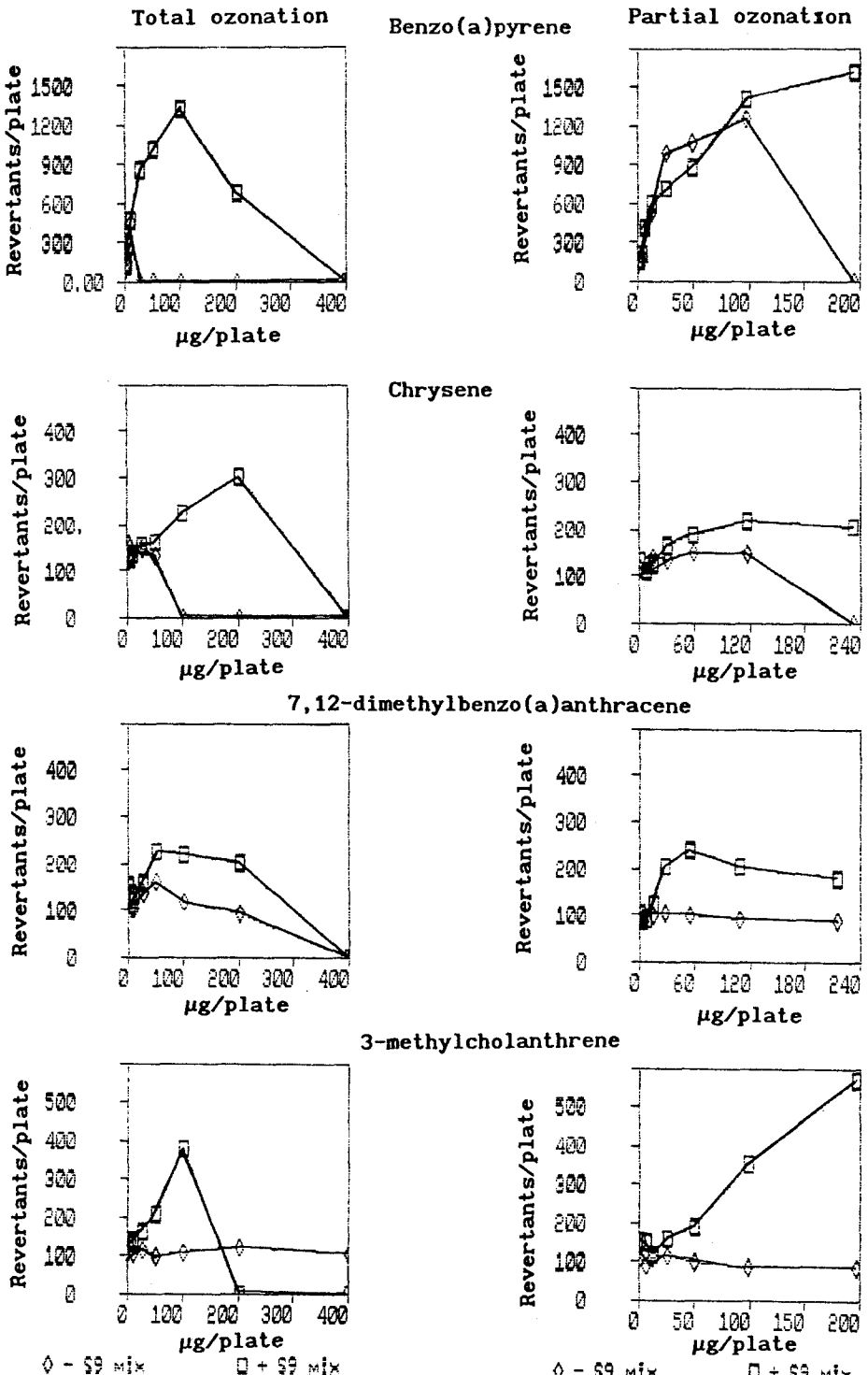

Figure 2. Dose-response curves of mutagenicity (revertants/plate) toward *Salmonella Typhimurium* TA 100 of PAH ozone byproducts (µg/plate).

The plate incorporation assay was carried out as described previously (Ames *et al.*1975; Maron and Ames 1983) . The assays were performed in duplicate for each concentration, with and without metabolic activation.

RESULTS AND DISCUSSION

Typical chromatograms of individual polycyclic aromatic hydrocarbons samples and corresponding ozonation by-products are shown in Figure 1.

As shown in Figure 2, under our conditions (see materials and methods), 7,12-dimethylbenzo(a)anthracene ozone by-products did not show mutagenic activity in the TA 100 strain. Revertants obtained were not twice the number of spontaneous revertants. On the contrary, ozonation of chrysene and 3-methylcholanthrene showed weak mutagenic activity with metabolic activation.

As for benzo(a)pyrene, immediately after partial and total ozonation , the ozone by-products of B(a)P show direct mutagenic activity (43 revertants/μg) which persists with metabolic activation at about 30 revertants per μg. However, when the mutagenicity test was done the day after, no mutagenic activity could be observed . Thus the lack of stability of PAH ozone by-products is clearly shown.
In all cases , the mutagenicity of by-products arising from partial ozonation appear to be weaker than that of by products from total ozonation.

Our results concerning benzo(a)pyrene ozonation are altogether in agreement with those from Pitts et al. (1980) who noted the development of direct mutagenic activity attributable to benzo(a)pyrene 4,5-oxide after ozonation of B(a)P. This work also shows that ozone treatment may represent an interesting alternative method for the destruction of PAH from water as chlorination was shown to produce carcinogenic halogenated hydrocarbons including chloroform and mutagenic chloramine (Shih and Lederberg 1976).

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