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Influence of Ozonation on the Mutagenic Activity of Benzidine in Water

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In 1895, Rehn, in Germany described four cases of bladder cancer in workers employed in the manufacture of magenta, a dye from aniline. This occupationnal disease has been associated with exposure to benzidine (I.A.R.C.1974).Since this time, benzidine has been shown mutagenic in a battery of tests and carcinogenic in experimental animal and in human (Mc Cann et al.1975, I.A.R.C. 1982, Zavon et al. 1973).

The presence of this compound in municipal and industrial effluents raises concern for public health. Part of the problem of carcinogens in the drinking water may be due to the water treatment process itself. Chlorination of water has been shown to cause formation of halogenated hydrocarbons including chloroform and weakly mutagenic chloramine (Shih and Lederberg 1976, Maruoka and Yamanaka 1980). As an alternative to chlorine, ozone has been used for disinfection and color-removal of drinking water, particularly in Europe, but the effect of ozonation on the carcinogenic potential of various contaminants of drinking water is poorly defined.

Burleson et al. 1979 and Caulfield et al. 1979 used ozonation to degrade benzidine and 2-naphthylamine in water . Ozonation of benzidine revealed a transient increase in mutagenicity which was lost after longer treatment with ozone..

In our study ,benzidine was treated with ozone to determine the effectiveness of degradation and the genetic properties of ozone byproducts. Several types of ozonation were studied , partial ozonation, ozonation at about 99.9% and total ozonation.Disappearance of parent compounds and appearance of ozone byproducts were measured by high performance liquid chromatography (HPLC) coupled with spectrofluorimetry and UV spectrophotometry.A plate incorporation mutagenicity assay using *Salmonella typhimurium* strain was used to test the ozone byproducts in presence and in absence of metabolic activation system.

MATERIALS AND METHODS

Benzidine (BZ) Fluka was solubilized in water at a concentration

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of 50 mg/L. When necessary, solubilization was completed by ultrasonication. Methanol and acetonitrile were HPLC grade and ethylacetate, K_2HPO_4, KH_2PO_4 were analytical grade.
Ozone was produced with a Trailigaz air-fed unit, model labo 76, with a production of 10 to 15 mg O_3/normoliter of air. 100 mL of aromatic amine solution were ozonated in a bubble chamber. Several ozonations were performed for benzidine (Table 1).

Table 1. Ozonation of aromatic amine

Aromatic amine	Ozone (mg)	% of degradation
Benzidine	10	59.2
	50	99.3
	100	100
	200	100
	400	100

Analysis of the aromatic amine was performed on a HPLC system (Kontron) using a reverse phase spherisorb ODS_2 column (Chrompack) with particle size of 5 µm (25 cm x 4.6 mm id.). The isocratic mobile phase developed for benzidine was: acetonitrile : methanol : 1.5 mM phosphate buffer, pH 7.4 (1:3:2). Elution was carried out at a flow rate of 0.4 mL/min.. Under these conditions, the retention time was as follows: 8.3 min.. The eluted aromatic amine was detected with U.V. spectrophotometer at 254 nm and a spectrofluorimeter at variable wavelengths : Ex.= 300 nm, Em.= 406 nm.

After 99.3% ozonation, the remaining trace of benzidine doesn't interfere in the mutagenicity test. The ozonated solutions were concentrated to about 2-5 mL under reduced pressure using a rotary evaporator and evaporated to dryness in a vacuum oven (35°C) in presence of phosphorus pentoxide.

After partial ozonation, the separation of benzidine ozone byproducts were realized by extraction with ethylacetate . The parent aromatic amine was extracted by solvent and ozone byproducts remained in water . The latter was concentrated to about 2 mL under reduced pressure using a rotary evaporator then evaporated to dryness in a vacuum oven (35°C) in presence of phosphorus pentoxide. The residue was dissolved in DMSO (1.25 mL).

The *Salmonella typhimurium* histidine auxotroph strain TA 98 was kindly supplied by Dr B.N. Ames , Biochemistry department, University of California, Berkeley, CA (USA).
Livers excised from rats (Sprague Dawley), pretreated with PCB

(Aroclor 1254, Monsanto), at 500 mg/kg, intraperitoneally were homogenized. Liver homogenate (S9) and S9 mix were prepared according to the method described by Ames et al. 1975.
Ozone byproducts in DMSO, 0.5 mL S9 mix, 0.1 mL overnight cultured bacterial suspension and 2 mL soft top agar (0.7% Difco agar, 0.6% NaCl, 0.1 μmole biotine, 0.1 μmole histidine) were added in sequence in a test tube. The contents were mixed and poured onto a minimal agar plate (1.5% agar, 2% glucose in Vogel-Bonner medium) . As positive controls, nitrofluorene (NF) and 2-amino anthracene were used. All mutagenicity assays were performed on duplicate plates.

RESULTS AND DISCUSSION

Figure 1 represents some typical chromatograms of the benzidine sample and reaction mixtures after ozonation.
Benzidine ozone by-products in water showed direct mutagenicity with a weaker indirect mutagenic activity (Figure 2). Mutagenic activities were maximum after ozonation by 100 mg ozone i.e. twice the ozone amount necessary for the disappearance of benzidine (seen by loss of fluorescence at the initial retention time: 8.3 min.). No mutagenic activity was observed after ozonation by 400 mg ozone i.e. eight times the ozone amount necessary for the disappearance of benzidine

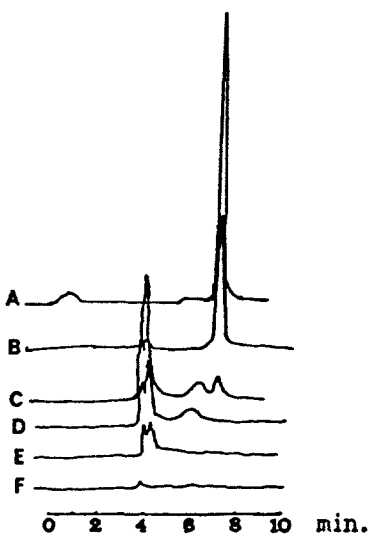

Figure 1. HPLC-UV chromatograms of Benzidine and reaction mixture after ozonation. BZ standard (A), BZ ozonated by 10 mg (B), 50 mg (C), 100 mg (D), 200 mg (E), 400 mg (F) of ozone.

The molar assessment of ozonation revealed that three moles of ozone are necessary to destroy one mole of benzidine.

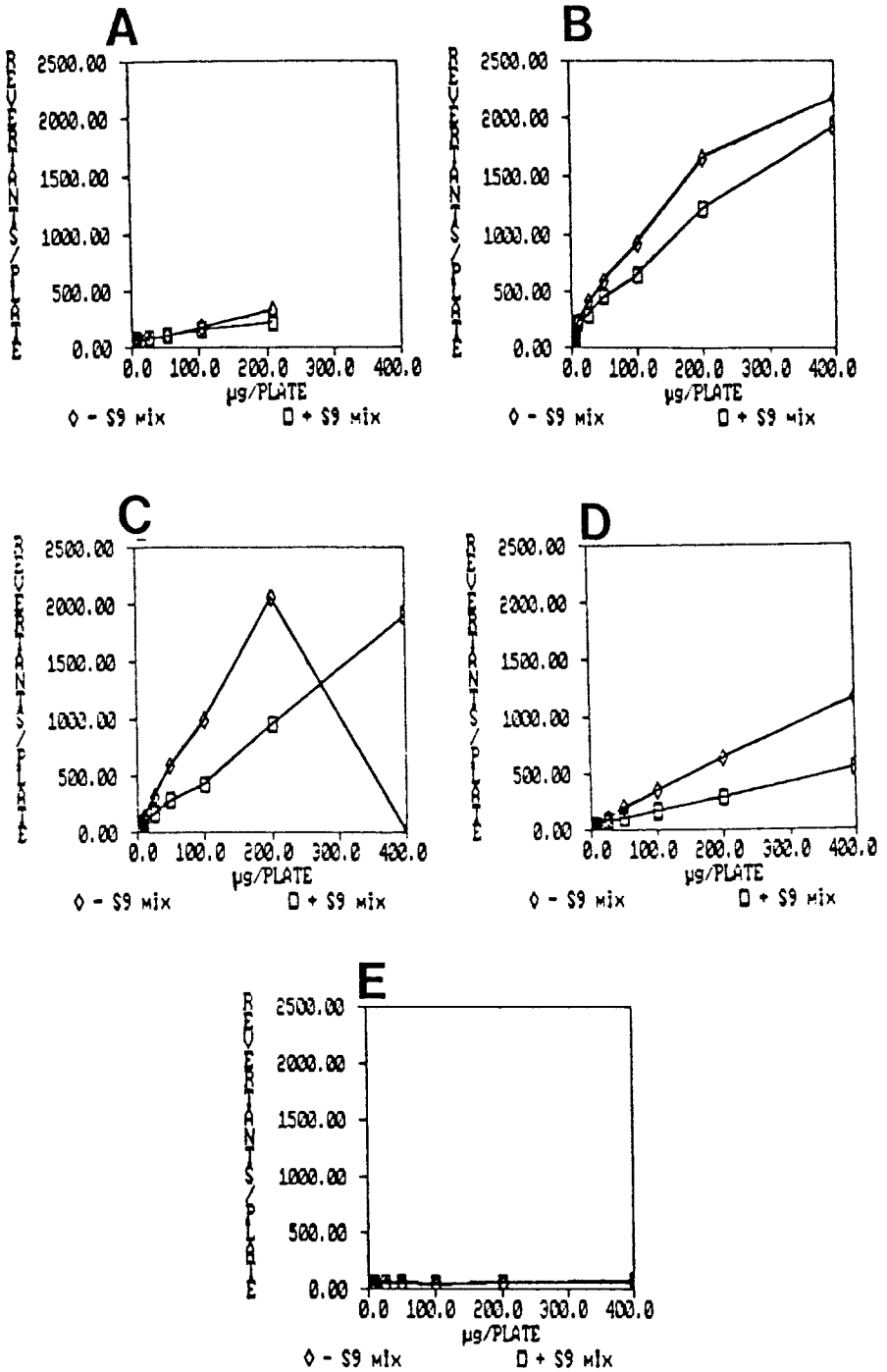

Figure 2. Dose-response curves of mutagenicity toward *Salmonella typhimurium* TA 98 of Benzidine ozone byproducts (A): 10 mg O_3(59%), (B): 50 mg O_3 (99%),(C): 100 mg O_3(100%),(D):200 mg O_3(100%),(E):400 mg O_3(100%).

Benzidine requires metabolic activation by S9 fraction to become mutagenic (Mc Cann et al 1975). However, according to Caulfield et al (1979), when benzidine was ozonated, there was a transient increase of mutagenicity due to the formation of an mutagenic intermediate without need of activation by S9 fraction. Upon further ozonation, mutagenicity of benzidine and its ozone by-products were eliminated.

Our results concerning benzidine ozonation are partly in agreement with those from Caulfield et al (1979). No mutagenicity was observed after ozonation by eight times the ozone amount necessary for total disappearance of benzidine.

Therefore, ozonation may represent an interesting alternative method for the destruction of Benzidine in water.

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