Ozonation of Mutagenic and Carcinogenic Polyaromatic Amines and Polyaromatic Hydrocarbons in Water

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ABSTRACT

The Salmonella-microsome assay for mutagenesis was used to determine the effect of ozone on the mutagenesis of selected carcinogens and mutagens in water. Short periods of ozonation were shown to completely inactivate the mutagenicity of several polyaromatic amine mutagens including acriflavine, proflavine, and β-naphthylamine. Selected polyaromatic hydrocarbons were also sensitive to ozonation. Kinetic studies revealed that the mutagenicity of benzo(a)pyrene, 3-methylcholanthrene, and 7,12-dimethylbenz(a)anthracene was destroyed after short periods of ozonation. To correlate loss of mutagenicity with loss of carcinogenicity, two polyaromatic hydrocarbons were treated with ozone, extracted from water with hexane, and tested for carcinogenicity in mice. When 7,12-dimethylbenz(a)anthracene and 3-methyl-cholanthrene were treated with ozone, there was a substantial reduction in carcinogenicity compared to control groups treated with oxygen alone. However, a small number of tumors developed in the group of animals receiving a hexane extract of ozonated 7,12-dimethylbenz(a)anthracene. This activity may be due to breakdown products of 7,12-dimethylbenz(a)anthracene that are not mutagenic.

INTRODUCTION

It has been estimated that as much as 90% of human cancer is caused by environmental factors (16). Carcinogenic organic chemical contaminants have been detected in many water sources (5, 14, 17). Therefore, contaminants in water have been suspected as a contributing factor in the etiology of cancer. Several studies have also indicated that suspected carcinogens are present in certain drinking water supplies (6, 13, 19, 29), e.g., 13 halogenated hydrocarbons were identified in the drinking water of New Orleans (13), and 5 halogenated hydrocarbons were found in pooled plasma samples of local residents. Tetrachloroethylene and carbon tetrachloride were found in both the plasma samples and the drinking water (13). On this basis, the drinking water was suspected as a possible cause of the high incidence of bladder cancer in the population of New Orleans (12, 23).

Part of the problem of carcinogens in drinking water may be due to the water treatment process itself. Chlorination of water has been shown to cause the formation of halogenated hydrocarbons including chloroform (6). In a survey of 80 cities, it was found that chloroform was present in much higher concentrations in chlorine-treated than in untreated water. Chlorination also results in the formation of chloramines which have been shown to be weakly mutagenic in a bacterial assay system (26).

Because of the problems associated with chlorination, ozone has been considered as an alternative water treatment practice. Ozone is a powerful disinfectant which can inactivate bacteria, bacterial spores, parasites, and viruses (7, 8, 22, 30). Ozone can react with unsaturated organic chemicals, splitting these molecules at the carbon-carbon double bond to form aldehydes and ketones (4, 20). Ozone has also been shown to oxidize aromatic and heterocyclic compounds (4, 5, 32). However, most ozonation studies were performed at low temperatures in organic solvents and with high concentrations of substrate. Few studies on the ozonation of organic chemicals in water have been performed (4, 10, 27, 28). It was the purpose of this investigation to study the biological effects of ozone on low concentrations of chemical carcinogens in water. This approach more closely duplicates the effects of ozone in large-scale water treatment facilities in which there is concern for the carcinogenic effects of low concentrations of pollutants.

There is a high degree of correlation between mutagenicity in the Salmonella-microsome assay and carcinogenicity in animal studies (21, 24). Therefore, the Salmonella-microsome assay was used to monitor the effects of ozonation on mutagens and carcinogens. The simplicity of the Salmonella-microsome assay and the short incubation period required for the assay allowed kinetic studies to be performed on each chemical treated with ozone.

Since polluted water contains only low concentrations of mutagens and carcinogens, the present studies were performed using distilled water to which known amounts of carcinogens and mutagens were added. Chemicals were chosen for ozonation studies on the basis of 3 criteria: (a) the chemical had been demonstrated to occur in water sources; (b) the chemical was a potential water pollutant; or (c) the chemical was structurally related to carcinogens or mutagens which were known to occur in water.

MATERIALS AND METHODS

The Salmonella typhimurium tester strains TA1535, TA1537, TA98, and TA100 were obtained as a gift from Dr. Bruce Ames from the Biochemistry Department, University of California, Berkeley, Calif., and were handled according to his methods (3) with the following exceptions. The cells were grown in Penassay Broth (Difco Laboratories, Detroit, Mich.) at 37° overnight with shaking, diluted 1:10 in fresh broth, and incubated at 37° for 2 hr before being used in mutagenesis testing. Liver homogenates were prepared from rats induced with the
polychlorinated biphenyl mixture Aroclor 1254 (1), a gift from W. B. Papageorge, Monsanto Chemical Co., St. Louis, Mo. The induction procedure was similar to that of Czygan et al. (11). Male Lobund Sprague-Dawley rats were given a single i.p. injection of Aroclor 1254 (diluted in corn oil to a concentration of 200 mg/ml) at a dosage of 500 mg/kg 5 days before sacrifice. The liver homogenate fraction (S-9) was prepared by the procedure of Garner et al. (15), and the S-9 mix was prepared according to the method of Ames et al. (2).

The mutagenesis assay is a modification of the method of Ames et al. (3). The sample to be tested (in volumes of 1 ml or less) was added to 4 ml top agar (0.75% Difco agar:0.5% NaCl: 0.025 mM L-histidine-HCl:0.025 mM biotin) at 45°. Just before plating, 0.5 ml of the S-9 mix (if required) and 0.1 ml of a 2-hr subculture of bacteria were added to the top agar, and the contents was mixed briefly by vortexing and then poured on minimal glucose agar plates (3, 31). The number of revertant colonies were counted after 2 days incubation in a dark 37° incubator.

Statistical Methods. The results are expressed as the mutation index which is the number of revertants in the experimental plates divided by the controls. An estimate of the variance of the spontaneous reversion rates of each tester strain was determined by inoculating 12 tubes of Penassay Broth with each tester strain, incubating the cells overnight at 37°, and then plating 0.1 ml of each tube in duplicate after a 2-hr subculture. Each strain was plated both with and without the addition of S-9 mix. Each plate was considered a separate observation and the means ± S.D. were determined for each strain. The upper confidence limit (L) was determined by the following equation:

\[ L = \bar{Y} + \left[ t \left( \frac{a}{2} \right) \right] \left( \frac{s}{\sqrt{n}} \right) \]

where \( \bar{Y} \) is the group mean, \( s \) is the S.D., and \( t \) is the value from Student’s \( t \) distribution for \( \nu \) degrees of freedom at the \( \alpha/2 \) level of probability. For this work, a one-tailed test was used because only increases in the mutation index were considered. \( \alpha \) was chosen to be 0.01. The base line index is defined as the ratio \( L/\bar{Y} \). Any mutation index greater than \( L/\bar{Y} \) is considered significant at the \( p \leq 0.005 \) level of probability. The base line indices for each strain are shown in Table 1. The ordinate of all charts has as its lowest value the base line index for the particular tester strain used for mutagen testing. Therefore, all values plotted above the base line index are considered significant at the \( p \leq 0.005 \) level.

Mutagens and Carcinogens. BP4 was obtained from Aldrich Chemical Co., Milwaukee, Wis.; MCA and DMBA were obtained from Eastman Kodak Co., Rochester, N. Y.; \( \beta \)-naphthylamine was obtained from Sigma Chemical Co., St. Louis, Mo.; acriflavine (a mixture of the hydrochlorides of 2,8-diamino-10-methylacridinium chloride and 2,8-diaminoacridine) and protaflavine (2,8-diaminoacridine sulfate) were obtained from Allied Chemical Co., Morristown, N. J.; croton oil was obtained from Consolidated Midland Corp., Brewster, N. Y. Chemicals were dissolved in either acetone, or DMSO obtained from Fisher Scientific Co., Pittsburgh, Pa.

Ozonation. Ozone was produced from oxygen by electrical discharge from a corona-type ozone generator and delivered into a modified gas-washing bottle with a fritted glass cylinder. Samples were ozonated in 100-ml starting volumes at a flow rate of 2 CFH with the ozone generator operating at 50 watts. All experiments were performed in a closed chemical hood.

Ozone was measured by the iodometric titration method of Saltzman and Gilbert (25). The effective ozone dosage was measured by delivering ozone at 2 CFH into neutral potassium iodide reagent [1% KI in 0.1 M KH2PO4:0.1 M NaHPO4 buffer (pH 6.6)] and collecting samples for assay at 15-sec intervals. After 45 min the absorbance was read in 1-cm quartz cuvettes at 352 nm, and ozone values were obtained by comparison with a standard curve. The standard curve was obtained by determining the A of dilutions of 0.01 N iodine solution in neutral potassium iodide reagent at 352 nm.

Residual ozone concentrations were determined by ozonating water at 2 CFH and collecting samples for assay at 15-sec intervals. The samples were immediately mixed with equal volumes of neutral potassium iodide reagent, and after 45 min, the absorbance was read at 352 nm.

Mutagenesis Assay of Ozonated Chemicals. The experimental protocol involved 3 steps: (a) each compound was screened on the 4 tester strains to determine which strain was most sensitive; (b) a dose-response experiment was performed to determine the optimum concentration of a chemical to be used in the assay; and (c) kinetic studies of the ozonation of a chemical were performed. To control for nonspecific losses of activity due to volatility and instability of the chemicals, all compounds were treated with oxygen for the same time period as the ozonation studies and tested for mutagenicity. Mutagens not directly soluble in water were first dissolved in either acetone or DMSO and subsequently diluted in water. This resulted in the chemical becoming miscible with or finely suspended in water. DMSO and acetone had no effect on the mutagenesis assay in concentrations used for assay.

Carcinogenesis Testing in Vivo. To correlate loss of mutagenicity with loss of carcinogenicity separate preparations of 2 PAH, MCA and DMBA, were treated with ozone and tested for carcinogenicity in mice. DMBA and MCA were dissolved in acetone at a concentration of 1 mg/ml and subsequently diluted in water at the same concentration as that used in mutagenesis testing. MCA was diluted to a concentration of 10 \( \mu \)g/ml, while DMBA was diluted to a concentration of 20 \( \mu \)g/ml. The samples, in volumes of 100 ml, were treated with ozone for 5 min at 50 watts and 2 CFH. Control samples were treated with oxygen for 5 min at 2 CFH. The treated samples, in 100 ml volumes, were then extracted twice with 10 ml of redistilled hexane for 5 min each in a 500-ml separatory funnel. The

<table>
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<th>Strain</th>
<th>S-9</th>
<th>( \bar{Y} )</th>
<th>L*</th>
<th>Base-line Index (L:( \bar{Y} ))</th>
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<td>154.5</td>
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* \( L = \bar{Y} + \left[ t \left( \frac{a}{2} \right) \right] \left( \frac{s}{\sqrt{n}} \right) \)

4 The abbreviations used are: BP, benz[a]pyrene; MCA, 3-methylcholanthrene; DMBA, 7,12-dimethylbenz[a]anthracene; DMSO, dimethyl sulfoxide; CFH, standard cu ft/hr; PAH, polynuclear hydrocarbons.
hexane layers were dried under air in a multiple sample evaporator. The dried contents were dissolved in acetone at a concentration of 500 µg/ml based on the amount of carcinogen originally suspended in water. The testing for carcinogenesis was done according to the method of Kinoshita and Gelboin (18). Each carcinogen was deposited on the shaved backs of both male and 7- to 8-week-old female Lobund CFW mice followed by twice weekly applications of 0.2 ml of a 1% solution of croton oil in acetone for the duration of the experiment. Control mice received croton oil in acetone only.

RESULTS

The kinetics of ozone production is shown in Chart 1A. The amount of ozone delivered to the solution was constant after the first 30 sec. As the power output of the ozone generator was increased from 50 to 150 watts, a higher percentage of the oxygen was converted to ozone. The system was saturated with ozone after 30 sec (Chart 1B). However, it cannot be ruled out that undissolved ozone could react directly with carcinogens and mutagens being tested.

A kinetic study of the ozonation of 3 polyaromatic amine mutagens is shown in Chart 2. Acriflavine and β-naphthylamine were both rapidly inactivated by treatment with ozone. Most of the mutagenic activity of proflavine was lost after ozonation for 30 sec, however, some activity remained until 4 min. This may indicate that proflavine reacted with ozone to form a weakly mutagenic intermediate which was inactivated by further ozonation. Treatment with oxygen had no effect on the mutagenicity of the polyaromatic amines tested.

Kinetic studies of the ozonation of 3 PAH are shown in Chart 3. BP was dissolved in acetone at a concentration of 1 mg/ml and diluted in water to a concentration of 10 µg/ml for testing. This resulted in the formation of an opaque suspension which did not settle out of solution after prolonged standing. The solution rapidly cleared when ozonated but remained opaque when treated with oxygen. Oxygenation had no effect on the mutagenicity of BP whereas ozonation caused complete inactivation within 1 min. DMBA and MCA reacted with ozone in the same manner as did BP. DMBA was dissolved in acetone at a concentration of 1 mg/ml and diluted in water to a concentration of 20 µg/ml for testing while MCA was dissolved in DMSO at 1 mg/ml and diluted in water to a concentration of 10 µg/ml. Both chemicals formed opaque suspensions in water which cleared rapidly with ozonation. The mutagenic activity of DMBA was inactivated after treatment with ozone for 30 sec while MCA was inactivated within 90 sec. Oxygenation had no effect on the mutagenicity of either compound.

Carcinogenesis Testing of Ozonated Chemicals. DMBA and MCA were suspended in water, treated with either ozone or oxygen, extracted with hexane, and tested for carcinogenicity on mouse skin as described in "Materials and Methods." The tumor incidence in mice treated with ozonated DMBA after 14 weeks is indicated in Table 2. Group 1 mice which received ozonated DMBA had significantly fewer tumors per mouse than did Group 2 mice which received oxygenated DMBA (P < 0.001). There was a slight difference between mice of Groups 1 and 3, the croton oil control group (P < 0.05). Therefore, it cannot be stated that there was complete inactivation of DMBA. However, ozonation caused a substantial reduction in tumor incidence from 4.65 tumors/mouse in the oxygen control group to 0.17 tumors/mouse in the group receiving ozonated DMBA.

The tumor incidence in mice treated with ozonated MCA is shown in Table 3. In the positive controls (Group 2 mice), MCA was treated with oxygen before extraction with hexane. The female mice receiving this extract failed to develop significantly more tumors than did the controls (Group 3) which received only croton oil. However, male mice from Group 2 did develop a significant number of tumors. Therefore, the tumor incidence in the males of Group 1 could be compared with the tumor incidence in the males of Group 2. Group 1-m developed significantly fewer tumors per mouse than did Group 2-m (P < 0.02). There was no difference between Groups 1-m and 3, the croton oil controls. Therefore, within the limits of sensitivity of this assay, ozonation caused complete inactivation of the carcinogenicity of MCA.

DISCUSSION

The mutagenicity of selected polyaromatic amines and PAH

![Chart 1](chart1.png)

**A** Ozone measurements at a power output of 50 (——) or 150 (— — — —) watts. A, ozone produced from oxygen and delivered at 2 CFH to a 100-ml volume of neutral KI reagent. B, residual ozone concentrations determined by ozonating a 100 ml volume of water and collecting samples for assay at 15-sec intervals. The samples were immediately mixed with equal volumes of neutral KI reagent. After 45 min, the A was read at 352 nm, and ozone values were obtained by comparison to a standard curve.

![Chart 2](chart2.png)

![Chart 3](chart3.png)
was inactivated by short periods of ozonation. The mutagenicity of acriflavine, \( \beta \)-naphthylamine, BP, and DMBA was abrogated after treatment with ozone for 30 sec, while MCA and proflavine required ozone treatment for 90 sec and 4 min, respectively.

There is a high degree of correlation between mutagenicity in the Salmonella-microsome test and carcinogenicity in animal studies (21, 24); therefore, certain ozonated chemicals were tested for carcinogenicity in animals in addition to tests for mutagenicity in the Salmonella-microsome assay. DMBA and MCA were suspended in water and treated with either ozone or oxygen for 5 min. Ozonation for 5 min was 3 to 4 min longer than was required to inactivate the mutagenicity of these chemicals in previous experiments.

To test for carcinogenicity, it was necessary to remove and concentrate the treated chemicals from water. This was accomplished by extracting the water with hexane and drying the hexane extract under air. However, it must be pointed out that this procedure may not have removed certain ozonation by-products that could potentially be carcinogenic even though no mutagenic activity could be detected. Therefore, the mutagenicity and carcinogenicity data may not be directly comparable. However, the results indicate that mice treated with a hexane extract of ozonated DMBA or MCA developed substantially fewer tumors than did mice treated with a hexane extract of oxygenated DMBA or MCA.

A small number of tumors (above background) developed in the group receiving a hexane extract of ozonated DMBA. This may have been the result of residual DMBA that did not react with ozone. However, this is unlikely since DMBA contains many unsaturated ring structures that are very reactive to ozone. It is more likely that the remaining carcinogenic activity is due to breakdown products of DMBA that are not mutagenic to the Salmonella tester strains used.

An observation made during this study was that the solubility of several PAH in water was greatly increased if these chemicals are first dissolved in acetone or DMSO before being suspended in water. When DMBA, BP, and MCA were treated in this manner, they did not precipitate out of solution and were still mutagenic after standing for 3 weeks. These observations suggest that potential carcinogens with low solubility in water may be readily dispersed in water supplies if a cosolvent such as acetone is present at the source of the pollution.

These results indicate that in addition to being an effective bactericidal and viricidal agent, ozone treatment of water inactivates the mutagenicity of several chemicals and the carcinogenicity of hexane extracts of water containing DMBA and MCA. However, further studies suggest that ozonation of certain carcinogens such as dimethylhydrazine may cause these chemicals to become mutagenic in the Salmonella-microsome assay (9). Therefore, the decision of whether to ozonate water supplies must consider the possibility that mutagenic by-products may be created.

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Chart 2. Treatment of selected polyaromatic amines with ozone (——) or oxygen (———). In A, acriflavine was dissolved in water at a concentration of 100 \( \mu \)g/ml and assayed with tester strain TA1537 with a mean of 7 spontaneous revertants in the absence of mutagen. Top structure, 2,8-diamino-10-methylacridinium chloride; bottom structure, 2,8-diaminoacridine. In B, proflavine was dissolved in water at a concentration of 200 \( \mu \)g/ml and assayed with tester strain TA1537 with a mean of 7 spontaneous revertants in the absence of mutagen. Structure, 2,8-diaminoacridine sulfate. In C, \( \beta \)-naphthylamine was dissolved in acetone at a concentration of 10 mg/ml and diluted in water to a concentration of 50 \( \mu \)g/ml. S-9 (0.05 \( \mu \)l) was added to each plate for assay with tester strain TA1535 with a mean of 27 spontaneous revertants in the absence of mutagen.
mutagen. in B, MCA was dissolved in DMSO at a concentration of 1 mg/ml and diluted in water to a concentration of 10 μg/ml. 5-9 (0.05 μl) was added to each plate for assay with tester strain TA100 with a mean of 129 spontaneous revertants in the absence of mutagen. In C, DMBA was dissolved in acetone at a concentration of 1 mg/ml and diluted in water to a concentration of 20 μg/ml for assay with tester strain TA100 with a mean of 138 spontaneous revertants in the absence of mutagen.

**REFERENCES**

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