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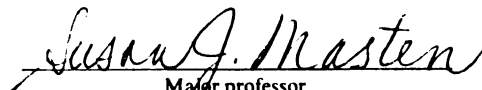
The Ozonation of Pyrene:
The Byproducts and Toxicological Implications

presented by

Holly Anita Herner

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**THE REACTION OF OZONE WITH PYRENE: THE BYPRODUCTS
AND THEIR TOXICOLOGICAL IMPLICATIONS**

By

Holly Anita Herner

A DISSERTATION

**Submitted to
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ABSTRACT

THE REACTION OF OZONE WITH PYRENE: THE BYPRODUCTS AND THEIR TOXICOLOGICAL IMPLICATIONS

By

Holly Anita Herner

Pyrene, a four ringed polycyclic aromatic hydrocarbon (PAH), was oxidized using ozone under varied conditions. Byproducts were studied as mixtures and, in some cases, individually using gap junction intercellular communication (GJIC) to assess the epigenetic toxicity of the byproducts. Several commercially obtained compounds, similar to the pyrene ozonation byproducts, were also evaluated using the same techniques.

Pyrene was oxidized using ozone dosages ranging from 0.42 to 3.62 mmol ozone per mmol pyrene. Phenanthrene type and biphenyl type byproducts were sequentially formed following the disappearance of pyrene. At an ozone dosage of approximately 1.8 mmoles ozone per mmole pyrene the majority of the pyrene was oxidized and approximately 14 byproducts were formed. The majority of experiments in this study were conducted at a low pH to observe the reaction of pyrene with molecular ozone. Experiments were also conducted at a high pH to observe the reaction of pyrene with the hydroxyl radical. The byproduct mixtures generated at both high and low pH were separated using several chromatographic techniques. The characterization of the byproducts generated at high and low pH showed that the byproducts are similar and sequentially formed. The high pH ozonation was less efficient as evidenced by the increase in residual pyrene and the lack of biphenyl type compounds formed.

In this study, byproduct mixtures at both high and low pH were evaluated for their ability to inhibit GJIC. The byproduct mixtures generated at high and low pH were separated into ten and six fractions, respectively. Three isolated pyrene ozonation byproducts and six commercially obtained compounds were evaluated using GJIC as the biological indicator.

It was hypothesized that some of the byproducts may be more inhibitory to GJIC than pyrene. However, the three purified byproducts studied were not more inhibitory to GJIC than the parent compound, pyrene. Of the impure byproduct fractions studied at both high and low pH, two fractions were completely inhibitory to GJIC. Compounds B and D were present in impure fractions inhibitory to GJIC. Compounds B and D are both three ringed phenanthrene type compounds that have both a bay region and at least one aldehyde group.

Six commercially obtained compounds similar to pyrene ozonation byproducts were studied, two phenanthrene type compounds and four biphenyl type compounds. None of the six compounds were more inhibitory than pyrene. However, one of the four biphenyl type compounds, 4BCH, was completely inhibitory to GJIC at a high concentration. This result is significant not because of the concentration at which it inhibits, but because of its toxicity in comparison with the other biphenyl type compounds studied. 4BCH contains both a bay region and an aldehyde group in contrast to the other three biphenyl type compounds which contain a bayregion and only acid type functional groups. These three biphenyl type compounds were not inhibitory to GJIC even at elevated concentrations.

I dedicate this work to my husband, Andy. His support was the critical ingredient required for my success.

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Chapter 1 - Introduction

1.1 Objectives

The ultimate goal of this project was to identify relationships between individual chemical structure of byproducts formed from the ozonation of the four ringed PAH, pyrene, and the epigenetic toxicity of these compounds. The specific goals of this project were:

- (1) To compare the rates of degradation of pyrene and the production of byproducts in aqueous solution by both the reaction of pyrene with molecular ozone and the hydroxyl radical. The hydroxyl radical was generated by ozonating the solutions at high pH.
- (2) To separate molecular ozone and hydroxyl radical byproducts of pyrene ozonated at a high and low pH using chromatographic techniques including medium pressure liquid chromatography, high pressure liquid chromatography, and thin layer chromatography.
- (3) To identify the major ozonation byproducts of pyrene at low pH using spectral analytical techniques including proton and carbon nuclear magnetic resonance (^1H and ^{13}C NMR) spectroscopy, ultraviolet and visible spectrophotometry (UV), and gas chromatography followed by mass spectroscopy (GC-MS). (Product characterization for impure fractions at high and low pH was completed using GC-MS.)

- (4) To complete toxicity studies for the parent compound, pyrene, and each purified ozonation byproduct using gap junctional intercellular communication (GJIC) as an indicator for potential tumor promotion.
- (5) To compare results of toxicity studies for byproducts of pyrene ozonation at pH=2 and 9.5.
- (6) To identify relationships between chemical structure or functional group and chemical toxicity.
- (7) To obtain compounds similar in chemical structure to the identified byproducts and perform toxicity studies to confirm or negate relationships.

1.2 Background

Polycyclic aromatic hydrocarbons (PAHs), compounds comprised of multiple fused rings, are derived from the incomplete combustion of organic matter. Major sources of PAHs include mineral fuels, coal derived oils, tobacco smoke and vehicle exhaust [1]. The release of PAHs to the environment has resulted in the presence of detectable levels of PAHs in air, water and soil [2]. As PAHs are sparingly or insoluble in water and are recalcitrant, they tend to accumulate on solid surfaces, including airborne particulate matter and soil organic matter. The accumulation of PAHs in the environment has resulted in the need for the development of a remediation process that

will reduce the risk of human exposure to these chemicals. In addition, many of these compounds are carcinogenic or potentially carcinogenic [2].

1.3 Significance: Results and Benefits Expected

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous in the environment and are particularly common at many contaminated sites in the United States [2]. The US-Environmental Protection Agency (EPA) has identified 16 PAHs as priority pollutants and at least 8 are carcinogens or possible carcinogens [3]. PAHs, found both in surface waters and soils, are characteristically nonpolar making them unavailable for biological destruction [4]. This persistence is of concern for water sources used for potable purposes [4].

Many mechanical, chemical and biological processes have been studied in order to develop an efficient method for PAH removal [5,6]. Each process has its advantages and disadvantages with respect to cost, process efficiency, complexity of process, and waste generation. The ideal process would be one that is efficient, inexpensive, and easy to operate with a negligible generation of waste. The use of ozone for oxidation of PAHs has proven to be an effective method for the degradation of these compounds [4,5,7-10]. Ozone, which is used both in water and wastewater treatment, is a very powerful oxidant that can react with numerous organic compounds, many of which are resistant to conventional treatment [11]. Unlike processes such as granulated activated carbon which has been used for PAH removal, ozonation destroys the compounds and, therefore, does not produce additional wastes [6]. Granulated activated carbon merely transfers the compounds from one matrix to another.

When ozone reacts with PAHs, it attacks either the bond with the lowest bond localization energy or the atom of lowest atom localization energy depending upon the PAH [12]. The degradation of multiple ringed PAHs using ozone involves a series of oxidations which reduce the number of fused rings. Eventually, as the ozone dosage increases, these compounds are oxidized to straight chain aliphatics [4]. Some PAHs show an immediate decrease in toxicity as a function of ozone dosage while other PAHs show an initial increase in toxicity followed by an eventual decrease in toxicity [1,7]. Upham et al. [13] found that certain three ringed PAH byproducts of ozonation were more toxic than the four or five ringed parent PAH compounds. The resulting byproducts of ozonation are a function of the ozone dosage. These may be harmful if the PAH is transformed into an intermediate compound that is still harmful.

Pyrene, a PAH with a four ringed structure, can be completely degraded using ozone. At least 10 major byproducts are formed [4,7,14]. However, some of the initial byproducts of pyrene ozonation in aqueous solution may be more toxic than the parent compound, pyrene [7,13]. It was demonstrated that an ozone dosage of 1.6 mmoles of ozone per mmole of pyrene was required to oxidize pyrene entirely whereas a dosage of 4.5 mol ozone per mol pyrene was required to destroy all byproducts inhibitory to gap junction intercellular communication (GJIC) [7]. Sasaki et al.[15] also showed that some byproducts of pyrene oxidation have a higher mutagenic activity than pyrene. It should be noted that the experimental conditions of the Sasaki study simulated photooxidation conditions causing the addition of a nitrogen group to the byproduct structures. These compounds are unlike products found from ozonation in aqueous solution.

Yoshikawa et al.[1], using blood chemistry in rats as the toxicity indicator, found that the byproducts of pyrene ozonation were not more toxic than the parent compound. Neither pyrene nor its byproducts were considered toxic in this Yoshikawa study. However, other PAHs analyzed in this study were found to have byproducts that were more toxic than their respective parent compounds.

Current engineering practice is to assess the success of a remediation project by monitoring only the removal of the parent compound PAHs. It is apparent, however, that due to the toxicity of some of the ozonation byproducts formed from PAHs, remediation projects must be evaluated based upon the degradation of both the parent compound and these byproducts with a concomitant decrease in toxicity [1,7,15].

The risk of cancer as a result of exposure to environmental contaminants has been a major concern. In the past, most chemicals were evaluated for their potential to cause cancer using genotoxic bioassays. Studies have shown, while many chemicals may not be genotoxic or complete carcinogens, they can still contribute to carcinogenesis by an epigenetic mechanism. Epigenetic toxicants have been implicated in tumor promotion during carcinogenesis [16-18], in teratogenesis [19], and in reproductive dysfunction [20-22]. Epigenetic toxicants are known to inhibit the communication between cells through their gap junctions.

Intercellular communication, the major mechanism for control of cell homeostasis, is a result of the transfer of ions and small molecules through membrane gap junctions. The gap junction is a hexameric channel or connexon comprised of six subunits called connexins which traverses the plasma membrane and is joined with the connexon of an adjacent cell membrane [16,23]. Most cancer cells have dysfunctional

gap junction intercellular communication (GJIC) [17]. The inhibition of GJIC has been used as a biological indicator for potential tumor promotion [16,24].

The PAHs as a whole are a fairly inert class of compounds. Although they are not highly reactive chemicals, studies have shown that they commonly exert their carcinogenic activity through metabolites which are able to damage DNA, RNA, and protein [25]. Several theories have been proposed which suggest the existence of a relationship between chemical structure and carcinogenesis. One common theory is the relationship between the bay region, which many PAHs contain, and carcinogenic activity [25-27]. Functional groups such as the hydroxy, the diol-epoxide, and the methyl group which are adjacent to or a part of the bay region have also been documented as enhancing the carcinogenic activity of PAHs [26, 28-32]. Upham et al. demonstrated that PAHs containing a bay region were inhibitory to GJIC while some PAHs without a bay region were not. For example, anthracene which does not have a bay region did inhibit GJIC when it was methylated in a position that formed a bay region [33]. Conversely, pyrene which does not have a bay region did inhibit GJIC without any modifications to its structure [7].

To date, most studies have been performed to detect cell initiators or complete carcinogens. However, many PAHs are known to have carcinogenic or tumor promoting properties and are not initiators or complete carcinogens [33]. A correlation has been made between compounds that exhibit tumor promoting activity and the down regulation of GJIC [7]. Pyrene, the focus PAH of this study, is known to down regulate GJIC [7].

Chapter 1 describes the goals and hypotheses of this research project. The primary goal is to investigate the relationships between the structures of pyrene and its ozonation

byproducts and compare with the chemical toxicity associated with each compound using GJIC as an indicator for toxicity. The identification of major byproducts and their individual toxicities is crucial for the development of structure-function relationships which could be helpful in the prediction of epigenetic toxicity of PAHs not studied. This project is different from previous research in the following ways:

- 1) Toxicological studies using GJIC as a biological indicator for potential tumor promotion will be performed using the scrape load/ dye transfer (SL/DT) technique in dose response, time response, and time recovery bioassays to evaluate pyrene, the purified byproducts and impure fractions of pyrene ozonation. The neutral red dye uptake bioassay will be used to evaluate the cytotoxicity of these compounds at a range of dosages. Previously, byproducts were studied as unknown mixtures.
- 2) Identification of pure byproducts will be completed using nuclear magnetic resonance (NMR) spectroscopy, an analytical technique applicable only for pure compounds. Compounds identified using NMR will be confirmed using GC-MS. Previously, byproduct mixtures studied were not characterized by NMR or GC/MS.
- 3) The data collected will be used to develop relationships between chemical structure and their individual toxicity which could in the future be used as a screening tool to predict toxicity in other chemicals with similar structures.

1.4 Research Goals

Ozone attacks PAHs by either substitution by atom or ring cleavage by bond attack and produces a multitude of ozonation byproducts [12]. Some of the byproducts may be more toxic than the parent compound [7]. As the ozone dosage is increased, chemical toxicity for most PAH byproducts eventually decreases. Toxicological data for pyrene and byproducts of pyrene ozonation is needed, as well as structural data for these compounds. The study was divided into several phases.

Phase I focused on the ozonation of pyrene. The purpose of phase I was to generate byproducts of pyrene ozonation in sufficient quantities for study in later phases. Two variables for this optimization were of primary importance: ozone dosage and pyrene concentration. It was hypothesized that the ozonation of pyrene generates two groups of distinct products - distinct in structure and order of production. The initial group formed being three ringed phenanthrene type compounds and the later group formed being two ringed biphenyl type compounds. In addition, ozonation experiments were conducted at both high and low pH. At a low pH, the interaction between ozone and pyrene is optimized. However, a high pH mimics actual conditions in water treatment at the stage where ozone would be used. It was hypothesized that the ozonation efficiency would be greatly reduced at a high pH.

Experiments were conducted using a batch reactor with a UV detection system. The required ozone dosage was that which produced a mixture of byproducts yet minimized the mass of original compound remaining. This mixture of compounds represented the first group of ozonation byproducts formed, the three ringed type

primarily, in addition to smaller quantities of the second group of byproducts, the biphenyl type products. The three ringed structures were examined first.

In order to conduct toxicological studies in a later phase of this research, a large amount of each byproduct was needed and, therefore, an unusually high concentration of pyrene was ozonated. PAHs are characteristically insoluble compounds which limited the pyrene concentration in solution. The conditions of the experiment were optimized to meet the goal of this phase.

In some cases, solutions containing byproduct mixtures were stabilized following ozonation due to organic peroxides generated during the ozonation. Organic peroxides were quenched using a sodium bicarbonate wash and byproducts were recovered from the solution using a liquid-liquid extraction method. The completion of phase I yielded a solution of pyrene ozonation byproducts ready for study in phase II.

Separation of the byproducts was the focus of **Phase II**. It was hypothesized that the byproducts of pyrene ozonation could be separated and purified using chromatographic methods such as medium pressure liquid chromatography (MPLC), high pressure liquid chromatography (HPLC), and thin layer chromatography (TLC). A reverse phase (C18) column was employed in each case. A normal phase system was unsuitable because of the strong affinity of the compounds for the column media. In addition, the reverse phase system allowed a greater range in solvent polarity to be used and thus made separation of compounds with a large range in polarity more likely. The separation of the pyrene ozonation byproducts presented a challenge for two reasons. First, the byproducts encompassed a large range in polarity. And second, the compounds which were classified as having similar polarities also had very similar structures with

perhaps only one different functional group. Pure compounds were identified in phase II using NMR spectroscopy. Both proton (^1H) and carbon (^{13}C) NMR spectroscopy were performed for each pure compound. Proton NMR provided essential information about the structure's functional groups where as carbon NMR provided information regarding the backbone of the structure [35]. Most structures were verified using gas chromatography followed by mass spectroscopy (GC/MS). Impure fractions were characterized using only GC-MS.

Phase III focused on the toxicological study of the pyrene ozonation byproducts. It was hypothesized that pyrene and some of its byproducts were inhibitory to GJIC. The scrape loading dye transfer (SL/DT) technique was used for dose response, time response, and time recovery studies. The neutral red uptake bioassay was used to test for cytotoxicity at selected chemical dosages.

Toxicological studies were performed for the parent compound, the purified ozonation byproducts, and some impure fractions. In order to measure the dose-response of each compound, cells were exposed to chemicals at varying concentrations for identical time periods whereas time-response experiments employed constant chemical concentrations with varying time of exposure. In time recovery studies, cell cultures exposed to compounds which inhibited GJIC were monitored to see how much time was required for communication between cells to be restored after the termination of the chemical exposure. Cytotoxicity experiments were performed to determine whether lack of cell to cell communication was due to closing of gap junctions or actual cell death. All GJIC experiments were be conducted using concentrations of the target chemicals that were not cytotoxic.

The purpose of **Phase IV**, was to evaluate toxicological data and identify parallels between chemical structure and chemical toxicity. It was predicted that there would be structural characteristics that could be associated with the toxicity of the individual chemical. The byproducts generated from PAH ozonation tended to have similarities in structure and thus provided the subtle differences needed to examine changes in toxicity caused by a portion of a structure , i.e. bond regions, functional groups.

In an effort to generate additional data to examine these proposed relationships, experiments were performed using commercially available compounds that were similar to the PAHs previously examined in this study. Toxicological studies were conducted in the same manner as that previously described. Results were used to confirm or negate parallels made from data generated in phase III.

1.5 Organization of Thesis

Chapter 2 describes the ozonation of pyrene in solution. Large quantities of byproducts were generated for later study. Chapter 3 describes the concentration and separation of pyrene byproducts by high pressure liquid chromatography and subsequent purification by various chromatographic techniques. Pure compounds were identified using NMR spectroscopy and confirmed in most cases using GC/MS. Impure fractions were characterized using GC/MS.

Results of a mass balance which accounts for the degradation of pyrene is included in Chapter 3. The efficiency of the ozonation of pyrene at a high pH (9.5) was evaluated using this mass balance. The results were compared to those observed at a low pH (2).

Chapter 4 describes the analysis of impure fractions and whole mixtures using GJIC to measure epigenetic toxicity.

1.6 Materials

Ozonation. The PAHs selected for this study were purchased from Sigma Chemical (Sigma Chemical, St. Louis, MO). Ozone was generated in a dried oxygen electric discharge using a Polymetrics Model T - 408 ozone generator (San Jose, CA). A 250 mL gas washing bottle was used as the ozonation reactor. Sodium sulfite (Sigma Chemical, St. Louis, MO) was used to quench organic peroxides generated during ozonation.

Byproduct Separation. All solvents used in chromatographic experiments were purchased from J.T.Baxter (Phillipsburg, NJ) or Aldrich Chemical Co. (Milwaukee, WI). A 0.2 μ m Whatman filter (Arbor Technologies, Ann Arbor, MI) was used to filter samples before injection into the HPLC.

Byproduct Identification. Dried samples for were prepared for identification by NMR using deuterated solvents purchased from Cambridge Isotope Laboratories (Woburn, MA). Samples run on GC/MS were derivatized by silylation using bis-trimethylsilyl/ trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) from Regis Technologies, Inc. (Morton Grove, IL) at 100 °C for one hour.

Toxicological Studies. The chemicals required to perform these studies were purchased from a variety of sources. Sodium dodecyl sulfate (SDS), Tween 20, TRIS glycine, acrylamide, and NNNN-tetramethylethylenediamine were purchased from Bio-Rad Laboratories (Hercules, CA). Sodium chloride, sodium phosphate, and ammonium persulfate were obtained from EM Science (Gibbstown, NJ), Columbus Chemical Industries (Columbus, WI), and Life Technologies, Inc. (Gaithersburg, MD), respectively. WB-F344 rat epithelial cell lines were obtained from Drs. J.W. Grisham and M.S. Tsao of the University of North Carolina (Chapel Hill, NC) [33]. Cells were cultured in 2 mL of D Medium (Formula No. 78-5470EG, GIBCO Laboratories, Grand Island, NY) and supplemented with 5% fetal bovine serum (GIBCO Laboratories, Grand Island, NY). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air [33]. 5,6-Carboxyfluorescein diacetate were purchased from Molecular Probes. Cell cultures were photographed using Nikkon Diaphot-TMD epifluorescence phase-contrast microscope illuminated with an Osram HBO 200W lamp and equipped with a 35-mm FA camera (Nikkon, Japan).

1.7 Methods

Ozonation. Ozone was bubbled into 200 mL of an acetonitrile and water (90:10 v/v) solution containing dissolved PAH. Acetonitrile is an ideal solvent because it is able to solubilize PAHs, it is miscible in water, it does not inhibit GJIC, and it has a low reactivity with ozone (half-life of 18 years at a concentration of 1400 mM) [36]. Water was required in solution because it is a participating solvent in the degradation of pyrene using ozone in an aqueous environment [14]. A 10% water solution is sufficient to assure

that the reaction is not water limited, but also dilute enough to avoid solubility problems due to the nonpolar characteristics of PAHs [7]. The pH of the solution in the reactor was acidified to approximately 2 using phosphoric acid. A solution pH of 2 was used to decrease the extent of decomposition of ozone to hydroxyl radicals [37,38]. In a comparison study, the reactor solution pH was increased to 9.5 using sodium tetraborate to mimic water treatment conditions. The flow of ozone was regulated at 100 mL/ min with a side track flow controller (Sierra Instruments Inc., Monterey, CA). All tubing (i.d. 1/8”), connectors and valves were constructed of Teflon ®.

During ozonation, solutions were continually mixed with a magnetic stirrer and stir bar. Reactions were terminated by flushing the reactor solution of ozone using helium gas. In some cases, organic peroxides were quenched using sodium sulfite. Ozonated samples were washed of excess sodium sulfite using a liquid-liquid extraction method. Washed samples were concentrated using rotary evaporation and dried using nitrogen gas.

The concentrations of ozone in the influent and effluent gas streams were measured spectrophotometrically at 258 nm using a UV-Vis spectrophotometer (Model 1201 Shimadzu, Scientific Instruments, Japan). A molar absorptivity coefficient for ozone of $3000 \text{ M}^{-1} \text{ cm}^{-1}$ was used [39]. Quartz flow cells with a path length of 0.2 cm was used. The effluent gaseous ozone was trapped in an aqueous 2% (w/v) potassium iodide solution.

Byproduct Separation. A reverse phase medium pressure column (C_{18} silica, 500 x 20 mm glass column, pressure rated 150 psi, DyChrom, Santa Clara, CA) was used to separate the byproducts into groups based on polarity. The mobile phase was introduced into the column using a low pressure pump (Model 81-M-2, Chemco, Sunnyvale, CA). Samples were solubilized in a mixture of acetonitrile and water and injected into a column which was preconditioned in the same solvent combination. Either an isocratic or gradient solvent system was employed depending upon the method.

Fractions were further separated using preparative or semi-preparative high pressure liquid chromatography. A reverse phase C_{18} column with dimensions of 250 by 20 mm (Jaigel, S-343-15, 15 μ m) or 250 by 10 mm (Phenomenex, Capcell Pak, 5 μ m) system with a C_8 guard column (Phenomenex, Capcell Pak, 5 μ m) was used. Again, samples were solubilized in the same solvent in which the column was preconditioned. The separation was completed using either an isocratic or gradient solvent system.

Compounds were detected in the preparative HPLC using a variable wavelength detector (S-310A Model II, Soma Optics, LTD., Dichrom, Santa Clara, CA). Semi-preparative experiments employed a dual variable wavelength absorbance detector (Waters, Model 2487, Milford, MA. Evaluation of the separation of the byproducts using HPLC was done using thin layer chromatography (TLC) with a Spectroline UV detector at 254 nm and 366 nm. Separated fractions were solubilized using a mixture of chloroform, benzene, and methanol (10:10:1, respectively) and applied to silica gel glass plates (20 by 5 cm, 250 μ m with organic binder). The separation of the components in

the fraction on the plates was accomplished using the same solvent mixture for 60 minutes. Some fractions were re-injected onto the HPLC for any further purification.

Byproduct Identification. Identification of byproducts was accomplished using both proton (^1H) and carbon (^{13}C) NMR spectroscopy. Spectra were recorded using Varian 300 and 500 MHz spectrometers at 25°C. GC/MS was performed using a JEOL AX-505H double focusing mass spectrometer coupled with a Hewlett - Packard 5890S GC (Norwalk, CT). A DB5MS (30 m length x 0.32 mm i.d. x 0.25 μm film thickness) fused silica capillary column (J & W Scientific, Rancho Cordova, CA) will be employed for GC separation. A splitless injector was used with a column head pressure of 10 psi using helium as the carrier gas, producing a flow rate of 1 mL/min. The initial column temperature was held for 2 minutes at 100°C, ramped at 20°C/min to 220°C, ramped at 5°C/min to 280°C, and finally ramped at 20°C/min to 300°C. The mass spectra were collected in the electron impact mode. Mass calibration of the spectrometer was completed using perfluorokerosine.

Toxicological Studies. Toxicological studies were conducted using GJIC as the biological indicator for potential tumor promotion. The SL/DT technique was used in dose response, time response, and time recovery experiments. The neutral red uptake bioassay was used to test cytotoxicity [40]. Target compounds were added to 2 mL of cell culture medium from stock solutions of chemical dissolved in acetonitrile. In dose-response studies, the volume of chemical added to cells varied with a constant exposure

time. In time-response studies, the time of exposure varied, with chemical dose remaining constant. Acetonitrile was used as the vehicle control in all experiments. SL/DT was performed as described by El-Fouly et al. [41]. Studies using the SL/DT technique were conducted at doses deemed non-cytotoxic by the Neutral Red Dye Uptake Assay.

Cell growth and conditions in the neutral red uptake assay were the same as in the SL/DT assay [40]. Following chemical treatment, cells were rinsed with PBS three times. Next, 2 mL of fresh growth medium containing 0.033% neutral red was added to cells for 1 hour. Neutral red solution was incubated with D Medium for 2 hours and then centrifuged and filtered through a 0.22 μ m Millipore syringe filter (Millipore Corp., New Bedford, MA) prior to its addition to the cells. After 1 hour, the cells were rinsed with PBS. Cells were lysed with 2 mL of a solution containing 1% acetic acid and 50% ethanol. Measurement of neutral red in lysed cells was accomplished using a UV spectrophotometer at 540 nm. Background absorbance was measured at 690 nm.

Data Analysis. From this study, data was generated detailing the chemical structure of each individual compound as well as their individual effect on gap junction intercellular communication. Using both the chemical structures and toxicological data, structure-activity relationships were identified. This data may be used in the future for the identification of quantitative structure-activity relationships (QSAR). However, any QSARs identified would be determined empirically because the mechanism causing the inhibition of GJIC is not fully understood in this case.

1.8 References

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Chapter 2 - Pyrene Ozonation

2.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs), one family of environmental contaminants, are derived from the incomplete combustion of organic matter and may be found in detectable quantities in soil, water and air [1,2]. These compounds are of concern because several are known or suspected carcinogens and at least 16 are considered priority pollutants by the United States - Environmental Protection Agency [3]. PAHs are ubiquitous in the environment and are resistant to conventional treatment technologies. This coupled with their low aqueous solubility and recalcitrance [6] leads to their accumulation in the environment.

Remediation techniques that protect human health as well as clean up polluted areas efficiently and economically are sorely needed. Chemical treatment is one remediation alternative which can be efficient, inexpensive, and easy to operate with negligible generation of waste. Ozone and other advanced oxidation processes (AOP) have been used in water and wastewater treatment for years [4,5]. Ozonation of PAHs and other recalcitrant compounds has proven to be an effective method for the degradation of these compounds [6-14].

Ozone is a strong ($E^\circ = +2.07 \text{ V}$) and selective oxidant which reacts with PAHs with rate constants on the order of $> 10^3 \text{ M}^{-1}\text{s}^{-1}$ [11,15]. The degradation of ozone yields hydroxyl radicals, a more powerful, non-selective oxidant, also capable of reacting with PAHs. The decomposition of ozone can be initiated by an increased pH with hydroxyl radical formation the result [16]. Although the hydroxyl radical is the most reactive

aqueous oxidant, the degradation of target pollutants may be inefficient. The formation of each mole of hydroxyl radical requires the decomposition of approximately double the number of moles of ozone [16]. In addition, the lack of selectivity characteristic of the hydroxyl radical ensures that other organics present will be very quickly oxidized, potentially leaving few radicals left to oxidize target compounds.

Classes of PAH compounds are differentiated from each other by the number and arrangement of the fused aromatic rings (Figure 2.1). The reaction between ozone and a PAH involves either the bond with the lowest bond localization energy or the atom of lowest atom localization energy, depending upon the PAH [17]. Table 2.1 describes the mechanism of attack for several classes of PAH compounds. The degradation of pyrene using ozone entails a series of oxidations which reduces the number of fused rings and eventually, at higher ozone dosages, produces straight chain aliphatics [6].

Pyrene is a PAH comprised of four fused benzene rings and is the subject of this study. Pyrene can be completely degraded with ozone at a dosage of 1.6 mmoles of ozone per mmole of pyrene in an acetonitrile and water (90:10) solution [8]. The ozonation of pyrene produces more than 10 byproducts, the majority of which contain multiple rings with aldehydes, acids, and hydroxyl groups [6,8,17,19].

Several research groups have evaluated the toxicity of the ozonation byproducts. Upham et al. [14] found that some of the initial byproducts of PAH ozonation, including those formed from pyrene, appear to inhibit GJIC more than the parent compounds [8]. In addition, Sasaki et al. [20] found that some byproducts of pyrene oxidation have a higher mutagenic activity than pyrene itself. Although it should be noted that this study simulated photooxidation conditions and, therefore, the byproducts were nitrogenated and

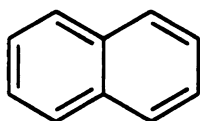
unlike products found from ozonation in aqueous solution. It is difficult to assess which compounds resulted in increased toxicity because byproduct mixtures were used in this study instead of pure compounds.

In this study, large quantities of pyrene were ozonated for future examination. Ozone dosages were chosen to ensure complete removal of the parent compound. The experiments were carried out at a low pH to guarantee that the majority of the reactions would be due to direct oxidation of pyrene by molecular ozone. The by-product mixtures were preserved for later separation by chromatographic techniques, identification by nuclear magnetic resonance (NMR) spectroscopy and gas chromatography followed by mass spectrometry (GC/MS), and toxicological study.

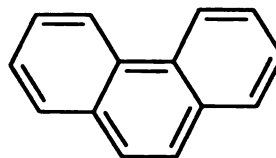
In addition, ozonation experiments were conducted at both low and high pH to determine the effects of pH on oxidation efficiency and by-product formation. However, there are practical circumstances in which the pH could be much higher (i.e., water treatment). The by-product mixtures were preserved for later separation by chromatographic techniques, identification by GC/MS, and toxicological study.

2.2 Methods

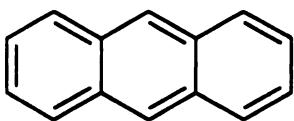
Ozone was generated by corona discharge using a Polymetrics Model T-408 ozone generator (San Jose, CA). Oxygen was first dried using a molecular sieve. Figure 2.2 shows the configuration of the ozonation system. A 250 mL gas washing bottle was used for the ozonation reactor. Ozone was bubbled into 200 mL of an acetonitrile and water (90:10 v/v) solution containing 5 mM dissolved pyrene (99% purity, Sigma Chemical Co., St. Louis, MO).



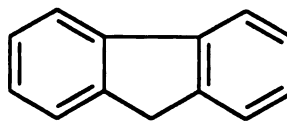
Naphthalene



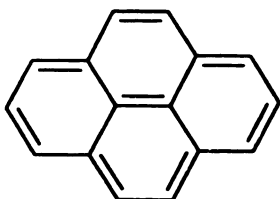
Phenanthrene



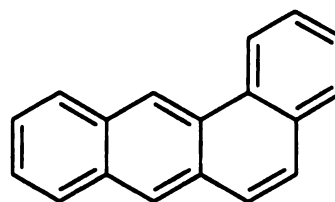
Anthracene



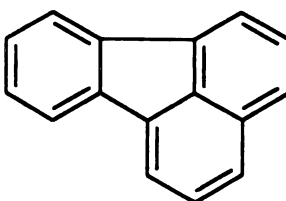
Fluorene



Pyrene



Chrysene



Fluoranthene

Figure 2.1 Generic classes of PAH compounds

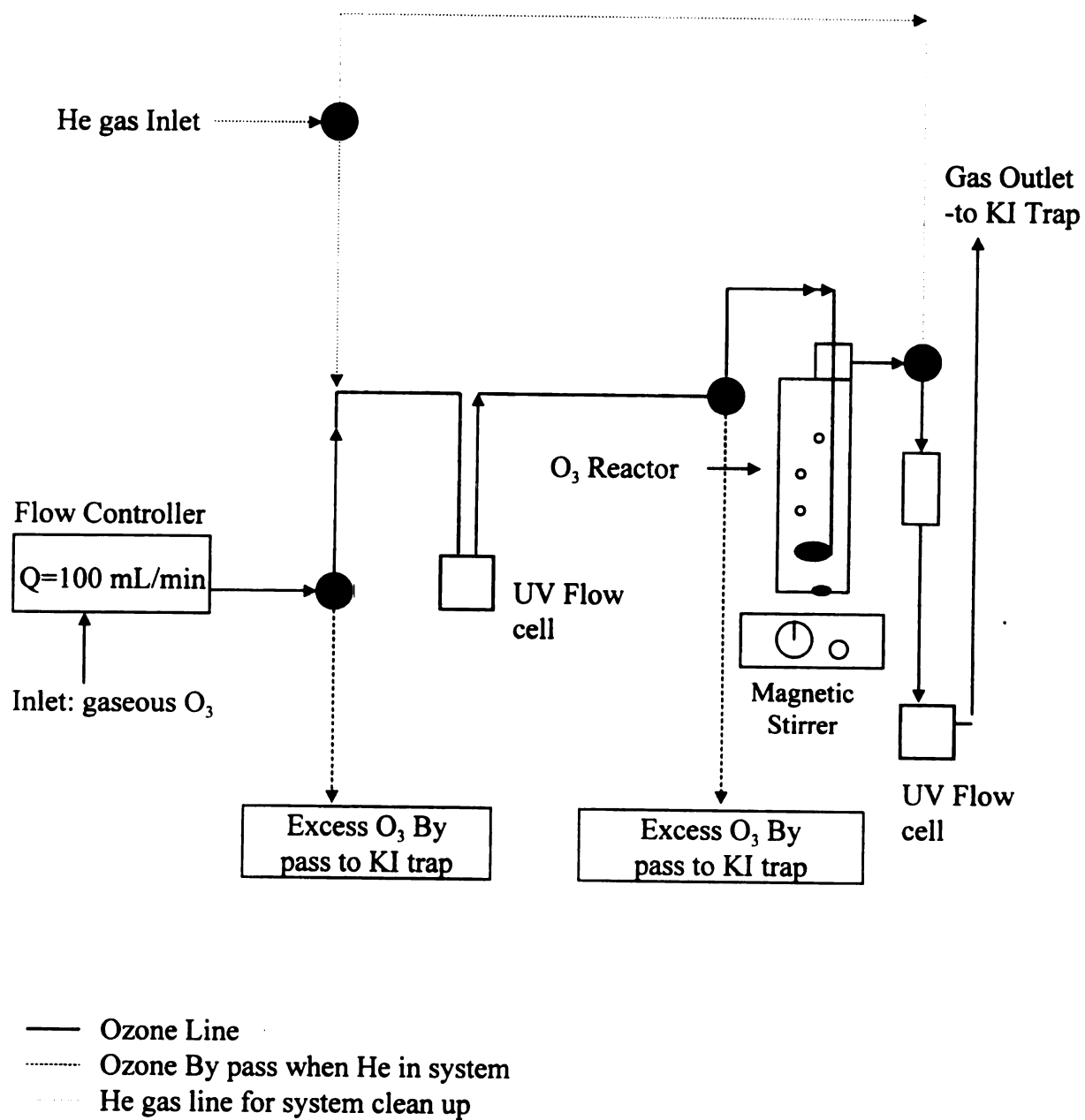


Figure 2.2. Configuration of ozonation system

Table 2.1 Mechanism of Attack by Ozone

PAH Class	Mechanism of Attack
Naphthalene	Near exclusive bond attack first at the 1-2 bond followed by attack at the 3-4 bond [17].
Phenanthrene	Bond attack at the 9-10 bond [17].
Anthracene	Major mechanism is attack at atoms 9 and 10 followed by ring cleavage [17].
Fluorene	Ozonolysis at methylene group with major product homophthalic acid [17].
Pyrene	Bond attack at the 4-5 bond followed by attack at the 9-10 bond [17].
Chrysene	Bond attack at 5-6 bond [17].
Fluoranthene	Atom or bond attack producing quinones and acids [18].

Acetonitrile (99.8% purity) was purchased from EM Science (Gibbstown, NJ).

Acetonitrile was chosen as the organic solvent because it will solubilize PAHs, it is miscible in water, it causes no interference with biological assays, and it has a low reactivity with ozone (half life of 28 years at a concentration of 1400 mM) [21]. Water acts as a participating solvent in the degradation of pyrene using ozone in an aqueous environment [19]. A 10% water solution is sufficient to ensure that the reaction is not water limited, but also dilute enough to avoid solubility problems due to the nonpolar characteristics of pyrene [8]. The pH of the solution in the reactor was acidified to approximately 2 using phosphoric acid. This decreased the extent of decomposition of ozone to hydroxyl radicals [5,16]. For experiments conducted at a pH of 9.5, a borate buffer solution (15.6 mM) replaced the acidified water solution. The borate buffer solution was prepared as described by Adams [22]. The flow of ozone was regulated at 100 mL/min with a side track flow controller (Sierra Instruments Inc., Monterey, CA). All tubing (i.d. 1/8"), connectors, and valves were constructed of Teflon®.

During ozonation, solutions were continually mixed with a magnetic stirrer and stir bar. Reactions were terminated by flushing the ozone from solution using helium gas. Organic peroxides were quenched using 0.2 M sodium sulfite (Sigma Chemical, St. Louis, MO). Ozonated samples were washed of excess sodium sulfite using a liquid-liquid extraction method. The extraction was performed by washing the solution with chloroform three times. Washed samples were concentrated using rotary evaporation and dried using nitrogen gas.

The concentrations of ozone in the influent and effluent gas streams were measured spectrophotometrically at 258 nm using a UV-Vis spectrophotometer (Model 1201 Shimadzu, Scientific Instruments, Japan). A molar absorptivity coefficient for ozone of $3000 \text{ M}^{-1}\text{cm}^{-1}$ was used [23]. Quartz flow cells with a path length of 0.2 cm were used. The effluent gaseous ozone was trapped in an aqueous 2% (w/v) potassium iodide solution.

2.3 Results

Pyrene was oxidized using ozone at dosages ranging from 0.42 to 3.62 mmoles of ozone per mmole of pyrene (Table 2.2). The pyrene concentration was 5 mmoles/L (or 1000 ppm) for all experiments. The ozone dosage was calculated using the following equation:

$$\text{Ozone Dosage} = (C_i - C_e) \times Q \times t$$

where C_i and C_e are the influent and effluent gaseous ozone concentrations, respectively. Q is the gas flow rate and t is the reaction time.

During the majority of experiments, the ozone dosage was maintained between 1.6 and 4.5 mmoles ozone/ mmole pyrene. At this range it had been previously observed that while the majority of the pyrene had oxidized, compounds that may be toxic were still present [8]. Figure 2.3 shows byproduct structures of pyrene ozonation previously identified by Yao [24]. When ozone initially reacts with pyrene the bond at the 4-5 position is broken, creating several three ringed variations. As the ozone dosage increases, the 9-10 bond is broken creating several two ringed or biphenyl type compounds from the initial three ringed byproducts [8,17]. Experiments conducted at

ozone dosages less than 2 mmoles ozone/ mmole pyrene were conducted to generate larger quantities of the three ringed byproducts. Experiments conducted at ozone dosages

Table 2.2 Summary of Ozonation Experiments

Sample Date	Sample Volume (mL)	Pyrene Concentration (ppm)	Ozone Dosage (mmol O ₃ / mmol PY)	pH
10/24/95	200	1000	1.74	1.8
	200	1000	1.89	1.8
	200	1000	2.36	1.8
	200	1000	2.54	1.8
1/18/96	200	1000	1.72	1.8
	200	1000	1.72	1.8
5/28/96	200	1000	1.40	1.8
	200	1000	1.77	1.8
	200	1000	1.67	1.8
	200	1000	1.63	1.8
5/12/97	200	1000	2.71	1.8
	200	1000	2.64	1.8
	200	1000	2.64	1.8
	200	1000	2.56	1.8
5/13/97	200	1000	0.42	1.8
	200	1000	0.43	1.8
	200	1000	0.81	1.8
	200	1000	0.82	1.8
10/2/97	200	1000	3.20	1.8
	200	1000	2.13	1.8
2/23/98	200	1000	1.81	1.8
	200	1000	1.84	1.8
	200	1000	3.62	1.8
6/12/98	200	1000	1.88	9.5
	200	1000	0.37	7.0

between 2 - 4 mmoles ozone/ mmole pyrene were completed in an effort to generate larger quantities of two ringed byproducts, should they be needed in later toxicological studies.

In previous studies, compound C was shown to be the major by-product of pyrene ozonation [24]. The formation of this product was initiated at ozone dosages as low as 0.04 mmoles ozone/ mmole pyrene [24]. Experiments were performed in this study at an ozone dosage of 0.42-0.43 mmoles ozone/ mmole pyrene in an attempt to generate large quantities of compound C byproduct for later studies.

The last series of experiments were conducted using the methods utilized for solutions ozonated at a pH of 2 with one exception. A borate buffer solution with a pH of approximately 9.5 replaced the acidified water (pH=1.8). The new buffer solution was used to mimic water treatment conditions and minimize scavenger formation. The purpose of this experiment was to analyze the resulting mixture of byproducts and pyrene oxidation efficiency and compare with the results when using a pH = 1.8 buffer solution. The ozone demand may be affected by the acetonitrile which acts as hydroxyl radical scavenger. However, this interference may be negligible due to a slow reaction rate ($k=2.2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$) [21].

2.4 Conclusions

The successful removal of pyrene from aqueous solution and subsequent production of ozonation byproducts can be assumed with some certainty as the laboratory methods utilized have been proven in previous studies [8,24]. This assumption is confirmed in Chapter 3.

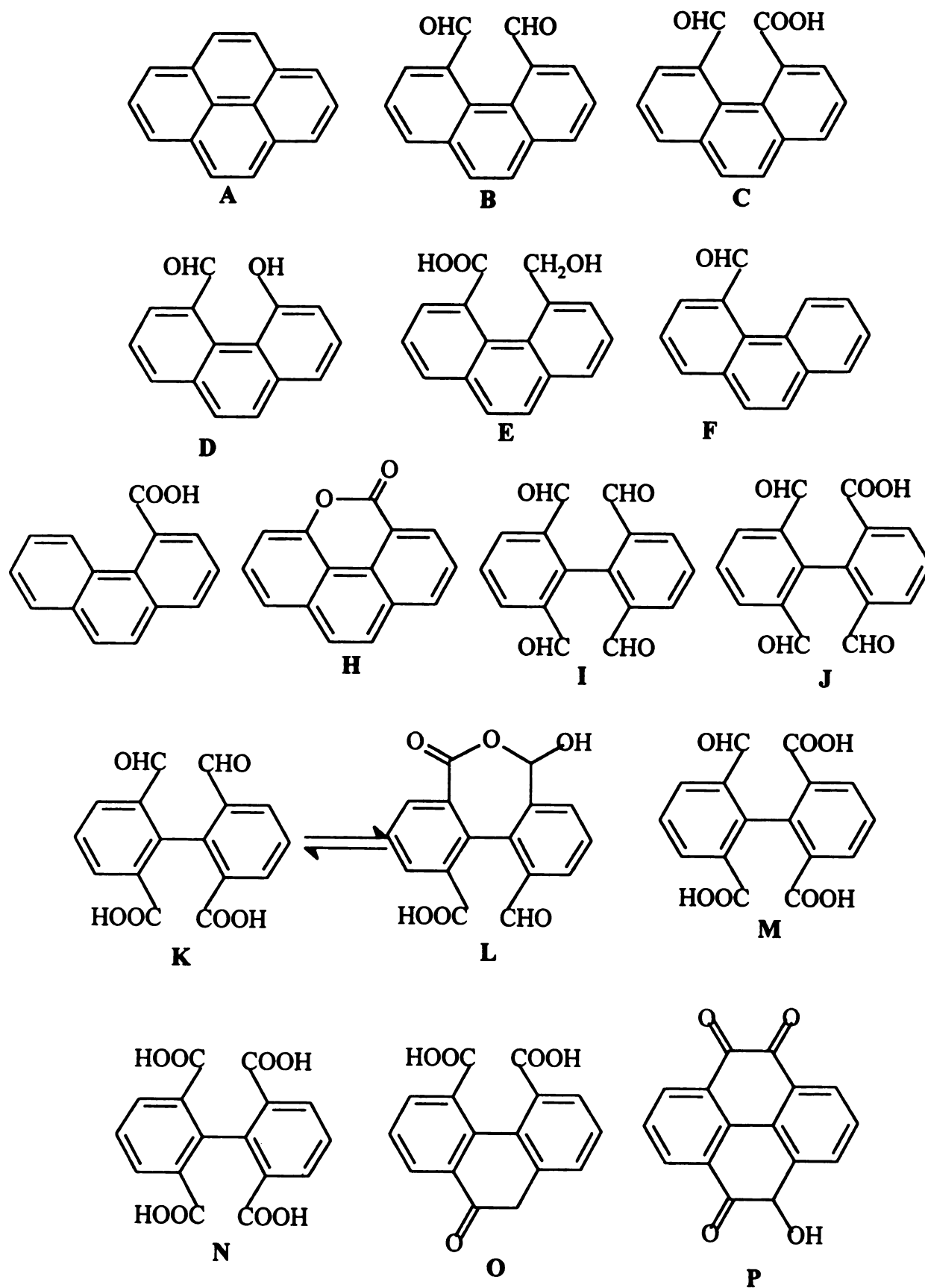


Figure 2.3 Previously identified byproducts of pyrene ozonation [23]

2.5 References

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Chapter 3 - Byproduct Separation and Identification

3.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs), compounds comprised of multiple fused benzene rings, are derived from the incomplete combustion of organic matter [1]. These compounds are ubiquitous in the environment and pose a health threat due to the carcinogenic properties of certain PAHs.

The removal of PAHs from the environment has been difficult in the past because conventional treatment technologies have not been extremely successful [2]. Chemical treatment and, in some cases, chemical treatment in conjunction with biological treatment have been favorable in degrading these recalcitrant compounds without the generation of additional waste products [2].

Biological degradation is a common technique employed in wastewater treatment processes for the removal of many organic compounds. In recent years, the use of this technique has been expanded to include soil and groundwater remediation projects for removal of compounds such as chlorinated solvents, pesticides, gasoline components, and larger hydrocarbons such as PAHs [3-11].

One of the most important factors which determine the success of biological treatment is the bioavailability of the compounds to the microorganisms. In general, as the PAH increases in ring number, the solubility decreases. This characteristic makes bioremediation of high molecular weight PAHs very difficult because the mass transfer of PAHs from soils to water is very small and, therefore, bioavailability is low [12,13]. The smaller PAHs, one to three aromatic rings have been biodegraded in liquids, soil slurries,

and, in some cases, soils; while biodegradation of four to six aromatic ring PAHs in these media has been fairly unsuccessful [14]. In cases where oxidation or other chemical treatments were performed prior to biological treatment, large PAHs were degraded to a much greater extent [14,15].

The use of ozone for the oxidation of PAHs is an effective method for the degradation of these compounds. Ozone can degrade these high molecular weight compounds rapidly in liquids and soils [15-18]. *In situ* ozonation, while not common place, has been used by a few companies with promising results [19]. *In situ* ozonation of soils and groundwater contaminated with PAH and BTEX was used at a site in Iowa. Ozone sparging at this site significantly reduced the PAH and BTEX concentrations [20].

The degradation of PAHs using ozone involves a series of oxidations which reduces the number of fused rings. Several intermediates are formed and subsequently oxidized during PAH ozonation. Eventually, these compounds can be oxidized to straight chain aliphatics given a large enough ozone dosage [21].

Engineering remediation projects tend to base the success of a site clean up on the removal of the parent compounds. However, many studies have shown that some of the byproducts may be as or more harmful than the parent compound. Upham et al. [17] found that some three ringed PAHs were more toxic than the four or five ringed PAH compounds.

Pyrene, a PAH comprised of four benzene rings is the object of this study and can be completely degraded using ozone with at least 10 byproducts formed [17,21,22]. According to Upham et al. [22], some of the initial by-products of pyrene ozonation may be as or more harmful than the parent compound, pyrene. Sasaki et al. [23] also showed

that some of the by-products of pyrene oxidation had a higher mutagenic activity than the parent compound. Although the oxidation products in the Sasaki study were dissimilar to the products in the Upham studies, both have similar conclusions [22]. The byproducts in addition to the parent compound must be evaluated due to the possible threat to human health.

Following the ozonation of pyrene in aqueous solution, the resultant mixture contains more than 10 byproducts. It is important to know which compounds contribute to the mixture's associated toxicity. Due to the unavailability of commercial metabolites, the goal of this study was to separate the byproducts from one another using chromatographic techniques described in the literature. These byproducts could then be studied individually as opposed to the whole mixture. Solutions that were ozonated as described in Chapter 2 were separated into fractions. Both low and high pH solutions were separated chromatographically. Three compounds were isolated and identified by NMR. All impure fractions and some isolated compounds were identified using GC/MS. These fractions were dried and preserved for later toxicological studies.

3.2 Methods

3.2.1 Byproducts of Pyrene Ozonation

Byproducts of pyrene ozonation were produced as described in Chapter 2 [24]. Following saturation using sodium sulfite (Sigma Chemical, St. Louis, MO), samples were wrapped in aluminum foil and refrigerated for 24 hours at 10°C.

3.2.2 Liquid-Liquid Extraction (removal of excess sodium sulfite)

Samples saturated with sodium sulfite were concentrated using rotary evaporation at 35°C to remove the acetonitrile. The dry material was subjected to a liquid-liquid extraction detailed in Figure 3.1. The majority of the dry material was dissolved in chloroform. Due to the range in polarity of the byproducts, there was a small portion of material insoluble in chloroform. The chloroform extract was washed three times with distilled water in a 250 mL separatory funnel. Each 15 mL aliquot of the chloroform soluble portion was washed with 30 mL of distilled water three times to remove excess sodium sulfite. The water soluble portion was washed three times with chloroform to extract ozonation byproducts. Each 15 mL aliquot of water soluble portion was then washed with 30 mL of clean chloroform three times in a 250 mL separatory funnel. The chloroform extracts were combined together, evaporated *in vacuo* at 35°C, dried under nitrogen gas, and weighed. Dry samples were stored at 0°C.

3.2.3 Ethyl Acetate/ Sodium Bicarbonate Extraction

Following ozonation, samples were evaporated *in vacuo* at 35°C, dried under nitrogen gas, and weighed. The separation of acids from the crude material is detailed in Figure 3.2. Dry samples were dissolved in ethyl acetate and extracted with a saturated solution of sodium bicarbonate. The ethyl acetate portion was washed with the sodium bicarbonate solution three times in a 250 mL separatory funnel. The ethyl acetate portion was evaporated *in vacuo* at 35°C, dried under nitrogen gas, and weighed.

The sodium bicarbonate portion was acidified using 3N hydrochloric acid; This brought the solution pH down to approximately 2. The acidified material was applied to

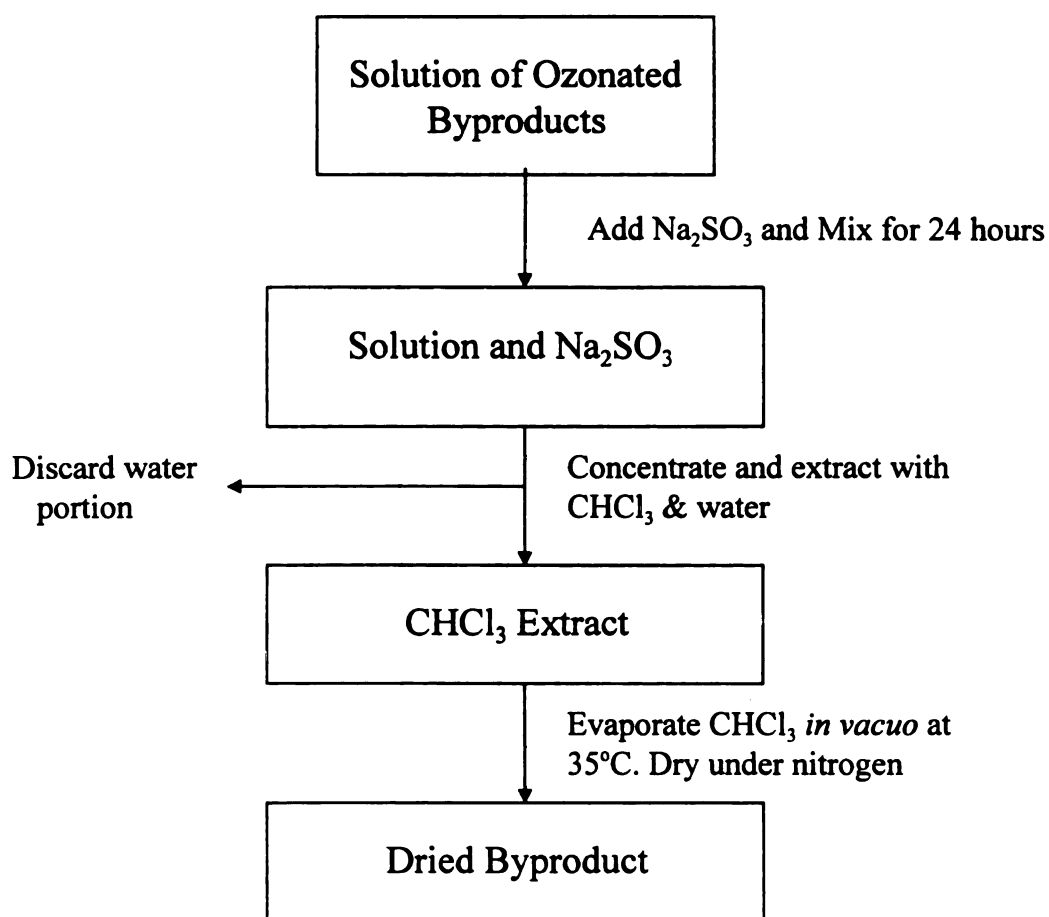


Figure 3.1 Protocol for organic peroxide quench and excess sodium sulfite removal

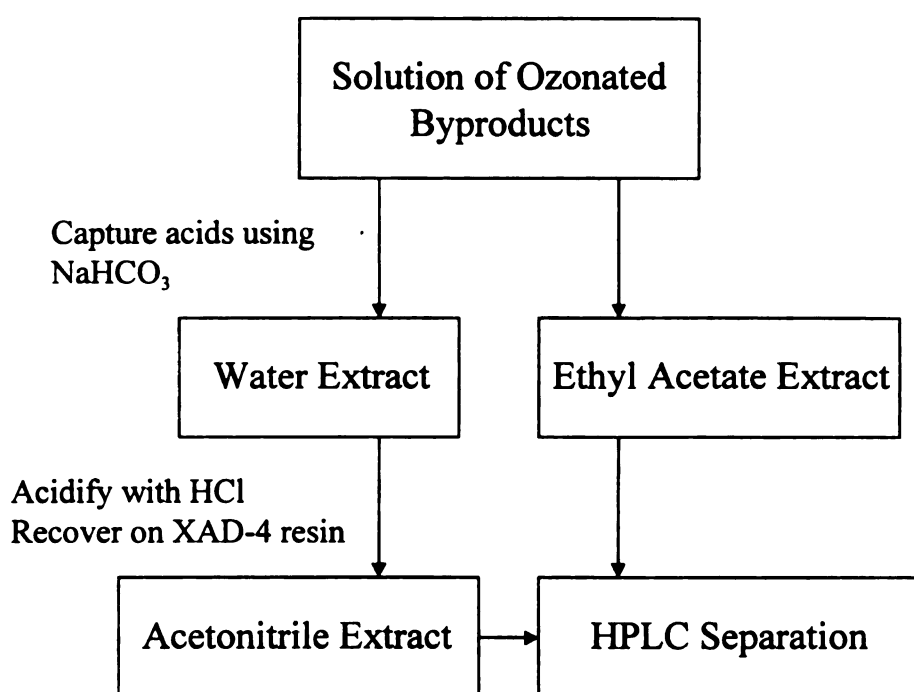


Figure 3.2 Protocol for the separation of acidic byproducts from the mixture of byproducts

Amberlite XAD-4 resin (Supelco, Bellefonte, PA) in 25 mL quantities. The XAD-4 resin was contained in a 200 mL glass column with a stop cock. The bed volume of the XAD-4 resin was approximately 50 mL. The XAD-4 material was conditioned in pure methanol for one hour and subsequently washed with distilled water. The volume of distilled water was three times the bed volume. Following application, the acidified sample was allowed to stand for 15 minutes. Distilled water was run through the column to remove excess sodium chloride. The distilled water was run through the column until the pH of the effluent was neutral. Byproducts were eluted from the XAD-4 resin by running two bed volumes of acetonitrile/water (50:50) and two bed volumes of acetonitrile (100%) through the column. The acetonitrile/water and acetonitrile solutions were combined and evaporated *in vacuo* at 35°C, dried under nitrogen gas, and weighed.

3.2.4 Thin Layer Chromatography

Analytical TLC. To accomplish a rough analysis of crude material or fractionated material following ozonation, preparative TLC, medium pressure liquid chromatography (MPLC), and high pressure liquid chromatography (HPLC) were performed using thin layer chromatography (TLC). Concentrated solutions of crude material or fractionated material was prepared in chloroform or methanol, depending upon solubility. The solutions were applied to silica plates (20 x 5 cm, 250 µm with inorganic binder and UV 254) with 5 µL capillary pipettes. The material applied to the plates in dots was allowed to dry by evaporation prior to placement of the plates in a solvent chamber. Solvent chambers were equilibrated with 15-20 mL of the following solvent system:

$\text{CHCl}_3/\text{C}_6\text{H}_6/\text{MeOH}$ (10:10:1). Plates were processed in solvent chambers for 55 minutes and dried under low heat in a ventilated hood.

Preparative TLC. Preparative TLC was performed in a similar manner.

Approximately 25 mg of material dissolved in methanol or chloroform were applied to each preparative plate (20 x 20 cm, 250 μm , silica gel HF, binder free) as a thin band along the bottom of the plate (Analtech Inc., Newark, DE). The material was allowed to dry on the plate before placement in a solvent chamber equilibrated with chloroform/benzene/methanol (10:10:1). Solvent chambers contained 175 mL of solvent and plates were processed in tanks for 55 minutes. Following exposure of silica plates to the solvent, plates were allowed to dry under low heat or by evaporation in a ventilated hood. Under UV light (254 nm and 366 nm), bands of separated material were identified and subsequently each band was scraped from the glass plate. Each band of silica removed from the plate was eluted with methanol and chloroform and filtered using 30 mL sinter glass funnels (10-20 μm). Solvents for each band containing recovered material were combined, concentrated *in vacuo* at 35°C, dried under nitrogen gas, and weighed.

3.2.5 Medium Pressure Liquid Chromatography

Reverse phase medium pressure liquid chromatography (MPLC) was conducted using a reverse phase medium pressure column (C18 silica, 500 x 20 mm glass column, pressure rated 150 psi, DyChrom, Santa Clara, CA) with a bed volume of approximately 300 mL. The mobile phase was introduced into the column using a low pressure pump

(Model 81-M-2, Chemco, Sunnyvale, CA). Material to be separated was solubilized in methanol and water. The column was conditioned in the same solvent system (methanol/water (70:30)). Samples were filtered using a 0.22 μm filter (Millipore Corp, Bedford, MA) prior to injection onto the column. The separation was accomplished using a stepwise isocratic solvent system at a flow rate of 4 mL/min. Two different systems were used as seen in Table 3.1.

Table 3.1 MPLC Stepwise Isocratic Solvent Systems

Solvent System A	Solvent System B
70:30 methanol/water - 300 mL	70:30 methanol/water - 250 mL
95:5 methanol/water - 300 mL	80:20 methanol/water - 150 mL
100:0 methanol/water - 500 mL	90:10 methanol/water - 200 mL
	95:5 methanol/water - 200 mL
	100:0 methanol/water - 300 mL

The experiment was monitored using a hand held UV light (254 nm). Samples were collected, evaporated *in vacuo* at 35°C, dried under nitrogen gas, and weighed.

3.2.6 High Pressure Liquid Chromatography

Preparative HPLC. Separation of crude materials and fractions previously separated using MPLC were further separated using a recycling preparative HPLC Model LC-20 (Japan Analytical Industry, Dichrom, Santa Clara, CA) with a Jaigel S-343-15 C₁₈ (15 μm) column (i.d. 20 x 250 mm). An isocratic solvent system was used (methanol/water (70:30)) at a flow rate of 3 mL/min. Samples were prepared in the same solvent in which the column was conditioned. Prior to injection, all samples were filtered

through a 0.22 μm filter (Millipore Corp., Bedford, MA). A variable wavelength UV detector (S-310A Model II, Soma Optics, LTD., Dichrom, Santa Clara, CA) set at 254 nm was used to monitor the analytes. Injection volumes ranged from 1 to 2 mL. All fractions collected were evaporated *in vacuo* (Buchii RE111 Rotovapor, Brinkman, Westbury, NY) at 35°C, dried under nitrogen gas, and weighed.

Semi-preparative HPLC. Separation of crude materials and/or fractions previously separated using MPLC were further separated using a semi-preparative HPLC (Perkin Elmer, series 200, Cupertino, CA) using a Phenomenex, Capcell Pak, C₁₈ (5 μm) column (i.d. 10 x 250 mm). A gradient solvent system using acetonitrile and water (Table 3.2) was employed with a flow rate of 1.5 mL/min.

Table 3.2 Semi-Preparative HPLC Gradient Solvent System

Solvent	Duration
70:30 to 75:25 Acetonitrile/water	23 minutes
90:10 Acetonitrile/water	15 minutes
70:30 Acetonitrile/water	10 minutes

Samples were solubilized in the same solvent in which the column was conditioned. Prior to injection, all samples were filtered through a 0.22 μm filter (Millipore Corp., Bedford, MA). A dual variable wavelength absorbance detector (Waters, Model 2487, Milford, MA) was set at 240 nm and 254 nm and used to monitor the analyte elution. All fractions collected were evaporated *in vacuo* (Buchii RE111 Rotovapor, Brinkman, Westbury, NY) at 35°C, dried under nitrogen gas, and weighed.

3.2.7 Identification Techniques

Nuclear Magnetic Resonance Spectroscopy. Identification of pure byproducts was accomplished using proton (^1H) and carbon (^{13}C) NMR spectroscopy. In some cases a Distortionless Enhancement by Polarization Transfer (DEPT) was performed. NMR spectra were recorded on a Varian VXR 300 MHz spectrometer and a Varian VXR 500 MHz spectrometer (Varian, Palo Alto, CA) at 25°C. NMR analyses were conducted at the Max T. Rogers facility, Chemistry Department, Michigan State University.

Gas Chromatography/ Mass Spectrometry. GC/MS was performed using a JEOL AX-505H double focusing mass spectrometer coupled with a Hewlett-Packard 5890S GC (Norwalk, CT). A DB5MS (30 m length x 0.32 mm i.d. x 0.25 μm film thickness) fused silica capillary column (J & W Scientific, Rancho Cordova, CA) was used for the GC separation. A splitless injector was used with a column head pressure of 10 psi using helium as the carrier gas, producing a flow rate of 1 mL/min. The initial column temperature was held for 2 min at 100°C, ramped at 20°C/min to 220°C, ramped at 5°C/min to 280°C, and finally ramped at 20°C/min to 300°C. The mass spectrometer was operated in the electron impact mode. Mass calibration of the spectrometer was completed using perfluorokerosine. GC/MS experiments were performed at the MSU-NIH Mass Spectrometry facility, Biochemistry Department, Michigan State University.

3.3 Results & Discussion

Several samples containing dissolved pyrene were ozonated for later byproduct analysis. The ozone dosage was kept between 1.6 to 2.0 mmoles ozone per mmole pyrene in an effort to obtain the byproducts present following the removal of the majority of the parent compound, pyrene. Multiple techniques for fractionation and purification of the byproducts of pyrene ozonation were investigated. Five procedures in particular showed the most promise and are described and discussed in this section.

3.3.1 Procedure One

The byproducts of pyrene ozonation were prepared as described in Chapter one at low pH. Organic peroxides that may have been present were quenched using sodium sulfite (0.2 M solution). The glass, teflon-capped bottle containing the solution was wrapped in foil and placed on the shaker (Labline, Model 3590, Melrose Park, IL) for 24 hours. The majority of the aqueous portion was dried *in vacuo* at 35°C. The dried portion was extracted with chloroform. The undissolved sodium sulfite present at the bottom of the sample jars was dissolved later with distilled water and saved for liquid-liquid extraction.

Byproducts contained in the chloroform soluble portion and the left over water portion were recovered using liquid-liquid extraction. Unlike acetonitrile and water, chloroform and water are immiscible. This property allows the byproducts to be separated from the sodium sulfite, which is soluble in water. This process does not work well for very polar compounds that are more soluble in water than in chloroform. Many of the byproducts were soluble in chloroform. However, there were several polar

compounds which made the byproduct recovery from liquid-liquid extraction very difficult (60-70%). In addition, dissolved sodium sulfite in clean samples obscured the actual recovery of the byproducts. However, the residual sodium sulfite was removed during subsequent separation steps.

An analytical TLC was performed which showed 7 major bands, one of which was residual pyrene. Further purification of the byproduct mixture was attempted using preparative TLC. Eight bands were identified and collected. A poor recovery (about 50%) was again encountered. A repeat TLC of the fractions collected from preparative TLC revealed they contained two or more compounds each per fraction. In addition, analytical TLC showed the presence of several compounds not present in the original crude material. This showed possible decomposition of some byproducts by during preparative TLC. Studies documenting photooxidation of PAHs on glass surfaces and degradation of PAHs using ozone on silica gel carrier suggest that decomposition of these compounds on silica gel plates may be unavoidable [25, 26]. In addition, Cope *et al.* commented that photooxidation of PAHs on TLC plates could explain the decomposition of compounds [26]. Due to the problems encountered following preparative TLC, this procedure was discontinued.

3.3.2 Procedure Two

The byproducts of pyrene ozonation were again prepared as described in Chapter one at low pH. However, a large quantity (4 x 200 mg samples) of pyrene was ozonated so as to have adequate quantities of byproducts for purification. Organic peroxides that may have been present were quenched using sodium sulfite (0.2 M solution) as described

in procedure one. The liquid-liquid extraction for excess sodium sulfite removal was performed with a recovery of approximately 80%. The increased byproduct recovery was due to a slight decreased ozonation dosage which left a greater amount of unoxidized pyrene and other nonpolar byproducts in solution. These compounds were more soluble in the extraction solvent. Analytical TLC showed approximately 7 major bands present which was consistent with procedure one.

The first step in the separation of the crude material was medium pressure liquid chromatography (MPLC). The separation was performed using MPLC solvent system A described in the methods section of this chapter. Five major bands were identified during the MPLC separation. Forty-six samples ranging in volume from 10 to 60 mL were collected. Analytical TLC of the fractions showed the separation was not successful. Similar to the preparative TLC, many of the same compounds were present in two or more fractions. Although, unlike the preparative TLC, compound decomposition was not a problem in the MPLC experiment. The 46 samples collected were combined into 5 fractions which were further separated using preparative HPLC.

The HPLC experiment was performed as described in the methods section of this chapter. The chromatogram showed 11 major peaks (Figure 3.3) which is consistent with previous studies performed by Yao [24]. The analytical TLC showed only 4 major bands which meant that each of these bands contained more than one compound. Of the fractions collected, peaks 9 (7.5 mg) and 10 (9 mg) were the largest in mass and the most pure. From the analytical TLC, peak 11 (2.1 mg) was determined to be residual pyrene. The total mass of the fractions collected was 47.6 mg (60 mg starting material).

Inefficient recovery and mass lost during filtration prior to HPLC fractionation may account for the rest.

Figure 3.4 shows the chemical structures for compounds D, Q, and pyrene. Peak 9 contained both compound D and Q. Peak 10 contained compound Q. Peak 11 consisted of residual pyrene.. Results from NMR spectroscopy for compounds D, Q, and pyrene are shown below:

Compound D: ^1H NMR (CDCl_3): δ 3.7 (3H, OCH_3), 7.6-7.9 (8H, aromatic protonated and unprotonated carbons), 10.05 (1H, CHO). ^{13}C NMR (CDCl_3): δ 56.40 (OCH_3), 125-139 (14C, aromatic protonated and unprotonated carbons), 191 (CHO).

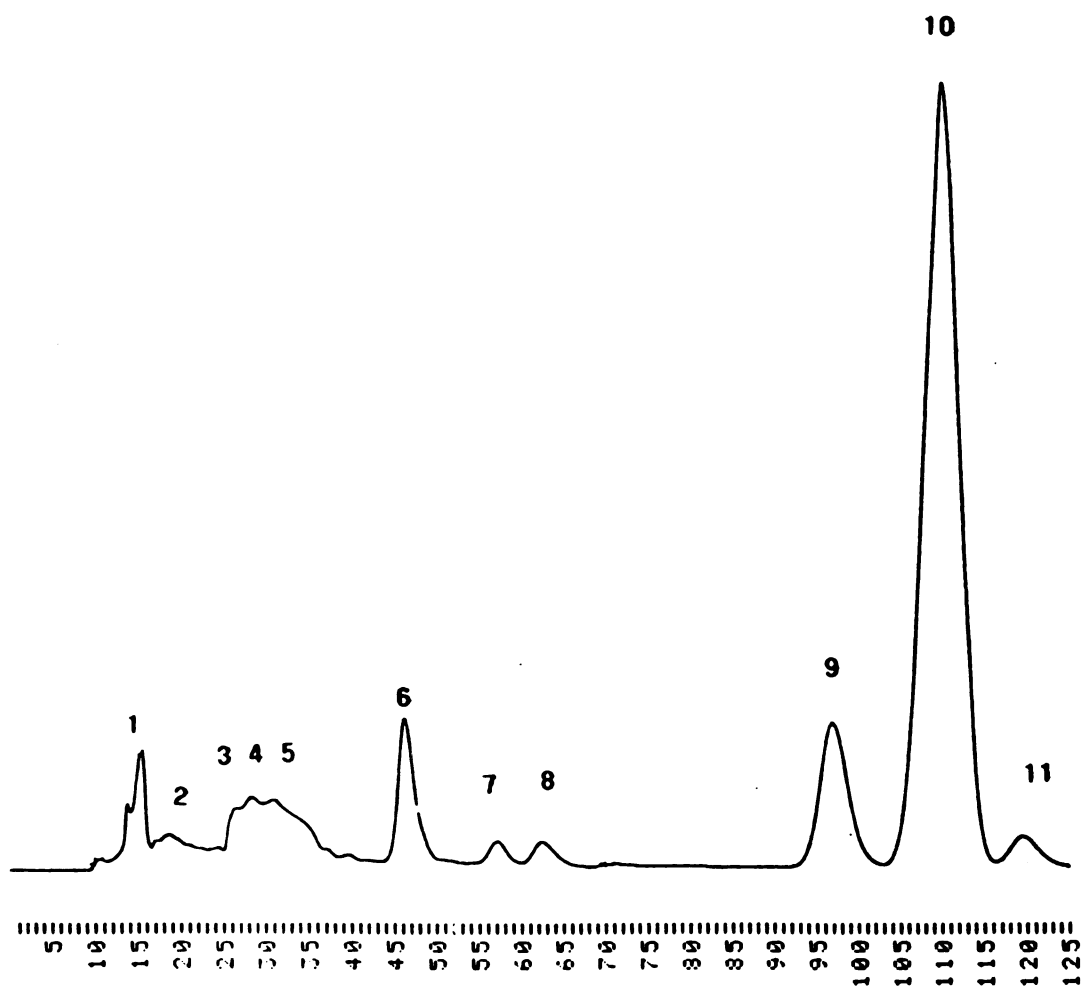
Compound Q: ^1H NMR (d_6 -DMSO): δ 3.46 (6H, s, OCH_3), 5.77 (2H, s, 15-H, 16-H), 7.64 (2H, t, $J=7.65$ Hz, 3-H, 6-H), 7.68 (2H, d, $J=6.90$ Hz, 2-H, 7-H), 7.84 (2H, s, 1-H, 8-H), 7.98 (2H, d, $J=7.80$ Hz, 9-H, 10-H). ^{13}C NMR (d_6 -DMSO): δ 55.37 (OCH_3), 101.07 (15-C, 16-C), 125.69 (9-C, 10-C), 126.17 (1-C, 8-C), 127.11 (2-C, 7-C), 127.40 (13-C, 14-C), 128.69 (3-C, 6-C), 132.88 (11-C, 12-C), 138.30 (4-C, 5-C).

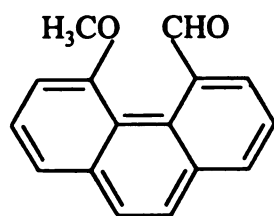
Pyrene: ^1H NMR (CDCl_3): δ (7.9-8.2, 10H).

The ^1H NMR spectrum for the compound contained in peak 11 is identical to the spectrum for pyrene [27,28].

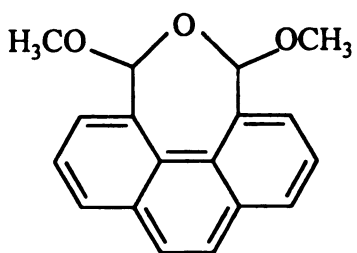
The compounds contained in peaks 9, 10, and 11 were also characterized by GC/MS. Table 3.3 contains the important GC/MS characteristics for the three peaks. Mass spectra for compounds which have been previously identified are not included, but can be viewed in Appendix A of reference 24.

Figure 3.3 Preparative HPLC chromatogram

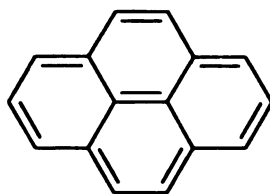




Compound D



Compound Q



Pyrene

Figure 3.4 Chemical structures of compounds characterized in peaks 9, 10, 11

Chemical structures for these compounds are listed in Figure 2.1. The mass spectrum and fragmentation pattern for Compound Q contained in peak 10 are shown in Figures 3.5 and 3.6. Some overlap in peaks occurred during the HPLC separation which caused Compound Q in peak 10 also to be present in small amount in peak 9.

In all three cases, the NMR and GC/MS data were in agreement with each other. Pyrene, contained in Peak 11, contained the typical peaks between δ 7.9 and 8.2 in the proton NMR spectra which represented the aromatic protons in the pyrene ring structure. The mass spectrum matched that of the parent compound, pyrene with the characteristic M^+ ion at m/z 202 and 101.

Table 3.3 GC/MS Characteristics of peaks 9,10,11

Peak I.D. Number	GC Retention time	Molecular Weight of Parent Cmpd	Molecular Weight of TMS Deriv. ^a	Important Ion Peaks, m/z	Product Spectra
9	10'03"	236	----- ^b	236 (M+.) 205 176 151	D [ref 24]
9	10'30"	222	294	294 (M+.) 293 205 176 151	D [ref 24]
9	12'29"	280	----- ^b	280 (M+.) 249 235 205 189 177	Fig. 3.5
10	12'19"	280	----- ^b	280 (M+.) 249 235 205 189 177	Fig. 3.5
11	9'54"	202	----- ^b	202 (M+.) 101	A [ref 24]

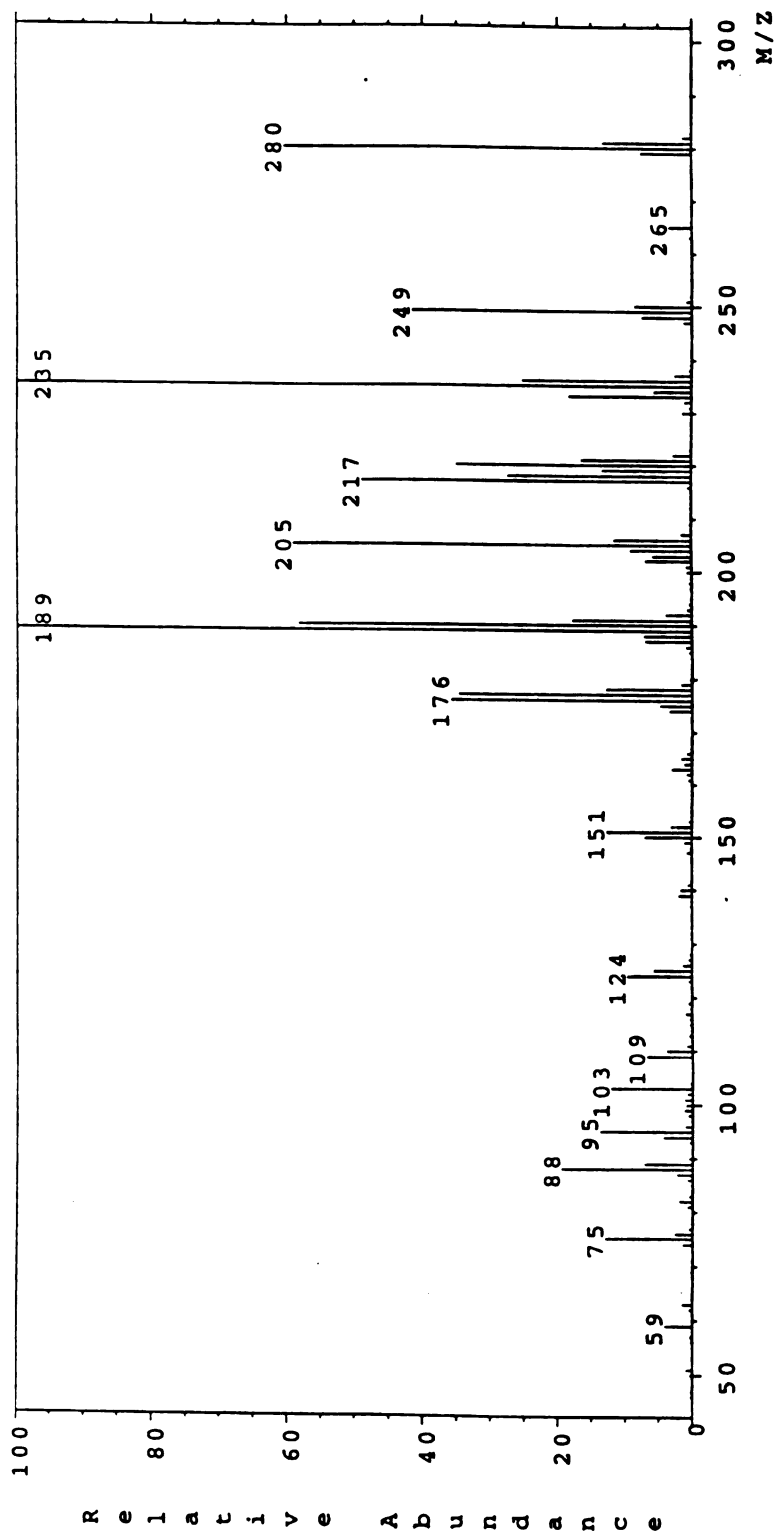
^a theoretical value based on molecular weight of proposed TMS derivative

^b no derivatives for the compound

Peak 10, which contained compound Q, was a very interesting compound to identify due to the structure's symmetry.

Figure 3.5 Mass spectrum of compound Q in peak 10

MASS SPECTRUM Data File: [100,100]A09309802 30-SEP-98 13:04
 Sample: HERNER/MASTEN 8/10-TMS DB5MS 100-2,20/M-220,5/M-26
 RT 12.19" EI (Pos.) GC 450.6c BP: m/z 189.0000 Int. 930.4697 LV 0.00
 Scan# (741) - (729) [coef. 1.00]



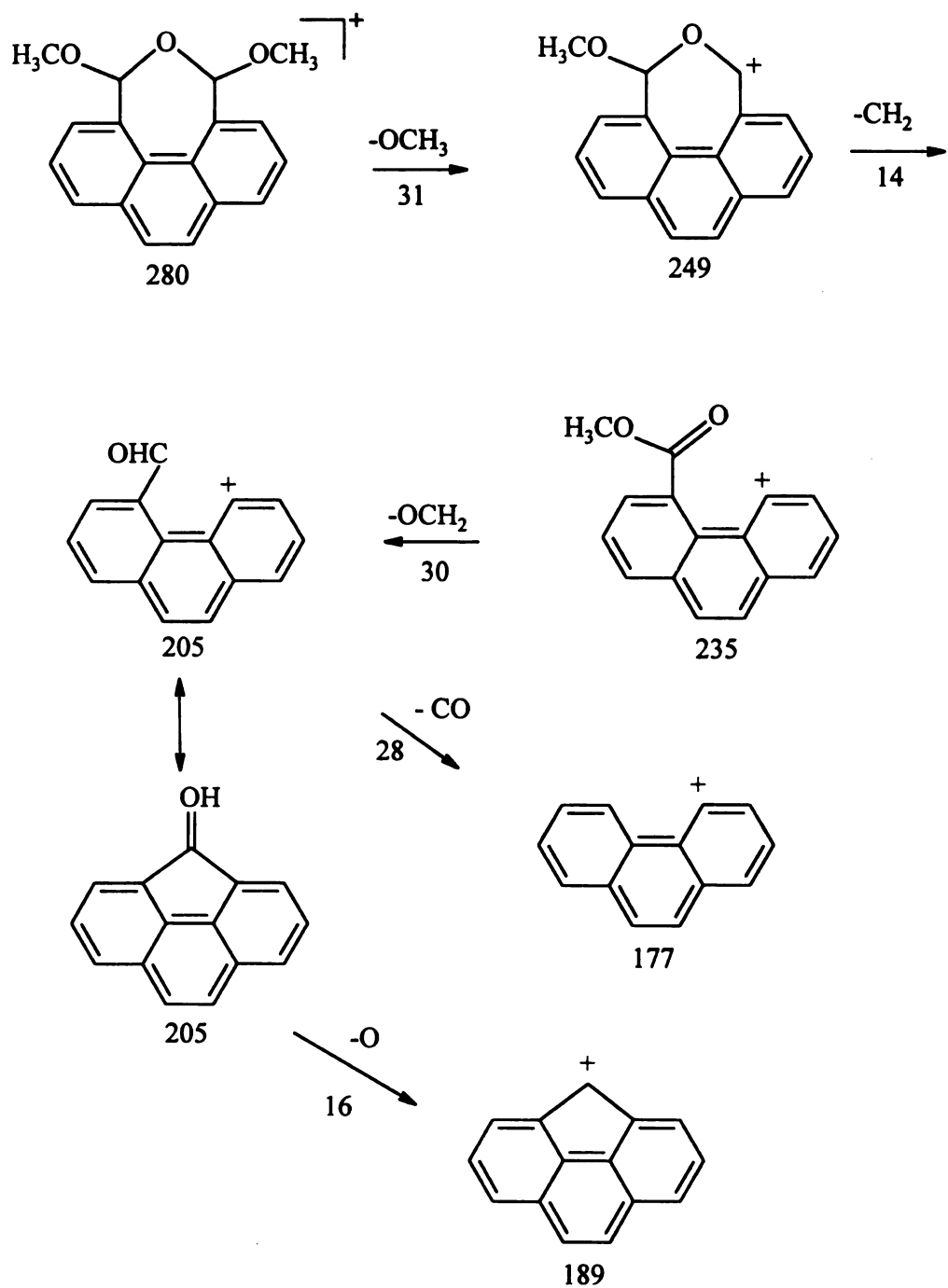


Figure 3.6 Proposed fragmentation pattern for Compound Q in Peak 10

Due to the nature of the chemical process performed during ozonation, it was suspected that the compound was aromatic in nature with 3 rings and 8-10 CH carbons. The DEPT spectrum showed the compound is symmetrical and gave only 5 CH carbons. The ^1H NMR spectrum indicated two OCH_3 functional groups, which integrated for 6H, at δ 3.46. The singlet at δ 5.77, which integrated for 2H, was assigned to the two non-aromatic CH carbons in the structure. The remaining three singlets, two doublets, and one triplet in the δ 7.64-7.98 range, each integrated for 2H, were easily assigned to the CH carbons of the aromatic rings.

Several features of the ^{13}C NMR spectrum for Compound Q should be noted. The chemical shift at δ 55.37 represents the two OCH_3 moieties. The chemical shift at δ 101.07 represents the two non-aromatic CH carbons while the aromatic CH and unprotonated carbons are represented by the peaks in the range of δ 125.69 - 138.30. Assignment of carbons for compound Q was determined using the data from the DEPT, the ^{13}C NMR, and the rules of aromaticity.

The M^+ ion and proposed fragmentation pattern for the Compound Q mass spectrum confirmed the proposed structure in Figures 3.4. (Although the fragmentation pattern proposed for compound Q confirmed the proposed structure, an additional experiment to confirm the fragmentation from m/z 205 to m/z 189 is necessary.) One additional point of interest was the presence of the OCH_3 groups. The nature of the ozonation of pyrene is such that three ringed aromatic byproducts with functional groups at the ring cleavage are expected. However, the functional groups which should evolve are carboxylic acids, aldehydes, and hydroxyl groups [29]. The presence of the OCH_3

groups indicated that a methylation of OH groups had taken place during the purification steps and not during the ozonation. Because the purified byproducts are to be used for toxicological studies and are intended to be the natural byproducts of pyrene ozonation, methanol was removed from the separation and purification steps during later experiments and replaced with acetonitrile.

The ^1H NMR, ^{13}C NMR and GC/MS results showed that peak 9 was a mixture of compound D and one minor compound, which was the same compound in peak 10, compound Q. The ^1H NMR showed signals which represent both compounds Q and D. The OCH_3 signal at δ 3.4 and non-aromatic CH signal at δ 5.77 were present in the ^1H NMR of peak 10. Two signals at δ 3.7 and 10.05 were assigned to the OCH_3 and the aldehyde group, respectively. The only other signal left in the ^1H NMR spectrum was a multiplet at δ 7.85 and accounted for the aromatic protons.

The ^{13}C NMR also confirmed the presence of compounds Q and D in Peak 9. The compounds are consistent with those detailed in Figure 3.4. Features of the spectrum that should be noted are the chemical shifts of the OCH_3 moieties for both compound Q and D (δ 55.80, 56.40, respectively), the non-aromatic CH carbons for compound Q (δ 101.07), and the CHO carbon for compound D (δ 191). The signals between δ 125 - 139 represent the aromatic carbons and were not assigned due to the impurity present in the sample. The mass spectra showed 2 major peaks and one very small peak. The small peak, retention time of 12'29", was identified as compound Q. The two major peaks are actually due to the same byproduct (compound D) in two different forms (Fig. 3.7). It appears that the hydroxyl group on the byproduct compound was methylated during the

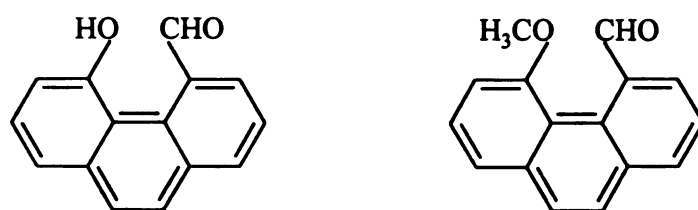


Figure 3.7 Two configurations of Compound D

separation and purification processes. However, the fragmentation patterns for the two compounds are identical. The only difference being the M^+ ion values which are consistent with the values expected for the molecular weights of the compounds prior to fragmentation.

Attempts were made to further purify fractions separated during MPLC using preparative HPLC in order to accumulate additional quantities of the compounds Q and D and also other byproducts which are generated in a small quantity that had not yet been characterized. However, they were unsuccessful due to a change in the selectivity or efficiency of the HPLC column. The separations which had been accomplished were no longer possible.

It appears that structure for compound Q is different enough from that of the other types of pyrene ozonation byproducts that isolation was possible. Compound Q has three aromatic rings and a cyclized fourth ring at the fourth and fifth positions. The majority of the other byproducts are structures with three aromatic rings and various combinations of functional groups attached to the fourth and fifth positions. With additional ozonation, major byproducts were biphenyl type compounds with various combinations of functional groups at the fourth, fifth, thirteenth and fourteenth positions.

3.3.3 Procedure Three

The byproducts of pyrene ozonation were again prepared as described in Chapter one at low pH. Approximately, 800 mg of pyrene was ozonated for byproduct purification. Organic peroxides that may have been present were quenched as described in procedure one. The liquid-liquid extraction for excess sodium sulfite removal was

performed with a recovery of approximately 60%. Analytical TLC showed at least 7 major bands which was consistent with procedure one and two.

The first step in the separation of the crude material was medium pressure liquid chromatography (MPLC). The separation was performed using MPLC solvent system B described in the methods section of this chapter. Although MPLC was not extremely successful in procedure one, it was used again because a new column was purchased (same type and model). Six major bands were identified during the MPLC separation. Seventy-seven 10 mL samples were collected. Similar to the preparative TLC, many of the same compounds were present in two or more fractions. The 77 samples collected were combined into 23 fractions.

Analytical TLC showed 8 major bands and several minor bands in these 23 fractions. However, it is not believed that the 8 major bands contained only 8 major compounds. It is very possible that compounds with similar structures may have been seen on analytical TLC as one band. Several fractions containing bands 3 and 8 appeared to be pure and were combined. After combining the fractions, the sample containing band 3 (called compound G) weighed approximately 28.3 mg. Compound G consisted of a very light tan to white powder and appeared to be pure. The sample containing band 8 (pyrene) weighed approximately 40.5 mg. Analytical TLC and ^1H NMR proved the compound representing band 8 was unreacted parent compound, pyrene, and was not further studied. The ^1H NMR spectra is discussed in detail in procedure two.

Figure 3.8 shows the tentative chemical structure for compound G. Results from NMR spectroscopy for compound G are shown below:

^1H NMR (d_6 -DMSO): δ 7.73 (2H, t, $J=7.65$), 7.93 (3H, t, $J=11.85$), 8.09 (2H, t), 8.13 (1H, d), 8.17 (1H, d), 8.50 (1H, s, COOH).

^{13}C NMR (d_6 -DMSO): δ 123.97 (2C), 125.79 (1C), 126.84 (1C), 126.90 (1C), 126.94 (1C), 127.43 (1C), 127.72 (1C), 130.33 (1C), 130.64 (1C), 131.17 (1C), 132.97 (1C), 133.18 (1C), 136.13 (1C), 169.34 (COOH).

Compound G had distinct features in its ^1H NMR and ^{13}C NMR which tentatively led to the chemical structure in Fig.3.8. However, GC/MS has not confirmed this structure and, therefore, the structure is considered tentative. In addition, not enough information was available to assign many of the peaks to their respective aromatic protons and carbons in the chemical structure. Additional two dimensional NMR experiments need to be performed to assign the structure completely.

Integration of the ^1H NMR showed the presence of 9 aromatic protons in the range of δ 7.73 - 8.12. It also showed a peak with a chemical shift of δ 8.50 which represented the proton in the COOH moiety. These features are consistent with the structure in Fig.3.8. The ^{13}C NMR of compound G showed the presence of 14 aromatic carbons in the range of δ 123.97 - 136.13. It also showed a peak at δ 169.34 which represented the carbonyl carbon of the COOH moiety. In addition, the DEPT spectrum of compound G confirmed that 9 CH carbons were present. It also showed that there were no CH_2 or CH_3 carbons present. The rest of the carbons must, therefore be unprotonated. The information acquired from the DEPT is consistent with the proposed chemical structure.

As discovered in procedure two, it seems that isolation of this particular byproduct was possible due to the dissimilarities to other byproducts. In this case,

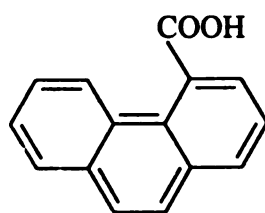


Figure 3.8 Chemical structure for Compound G

compound C has three aromatic rings with one functional group at the fourth position. The majority of other three ringed byproducts have two functional groups located at the fourth and fifth positions.

Additional attempts were made to isolate other byproducts from MPLC fractions using preparative HPLC as described in procedure two. However, these attempts were unsuccessful perhaps due to the similarities in structures from one byproduct to another. In some cases, repeated separation and purification caused fractions to dwindle to quantities too small for NMR analysis and certainly too small for later toxicological studies.

3.3.4 Procedure Four

The byproducts of pyrene ozonation were again prepared as described in Chapter one at low pH. Approximately, 400 mg of pyrene split into two samples was ozonated for byproduct purification. The sodium sulfite organic peroxide quench was not performed. In previous experiments, the recovery of byproducts from the liquid-liquid extraction which removed excess sodium sulfite was poor. In this case, samples were dried and preserved just as described previously following the liquid-liquid extraction step.

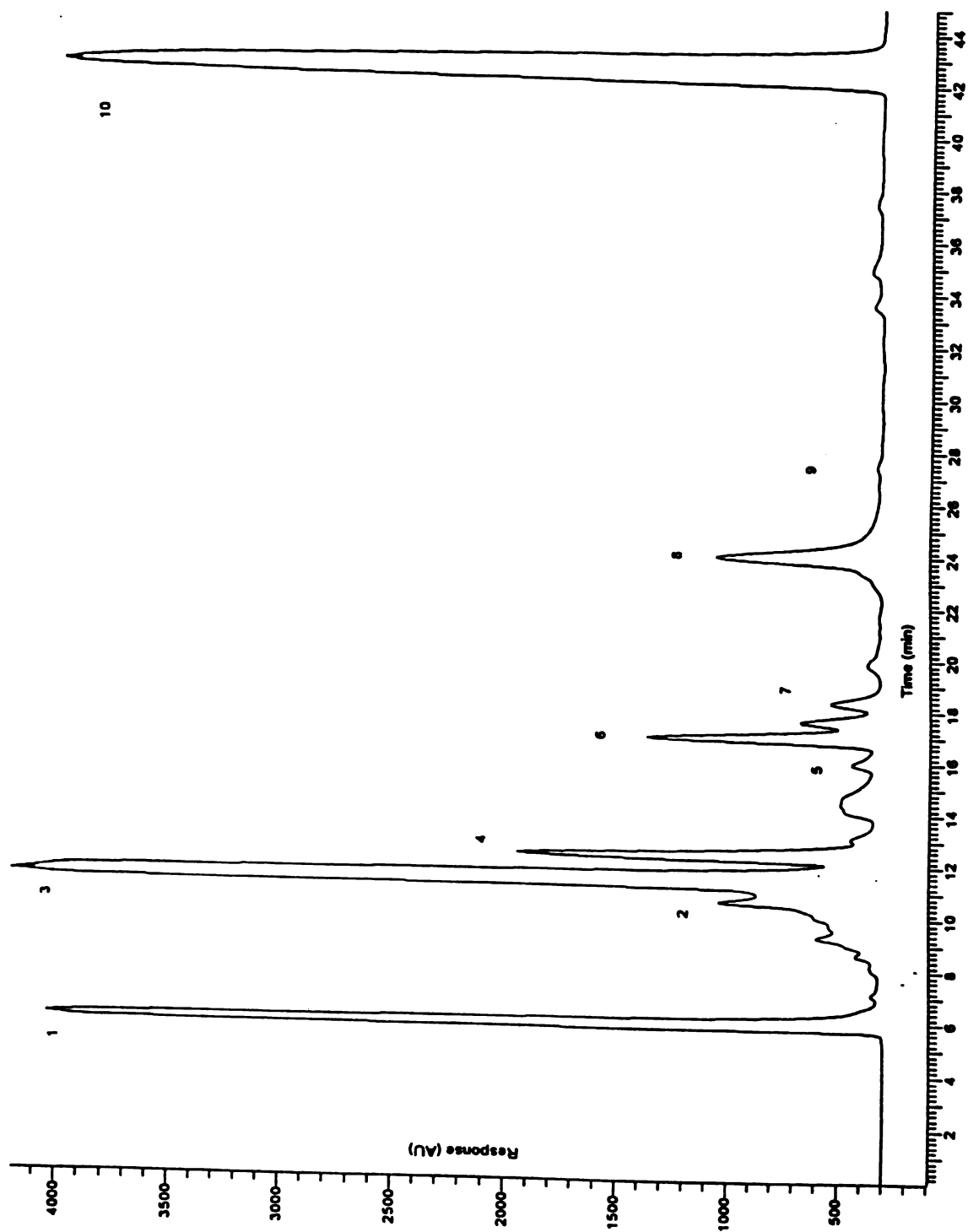
Because many of the byproducts had such similar structures, it was hypothesized that an extraction step to separate the acid containing byproducts from the aldehyde and phenol containing byproducts prior to HPLC might enhance the purification process. An ethyl acetate/sodium bicarbonate extraction was performed. However, several of the byproducts have both an acid and an aldehyde group which may be the reason that this

step was not extremely successful. From the extraction process, three fractions emerged, the ethyl acetate soluble portion (84.4 mg) , the water soluble portion (40.6 mg) , and the portion not greatly soluble in either (64.9 mg).

The ethyl acetate portion was further separated using semi-preparative HPLC as described in the methods section of this chapter. A gradient solvent system using acetonitrile and water was employed. Ten fractions were collected from the ethyl acetate portion (Fig.3.9). The portion not soluble in ethyl acetate or water was washed several times with acetonitrile. The portion soluble in acetonitrile was also separated on the HPLC using the same parameters as the ethyl acetate soluble portion. The HPLC chromatograms for the ethyl acetate soluble portion and the acetonitrile soluble portion were the same (Fig.3.9). The water soluble portion was also profiled on the HPLC and appeared to contain some of the most polar byproducts of pyrene ozonation. This portion was not further fractionated. The remaining mass from the portion not particularly soluble in ethyl acetate, water, or acetonitrile (40.1 mg) was found to be rather pure as indicated by analytical TLC and later NMR spectroscopy and GC/MS spectrometry.

From the HPLC separation, ten fractions were collected and analyzed by TLC. Again, the same 8 bands were present, but the purification was not much better. Several of the fractions were further purified using the HPLC and analyzed using NMR. Of the fractions further separated, only fraction 4 appeared to be pure. The insoluble portion which was not purified using HPLC was also analyzed by NMR and results showed that both fraction 4 and the insoluble portion were in fact the same compound. This compound, 4-carboxy-5-phenanthrene carboxaldehyde, is known to be the major byproduct formed by ozonation of pyrene [24]. This compound is also known to exist in

Figure 3.9 Semi-preparative HPLC chromatogram for ozonation byproducts generated at low pH



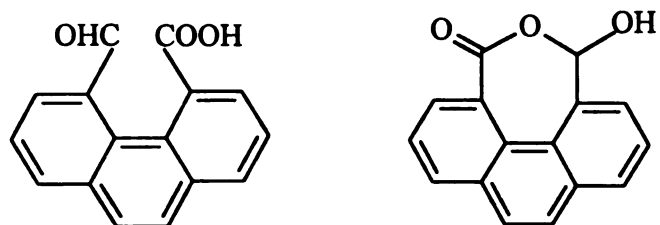


Figure 3.10 Chemical structures for both configurations of Compound C
(4-Carboxy-5-Phenanthrene Carboxaldehyde)

two different forms which cannot be separated from one another. Figure 3.10 shows the chemical structures for this compound. Results from NMR spectroscopy are shown below:

^1H NMR (d_6 -DMSO): δ 6.12 (1H, dioxygenated H), 6.66 (1H,OH), 7.78-8.32 (aromatic protons), 9.62 (1H, s, CHO), 9.96 (1H, s, COOH).

^{13}C NMR (d_6 -DMSO): δ 90.22 (CHOH), 126.11, 126.17, 127.12, 127.48, 127.54, 127.58, 127.83, 128.62, 129.50, 132.20, 132.42, 133.10, 133.41, 135.92, 167.9 (C=O).

Results from the NMR experiments were difficult to assess in terms of assignment of protons and carbons because the two different configurations of the compound were present. Functional groups for both configurations were present in the proton NMR. The carboxylic acid and aldehyde peaks were weak in comparison to the peaks which represented the protons on the dioxygenated carbon. The carbon NMR showed the ester carbon, the dioxygenated carbon, and 14 protonated and unprotonated carbons of the aromatic rings. The carboxylic acid and aldehyde peaks were not large enough to distinguish from background noise.

GC/MS was performed and confirmed the sample contained both configurations of the compound 4-carboxy-5-phenanthrene carboxaldehyde, compound C (Table 3.4). It is possible that again this compound was isolated from the other byproducts due to its differences in structure. One of the configurations of the compound has three aromatic rings with a cyclized fourth ring instead of the typical three ring structure. It is possible that the majority of the compound exists in this configuration.

The ten fractions collected from the semi-preparative HPLC experiments along with the water soluble fraction from the ethyl acetate extraction were analyzed by GC/MS and saved for later toxicological study.

Table 3.4 GC/MS Characteristics of 4-carboxy-5-phenanthrene carboxaldehyde

GC Retention time	Molecular Weight of Parent Cmpd	Molecular Weight of TMS Deriv. ^a	Important Ion Peaks, m/z	Product Spectra
12'54"	250	322	322 (M+.) 293 205 189 176	C [ref 24]
13'45"	250	322	322 (M+.) 307 294 205 189	C [ref 24]

^a theoretical value based on molecular weight of proposed TMS derivative

Table 3.5 contains compound identification information obtained from GC/MS for the fractions and water soluble portion. From the GC/MS it is clear that the majority of the fractions were mixtures of two compounds.

Mass spectra for compounds which have been previously identified are not included, but can be viewed in Appendix A of reference 24. Compound R of fraction 3 is the only compound that has not been previously identified. The mass spectrum and fragmentation pattern are shown in Figures 3.10 and 3.11. (Although the mass spectrum confirmed the structure for compound R, the fragmentation step from m/z 205 to m/z 189 must be further investigated.)

3.3.5 Procedure Five

In the fifth procedure, the ozonation experiment was performed with slight changes. Instead of using an acidified water of pH=1.8, a borate buffered water of pH=9.5 was used. The change in pH caused the ozone to decompose rapidly in solution and form hydroxyl radicals ($\cdot\text{OH}$) which interacted with the pyrene as opposed to direct ozone reactions at a low pH. A high pH during ozonation is of interest because it mimics water treatment conditions.

Table 3.5 GC/MS Characteristics for HPLC fractions and water soluble portion

Peak No. water portion	Compound I.D. (Fig. 2.1) ^a J, K/L
1	I, J, K/L
2	A, C, E, I, J
3	A, B, C, E, G (weak), J, K/L, R (Fig.3.10) ^b
4	C
5	C, E
6	C, D, E
7	C
8	C
9	C, D, E, I, J, K/L (weak)
10	A

^a mass spectra in reference 24

^b compound R was not identified previously and the mass spectrum is located in Figure 3.11

The products formed under these conditions in contrast to those formed at low pH and later comparative toxicological studies to low pH byproducts were the driving force behind procedure five.

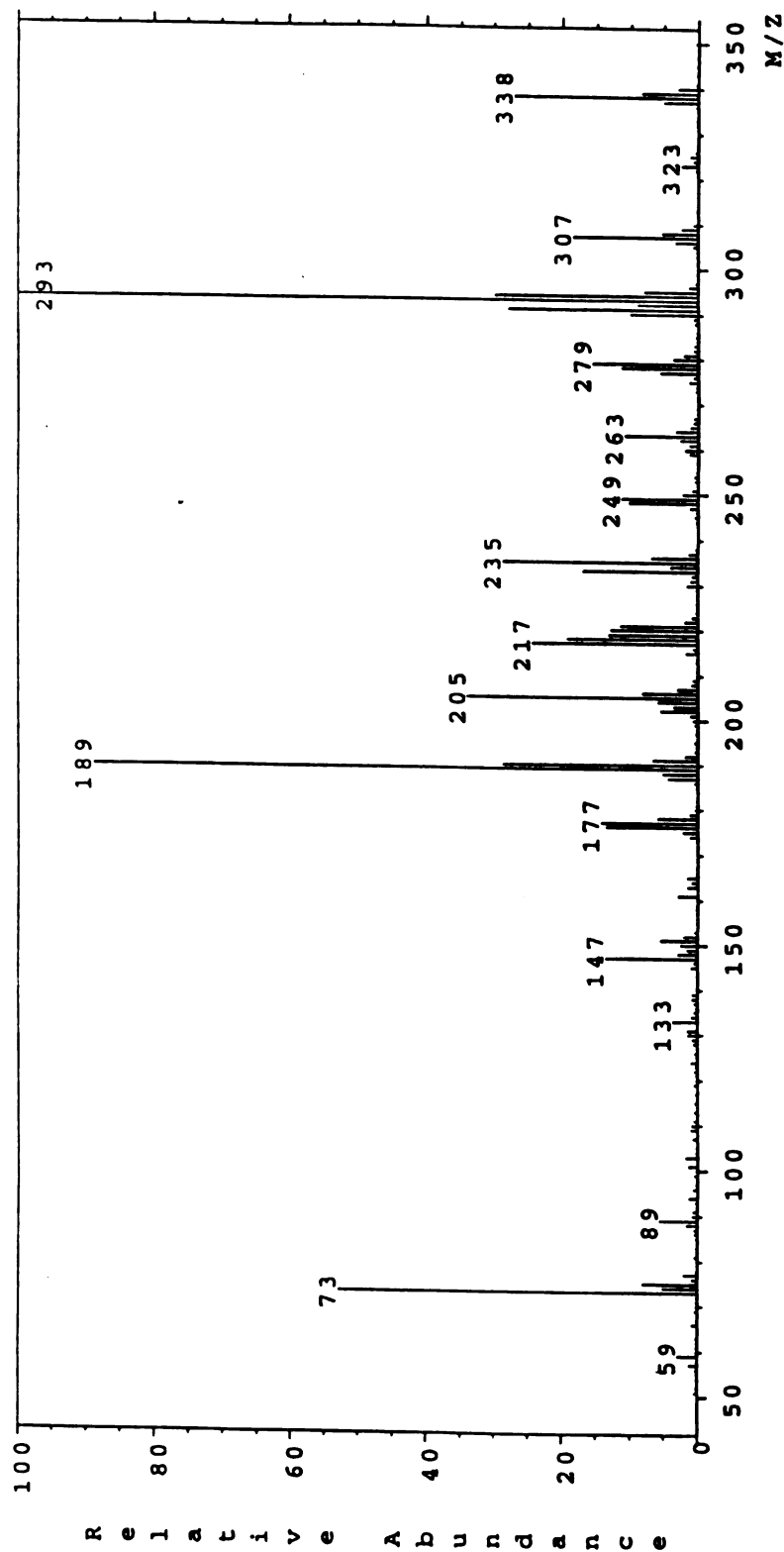
Following ozonation, samples were dried as previously reported and prepared for semi-preparative HPLC. The ethyl acetate extraction was not performed due to minimal success found in procedure four. All conditions for the HPLC separation were kept consistent with procedure four. Six fractions were collected from the HPLC experiment (see chromatogram in Fig.3.13).

Each fraction was a mixture as seen in analytical TLC with 7 major bands present. GC/MS was performed on each fraction. Table 3.6 contains compound identification information obtained from GC/MS for each fraction. From the GC/MS it is clear that all of the fractions are at least two compound mixtures.

All the compounds in Table 3.6 except two have been previously identified and mass spectra are located in Appendix A of reference 24. Structures are shown in figure 2.1. Compound R of fraction 3 and compound S of fraction 4 are the only compounds that have not been previously identified. The mass spectrum and fragmentation pattern for compound R are shown in Figures 3.11 and 3.12. The mass spectrum and proposed structure for compound S are shown in Figures 3.14 and 3.15.

Figure 3.11 Mass spectrum for Compound R

MASS SPECTRUM Data File: [100,100]A10129806 12-OCT-98 12:57
Sample: HERNER/MASTEN 129/3-TMS DB5MS(JJ)100-2,20/M-220,5/
RT 12.01" EI (Pos.) GC 450.6c BP: m/z 293.0000 Int. 274.8293 LV 0.00
Scan# (723)



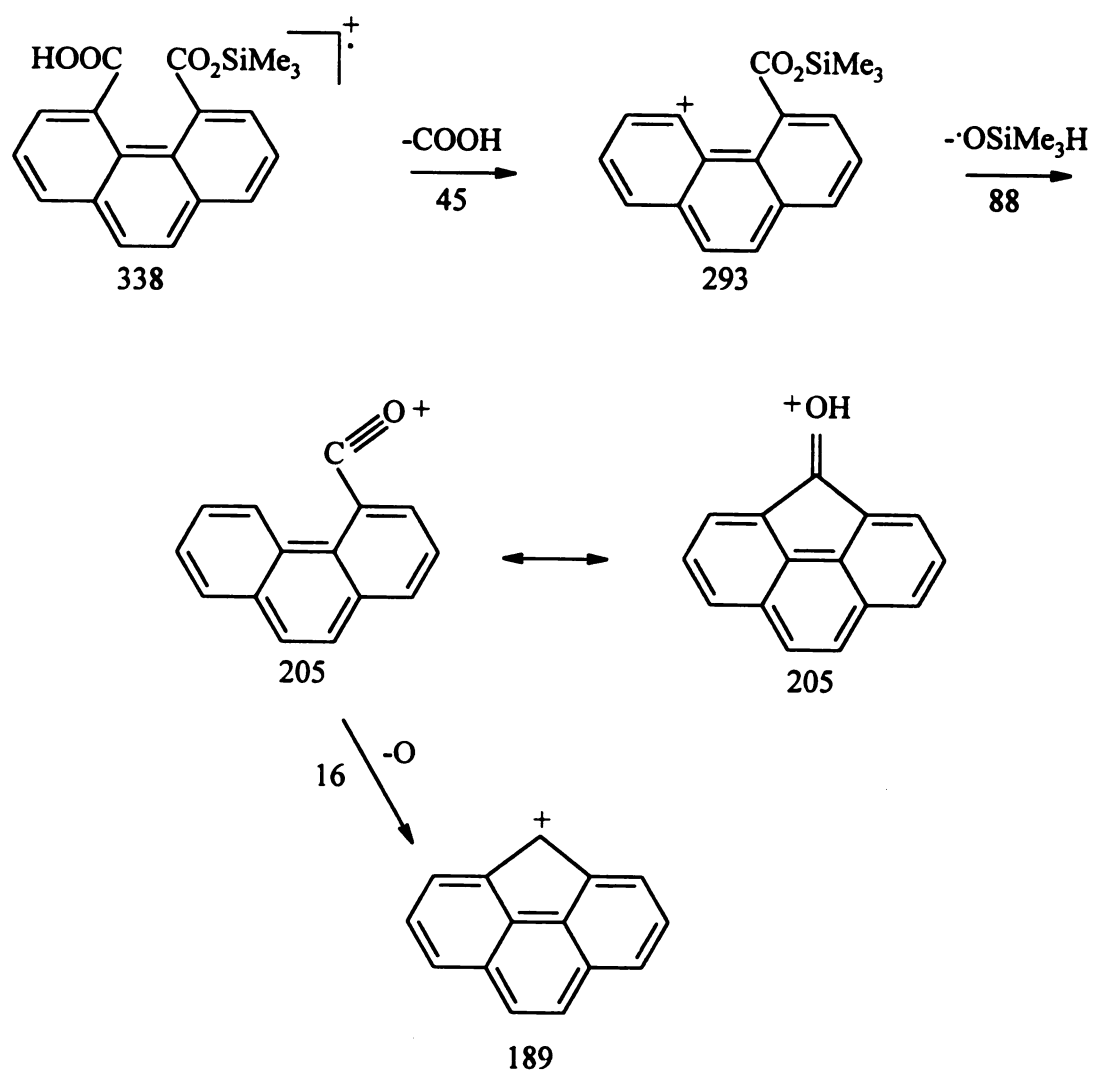


Figure 3.12 Proposed fragmentation pattern for Compound R

Figure 3.13 Semi-preparative HPLC chromatogram for ozonation byproducts generated at high pH

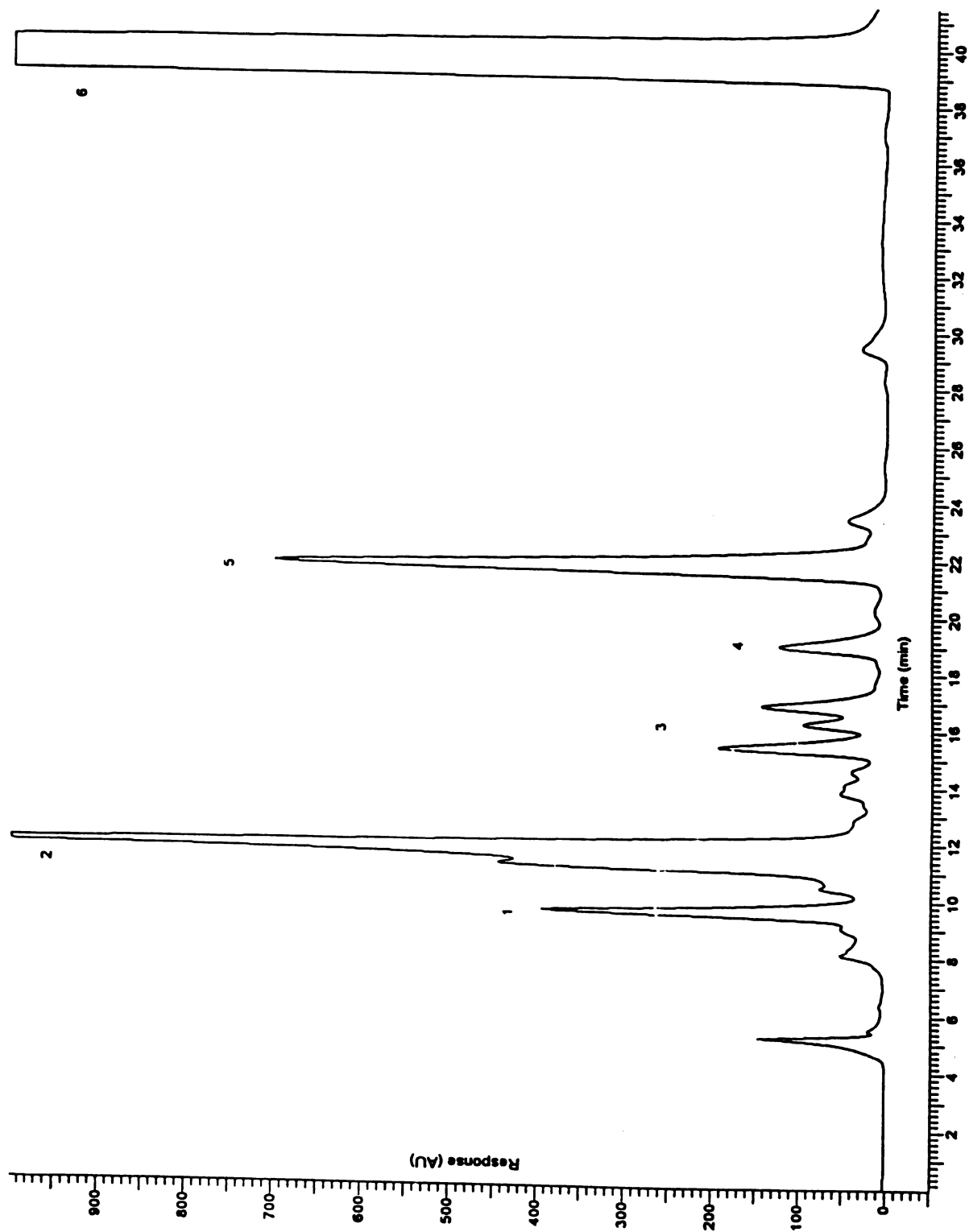
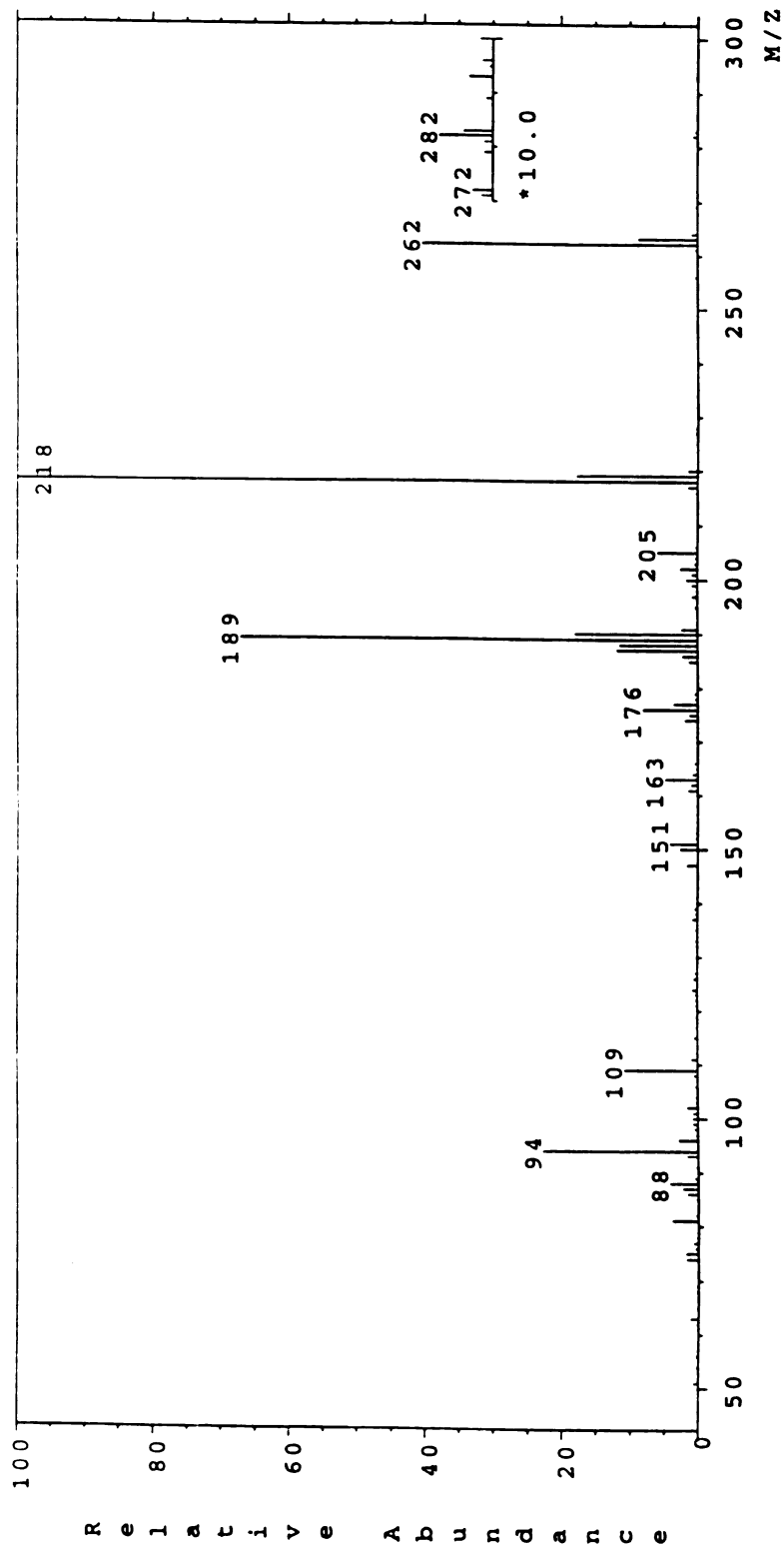
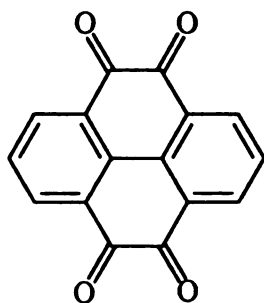


Figure 3.14 Mass spectrum for compound S

MASS SPECTRUM Data File: [100,100]A10129811 12-OCT-98 15:29
 Sample: HERNER/MASTEM 40/4-TMS DB5MS(JJ)100-2,20/M-220,5/M
 RT 14.12- EI (Pos.) GC 450.6c BP: m/z 218.0000 Int. 214.9906 Lv 0.00
 Scan# (854) - (848) [coef. 1.00]





MW 262

Figure 3.15 Proposed structure for Compound S

Table 3.6 GC/MS Characteristics for HPLC fractions ozonated at high pH

Peak No.	Compound I.D. (Fig. 2.1) ^a
1	A (weak), C
2	C, E (weak)
3	B, C, D, E, R (Fig.3.10) ^b
4	E (weak), S (Fig.3.13) ^c
5	C, D (weak), E (weak)
6	A

^a mass spectra in reference 24

^b compound R was not identified previously in reference 24 and the mass spectrum is located in Figure 3.11

^c compound S was not identified previously in reference 24 and the mass spectrum is located in Figure 3.14

There are at many possibilities for the fragmentation of compound S. Additional studies are necessary to confirm the pattern. Compound S contained the biphenyl structure with two cyclized rings at the fourth and fifth positions and at the thirteenth and fourteenth positions. Each cyclized ring contained two ketone groups.

The byproducts formed from the ozonation at a high pH were almost all of the three ring type, the first products formed. Only one biphenyl type compound, compound S, was formed. In addition, nearly 16% of the original pyrene remained following ozonation at high pH in contrast to the 3-4 % remaining following ozonation at low pH in procedure four. This evidence shows that ozonation at a higher pH is much less efficient and an increased ozone dosage would be required to remove the majority of the pyrene.

3.4 Conclusions

The primary objective of this phase of the research was to isolate byproducts of the ozonation of pyrene. Various methods were employed to accomplish this task, some successful and some not. Results showed that preliminary separation steps prior to preparative and semi-preparative HPLC were not remarkably successful. The best separation was achieved when samples were taken directly to HPLC separation following ozonation. Compound Q was isolated using preparative HPLC. Compound D was not purified completely, but in conjunction with compound Q also using preparative HPLC. The major byproduct of pyrene ozonation, 4-carboxy-5-phenanthrene carboxaldehyde, was isolated using semi-preparative HPLC. The MPLC separation enabled the isolation of compound G. The difficulty encountered in the separation of these compounds is due to the similarity in the structures. The compounds isolated had significant differences in structure from the majority of other byproducts which allowed successful purification.

Future studies conducted to isolate additional byproducts may proceed in several directions. A specially made column made to isolate these compounds specifically might be one option. However, the cost and existence of such a column may make this route unrealistic.

Another option might be to approach this problem from the opposite direction. The synthesis of known byproducts may be the option with the best chance of success. Although fewer compounds were isolated than anticipated from these studies, other important information was revealed. In addition to the byproducts identified by Yao [24], two other byproducts were identified by GC/MS. The first, compound R, is a three ringed compound with an acid at both the C4 and C5 position. According to Bailey [29],

this product is certainly possible theoretically. This compound is produced in small quantity which may be the reason it had not been identified previously. The ozonation carried out at high pH produced one compound, compound S, which was not seen at low pH, but resembled compound P, produced at low pH. The ozonation experiments conducted at high pH also showed that indeed the degradation of pyrene was less efficient under the modified conditions and that the majority of the byproducts formed were the same whether ozonated at high or low pH.

3.5 References

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Chapter 4 - Toxicological Study

4.1 Introduction

The threat to human health and to the environment has been the driving force behind the successful clean up of many contaminated sites in this country. However, the need for improved remediation processes still exists. Initially, remediation processes were performed with the intention of removing risk from the population without long term consideration. Often times, contaminated materials were landfilled which essentially moves the problem from one place to another without true resolution.

Today, long term solutions to polluted sites are popular. Chemical and biological processes which degrade harmful compounds are common choices. For example, polycyclic aromatic hydrocarbons, (PAHs), are one family of compounds which have been successfully treated using various chemical and biological technologies [1-4]. PAHs, 16 of which have been identified as priority pollutants and 8 as carcinogens or possible carcinogens, are found in air, soil, and water [5,6].

Ozone has been used in the degradation of PAHs as well as other compounds resistant to conventional treatment methods [7]. Ozone is a strong oxidant capable of degradation of PAHs to carbon dioxide, water and straight chain aliphatics, given a proper ozone dosage [3]. However it must be noted that the ozone dosage required to obtain these simple byproducts is much larger than the ozone dosage to remove the parent compounds. In addition, studies have shown that certain PAH byproducts of these processes can be as or more harmful than the parent compounds themselves [1,8,9].

Unfortunately, the progress of remediation projects is usually gauged solely by the removal of the parent compounds, the original compounds which caused the health risk.

Pyrene, a four ringed PAH and the subject of this study, is almost entirely degraded (>90%) by ozone at a dosage of 1.6 moles ozone per mole pyrene [1]. However, the mixture of compounds produced was found to be more inhibitory to gap junction intercellular communication (GJIC) than was the parent compound pyrene [10]. The byproduct mixture was fractionated by HPLC into 13 fractions and one was found to be more inhibitory to GJIC [1]. An increased ozone dosage of 4.5 moles ozone per mole pyrene eliminated all products inhibitory to GJIC [1]. Another study showed that some products of pyrene oxidation had higher mutagenic activity than the parent compound [9]. Although, this study mimicked photooxidation conditions which produced nitrogenated compounds unlike those of ozonation in aqueous solution. Also, the study was completed using a mutation as the toxicity endpoint where as the GJIC studies monitor a nongenotoxic endpoint.

In a study by Yoshikawa, byproducts of pyrene ozonation were found not to be more toxic than the parent compound [8]. The marker for toxicity was blood chemistry in rats which differs greatly from GJIC. Other PAHs in the study were found to have byproducts that were more toxic than the respective parent compounds.

Due to the possible risk still present following the removal of the parent PAHs, a remediation project must be evaluated based on the degradation of both the parent compounds and the byproducts with a concurrent decrease in toxicity.

Decreasing the risk of cancer by reducing environmental exposures has been a major priority. This type of risk is seen by the public as unnecessary and one that can be

eliminated. However, it is difficult in many cases to know which compounds actually cause or contribute to the cancer process. Historically, most chemicals were evaluated for their potential to cause cancer using genotoxic assays. Today we know that a carcinogen isn't necessarily a mutagen. In addition, many compounds which were not mutagens or complete carcinogens were able to contribute to carcinogenesis by an epigenetic mechanism. Epigenetic toxicants have been implicated in tumor promotion during carcinogenesis [11-13], in teratogenesis [14], and in reproductive dysfunction [15-17]. Epigenetic toxicants have been shown to inhibit communication between cells through their gap junctions. Intercellular communication is the major mechanism for control of cell homeostasis and is accomplished by the exchange of ions and small molecules through the membrane gap junctions.

The gap junction is a hexameric channel or connexon comprised of six subunits called connexins which traverses the plasma membrane and is joined with the connexon of an adjacent cell membrane [11,18]. Most cancer cells have dysfunctional GJIC [12]. The inhibition of GJIC has been used as a biological indicator for potential tumor promotion, and indirectly as an indicator for potential carcinogenicity [11,19].

Although PAHs are not a particularly reactive group of chemicals, they have been known to be most harmful in the form of metabolites which are able to damage DNA, RNA, and protein [20]. Various theories exist which link compound structure and carcinogenesis. A common theory links the bay region, which many PAHs contain, to carcinogenesis [20-23]. Functional groups such as the hydroxy, the diol-epoxide, and the methyl group which are adjacent to or part of the bay region have also been shown to enhance activity of PAHs [21,24-27].

Several studies were conducted which focused on the inhibition of GJIC and the PAH structure. Upham et al. [9] showed that certain four and five ringed PAHs were more inhibitory to GJIC than some three ringed PAHs. For example, pyrene, a four ringed PAH, was found to be more inhibitory than benzo[a]pyrene and benzo[e]pyrene, each with five rings [9]. Also included in this study was a comparison of PAHs which contained a bay region and those which did not. Phenanthrene, a three ringed PAH with a bay region, was much more inhibitory to GJIC than anthracene, three rings without a bay region [9]. Phenanthrene completely inhibited GJIC where as fluorene, another three ringed PAH, only partially inhibited GJIC. In another study, the effect of a methyl group attached to a PAH was evaluated with respect to inhibition of GJIC. The parent compound studied was anthracene which did not inhibit GJIC. When a methyl group was located at C-1 or C-9 in the parent compound GJIC was inhibited where as a methyl group located in the C-2 position caused no inhibition of GJIC [22]. The position of the methyl group at C-1 or C-9 created a bay like region which was hypothesized as the cause of the change in GJIC.

Several issues were raised in these previous studies which make this research project an important addition to the available information pertaining to this subject. As previously discussed, it was discovered that at least one byproduct of pyrene ozonation was inhibitory to GJIC. In order to determine which compound(s) might be causing the toxicity, three compounds isolated from the mixture of ozonated byproducts were analyzed along with the parent compound, pyrene. One of the compounds isolated was available commercially and purchased for comparison purposes. Six additional compounds, which share similar structures to pyrene ozonation byproducts, were

purchased and also examined. GJIC was studied under various conditions for all eleven of these compounds.

With the exception of one compound, the ozonation byproducts of pyrene were not commercially available and not easily isolated. Therefore, all impure fractions collected from HPLC for ozonations conducted at both high and low pH were also analyzed for their affect on GJIC. Although these fractions were mixtures, GC/MS was performed which revealed the identity of the compounds in each mixture.

4.2 Materials and Methods

Cell Culture. The compounds used in this section were purchased from several chemical companies. Pyrene was purchased from Sigma Chemical Co. (St. Louis, MO). Diphenic acid, 2-biphenyl carboxylic acid, 4-biphenyl carboxylic acid, 4-biphenyl carboxaldehyde, and 37% formaldehyde were purchased from Aldrich Chemical Co. (Milwaukee, WI). 4-Carboxy-5-phenanthrene carboxaldehyde, 1,2,3,4-Tetrahydro-9-phenanthrene carboxaldehyde and 9-oxo-1-fluorene carboxaldehyde were purchased from Sigma-Aldrich's Library of Rare Chemicals (Milwaukee, WI). Neutral red dye and lucifer yellow dye were obtained from Sigma Chemical Co. (St. Louis, MO). Acetonitrile and sodium chloride were purchased from EM Science (Gibbstown, NJ). Sodium phosphate and ammonium persulfate were purchased from Columbus Chemical Industries (Columbus, WI) and Life Technologies, Inc. (Gaithersburg, MD), respectively.

WB-F344 rat liver epithelial cells were obtained from Dr. J. W. Grisham and Dr. M. S. Tsao of the University of North Carolina (Chapel Hill, NC) [22]. Cells were cultured in 2 mL of D-medium (Formula No. 78-5470EG, GIBCO Laboratories, Grand

Island, NY) and were supplemented with 5% fetal bovine serum (GIBCO Laboratories, Grand Island, NY). Cells were prepared for experimentation in 35 mm² plastic petri dishes (Corning Glass Works, Corning, NY) and were cultured in 150 cm² flasks (Corning, Corning, NY). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. Bioassays were conducted on confluent cultures obtained after 2 days growth. Cell cultures were photographed using a Nikon Diaphot-TMD epifluorescence phase-contrast microscope illuminated with an Orsam HBO 200W lamp and equipped with a 35-mm FA camera (Nikon, Japan).

Test Compound Preparation. The test compounds were dissolved in 100% acetonitrile for bioassay experiments. Concentrations of stock solutions ranged from 5 to 20 mM. Concentration of stock solutions was dependent upon both the solubility in acetonitrile and in D-medium. Concentration of impure ozonated fractions was determined using the molecular weight of the parent compound, pyrene. Previous studies showed that acetonitrile is neither cytotoxic nor inhibitory to GJIC to cells up to 2% by volume which is 40 µL of acetonitrile in 2 mL of D-medium [9]. Therefore, chemical dosages were limited to 35-40 µL stock solution. Vehicle controls were performed using 100% acetonitrile. The volume of acetonitrile was identical to chemically treated cell cultures. Vehicle controls were treated for the same amount of time as chemically treated cell cultures. Experimental controls which sustained no chemical treatment were also performed for each experiment. Controls, vehicle controls, and chemical treatments were performed in triplicate.

Scrape Load/Dye Transfer (SL/DT) Bioassay Protocol. GJIC was monitored using the SL/DT method as described by El-Fouly et al. [28]. Following chemical treatment, cells were rinsed 5 times with phosphate buffer solution (PBS). Approximately 2 mL of 0.05% lucifer yellow dye dissolved in PBS as added to cell cultures. A steel surgical blade (No.20, Bard-Parker, Franklin Lakes, NJ) was used to make 6 to 8 scrapes in each cell culture. Lucifer yellow was let stand on scraped cell cultures for 3 min at room temperature. The dye was then removed from the culture and the cell culture was again rinsed 5 times with PBS. Cell cultures were fixed with 0.5 mL of 4% formalin.

Dose Response Experiment. For each chemical studied, several dosages were selected which traversed the dose response curve, from a low dose which did not inhibit GJIC to a high dose which may inhibit GJIC. For compounds which were not inhibitory to GJIC, the dose was increased until the solubility for the compound in the cell's media had been reached. The range typical for the compounds of this study was 0 to 200 μ M. In addition, compounds were studied at dosages which were not cytotoxic as determined by the cytotoxicity assay. For each dosage selected, three plates were exposed to the target compound for a fixed period of time. Following chemical treatment, the SL/DT assay was performed on each plate and subsequently photographed.

Time Response. The time response experiment was similar to the dose response experiment. For each chemical studied, the chemical dosage was fixed, but the time of

exposure to the cells was varied. The dosage selected was one known to cause inhibition of GJIC. For compounds that did not appear to inhibit GJIC, the highest soluble dosage which was not cytotoxic was used in time response experiments. The variance in exposure time was determined based upon the compound's activity. Again, the exposure time was ranged from minutes to hours in order to determine after how long the inhibition of GJIC occurred, if at all. Following chemical treatment, each plate was subjected to the SL/DT assay and subsequently photographed.

Time of Recovery. Time of recovery experiments were performed to determine how much time was required for GJIC to resume following removal of the test compound from the cell culture media. Time of recovery experiments were performed for each target compound at a dosage which caused inhibition of GJIC as determined by the dose response experiment. The cells were exposed to the chemical for the amount of time required for the inhibition to occur as determined from the time response experiment. For compounds which did not inhibit GJIC completely or at all, the dosage and time which exhibited the most activity for the target compound was selected.

Following chemical treatment, each plate was rinsed 5 times with PBS. Approximately, 2 mL of D-medium was added to each plate. All plates were returned to the incubator for various lengths of time. The range of incubation time was to determine the length of time required for inhibited cells to recover cell-cell communication. The incubation times ranged from 0 minutes to several hours. Following incubation without chemical exposure, the SL/DT assay was performed on each plate. Each plate was photographed.

Cytotoxicity Bioassay. Cytotoxicity of target chemicals to cell cultures was monitored using the neutral red dye uptake assay [29]. Healthy cells take up neutral red dye where as nonviable cells do not. A solution of neutral red dye (0.033%) in D-medium was incubated for at least 2 h at 37°C. The dye solution was centrifuged for 10 min at 1300 rpm and filtered through a 0.22 µm syringe filter (Millipore Corp., New Bedford, MA). Filtration was performed to remove any excess dye that may have precipitated out of solution.

The first portion of the cytotoxicity assay was performed in the same manner as the dose response experiment. Following chemical treatment, all plates including vehicle and experimental controls, were rinsed with PBS five times. Approximately, 2 mL of neutral red dye solution was added to each plate. Plates were incubated in this condition for 1 h at 37°C. Following incubation, all plates containing cells were rinsed with PBS five times. To each plate, 2 mL of neutral red solubilizer was added to release the dye trapped within viable cells. Solubilizer consisted of 1% acetic acid, 50% ethanol, and 49% distilled water. At least 15 min was required to release the dye fully from the plated cells.

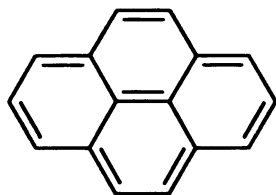
Cytotoxicity was monitored indirectly by monitoring the absorbance of dye per plate measured using a UV/vis spectrophotometer (Beckman, model DU-7400). The concentration of dye retained by healthy cells was determined indirectly by monitoring absorbance at 540 nm. The background absorbance was measured at 690 nm and was subtracted from readings recorded at 540 nm. Cytotoxicity was measured as a fraction of

the control. A value of 1.0 indicated a non-cytotoxic response. A value of less than 0.8 indicated that less neutral red dye solution had been retained by the cells and that the compound is cytotoxic at that dosage.

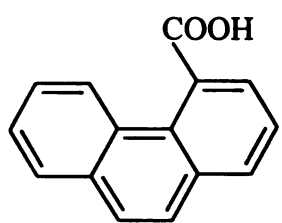
Analysis of Photographs. Each plate of cells used for dose response, time response, or time of recovery was photographed under UV light at a magnification of 200x. One scrape representative of the entire culture was selected to assess the migration of the lucifer yellow dye. Ten measurements were made at one centimeter intervals along the scrape. Perpendicular measurements from the scrape to the dye front were recorded for all ten places and averaged. GJIC was determined by comparing the distance the dye traveled in controls to that of the chemically treated cells. GJIC was reported as a fraction of the control. A value of 1.0 indicates complete communication and approximately 0.3 or lower, a complete inhibition of cell-cell communication. A value between 1.0 and 0.3 indicates partial inhibition of GJIC.

4.3 Results

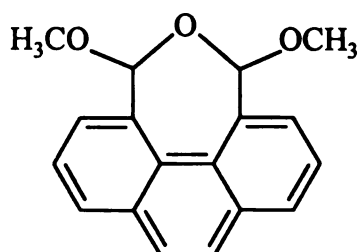
The experiments performed were divided into two studies. In the first study, experiments were conducted using the parent compound, pyrene, 3 byproducts of pyrene ozonation, and the 7 commercial compounds with structures similar to pyrene ozonation byproducts. Figure 4.1 contains structures of pyrene and isolated ozonation byproducts. Figure 4.2 contains structures of commercial compounds. Dose-response, time-response, time of recovery, and cytotoxicity experiments were completed for all 11 compounds.



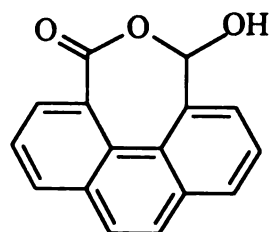
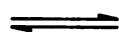
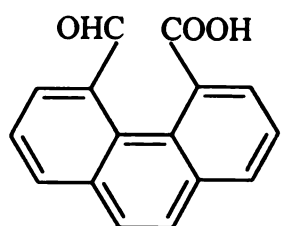
Pyrene



G

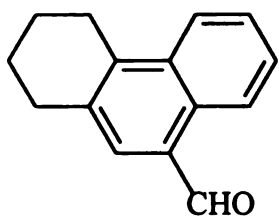


Q

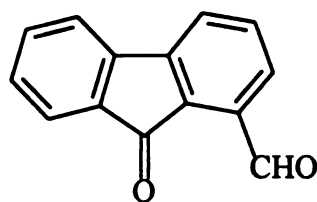


C

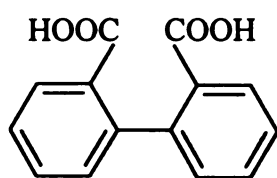
Figure 4.1 Pyrene and ozonation byproducts



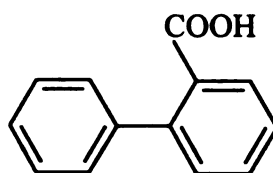
**1,2,3,4 - Tetrahydro-9-Phenanthrene
Carboxaldehyde**



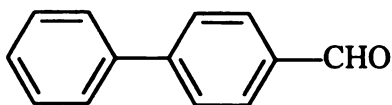
9-Oxo-1-Fluorene Carboxaldehyde



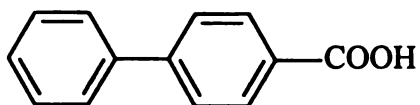
Diphenic Acid



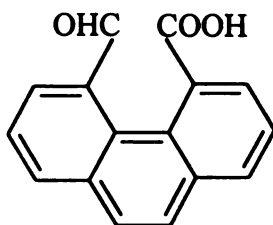
2-Biphenyl Carboxylic Acid



4-Biphenyl Carboxaldehyde



4-Biphenyl Carboxylic Acid



4-Carboxy-5-phenanthrene Carboxaldehyde

Figure 4.2 Chemical structures of commercial compounds used in bioassay studies

The second study consisted of experiments using pyrene, fractions from pyrene ozonation, and mixtures of ozonation byproducts generated at low and high pH. The fractions from pyrene ozonation were obtained from HPLC experiments detailed in Chapter 3. Dose-response experiments and some cytotoxicity experiments were performed. Due to the small quantities of material available, time-response and time of recovery experiments were generally not completed.

4.3.1 Study One

Dose Response. For all dose response experiments an exposure time of 30 min was used. The results of the dose response experiments showed that pyrene, the parent compound, inhibited GJIC completely at a dose of 40 μM with an fraction of control (FOC) equal to 0.2 (Figure 4.3). Compound G, 4-Carboxyphenanthrene (4CP), and compound C, 4-Carboxy-5-phenanthrene carboxaldehyde (4C5P), from figure 2.1 were both byproducts isolated from the ozonation of pyrene as described in Chapter 3. Both 4CP and 4C5P showed partial inhibition with an FOC of approximately 0.75 (Figure 4.4). 4CP showed the maximum inhibition at 70 μM . 4C5P showed maximum inhibition at 50 μM . Compound Q, the third isolated byproduct of pyrene ozonation, showed complete inhibition of GJIC with an FOC equal to 0.3 at 135 μM (Figure 4.5). The first commercial compound studied was 4C5P. This compound was purchased with the knowledge that the purity could not be guaranteed. An HPLC analysis (Chapter 3) showed that the 4C5P contained pyrene as one impurity. Pyrene is known to inhibit GJIC

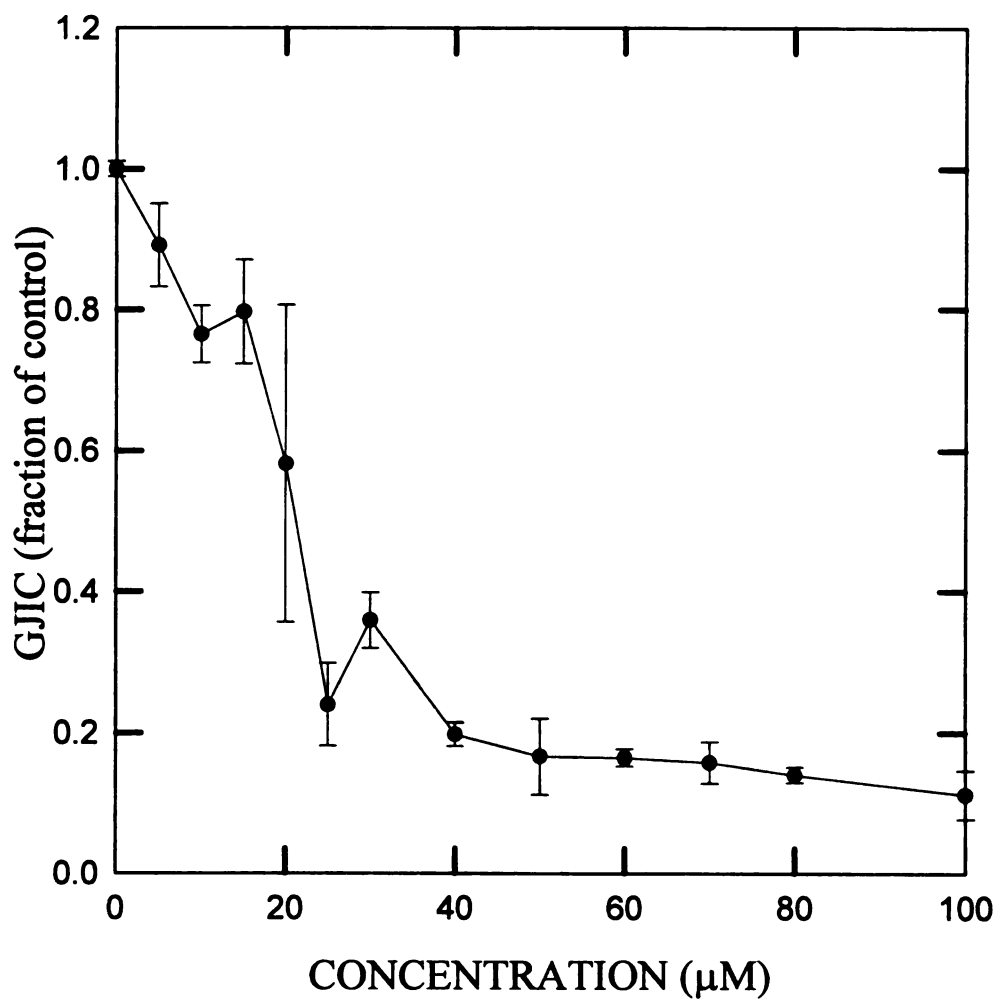


Figure 4.3 Dose response curve for Pyrene

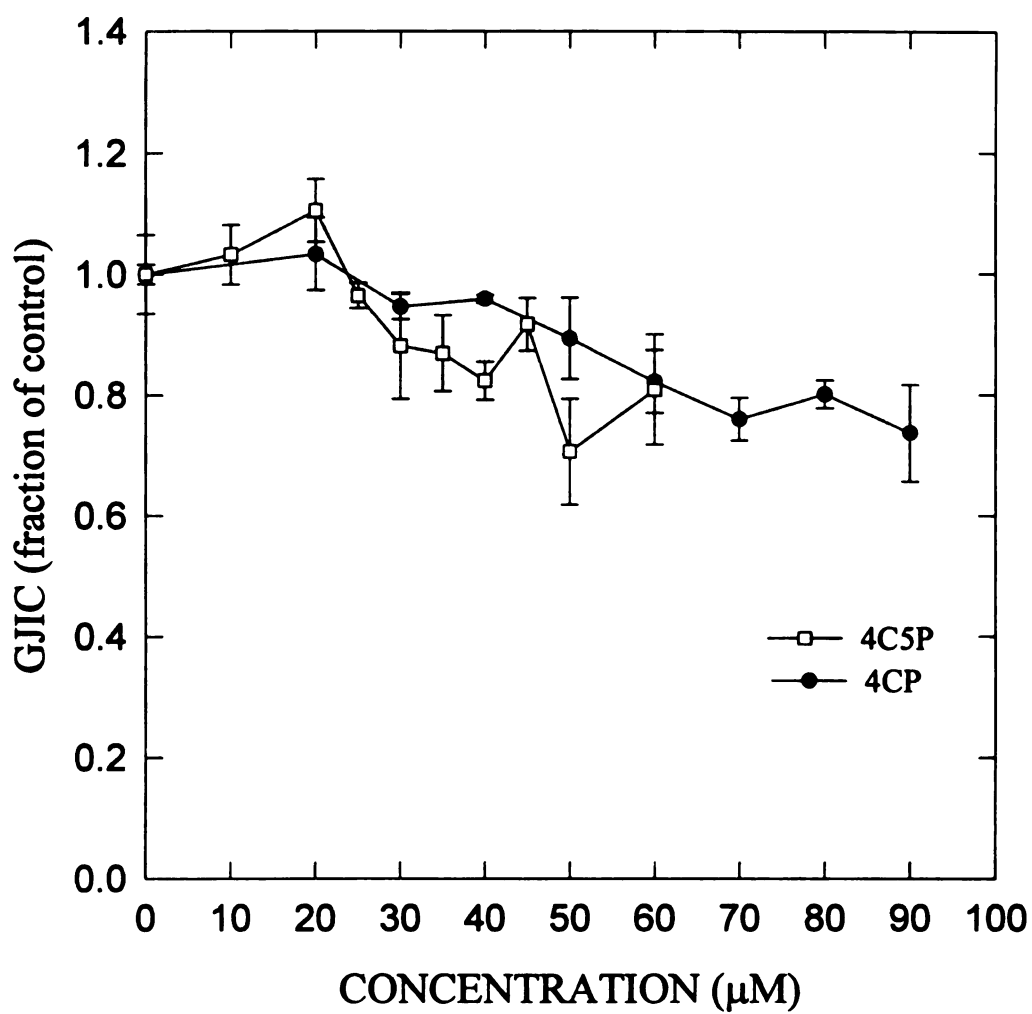


Figure 4.4 Dose response curve for 4-Carboxy-5-Phenanthrene Carboxaldehyde and 4-Carboxyphenanthrene

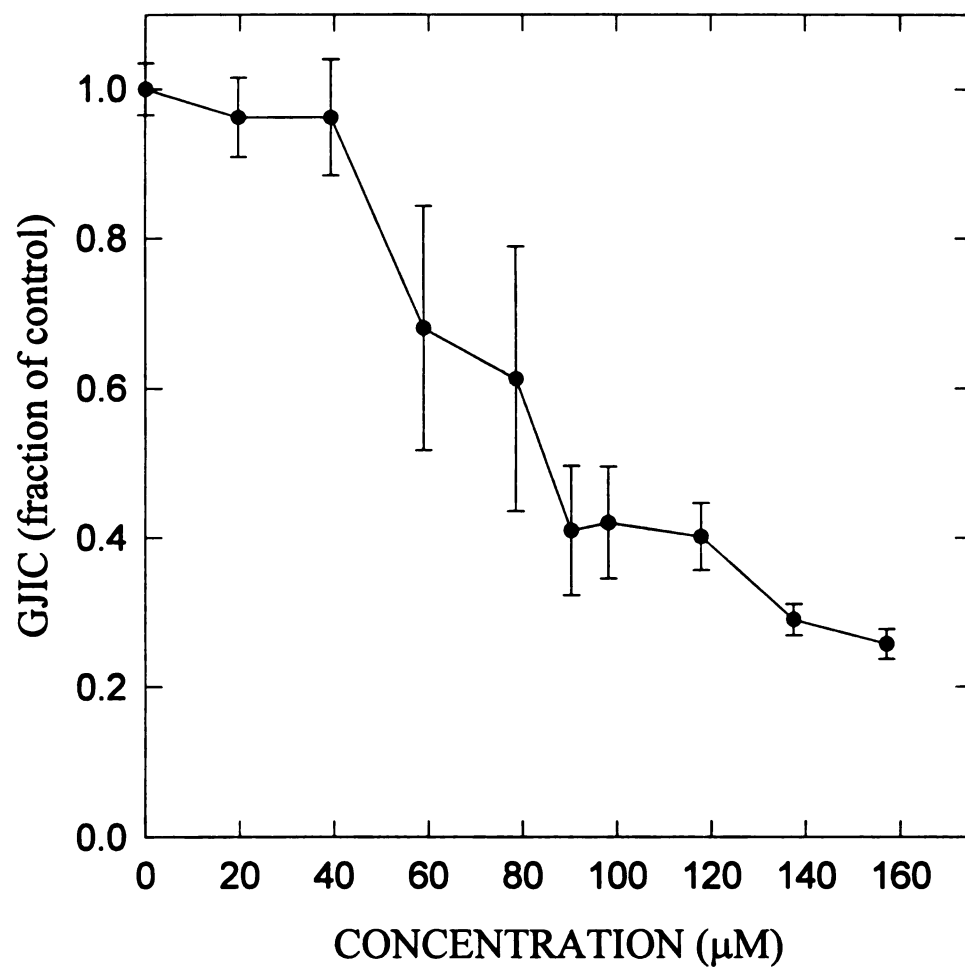


Figure 4.5 Dose response for Compound Q

[1,9]. The commercial 4C5P partially inhibited GJIC with an FOC equal to approximately 0.6 beginning at 60 μ M (Figure 4.6).

Of the remaining compounds analyzed (6 commercial compounds), two were three-ringed PAHs and four were biphenyl type compounds. The three ringed PAHs, 1,2,3,4-Tetrahydro-9-phenanthrene carboxaldehyde (TPC) and 9-oxo-1-fluorene carboxaldehyde (OFC), both inhibited GJIC completely beginning at approximately 35 μ M and 50 μ M, respectively (Figure 4.7). Both compounds had an FOC value of less than 0.2. Three of the four biphenyl type compounds had partial inhibition of GJIC (Figure 4.8). Both diphenic acid (DPA) and 2-Biphenyl carboxylic acid (2BCA) had a minimum FOC value of approximately 0.8 beginning at 175 μ M. 4-Biphenyl carboxylic acid (4BCA) had a minimum FOC value of approximately 0.7 at 200 μ M. A concentration of 200 μ M was the greatest dosage studied due to the solubility of the chemical under the conditions of the assay. The only biphenyl type compound that completely inhibited GJIC was 4-biphenyl carboxaldehyde (4BCH). This compound had a minimum FOC value of approximately 0.25 beginning at 110 μ M.

Time Response. The results of the time response experiments showed that pyrene inhibited GJIC completely within 20 min (Figure 4.9). Isolated byproduct compounds, 4CP and 4C5P, which inhibited GJIC partially, inhibited within 30 min and 60 min, respectively (Figure 4.10). Compound Q inhibited GJIC completely within approximately 15 min and began partial recovery of communication without removal of the chemical within approximately 50 min (Figure 4.11). The commercial version of

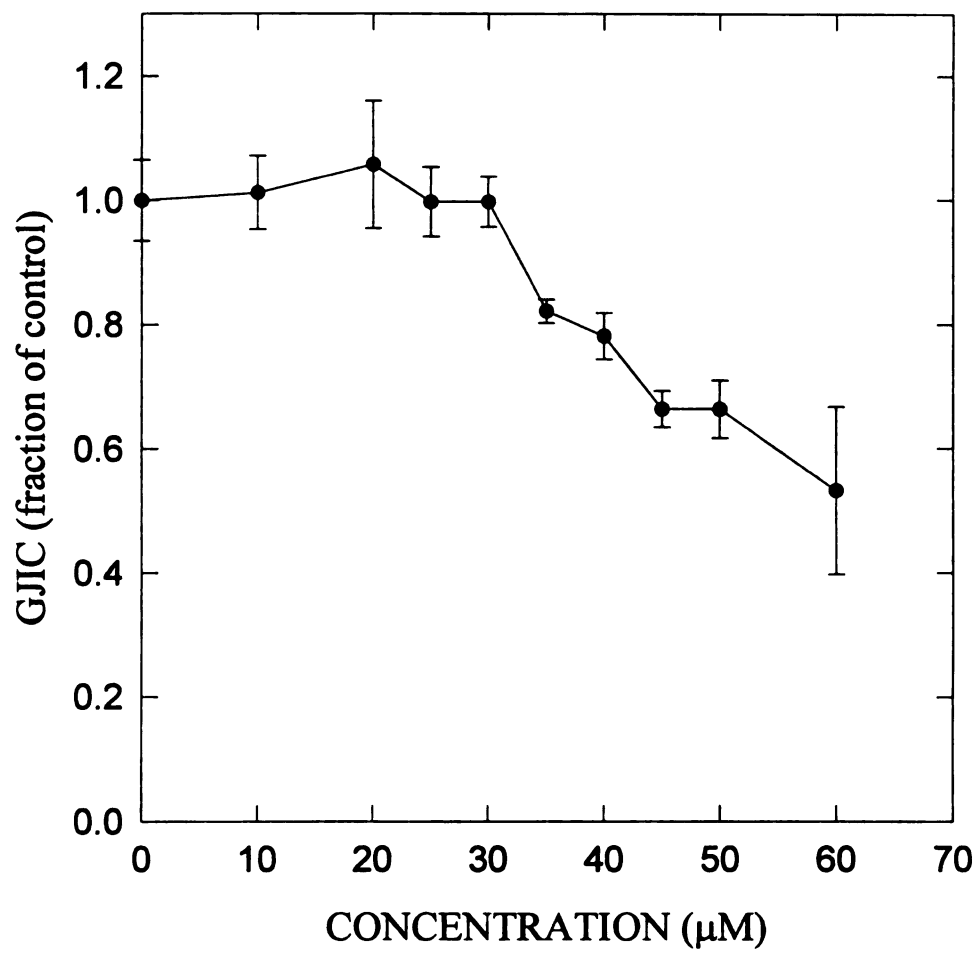


Figure 4.6 Dose response curve for commercially obtained 4-Carboxy-5-Phenanthrene Carboxaldehyde

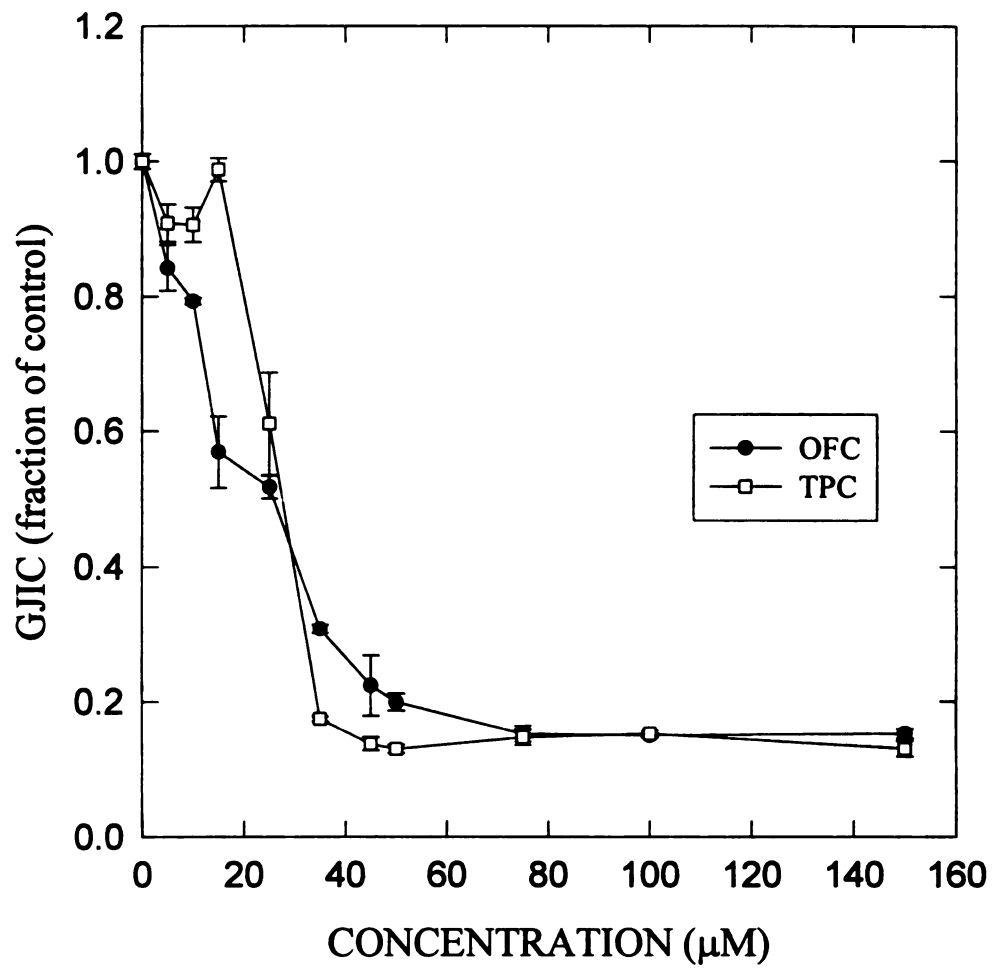


Figure 4.7 Dose response curve for 1, 2, 3, 4-Tetrahydro-9-Phenanthrene Carboxaldehyde and 9-Oxo-1-Fluorene Carboxaldehyde

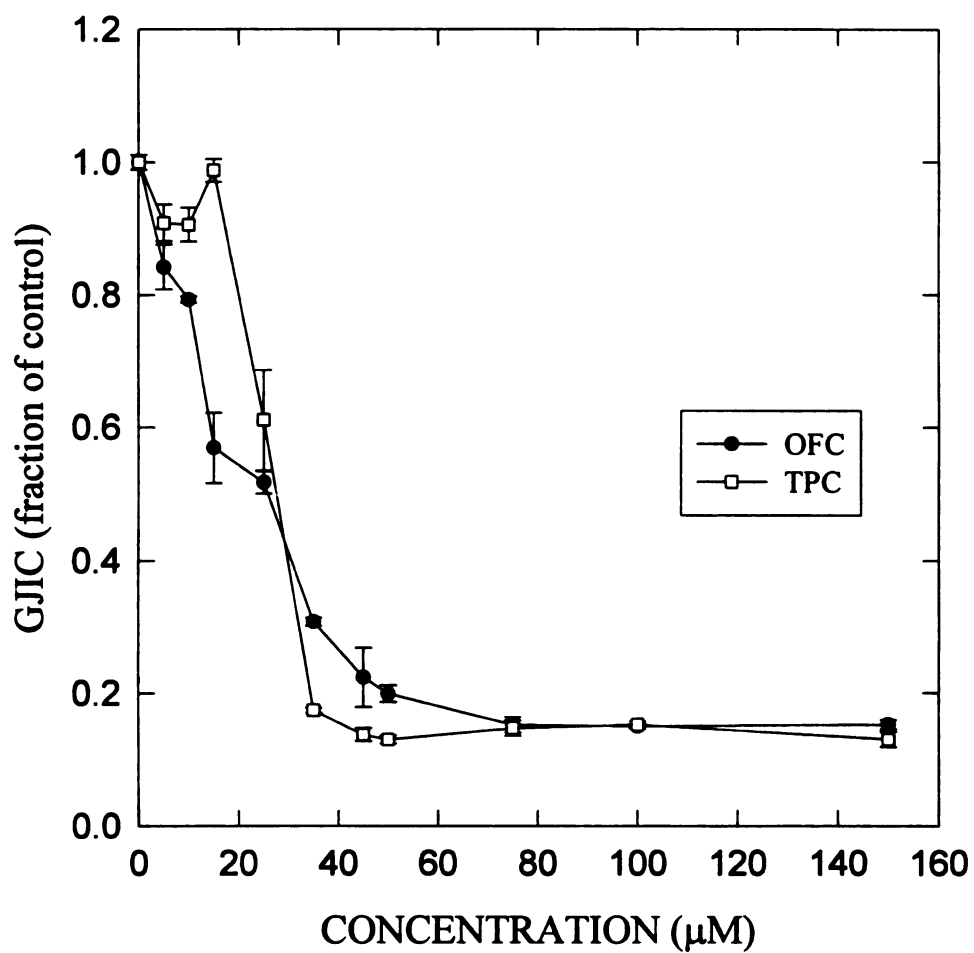


Figure 4.7 Dose response curve for 1, 2, 3, 4-Tetrahydro-9-Phenanthrene Carboxaldehyde and 9-Oxo-1-Fluorene Carboxaldehyde

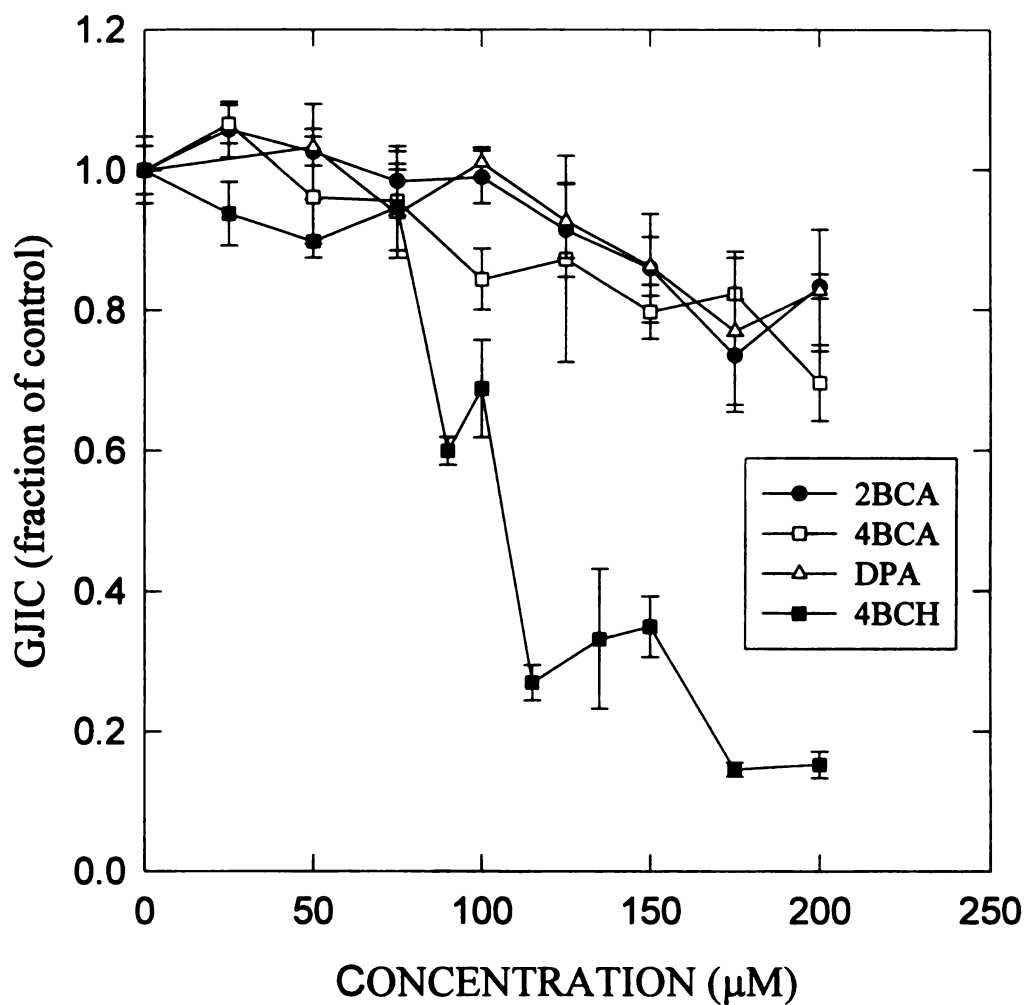


Figure 4.8 Dose response curve for Diphenic Acid, 2-Biphenyl Carboxylic Acid, 4-Biphenyl Carboxylic Acid, and 4-Biphenyl Carboxaldehyde

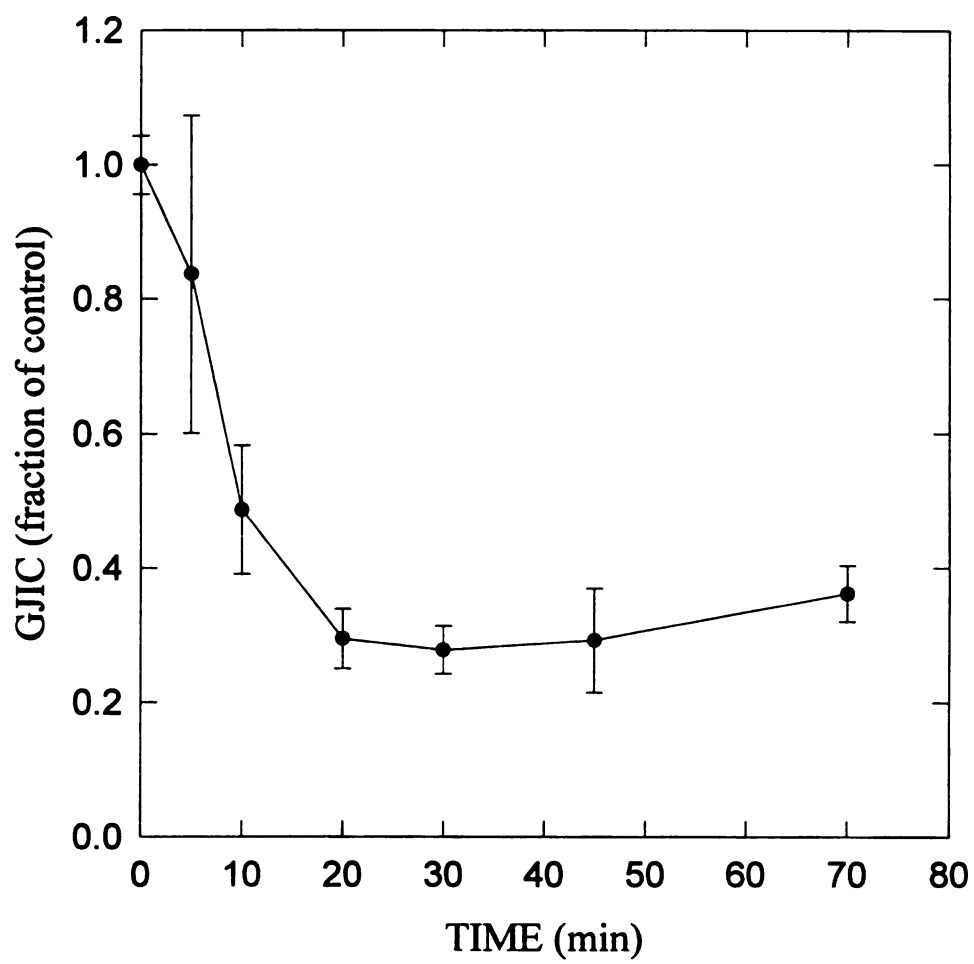


Figure 4.9 Time response curve for Pyrene

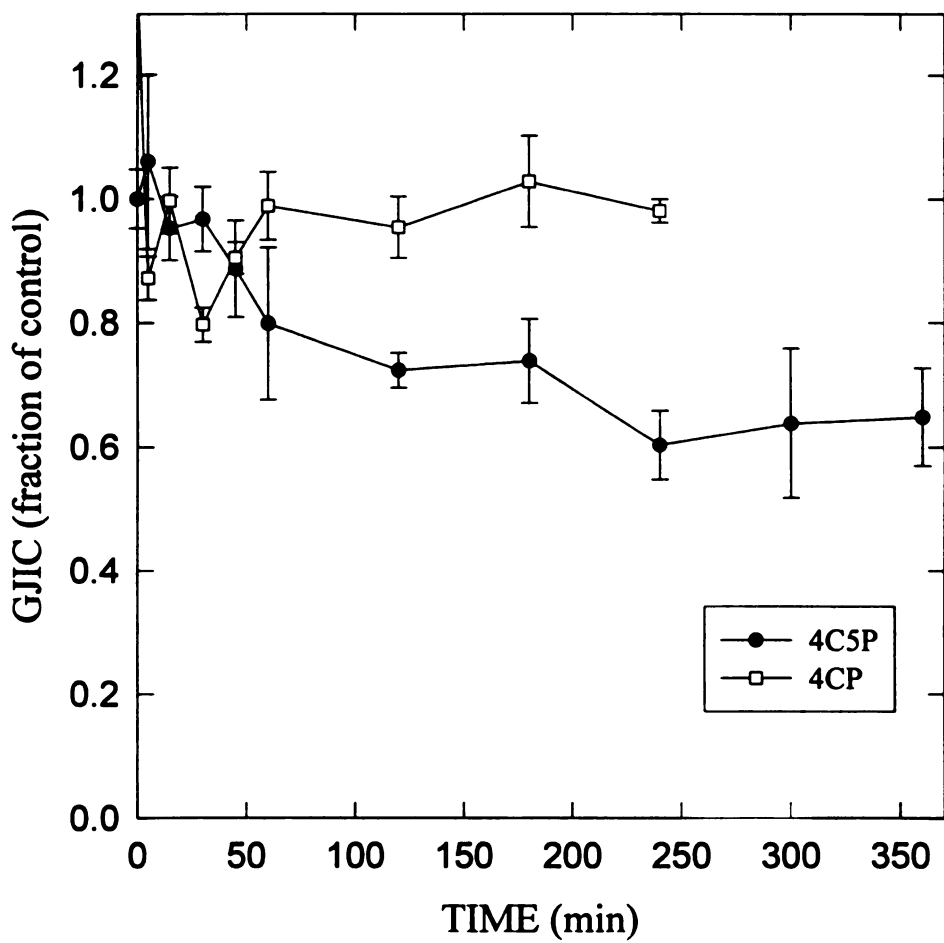


Figure 4.10 Time response curve for 4-Carboxy-5-Phenanthrene Carboxaldehyde and 4-Carboxyphenanthrene

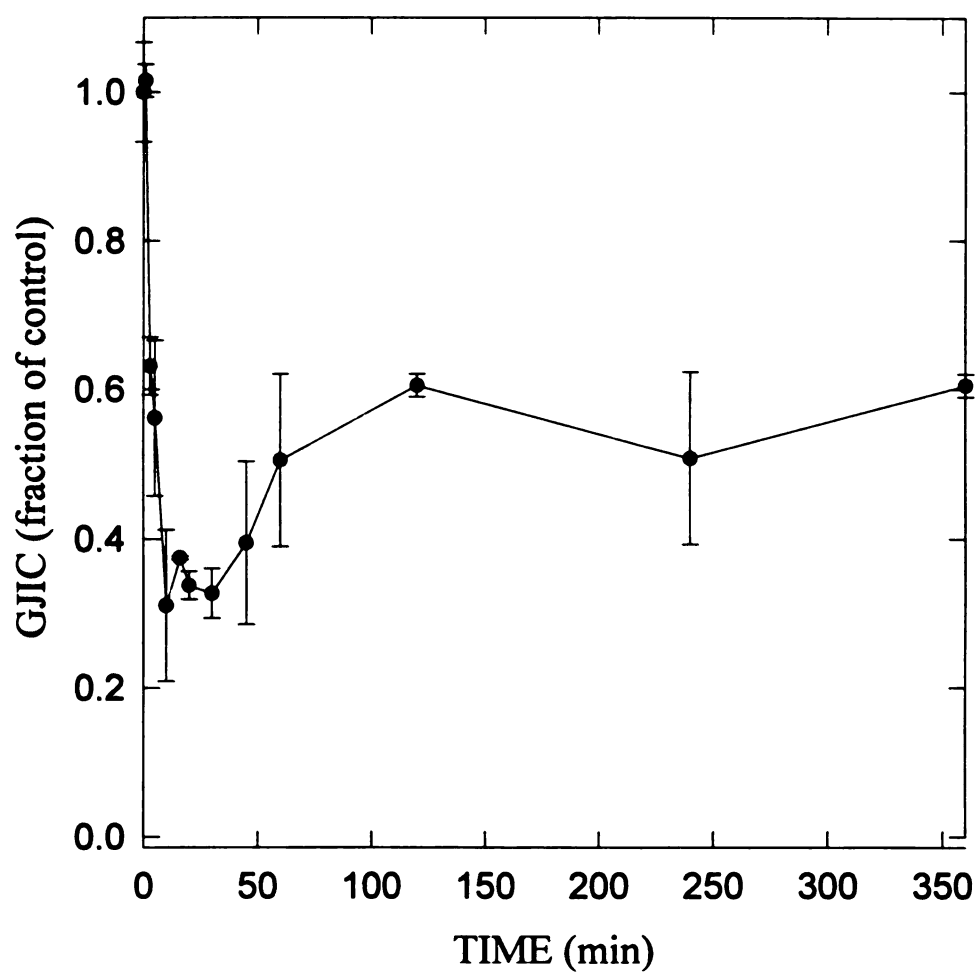


Figure 4.11 Time response curve for Compound Q

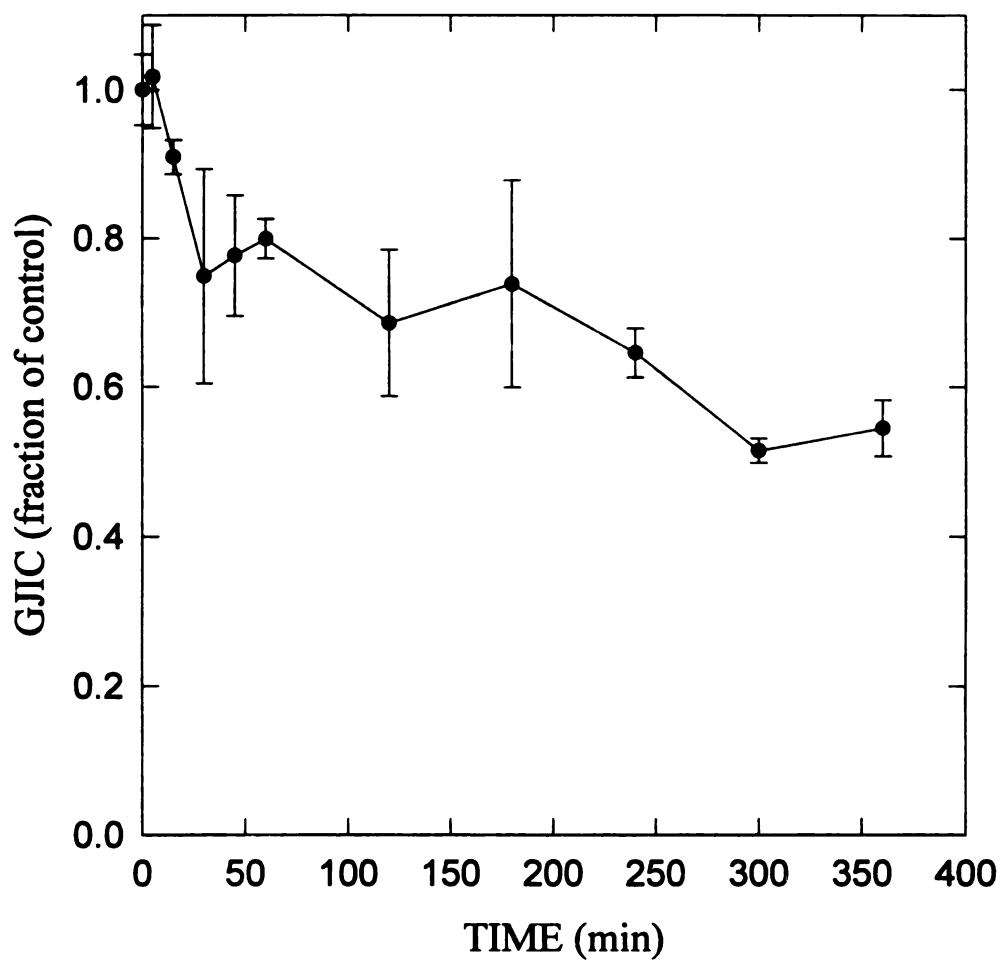


Figure 4.12 Time response curve for commercially obtained 4-Carboxy-5-Phenanthrene Carboxaldehyde

4C5P, which inhibited GJIC partially, inhibited to its maximum (FOC = 0.55) within approximately 300 min (Figure 4.12). TPC inhibited GJIC completely within 30 min and began partial recovery of cell-cell communication without removal of the chemical between 60 and 120 min (Figure 4.13). OFC inhibited GJIC completely within approximately 40 min and also began a partial recovery of cell-cell communication within approximately 60 min (Figure 4.13).

DPA, which inhibited GJIC only partially, showed its maximum activity within approximately 40 min (Figure 4.14). DPA exhibited a full recovery of cell-cell communication without the removal of the chemical within about 4 h. 2BCA inhibited GJIC partially within 30 min with a minimum FOC equal to 0.85 (Figure 4.14). 4BCA inhibited GJIC partially within 2 h and began to recover GJIC partially between 2 and 4 h (Figure 4.14). 4BCH inhibited GJIC completely within 5 min and began partial recovery of GJIC within 45 min (Figure 4.14).

Time of Recovery. Cell cultures originally exposed to pyrene recovered communication completely within approximately 2 h after the chemical was removed (Figure 4.15). 4CP required approximately 5 h before the FOC reached greater than 0.9 (Figure 4.16). The isolated 4C5P recovered communication within approximately 2 h following removal of the chemical from the media (Figure 4.16). Compound Q recovered GJIC within 90 min after the chemical was removed from the cell culture (Figure 4.17). The commercial version of 4C5P recovered complete cell-cell communication within 60 min after the chemical was removed (Figure 4.18). TPC recovered GJIC completely within approximately 6 h following removal of the chemical

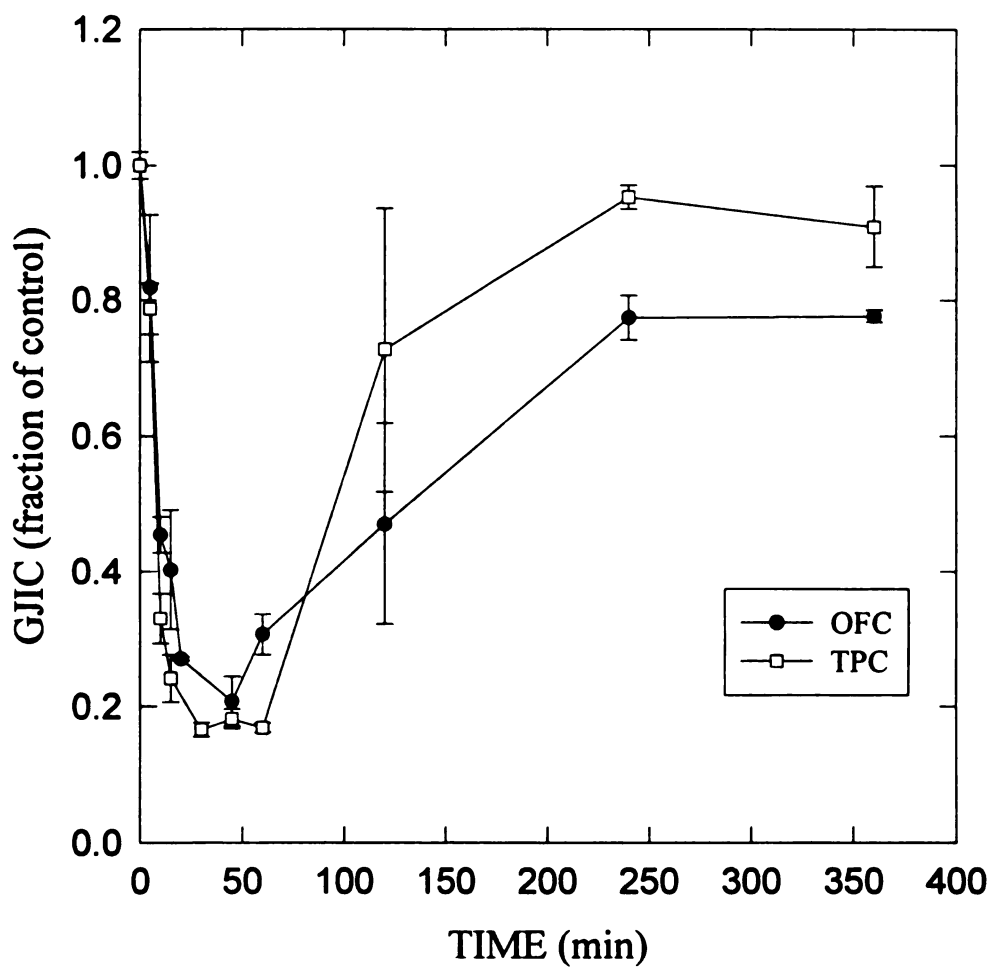


Figure 4.13 Time response curve for 1, 2, 3, 4-Tetrahydro-9-Phenanthrene Carboxaldehyde and 9-Oxo-1-Fluorene Carboxaldehyde

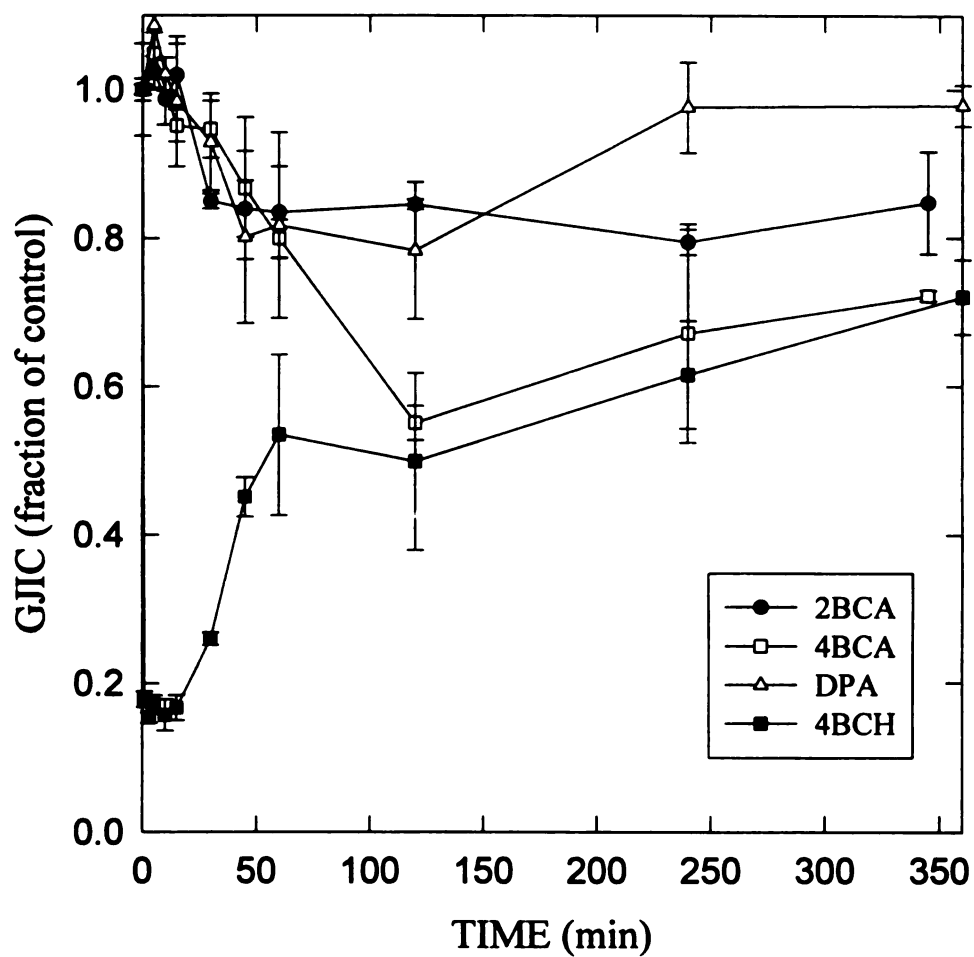


Figure 4.14 Time response curve for Diphenic Acid, 2-Biphenyl Carboxylic Acid, 4-Biphenyl Carboxylic Acid, and 4-Biphenyl Carboxaldehyde

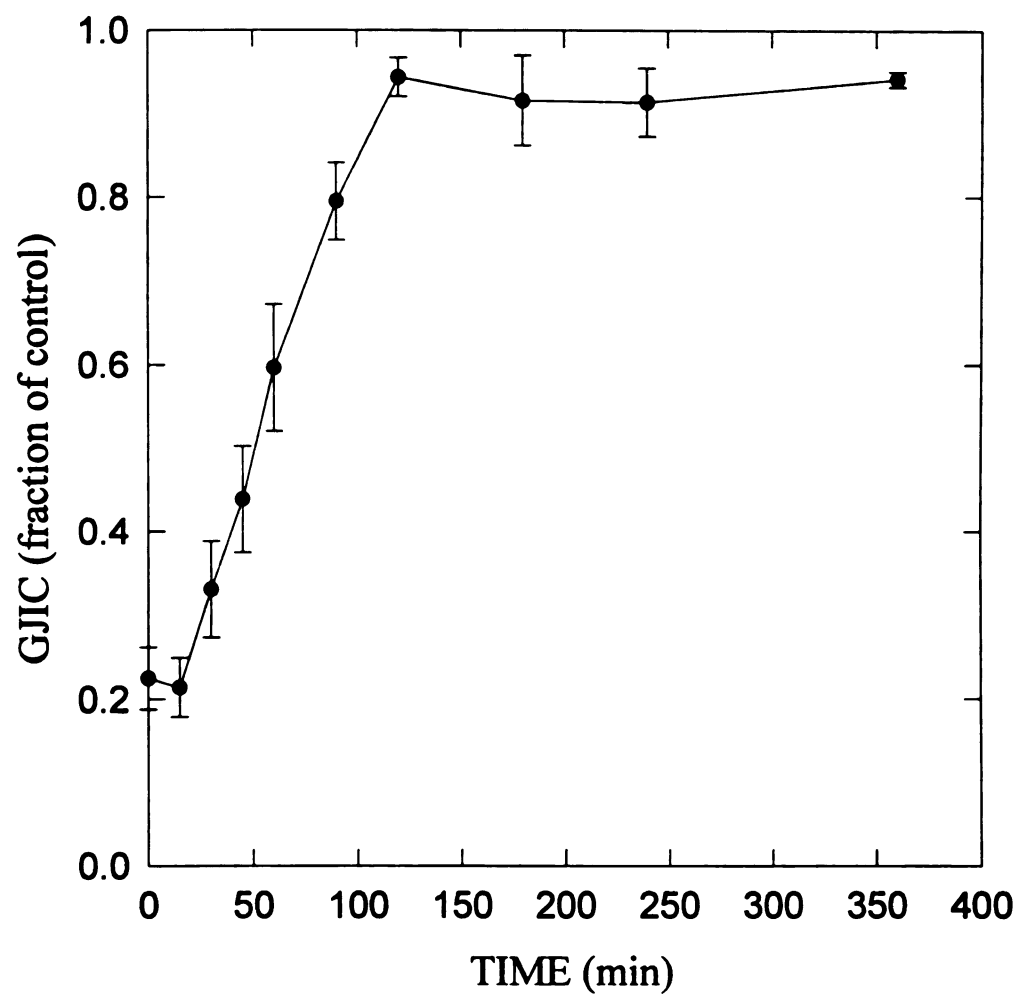


Figure 4.15 Time of recovery curve for Pyrene

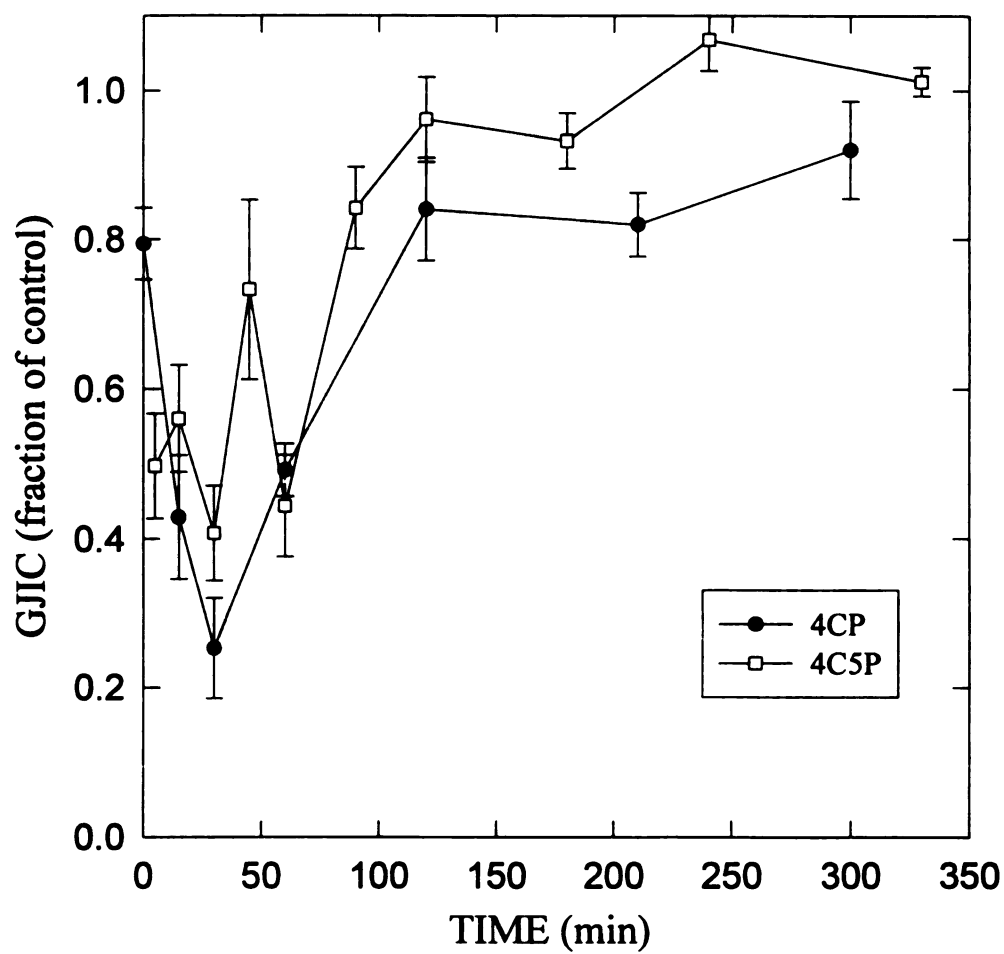


Figure 4.16 Time of recovery curve for 4-Carboxy-5-Phenanthrene Carboxaldehyde and 4-Carboxyphenanthrene

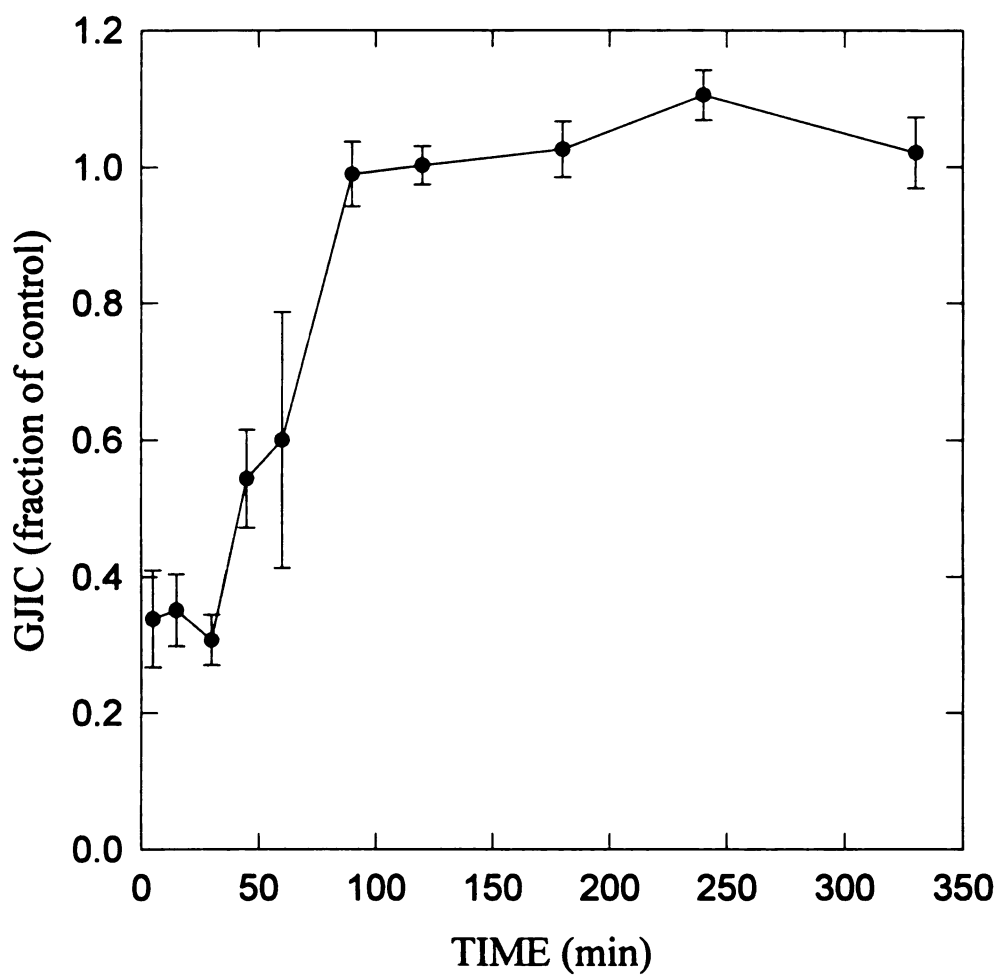


Figure 4.17 Time of recovery curve for Compound Q

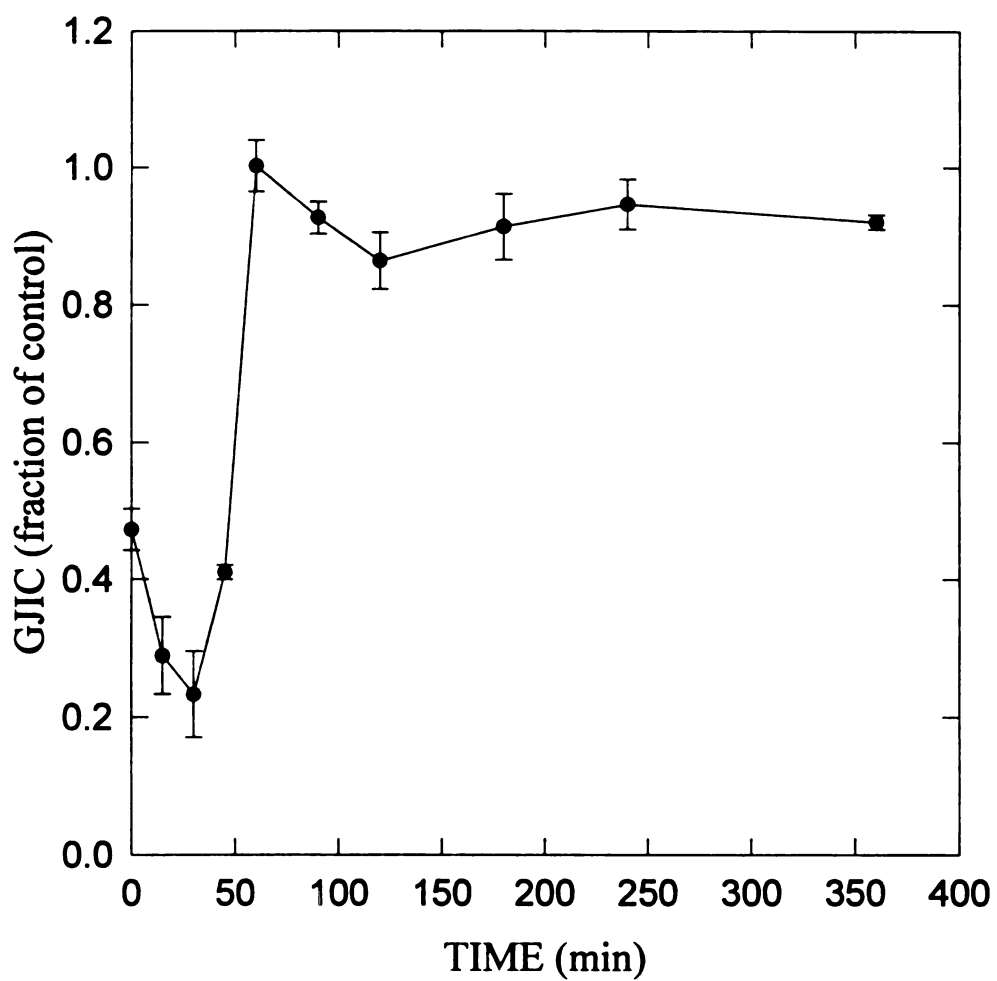


Figure 4.18 Time of recovery curve for commercially obtained 4-Carboxy-5-Phenanthrene Carboxaldehyde

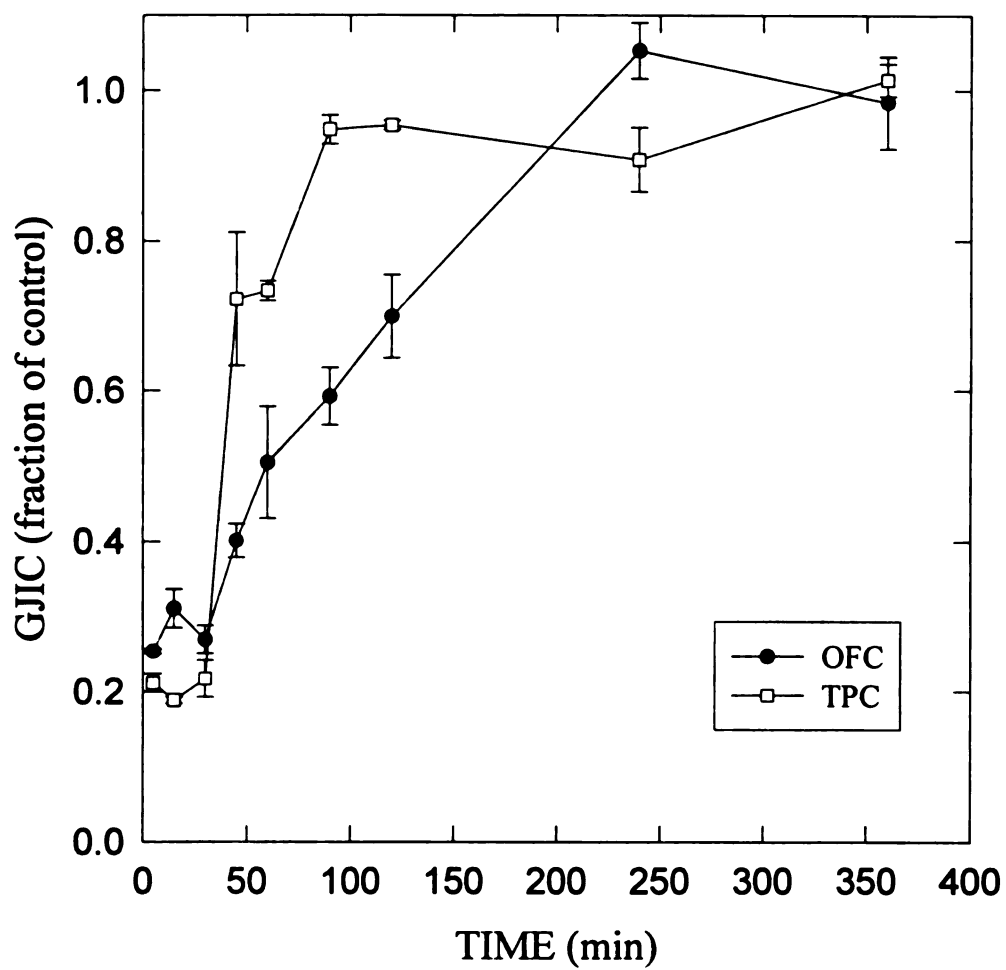


Figure 4.19 Time of recovery for 1, 2, 3, 4-Tetrahydro-9-Phenanthrene Carboxaldehyde and 9-Oxo-1-Fluorene Carboxaldehyde

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from the cell culture (Figure 4.19). OFC recovered GJIC completely within approximately 4 h following removal of the chemical (Figure 4.19). DPA required approximately 6 h to resume complete cell-cell communication (Figure 4.20). 2BCA and 4BCH required approximately 2 h to resume complete GJIC where as 4BCA required only 90 min (Figure 4.20).

Cytotoxicity. The results of the cytotoxicity experiments showed that the compounds analyzed were not cytotoxic at the concentrations studied during dose response experiments (Figures 4.21 to 4.24). Cytotoxicity experiments mimicked dose response experiments in dosage and exposure time. Cytotoxicity of chemicals after a 24 h exposure at the most inhibitory dosage was performed. The results showed that none of the eleven compounds studied were cytotoxic after 24 h (figure 4.25).

4.3.2 Study Two

Dose Response. Study two consisted of the investigation of two groups of pyrene ozonation byproducts, produced at a pH equal to 2.0 and 9.5, respectively. From previous work, it was known that the byproducts as a mixture generated under low pH conditions were still inhibitory to GJIC [9]. In this study, the procedure for the ozonation was performed as described by Upham et al.[1] and the mixture produced was separated into 10 fractions using semi-preparative HPLC (Figure 3.8). Because the fractions collected as major peaks during the HPLC separation were impure, the concentrations for this assay were calculated based upon the molecular weight of pyrene. A preliminary dose response experiment was conducted for each fraction and pyrene at a concentration

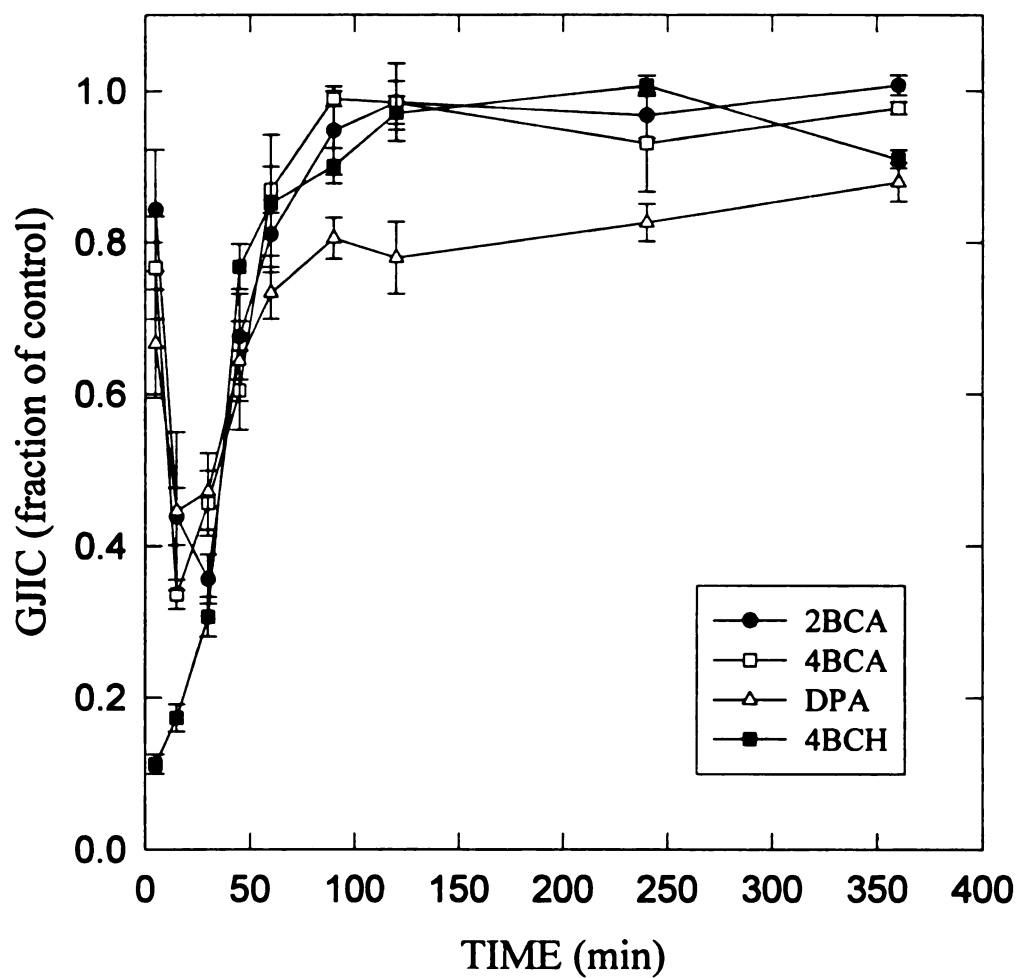


Figure 4.20 Time of recovery curve for Diphenic Acid, 2-Biphenyl Carboxylic Acid, 4-Biphenyl Carboxylic Acid, and 4-Biphenyl Carboxaldehyde

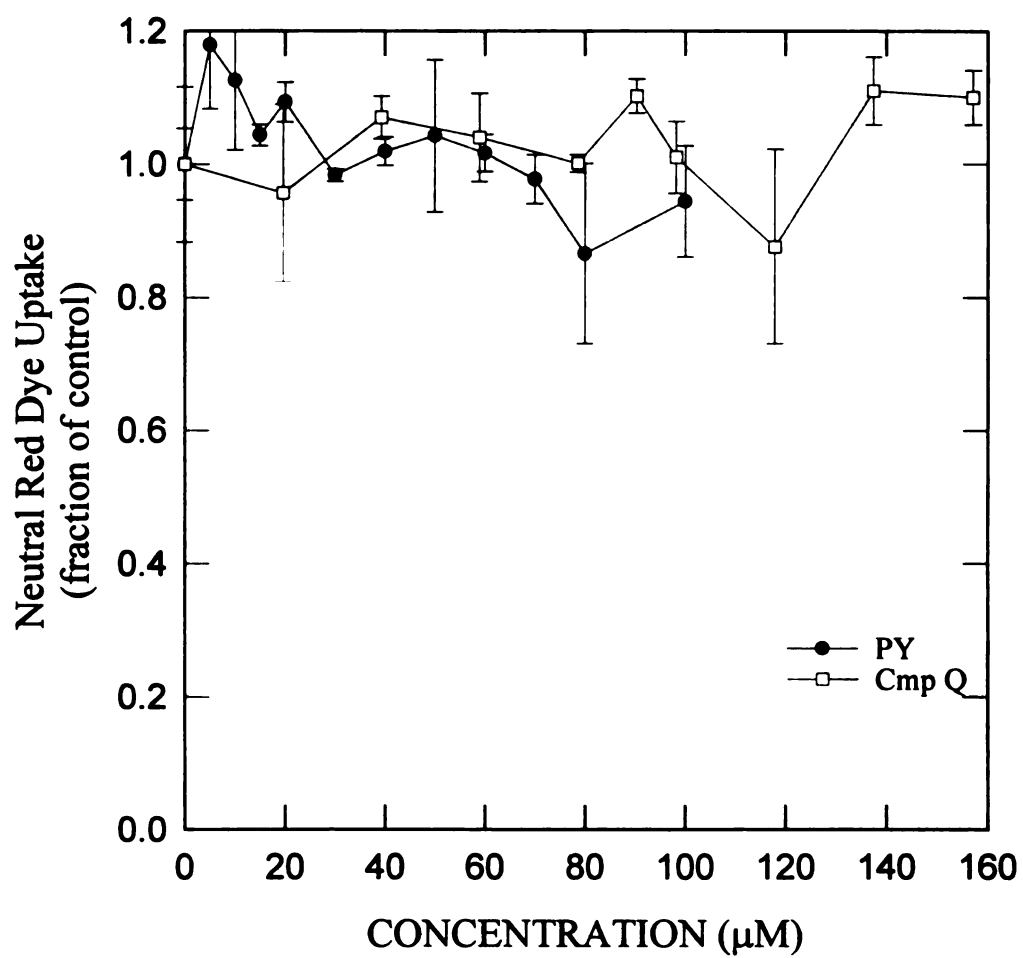


Figure 4.21 Results of cytotoxicity experiments for Pyrene and Compound Q

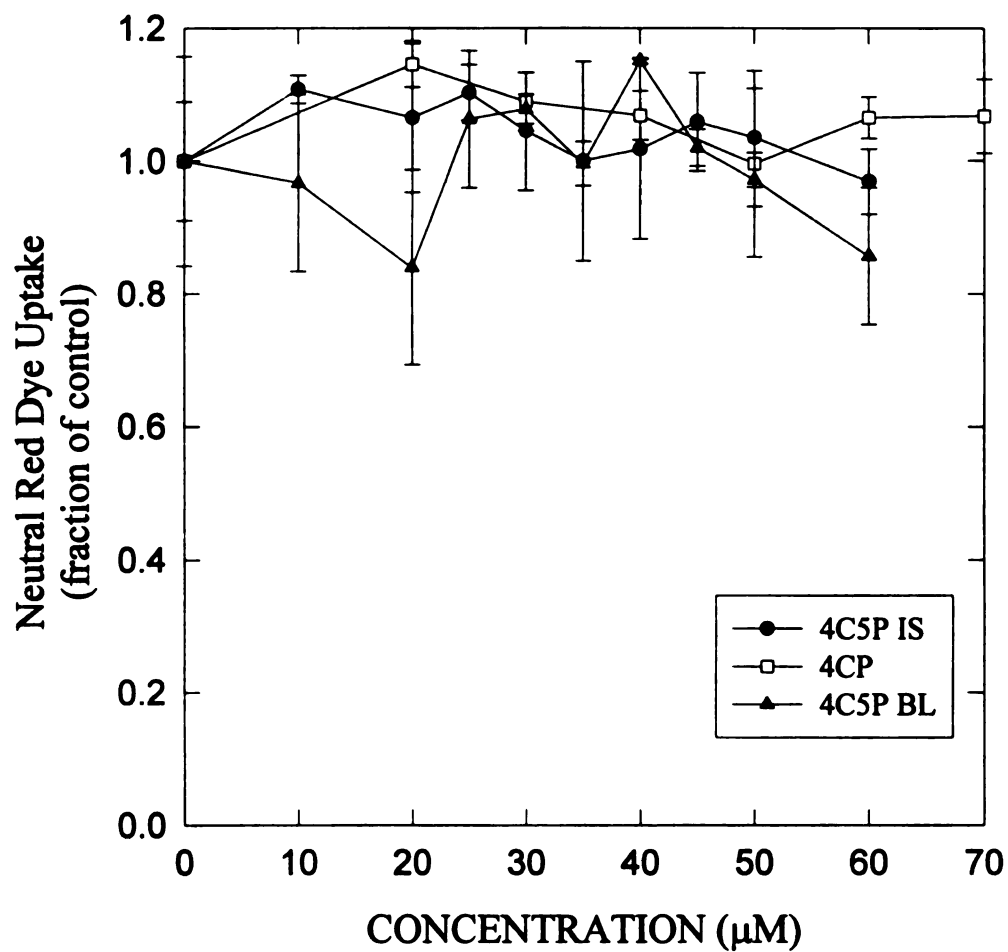


Figure 4.22 Results of cytotoxicity experiments for 4-Carboxyphenanthrene, isolated 4-Carboxy-5-Phenanthrene Carboxaldehyde, and commercial 4-Carboxy-5-Phenanthrene Carboxaldehyde

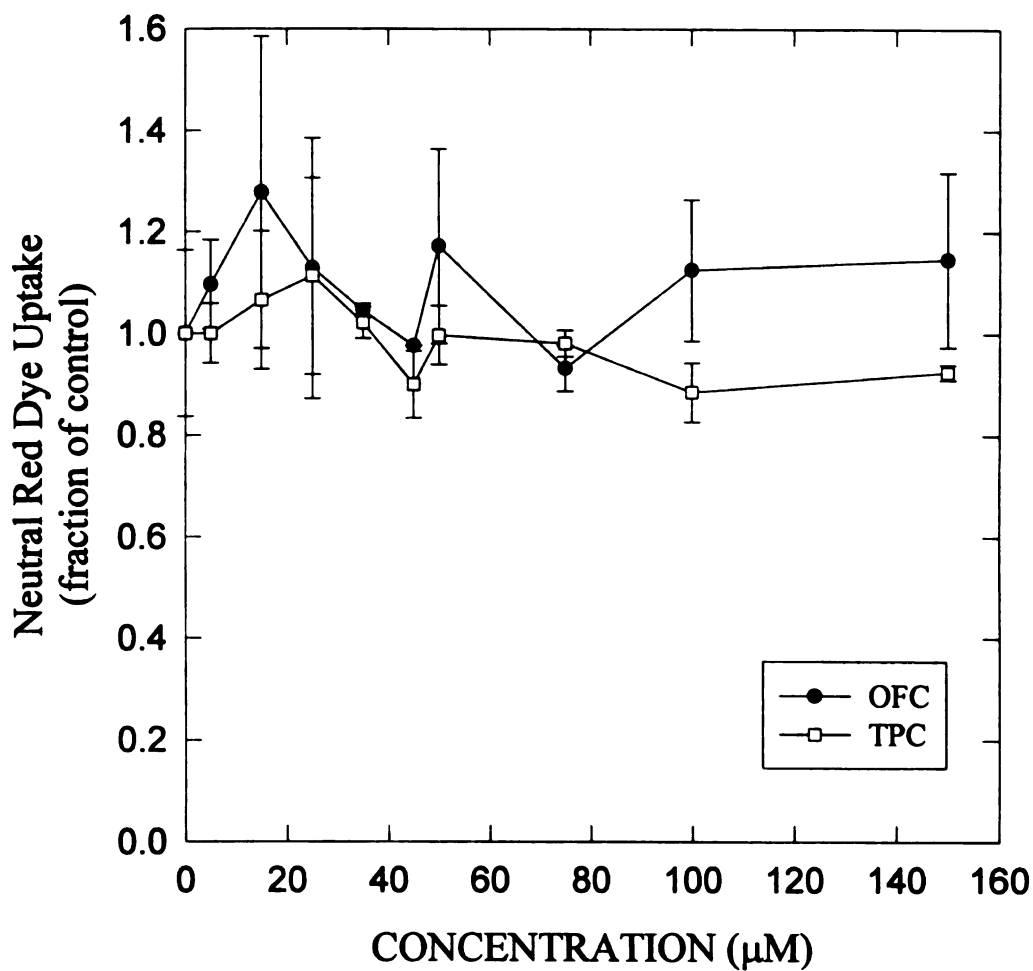


Figure 4.23 Results of cytotoxicity experiments for 1, 2, 3, 4-Tetrahydro-9-Phenanthrene Carboxaldehyde and 9-Oxo-1-Fluorene Carboxaldehyde

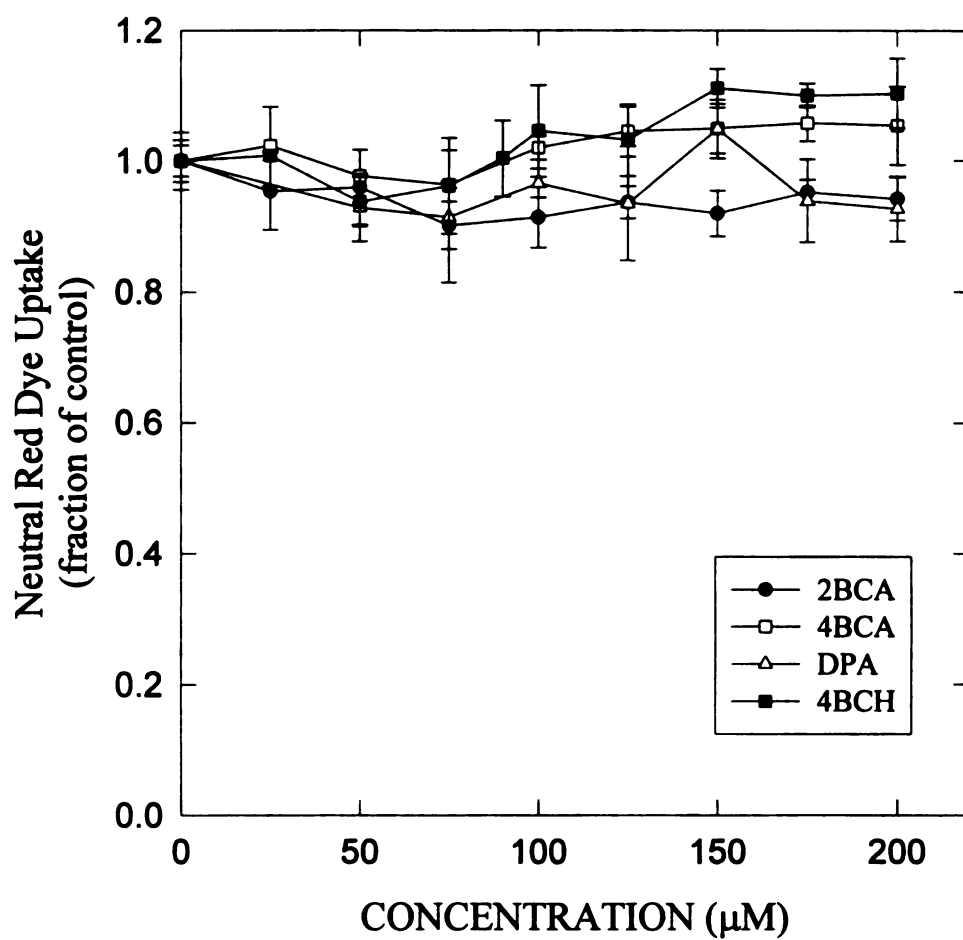


Figure 4.24 Results of cytotoxicity experiments for Diphenic Acid, 2-Biphenyl Carboxylic Acid, 4-Biphenyl Carboxylic Acid, and 4-Biphenyl Carboxaldehyde

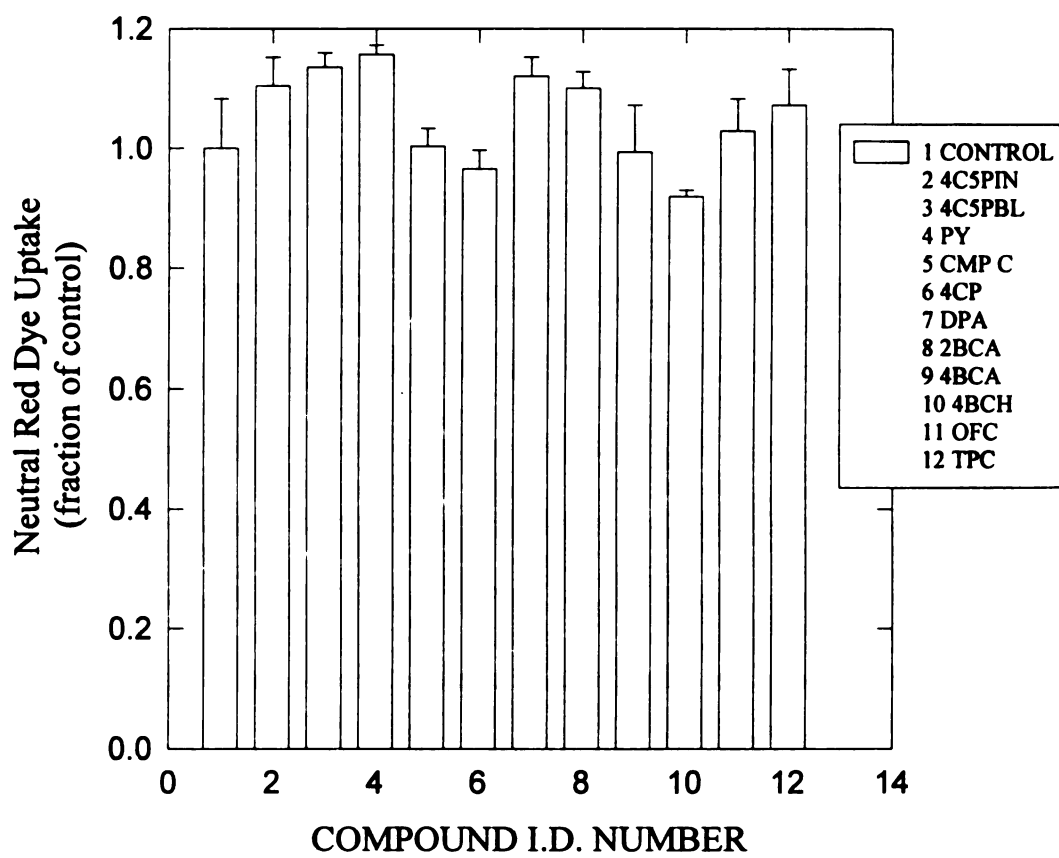


Figure 4.25 Results of 24 hour cytotoxicity experiments for pyrene, all isolated compounds, and all commercial compounds. Experiments were conducted at the most inhibiting concentration for each compound.

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of 75 μM as pyrene. This dose was chosen because pure pyrene completely inhibits GJIC at this level and it is noncytotoxic to the cell cultures.

Several of the fractions tested inhibited GJIC partially or completely (Figure 4.26). The water soluble fraction did not inhibit GJIC, but a complete dose response experiment was performed. Fractions 1-4, 6, and 9 partially inhibited GJIC and fraction 10 completely inhibited GJIC. Fraction 10 was proved to be residual parent compound, pyrene. Therefore, no further studies were performed using fraction 10. For the fractions that inhibited GJIC partially at 75 μM as pyrene, a complete dose response curve was generated to determine if the fraction could in fact inhibit GJIC completely at an increased dose. Fractions 5, 7, and 8 did not inhibit GJIC at 75 μM as pyrene, but were also evaluated at additional dosages to generate more complete dose response curves.

Dose response data for fractions 5, 7, 8, and the water soluble portion are shown in Figure 4.27. Fractions 5, 7, and the water soluble portion did not inhibit GJIC even with increased concentrations of the chemical. However, at 85 μM as pyrene, fraction 8 partially inhibited GJIC with an FOC of approximately 0.75. Further increases in concentration were not possible due to the solubility and mass constraints.

Figure 4.28 shows complete dose response data for fractions 1-4. Fraction 1 inhibited GJIC partially with a minimum FOC of approximately 0.75 beginning at 85 μM as pyrene. Fraction 2 and 3 also partially inhibited GJIC with a minimum FOC of approximately 0.65 and 0.55 at 100 μM as pyrene, respectively. Fraction 4 exhibited a

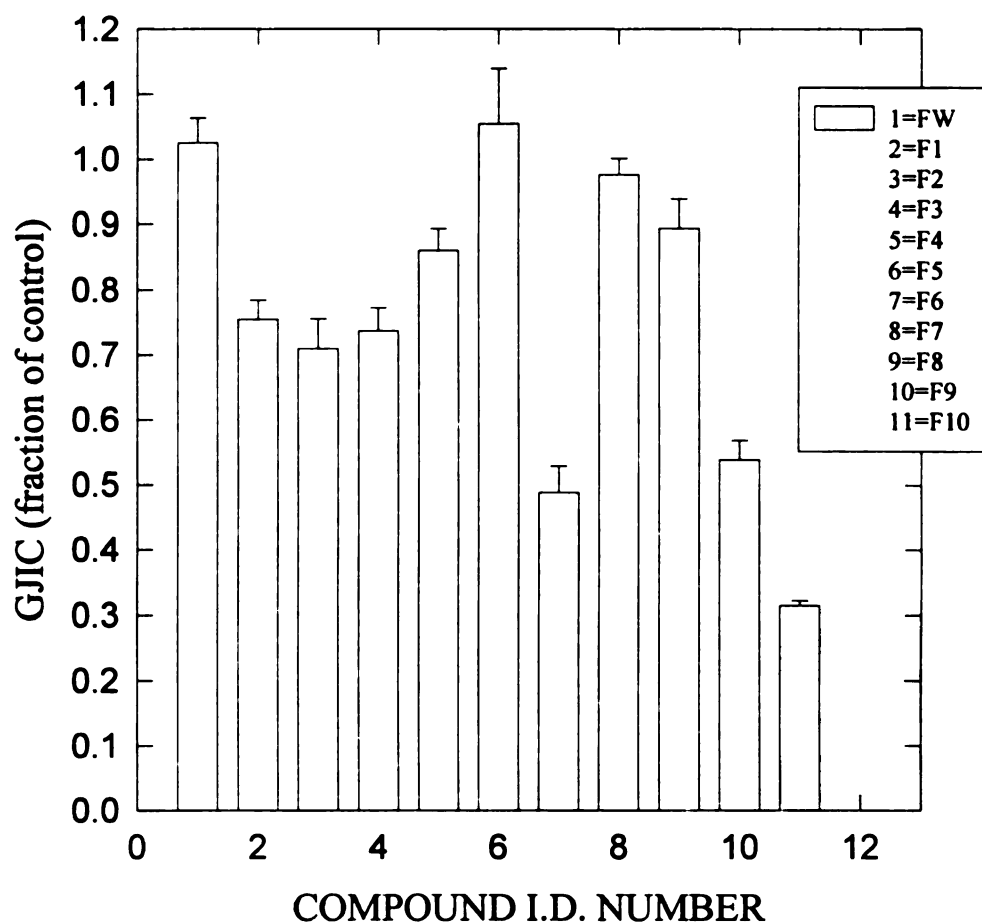


Figure 4.26 Dose response results for byproduct fractions generated under low pH conditions during ozonation. Concentration to cell cultures was 75 μ M as pyrene for all fractions.

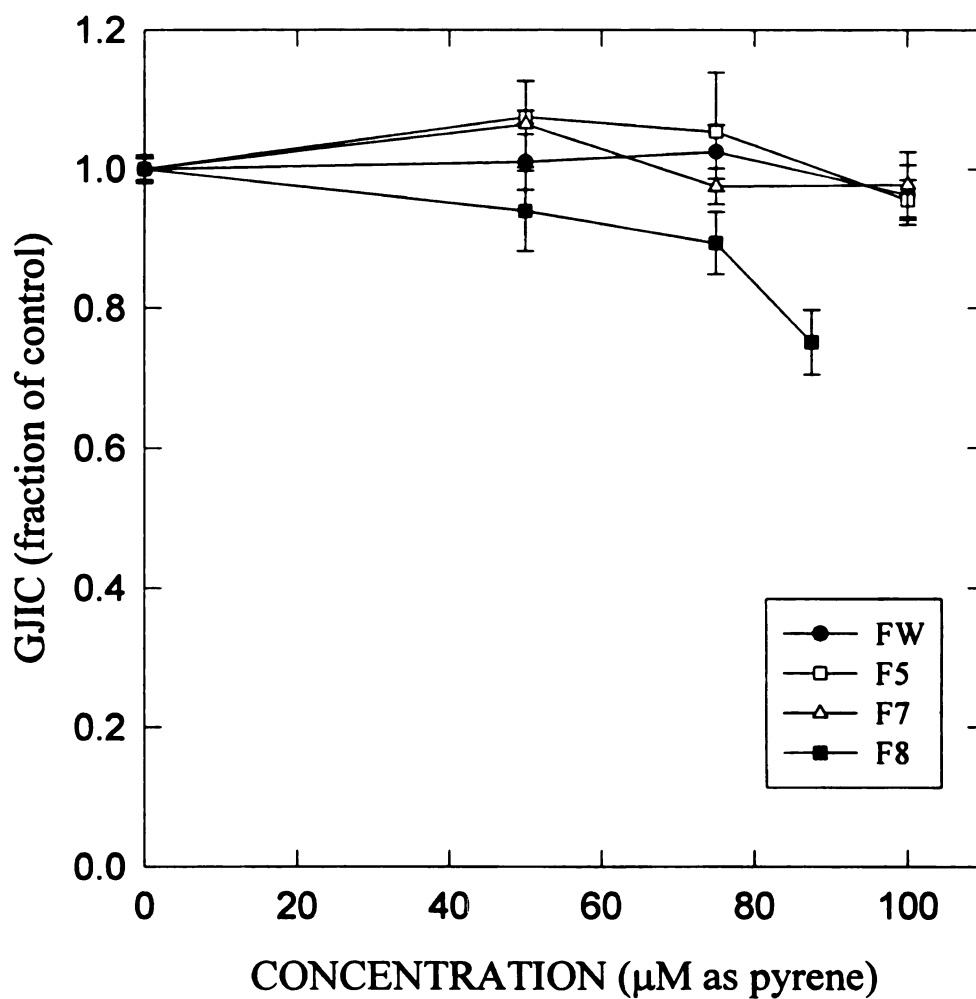


Figure 4.27 Dose response curves for fractions 5, 7, 8, and water soluble fraction. These fractions were byproducts generated during ozonation at low pH. All chemical concentrations applied to cell cultures were calculated as pyrene due to impure status.

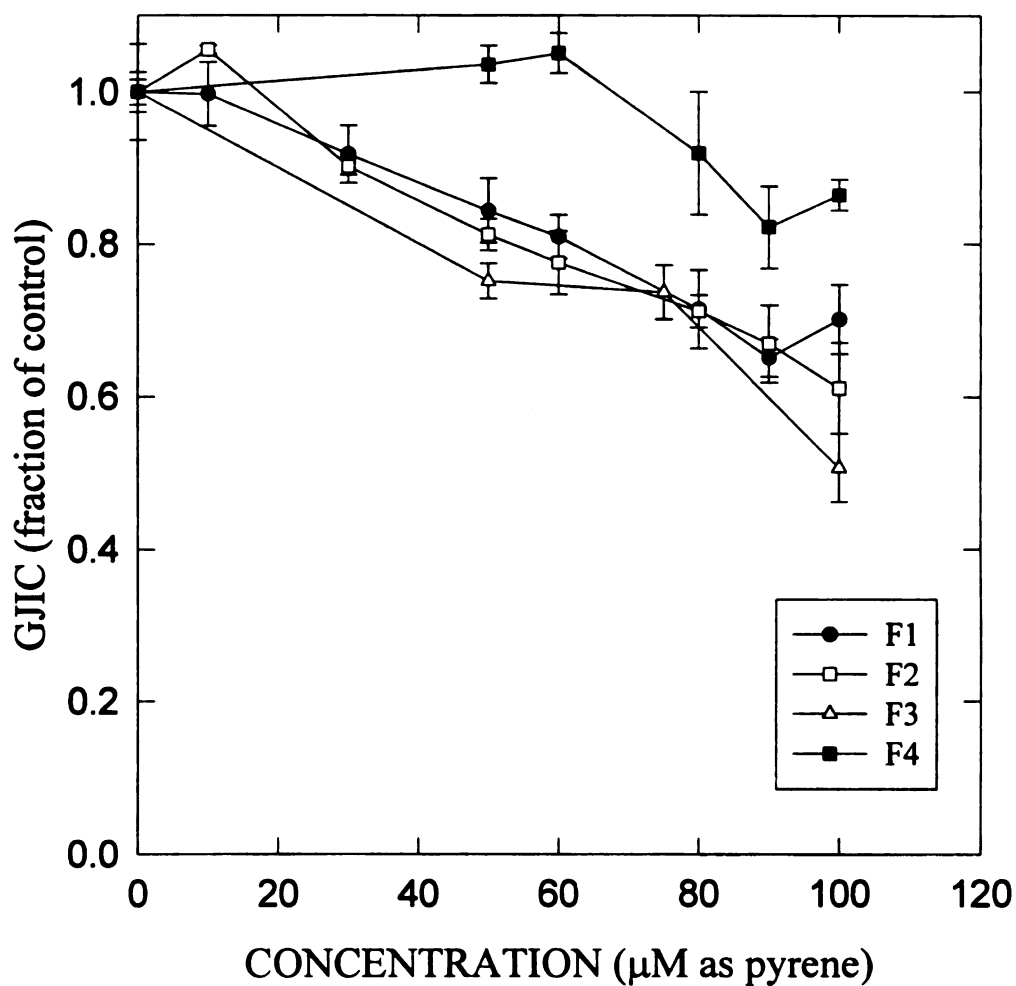


Figure 4.28 Dose response curves for fractions 1 through 4. These fractions were byproducts generated during ozonation at low pH. All chemical concentrations applied to cell cultures were calculated as pyrene due to impure status.

partial inhibition of GJIC with a minimum FOC of approximately 0.85 at 90 μM as pyrene.

Fractions 6 and 9 completely inhibited GJIC when the dose was increased (Figure 4.29). Fraction 6 and 9 inhibited GJIC completely with a minimum FOC of approximately 0.3 at 100 μM as pyrene and 90 μM as pyrene, respectively.

The second portion of the second study was different because the pH conditions during ozonation were much higher with a pH equal to 9.5. The separation of the components by HPLC was consistent with the low pH byproduct separation. Six fractions were collected as shown in Figure 3.12. At low pH, the byproduct mixture was reported to inhibit GJIC based on previous studies [1]. The effects of the increased pH on the toxicity of the byproduct mixture was unknown. Figure 4.30 shows the dose response curve for the byproduct mixture ozonated at high pH. The byproduct mixture completely inhibited GJIC with a FOC of approximately 0.25 at 100 μM as pyrene.

A preliminary dose response experiment was conducted again using a dose of 75 μM as pyrene for all 6 fractions, the crude mixture, and pyrene. Figure 4.31 shows that all fractions inhibited GJIC partially or completely. The crude mixture inhibited almost as much as the parent compound. Fractions 1, 2, 4, and 5 partially inhibited GJIC and fractions 3 and 6 completely inhibited GJIC. Complete dose response studies were again conducted on all fractions to determine if and at what dose GJIC was completely inhibited.

Figure 4.32 contains complete dose response data for fractions 1, 2, 4, and 5. All four compounds exhibited greatest activity at 100 μM as pyrene. Fraction 1 partially

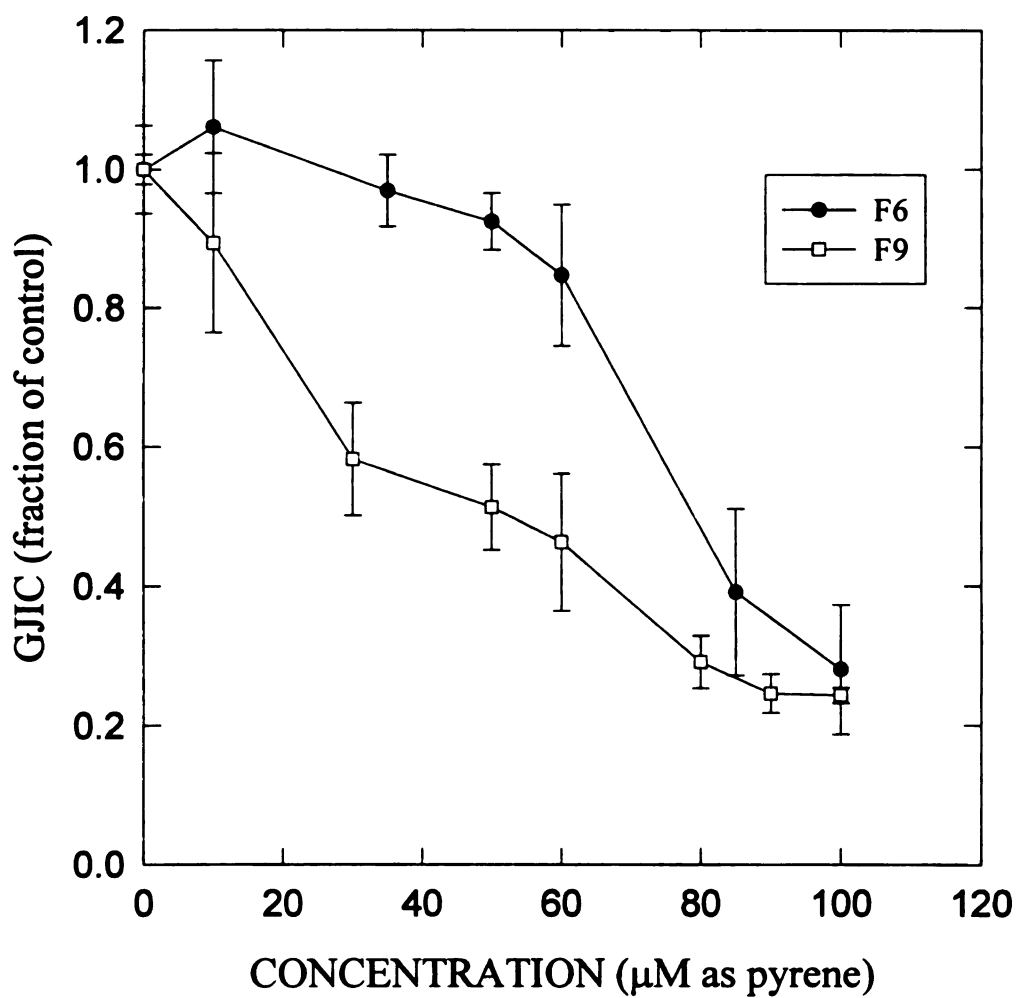


Figure 4.29 Dose response curves for fractions 6 and 9. These fractions were byproducts generated during ozonation at low pH. All chemical concentrations applied to cell cultures were calculated as pyrene due to impure status.

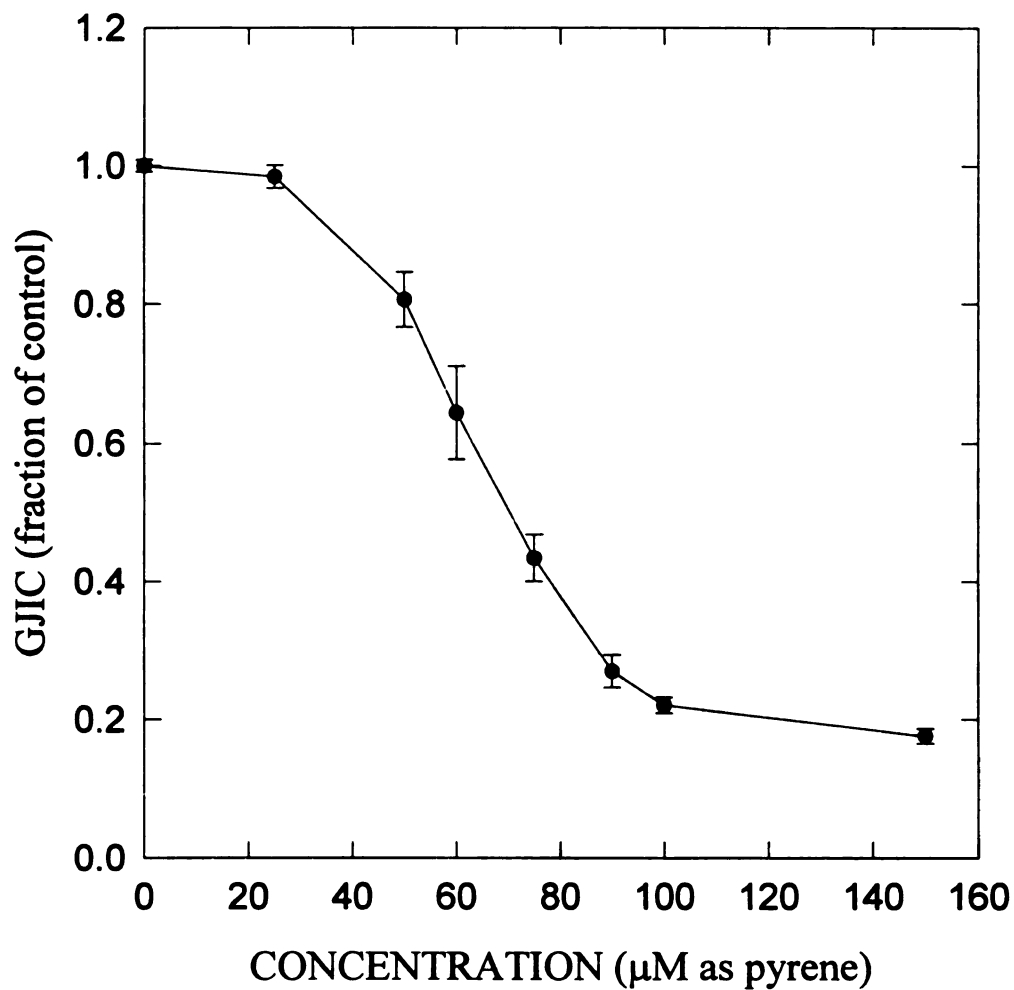


Figure 4.30 Dose response curve for crude mixture of byproducts generated during ozonation at high pH. Chemical concentrations applied to cell cultures were calculated as pyrene due to impure status.

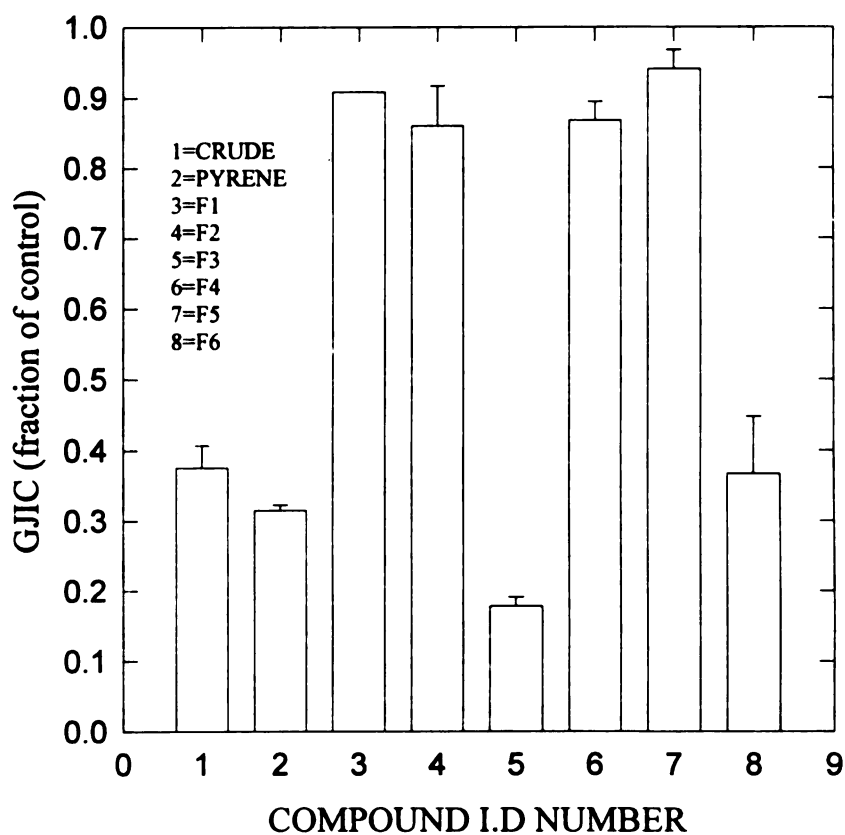


Figure 4.31 Dose response results for byproduct fractions generated under high pH conditions during ozonation. Concentration to cell cultures was 75 μ M as pyrene for all fractions.

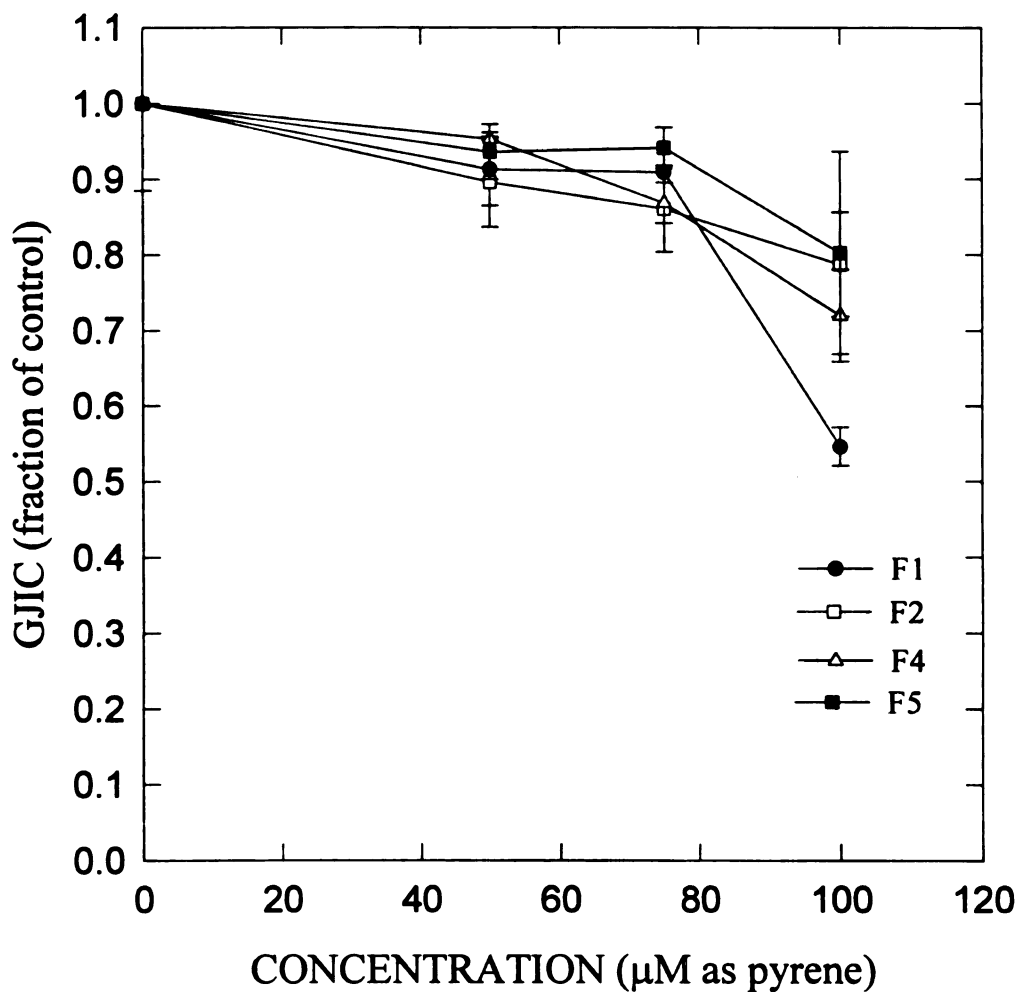


Figure 4.32 Dose response curves for fractions 1, 2, 4, and 5. These fractions were byproducts generated during ozonation at high pH. All chemical concentrations applied to cell cultures were calculated as pyrene due to impure status.

inhibited GJIC with a minimum FOC of 0.55. Fractions 2 and 5 each partially inhibited GJIC with a minimum FOC value of 0.8. Fraction 4 partially inhibited GJIC with a minimum FOC value of approximately 0.75. Figure 4.33 shows the complete dose response curve for fractions 3 and 6. Fraction 6, which was residual unreacted pyrene, inhibited almost completely which was consistent with the control pyrene (compound I.D. 2). Fraction 3 also completely inhibited GJIC between 50 and 75 μ M as pyrene.

Due to the small quantity of material available for each fraction, additional experiments were not performed. The lack of cytotoxicity for pyrene and several isolated byproducts was shown in study one. In addition, visual inspection of cell cultures following chemical exposure was performed to identify any signs of cytotoxicity. There were no indications that cytotoxicity played a role in the inhibition of GJIC during study two.

4.4 Discussion

4.4.1 Study One

Several compounds were investigated for their ability to inhibit GJIC. A variety of compounds ranging from 2 to four rings with various functional groups were analyzed using the SL/DT technique. Of the eleven compounds investigated, 5 compounds were strong inhibitors of GJIC and the remaining 6 compounds partially inhibited GJIC.

The parent compound, pyrene, one of the larger PAHs investigated, has a lower solubility in aqueous solution than some of the byproducts and other compounds tested.

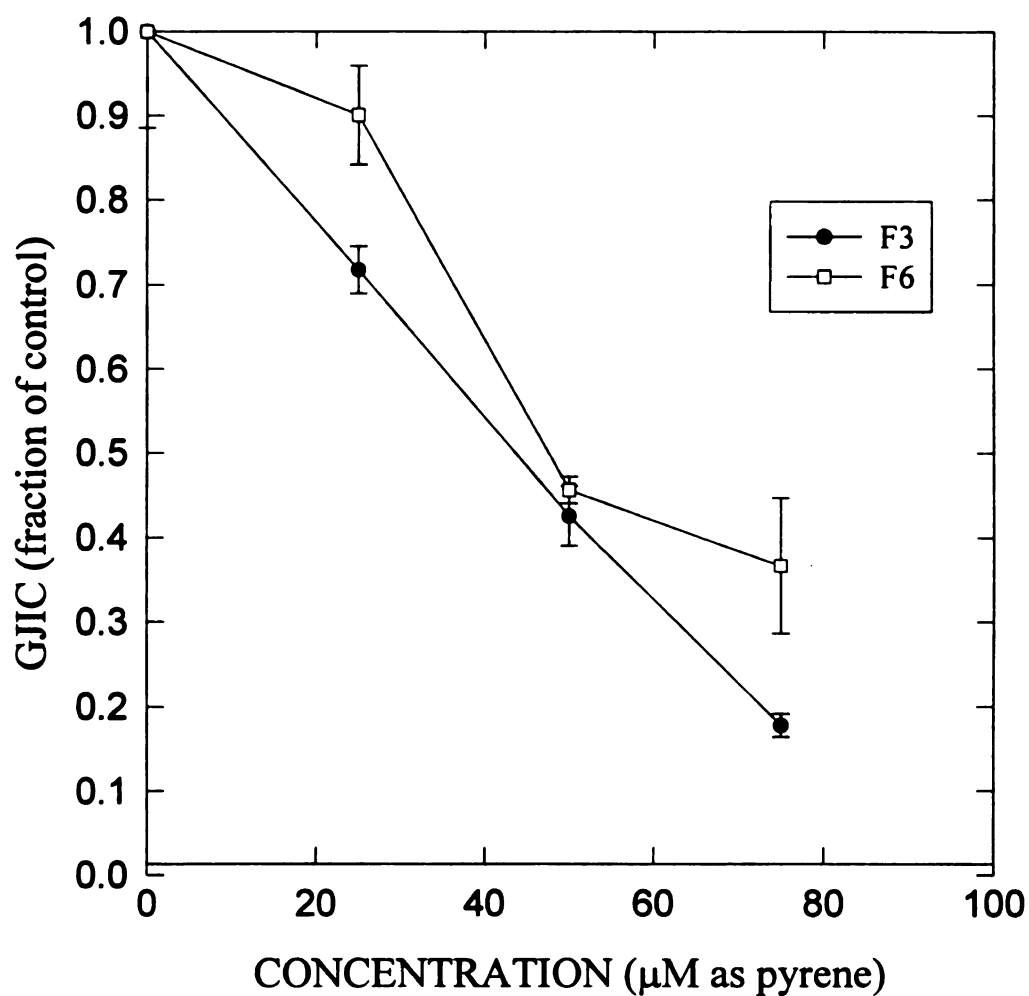


Figure 4.33 Dose response curves for fractions 3 and 6. These fractions were byproducts generated during ozonation at high pH. All chemical concentrations applied to cell cultures were calculated as pyrene due to impure status.

Pyrene completely inhibited GJIC within a short period of time at a low dose. Of the byproducts isolated, only one, compound Q, completely inhibited GJIC. Compound Q is a 3 ringed aromatic compound with a fourth non-aromatic ring. The methylations encountered during purification translated the structure of Compound Q from its original ozonated form. Although this compound does inhibit GJIC, it is not as potent as pyrene.

4CP and 4C5P which were isolated byproducts of pyrene ozonation both only partially inhibited GJIC and were not as inhibitory as the parent compound. Both compounds were phenanthrene type compounds each with a bay region. It should be noted that phenanthrene completely inhibits GJIC [9]. Interestingly, 4C5P which only partially inhibits GJIC is the most abundant byproduct of pyrene ozonation as determined by Yao [30].

Previous studies have shown that some byproducts of ozonation can be as or more toxic than the parent compound. However, during this study, none of the byproducts studied were found to be more inhibitory than the parent compound. However, only a limited number of byproducts were investigated due to the difficulty in byproduct isolation. It is very possible that one of the byproducts which constitutes a small amount of the byproduct mass is responsible for the more inhibitory results observed in studies by Upham et al. [1]. During the isolation process, byproducts with larger mass were the focus. In study two of this section, impure byproduct fractions characterized by GC/MS, were investigated to address this possibility.

The commercially obtained byproduct 4C5P partially inhibited GJIC, but more so than the isolated 4C5P. This may be due to the impurities in the commercial product

observed during HPLC. One of the major impurities in the commercial 4C5P appeared to be pyrene.

A large interest in the identification of relationships between structure and chemical activity prompted the investigation of 6 additional commercial compounds. These 6 compounds resembled the byproducts in ring number and functional groups. TPC and OFC both inhibited GJIC completely at low doses comparable to pyrene. Both compounds possess 3 rings, but with subtle differences (Figure 4.2). TCP and OFC each contained an aldehyde group and a bay like region. It is difficult to determine which of these factors cause the differences in activity. It should be noted that fluorene was not a strong inhibitor to GJIC [9]. However, many observations can be made. First, 4CP and 4C5P are both 3 ringed compounds containing bay regions and did not inhibit GJIC completely. Although, 4C5P is present in 2 forms one of which does not contain a bay region and the percent distribution is known. 4CP does have a bay region, as determined by NMR spectroscopy, but the structure has not been confirmed by GC/MS. OFC and TPC are also 3 ringed compounds with a bay like region which inhibited GJIC. The obvious difference in activity may be attributed to the aldehyde group in both OFC and TPC. 4C5P also contains a potential aldehyde group as determined by NMR and GC/MS, but is present as part of a cyclized fourth ring. When in an uncyclized form, the aldehyde group has interactions with the acid group due to the close proximity.

The breakdown of pyrene occurs with the formation of phenanthrene type compounds initially followed by the formation of biphenyl type compounds [30]. The remaining 4 compounds studied were biphenyl type compounds, all very close in structure. Three of the four compounds studied had carboxylic acid functional groups

and the fourth compound had an aldehydic group. 2BCA, 4BCA, and DPA partially inhibited GJIC at a high concentrations. In contrast, 4BCH which was examined at in the same concentration range as the other 3 biphenyls, completely inhibited GJIC. 2BCA and 4BCA differed in structure only in the placement of the acid moiety on the biphenyl structure. This did not appear to change the affect on cell-cell communication. DPA contained an additional acid group which separated its structure from that of 2BCA. Again, the difference in inhibition of GJIC was negligible. The structures representing 4BCA and 4BCH differed only in the type of functional group at the fourth position. The compound containing the aldehyde group, 4BCH, inhibited GJIC completely, and the compound containing the acid, 4BCA, inhibited GJIC only slightly. The chemical concentrations examined were much higher for these 4 biphenyl compounds as opposed to the 3 and 4 ringed compounds. The biphenyl compounds are more soluble in aqueous solutions and would be expected to be found in greater concentration in the environment. A much higher dose was required for 4BCH to display GJIC inhibitory properties.

Of the 5 compounds studied which inhibited GJIC completely, the inhibition was observed quickly within 30 min. Although 4BCH required a high concentration for inhibition of GJIC, this response was observed within 1 min of exposure. The remaining 2 and 3 ringed compounds which partially inhibited GJIC, appeared to increase inhibition slightly or stay the same with increased exposure duration. DPA was one exception because incomplete inhibition was partially recovered without the removal of the test compound. All 5 compounds which completely inhibited GJIC displayed a partial recovery characteristic without the removal of test compounds. Pyrene began recovery of GJIC slightly after 70 min. Compound Q exhibited partial recovery of GJIC after only 1

h (FOC = 0.5). TPC, OFC, and 4BCH all recovered cell-cell communication dramatically without the removal of the test compound. TPC regained almost 100% communication within 4 h of exposure.

There are several possible explanations for the transient nature in which the compounds block GJIC. It is possible that the chemical indirectly inhibits GJIC by way of protein kinase (PK) activation [31]. In this case, a PK phosphorylates the gap junction protein (connexin 43) which blocks cell-cell communication. At the same time, however, PK is also phosphorylating phosphatases which dephosphorylate the gap junction protein and restore communication. There is a lag time before the phosphatases are activated which allows the inhibition of GJIC to be observed. If this is indeed the mechanism by which the chemical inhibits, the addition of a phosphatase inhibitor would prohibit the recovery of GJIC.

It is also possible that the inhibiting compound works in a direct manner in its attack [31]. The membrane fluidity and probably other conditions of the cell's homeostasis may be altered which cause the initial inhibition of GJIC. The recovery of GJIC may be due to the cell's ability to adapt to the new conditions. Finally, metabolism of the chemical may explain the return of cell-cell communication. Certain cells produce enzymes which can metabolize these compounds. However, it is unlikely that the undifferentiated rat liver epithelial cells used in this study produce such enzymes.

The indirect inhibition by the PK production may be the proper explanation. 12-O-tetradecanoylphorbol-13-acetate (TPA), a known inhibitor of GJIC, works by this mechanism. In addition to the dephosphorylation of the gap junction, phosphatase dephosphorylates the protein kinase C (PKC) which initially causes the inhibition [32].

Studies showed that after communication has been restored, the addition of more TPA does not cause inhibition of GJIC. In this case, the PKC has been completely inactivated and the TPA cannot act again by the indirect mechanism to inhibit GJIC until the PKC has been replenished which takes approximately 24 h [32].

The time of recovery experiments showed that cell-cell communication could be restored following exposure with any of the compounds examined within approximately 2 h. This phenomena is consistent with the theory that inhibition of GJIC by a tumor promoter is a reversible process [12]. In addition, the recovery of cell-cell communication shows indirectly that the chemicals tested were not cytotoxic at the inhibiting doses.

Of the 11 compounds tested, all were noncytotoxic at the concentrations analyzed. This proved that the inhibition caused by certain compounds was not due to cell death. The 24 h cytotoxicity experiment showed that even after prolonged exposure, cell death did not play a role in the inhibition of cell-cell communication.

4.4.2 Study Two

Like study one, study two aimed to determine whether or not some of the byproducts of pyrene ozonation were as or more toxic than the parent compound, pyrene. Study one was limited in number of pure byproducts available for study, although the major byproduct, 4C5P, was evaluated. However, in study two impure fractions were further evaluated for GJIC activity. Although the concentration of each individual compound within the mixture could not be determined, the compounds themselves were characterized using GC/MS.

Preliminary dose response studies of fractions collected from the byproduct mixture generated at low pH showed that fractions 6, 9, and 10 inhibited GJIC completely or nearly completely. Compound 10 was unreacted pyrene which explains the inhibition observed. The complete dose response experiments for fractions 6 and 9 showed that both inhibited GJIC completely, but at a higher concentration than pyrene. However, this observation is deceiving due to the impurity of the compounds. It is possible that these compounds could be more inhibitory than pyrene if it were pure.

Both fractions 6 and 9 contained several compounds in common although at what concentration is unknown. Fractions 6 and 9 both contained compounds C, D, and E (figure 2.1). Pure compound C was studied in detail and was not responsible for the inhibition observed. It is possible that compound E played a role in the activity, although compound E present in other fractions did not cause great inhibition. It is also possible that compound E was present in greater concentration in the inhibiting fractions in contrast to some of the other less active fractions. Compound D was present only in fractions 6 and 9. This compound appears to be a good candidate for the GJIC inhibition observed. This compound contains both a bay region and an aldehydic group.

Fractions 1, 2, and 3 partially inhibited GJIC and contained many common compounds. Again the lack of knowledge regarding concentration of each compound made it difficult to pinpoint the active compound(s). Fractions 2 and 3 displayed the greatest partial inhibition and both contained traces of residual pyrene. Fraction 3 also contained compound B which contained 2 aldehyde groups. Compounds J, K, and L were present in both active and inactive fractions.

The byproduct mixture generated at high pH inhibited GJIC completely, but at an increased concentration compared to the parent compound. The observed inhibition of GJIC was partly due to greater residual pyrene and possibly to other byproducts. The byproducts formed were consistent with compounds formed when ozonated at low pH with the exception of compound S. Yao [30] showed that the ozonation of pyrene first produced 3 ringed compounds and as these compounds are degraded, 2 ringed compounds are formed. The ozonation at high pH was less efficient since only 3 ringed compounds were formed and a greater amount of pyrene remained.

Preliminary dose response experiments showed that fractions 3 and 6 were completely inhibitory to GJIC. Fraction 6 contained only residual pyrene. Fraction 3 appeared to be more inhibitory than fraction 6 or the parent compound, pyrene, and contained several compounds which may have caused the inhibition of cell-cell communication. Fraction 3 contained compounds B, C, D, E, and R. From the results of low pH fractions, it can be concluded that compounds C and E were not the likely cause of the inhibition. Also, compound R was present only in trace amount. Compounds B and D appeared to be the likely inhibitory compounds. Fraction 3 was the only fraction to contain compound D in large quantity which has both a bay region and an aldehydic group. Fraction 5 contained trace amounts of D in addition to its major compound C. As discussed previously, compound C was found to be rather inactive. Of the 6 fractions, fraction 3 was the only fraction which contained compound B. Compound B also contains a bay region and 2 aldehydic groups. Under the circumstances, compound B and D were most likely inhibitors of GJIC.

Fraction 1 showed an increased partial inhibition at a concentration of 100 μ M which was probably due to the residual pyrene present in the fraction. Fractions 2, 4, and 5 did not cause complete inhibition of GJIC even at increased concentrations.

4.5 Conclusions

The goal of this research was to first determine if any of the byproducts of pyrene ozonation were as or more inhibitory to GJIC than the parent compound, pyrene. The second goal was to identify any relationships between the compound structure and the ability to inhibit GJIC. The third goal was to determine whether byproducts generated at high pH were similar to those generated at low pH.

The purified byproducts tested, 4C5P, 4CP, and compound Q, were not more inhibitory than the parent compound. In addition, slight modifications in compound Q's structure rendered it unlike its original byproduct structure. Additional purified compounds must be studied to confirm or negate the first hypothesis.

The investigation of the impure byproduct fractions, generated at both high and low pH, suggested that some of the byproducts may be as or more inhibitory than the parent compound. Compounds B and D were present in fractions which were the most inhibitory to GJIC. Although, the evidence that compounds B and D are responsible for the observed inhibition is strong, the potential for additive effects and synergy must be considered. Until the compounds within the mixtures are studied individually, one cannot be certain which compound(s) are inhibitory to GJIC.

Of the isolated compounds and purchased compounds studied which inhibited GJIC, some similarities in chemical structure are apparent. First, many of the compounds have bay or bay like regions. In addition, inhibition was enhanced by the addition of an aldehydic group to the structure. In most instances the aldehydic group was in close proximity to the bay region, but was not in the case of 4BCH. Although the concentration required for 4BCH to inhibit GJIC was much higher than the other inhibitory compounds studied, the result is still very important. Of the four biphenyl type commercial compounds studied, 4BCH was the only one to inhibit GJIC and the only one to contain an aldehyde group where as the other three contained acid functional groups. 4BCH is much more soluble in aqueous solution than the other inhibitory compounds and it is more likely that a compound like 4BCH be encountered at such a level due to its increased solubility.

The characterization of byproducts at high and low pH showed that the byproducts are similar and sequentially formed. The high pH ozonation was less efficient as evidenced by the increase in residual pyrene and the lack of biphenyl type byproducts formed.

4.6 References

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Chapter 5 - Summary and Conclusions

5.1 Summary and Conclusions

The byproducts of pyrene ozonation were the primary focus of this study. The objective was to isolate and identify the byproducts for toxicological study. The interest in the chemical structure of these byproducts and the associated inhibition of GJIC prompted the investigation of six additional compounds with structures similar to the pyrene ozonation byproducts. The final goal of this study was the examination of byproducts generated under high pH conditions versus those generated under low pH conditions. Several of the byproducts comprised a very small percent of the total mass which made it difficult to examine them individually. During this study minor byproducts which could not be isolated were studied in fractionated mixtures. Although impure, there were fewer compounds in each mixture in comparison to the original ozonated mixture. Because ozonation may be used for treatment of these compounds under high pH conditions as opposed to the ideal low pH, the difference in degradation efficiency and byproduct generation was also analyzed.

The ozonation of pyrene was performed with high concentrations of pyrene in solution. High concentrations of pyrene were used due to the need for large quantities of byproducts and also because of the difficulty in controlling the progress of a reaction using dilute solutions. An ozone dosage large enough to remove the majority of the pyrene, but retain some of the byproducts initially formed was used. Because of the competition between pyrene and the byproducts for ozone, the ozone dosage used was

greater than a ratio of 1. The dosage used ranged from 1.6 to 1.9 mmol of ozone per mmol of pyrene.

An array of analytical techniques were employed during the isolation of the pyrene ozonation byproducts. Preparative TLC, MPLC, and HPLC were the major separation techniques studied. An ethyl acetate extraction was used as a preliminary step in an effort to separated acid containing compounds from the byproduct mixture. The best results were encountered with preparative and semi-preparative reverse phase HPLC without any preliminary separation steps. Preparative HPLC separations were performed using a C₁₈ column and an isocratic solvent system of methanol and water (70:30). Semi-preparative HPLC separations were conducted using a gradient solvent system of acetonitrile and water described in Table 3.2. Methanol was replaced by acetonitrile during later separations due to the methylations observed during preparative HPLC experiments. Compound Q was isolated using preparative HPLC and identified using ¹H and ¹³C NMR. GC/MS was used to confirm the proposed structure. Compound D was isolated in conjunction with compound Q also using preparative HPLC. Again, NMR and GC/MS enabled the identification of this compound. Compound C, 4-carboxy-5-phenanthrene carboxaldehyde was purified using semi-preparative HPLC and identified using NMR and GC/MS.

Although preparative TLC proved unsuccessful, MPLC provided enough selectivity to separate compound G from the other byproducts. The compound was identified using NMR only. MPLC experiments were performed using a C₁₈ column and an isocratic solvent system of methanol and water described in Table 3.1.

The compounds isolated during this study possessed chemical structures which differed sufficiently from other byproducts and enabled their isolation. Many of the remaining impure byproducts were inseparable under the purification conditions employed which made isolation very difficult under the circumstances. In addition, many of the remaining byproducts constituted a small percentage of the total mass.

Previous studies have been conducted on a selection of PAH compounds for their ability to inhibit GJIC, a tumor promoting in vitro assay. These studies showed a correlation between chemical structure and toxicity. The bay region theory which links activity to compounds containing a bay or bay-like region has been supported in several studies. Upham et al. also found a correlation between inhibition of GJIC and compounds with enhanced bay regions.

Compounds C, G, and Q, in addition to pyrene, were investigated for their ability to inhibit GJIC. Six additional compounds with structures similar to the ozonation byproducts were purchased and studied under the same conditions. Of the compounds investigated, 5 of them inhibited GJIC completely. Pyrene inhibited GJIC completely at a lower concentration than the isolated byproducts. The original hypothesis was that one or more of the byproducts of pyrene ozonation were more inhibitory to GJIC than the parent compound. However, it was not confirmed in the initial phase of this project.

OFC and TPC, two commercial compounds, inhibited GJIC completely at approximately 40 μM , the same concentration as pyrene. 4-BCH inhibited GJIC completely at approximately 175 μM , a much higher concentration than the parent compound. All three of these compounds contained a bay region and an aldehydic group.

All 5 compounds that inhibited GJCIC, did so quickly, within 30 min. 4BCH inhibited GJCIC within 1 min of exposure. All 5 inhibitory compounds also recovered partial communication without the removal of the test compound. As discussed in Chapter 4, this may be due to an indirect or direct blockage of cell-cell communication.

In all cases of partial or complete inhibition of GJCIC, cells recovered communication within approximately 2 h following removal of the test compound. The restoration of communication validated the hypothesis that inhibition of GJCIC is a reversible process. In addition, all studies were conducted at noncytotoxic concentrations which confirmed that the observed inhibition of GJCIC was not due to cell death.

The study of byproducts generated at low vs. high pH confirmed that ozonation efficiency is decreased with increased pH. Approximately, 16% of the original pyrene remained when ozonated at high pH in contrast to the 3-4% at low pH. Yao showed that the generation of byproducts is sequential with 3 ringed, phenanthrene type byproducts formed initially followed by 2 ringed, biphenyl type compounds. The ozonation at high pH also proved less efficient as evidenced by the presence of 3 ringed byproducts only. Treatability studies would be required for use of ozonation for degradation of PAHs under varied conditions.

All impure fractions collected were characterized using GC/MS. Of the fractions collected from HPLC that were ozonated at low pH, 3 were completely or nearly completely inhibitory to GJCIC. Fraction 10 was residual pyrene. Fractions 6 and 9 both contained compound D with a bay region and an aldehydic group. Compound E was also present in both fractions, but the presence of compound E in other fractions did not cause inhibition of GJCIC.

Of the fractions collected from HPLC that were ozonated at high pH, fractions 3 and 6 were completely inhibitory to GJIC. Fraction 6 was residual pyrene. Fraction 3 which was more inhibitory than fraction 6, contained two compounds which were similar in nature. Compounds B and D were present in fraction 3 and each have a bay region and at least one aldehydic group. In fact, fraction 3 was the only fraction collected from the byproduct mixture produced at high pH that contained compound B or D as a major product.

The results of the toxicology studies uncovered two consistent trends between chemical structure and GJIC inhibition. Compounds which contained an unobstructed bay region and an aldehydic group tended to be more inhibitory to GJIC than compounds without these features. In addition, 3-ringed byproducts were more inhibitory than 2-ringed byproducts. However, it should be noted that the addition of an aldehydic group to a 2-ringed compound significantly increased inhibition of GJIC in comparison to 2-ringed compounds without an aldehydic group.

The majority of ozonation byproducts had been previously identified. From this research, two additional compounds were identified. Compound R was identified in the byproduct mixture generated from ozonation experiments conducted at both low and high pH. Compound S was identified in the mixture generated at high pH.

5.2 Recommendations

One of the primary goals of this study was the isolation of the byproducts of pyrene ozonation. Although some of the major byproducts were isolated, there may be other alternatives for the isolation of additional compounds. One possibility is the

purchase of a very specific column made for the isolation of these compounds. The column primarily used in this study was a polymer coated, silica based, C₁₈, semi-preparative column. The column had excellent selectivity for polar compounds and nonpolar PAHs have been consistently separated on C₁₈ columns. However, this column was not suitable for some of the very closely related byproducts of this study. There may be a column available that has the proper selectivity to overcome this problem. However, the cost involved may be extreme due to the specificity and size required for a column of these qualifications.

Synthesis of the byproducts is another possibility. Dr. Maleczka, of the Chemistry Department, Michigan State University, expressed an interest in producing some of these compounds. Compound I and N of Figure 2.2 have been synthesized previously, but are unavailable commercially. Dr. Maleczka feels the synthesis of these compounds and many others in Figure 2.2 are possible. The cost to synthesize these compounds is unknown and must be investigated further.

Mechanistic studies on compounds which inhibited GJIC may also be of interest. The manner by which GJIC is inhibited is uncertain although various possibilities were discussed in Chapter 4. The indirect mechanism in which a chemical activates a protein kinase to phosphorylate the gap junction protein is one possibility. As discussed previously, 12-O-tetradecanoylphorbol-13-acetate (TPA) works by this mechanism. Previous studies showed that TPA inhibited GJIC in this indirect manner, but also caused the reopening of the gap junction after a period of time due to the dephosphorylation of both the gap junction protein and the protein kinase C. Because the PKC had been inactivated, additional TPA added to culture did not cause the gap junctions to be again

closed. Only when the PKC had been replenished could the addition of more TPA again cause inhibition of GJIC. It may be possible to determine whether the inhibitory compounds of this study work by the same mechanism.

Many other mechanisms are possible and should be investigated. The recovery of communication between cells without removal of the target compound has several implications. The duration of inhibition must be considered. Is the lag time between inhibition and recovery long enough to cause tumor promotion? In this study, only partial recovery without compound removal was observed. It may be of interest to determine whether GJIC could be completely recovered over an extended period of time.

From an engineering perspective, it is clear that ozonation is a viable option for degradation of pyrene and any harmful byproducts. This remediation technique is advantageous because it destroys the harmful contaminants and generates no additional wastes, unlike more frequently used options such as activated carbon. The cost of in-situ remediation of PAH in soils is not unreasonable when compared to other technologies. A study of PAH contaminated soils at Wurtsmith Air Force Base showed the energy cost associated with the degradation of PAHs was \$3.63 per ton of soil. Assuming a 200% increase in ozone demand to account for any toxic byproducts, a conservative energy cost of \$10.89 per ton contaminated soil would be incurred. When capital and operation and management costs are considered in addition to energy costs, in-situ remediation using ozone is an inexpensive and effective clean up alternative.

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