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THE EFFECT OF OZONATED CHRYSENE BYPRODUCTS ON GAP JUNCTIONAL INTERCELLULAR COMMUNICATION IN RAT LIVER EPITHELIAL CELLS

presented by

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has been accepted towards fulfillment of the requirements for

M.S. degree in Chemical Engineering

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Major professor

Date 8/9/2000

DATE DUE	DATE DUE	DATE DUE

THE EFFECT OF OZONATED CHRYSENE BYPRODUCTS ON GAP JUNCTIONAL INTERCELLULAR COMMUNICATION IN RAT LIVER EPITHELIAL CELLS

By

Stephanie LaVerne Luster-Teasley

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Chemical Engineering

2000

ABSTRACT

THE EFFECT OF OZONATED CHRYSENE BYPRODUCTS ON GAP JUNCTIONAL INTERCELLULAR COMMUNICATION IN RAT LIVER EPITHELIAL CELLS

By

Stephanie LaVerne Luster-Teasley

The use of ozone to remediate polycyclic aromatic hydrocarbon (PAHs) has proven effective, however, a number of unknown byproducts are produced from the oxidation of the original PAH contaminant. This study evaluated the effect of chrysene and the ozonated byproducts on *in vitro* cell communication using the scrape loading/ dye transfer (SL/DT) technique. A 1 mM solution of chrysene was ozonated to produce byproduct mixtures at 1.75, 3, 4.25, and 5 mol O_3/ mol as Chrysene (Chr). The toxicity of these byproducts was evaluated using SL/DT to measure gap junctional intercellular communication (GJIC) in rat liver epithelial cells. The studies included dose response, time response, time recovery, and cytotoxicity for chrysene and the mixtures. An increase in cellular communication blockage is seen at 1.75 mol O_3/mol as Chr and results indicate irreversible damage can be done to GJIC. At higher ozone doses, blockage of cellular communication decreased with increasing ozone concentration. The 3 mol O_3/mol as Chr byproducts inhibit communication at the same level as chrysene. The 4.25 mol O_3/mol as Chr initially inhibits communication, however, the cells are able to return to normal communication levels. The 5 mol O_3/mol as Chr sample showed little to no inhibition of communication at levels less than 100 μM. Concentrations ranging between 150 –210 μM were required to see inhibition for this sample.

ACKNOWLEDGEMENTS

I would like to thank my advisors, Dr. Susan Masten and Dr. Bruce Dale, and committee members, Dr. James Trosko and Dr. Dennis Miller, for their patience and guidance during this process. I would also like to thank my husband and son, Edward I and Edward II, for their faith and support.

Thank you to the following sources of funding: the NIEH - Superfund Grant and the Graduate Assistantship in Areas of National Need (GAANN).

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KEY TO ABBREVIATIONS

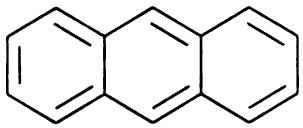
Ca^{2+}/Mg^{2+}	-	Calcium/Magnesium
Chr	-	Chrysene
EPA	-	Environmental Protection Agency
f	-	Fraction of Control
FBS	-	Fetal Bovine Serum
GJIC	-	Gap Junctional Intercellular Communication
HPLC	-	High Pressure Liquid Chromatography
KI	-	Potassium Iodide
M	-	Molarity
mol	-	mole
$Na_2S_2O_3$	-	Sodium Thiosulfate
PAHs	-	Polycyclic Aromatic Hydrocarbons
PBS	-	Phosphate Buffer Solution
SL/DT	-	Scrape Load/Dye Transfer

CHAPTER 1

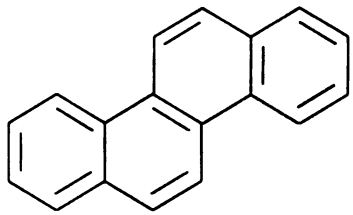
INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are compounds produced during the burning of coal, oil, gasoline, and garbage. In the environment, PAH exposure may occur by air, such as from vehicle exhaust or smoke, or through contact with soil or water contaminated with PAH hazardous waste. Major producers of PAH compounds include the petroleum industry, coking plants, and wood/paper processing facilities. Unfortunately, due to years of improper disposal of waste at these sites, PAH contamination has become a major environmental concern. The Environmental Protection Agency (EPA) lists 16 PAHs as priority pollutants and eight as carcinogens or potential carcinogens causing skin, liver, and/or lung cancer in humans. Figure 1 shows the structure of some representative PAH compounds. These compounds present a remediation challenge because they are highly recalcitrant, insoluble in water, and tend to accumulate on solid surfaces (Sontag, 1981). Many PAH contaminated sites are now slated for clean up by the EPA and are included in the list of Superfund Sites. It is imperative that environmental engineering develop technically feasible processes that will remediate contaminated sites and reduce the risk of human exposure to carcinogenic PAH compounds.

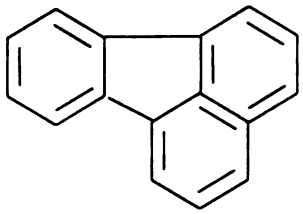
Chemical, biological, and mechanical means have all been employed to remediate sites contaminated with PAHs. Each process varies with respect to complexity of the system, cost, process efficiency, and waste generation (Herner, 1999). Ozone has proven to be a viable method in the reduction of PAH compounds in wastewater. Ozonation is



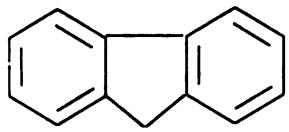
Anthracene



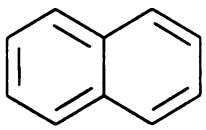
Chrysene



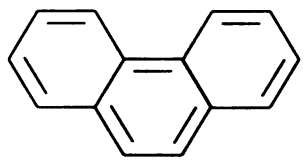
Fluoranthene



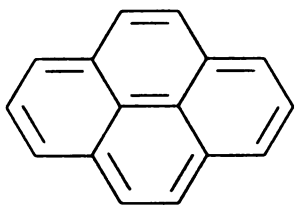
Fluorene



Naphthalene



Phenanthrene

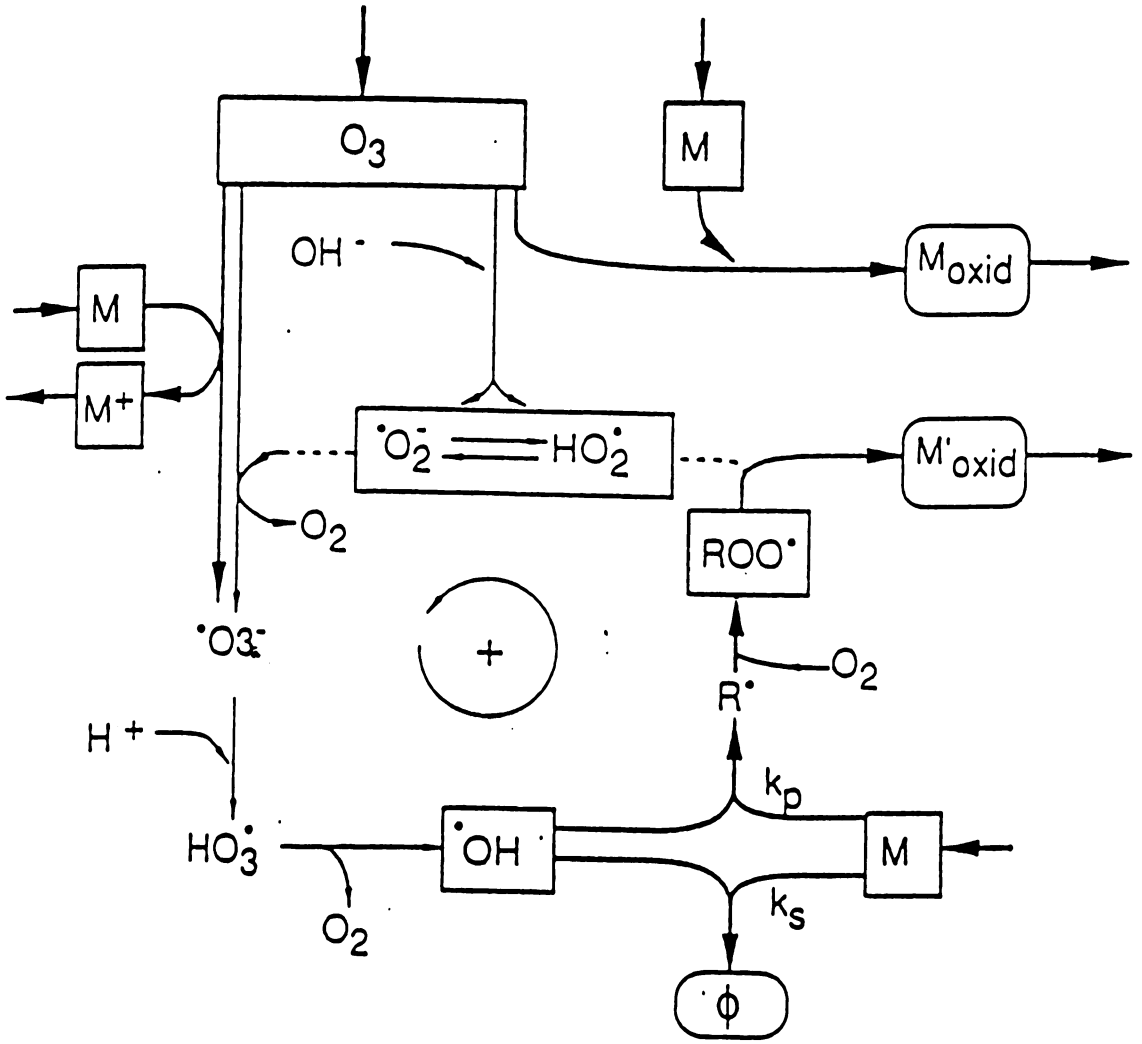


Pyrene

Figure 1. Representative PAH compounds

believed to promote a series of oxidation steps, which reduce the number of fused rings in PAHs. As the ozone dosage increases, destruction of the stable rings occurs at the site of lowest bond energy or the atom of lowest atom localization energy.

Initially, only the disappearance of the parent PAH was monitored in ozonation processes. Further experiments presented findings indicating that byproducts such as aldehydes, organic acids, diones, quinones, and lactones were produced during PAH reactions with ozone (Meineke and Klamber, 1978; Neff, 1979; Kuo and Barnes, 1985; Rodd, 1985; Legube et. al., 1986; Marley et. al, 1987; Dreher and Klamberg, 1988). Staehelin and Hoigné (1985) proposed the mechanism for ozone reaction in aqueous systems with a target compound (Solute M). In solution, the oxidizing species present in the solution are ozone (O_3), hydroxyl radicals ($^{\bullet}OH$) and superoxides ($^{\bullet}O_2^-$). Both $^{\bullet}O_2^-$ and $^{\bullet}OH$ radicals react to enhance the decomposition of ozone. Figure 2 is a depiction of this reaction. Ozone (O_3) reacts four ways in aqueous systems. Ozone can react with hydroxyl ions (OH^-) to form one superoxide anion ($^{\bullet}O_2^-$) and one hydroperoxyl radical ($HO_2^{\bullet}$). Ozone and $^{\bullet}O_2^-$ can react resulting in the formation of O_2 and an ozonide ion radical ($^{\bullet}O_3^-$). Solute M present in the system may directly react with ozone to form a new compound (M_{oxid}) or ozone can react with solute M to produce an $^{\bullet}O_3^-$ ion radical by electron transfer. Upon protonation, $^{\bullet}O_3^-$ in the system decomposes into $^{\bullet}OH$ radicals. The $^{\bullet}OH$ radicals and O_2 present in the system can then react with solute M to form other M'_{oxid} compound (Staehelin and Hoigné, 1985). The production of byproducts could continue to present an environmental concern if the toxicity of the new compounds is not less than the original PAH. The objective of this thesis was to evaluate the toxicity of the byproducts produced during the ozonation of the target compound Chrysene.



(adapted from Staehelin and Hoigné, 1985)

Figure 2. Mechanism of Aqueous Ozonolysis Reaction with a target solute

Understanding the Toxicity of PAH parents and Byproducts

Environmental carcinogens are always a major concern in remediation efforts. In the past, most chemicals were evaluated for their potential to cause cancer using genotoxic bioassays (Herner, 1999). While many chemicals may not be the principal triggers for causing cancer development, they may help contribute to carcinogenesis by affecting DNA, RNA, or other cellular mechanisms. PAH compounds are classified as epigenetic toxicants, or compounds able to damage cell mechanisms (Jerina, 1987). Epigenetic toxicants have been implicated in tumor promotion during carcinogenesis, teratogenesis, and in reproductive dysfunction (Yamasaki, 1990; Trosko, 1990, 1993; Gilula, 1976; Larsen, 1986; Ye, 1990).

One method of determining if a compound can damage cell mechanisms is to evaluate the effect the compound has on gap junctional cellular communication (GJIC). Trosko et. al. (1993) noted that chronic exposures to epigenetic toxicants implicated in tumor promotion during carcinogenesis are known to effect gap junctional intercellular communication. To maintain homeostasis, cells transfer information to each other through channels in the cell membrane. These channels, or gap junctions are composed of six hexameric subunits called connexins (Figure 3). The joined connexins form a channel called a connexon. A connexon traverses the plasma membrane of a cell and when the connexons of two opposing cells join, a continuous channel is formed between the cells. This channel allows for ions, low molecular weight molecules, and small regulatory and macromolecular substances to pass through the cytoplasm of one cell to the next cell (Trosko, 1993). Most cancer cells have dysfunctional gap junctional intercellular communication (Trosko, 1990). Disruption of gap junctional communication will inhibit

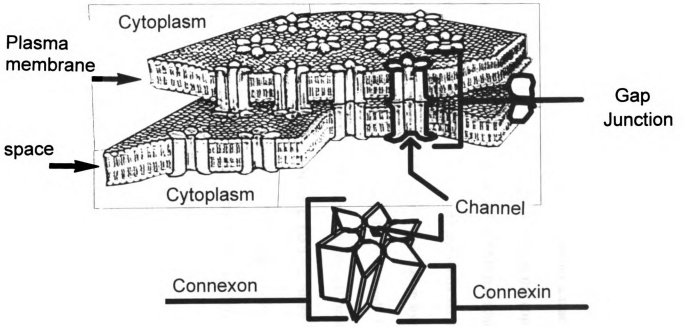


Figure 3. Schematic of Gap Junction

the regulatory roles within cells such as growth control, developmental and differentiation processes, synchronization, and metabolic regulation (Trosko et. al., 1990). Blockage of these processes could lead to uncontrolled growth, tumor formation, and possibly lead to the development of cancer.

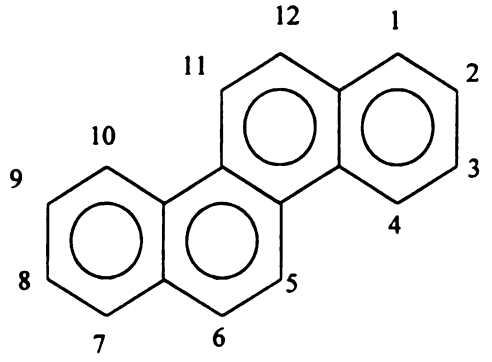
For ozonation to be a viable remediation technique, ozonation byproducts must be less toxic than the original parent compound, cannot cause an increased risk to the environment, and overall must reduce the potential of the contaminated matrix to cause cancer. Engineering application for ozone use in PAH remediation could lead to a viable means of contaminant removal, however, considerations for the fate of the byproducts produced must be investigated. Identification of ozonation byproducts for PAHs such as anthracene, pyrene, and chrysene are available (Copeland, 1961; Rodd, 1979; Bailey, 1982). Only a few studies have focused on investigating if ozonation actually reduces the carcinogenic potential of treated solutions containing these PAHs (Yoshikawa, 1985; Upham, 1994; Herner, 1999). Herner (1999) suggests that while ozonation may be effective in eliminating a PAH compound, such as pyrene, its removal does not necessarily result in the elimination of toxicity.

Objective and Scope

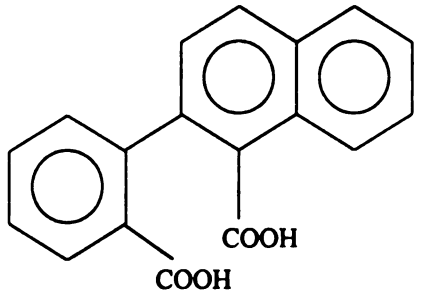
The mixture of byproducts produced during ozonation could increase overall toxicity, ultimately, resulting in the opposite desired effect for remediation efforts. Therefore, evaluating the toxicity of byproduct mixtures is extremely important. The objective of this study was to perform aqueous chrysene ozonation and test the mixture of byproducts produced for increases or decreases in toxicity. Byproduct toxicity was

evaluated based upon the ability of the mixture to block gap junctional intercellular communication. Detection of GJIC inhibition served as an indicator that a mixture demonstrates the potential for damage to normal cellular function.

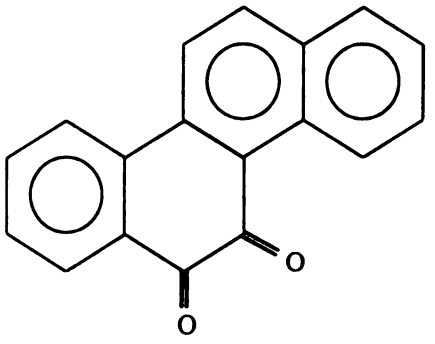
Chrysene is identified as a weak carcinogen that promotes primarily skin, liver, and lung carcinomas. This compound was selected for the study because literature searches revealed little to no information of whole or individual byproduct toxicity. Chrysene is composed of 4 rings and reacts with ozone at the bond with the lowest localization energy, the 5,6 bond. Several investigators have identified chrysene and ozone reaction byproducts (Copeland, 1961; Rodd, 1979; Bailey, 1982; Yao, 1999). Figure 4 presents the byproducts identified by Copeland (1961) and Rodd (1979). Copeland (1961) proposes chrysene reacts with ozone at either the 5,6 or 11,12 bond yielding a 48% formation of 2-2-carboxyphenyl-1-naphthoic acid. The 2-2-carboxyphenyl-1-naphthoic acid, together with a compound thought to be the lactone, are produced when chrysene is ozonated and treated with hydrogen peroxide in acetic acid. Rodd (1979) presents findings where oxidation of chrysene with sodium dichromate in acetic acid gives chrysene-5,6-quinone. Work done by Yao et. al. (1999) identified the same chrysene byproducts and two additional compounds during ozonation in acetonitrile (Figure 5). Now, knowing the compounds present following ozonation, the question remains as to whether the toxicity of the byproducts is less than that of the original parents. For successful engineering application, the synergistic effects of the potential byproducts produced in the field are more important than individual toxicity of a specific compound found within the matrix.



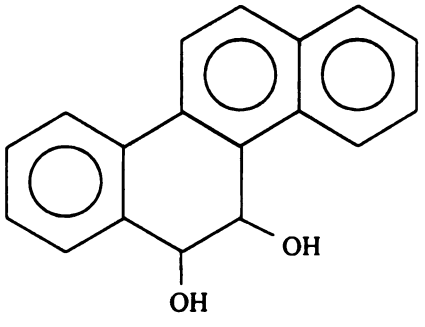
Chrysene



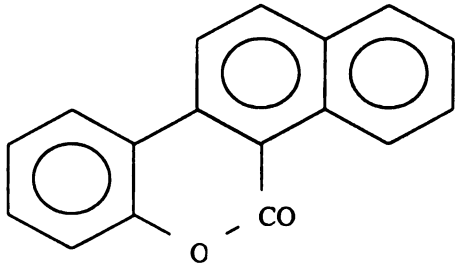
2-2-Carboxyphenyl-1-naphthoic acid



Chrysene-5,6-quinone

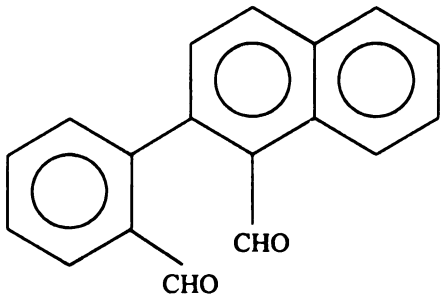


5,6-dihydro-5,6-dihydroxide chrysene

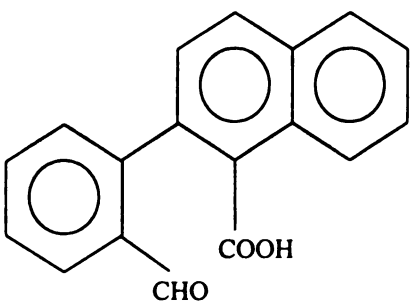


Chrysene lactone

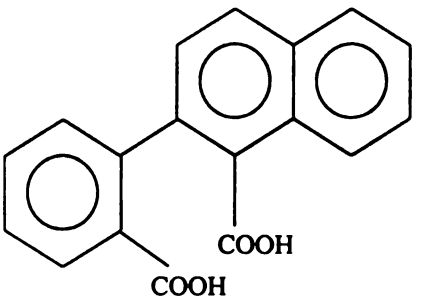
Figure 4. Chrysene Byproducts from Ozonation
(Copeland, 1961 and Rodd, 1979)



COMPOUND I:
2-(2'-formyl) phenyl-1-naphthaldehyde



COMPOUND II:
2-(2'-formyl)phenyl-1-naphthoic acid



COMPOUND III:
2-2-Carboxyphenyl-1-naphthoic acid

Figure 5. Chrysene byproducts from ozonation in acetonitrile (Yao et. al., 1999)

CHAPTER 2

EXPERIMENTAL METHODS

Materials

Chrysene (98% purity, Aldrich Chemicals) was dissolved in acetonitrile (99.8% purity) and adjusted to a pH 3 – 4 using acidified deionized water to make 1 mM solution. The low solubitiy of chrysene in pure water (0.006 mg/L) required using an acetonitrile/watermixture. Acetonitrile (99.8% purity, EM Science, Gibbstown, NJ) was selected as the solvent because it has low reactivity with ozone ($t_{1/2} \geq 18$ years at pH 7 and $[O_3] = 20.8$ mM). A 10% water concentration is sufficient to act as a participating solvent in ozonolysis (Yao and Haag, 1991). However, the acetonitrile/water ratio could not exceed 90%/10% to prevent chrysene from settling out of solution. The byproduct mixtures used in the toxicology study ranged from 1.75 – 5 mol O_3/mol as Chrysene.

Ozonation Experiments

A semi-batch system was used for ozonation (Yao et. al., 1999). Figure 6 shows a schematic of the experimental set-up. Ozone generated in dried oxygen electric discharge using a Polymetrics Model T-408 ozone generator (San Jose, CA). The flow of ozone into the reactor was regulated at 200 ml/min using a Sidetrack flow controller (Sierra Instruments Inc., Monterey, CA). The tubing (1/8" i.d.), connectors and valves were constructed of Teflon® or stainless steel. The concentration of ozone in the influent and effluent gas streams was measured spectrophotometrically at 258 nm using an UV-Visible light spectrophotometer (Model 1201, Shimadzu Scientific Instruments, Japan).

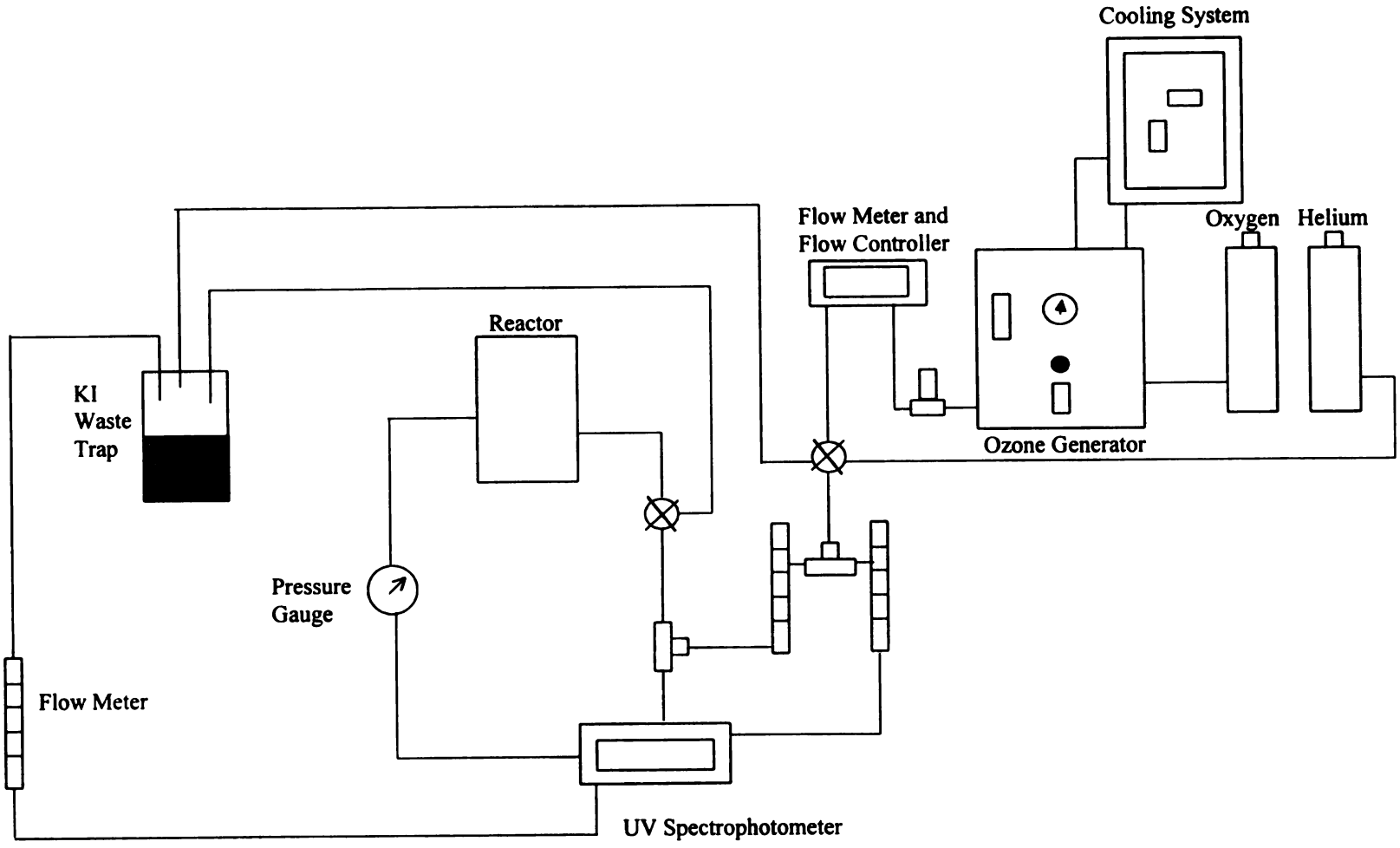


Figure 6. Schematic of Aqueous system Ozonation experimental set-up

The absorbance values for ozone were converted to the concentration using a molar absorptivity coefficient for ozone of 3000 M^{-1} cm^{-1} (Bader and Hoigné, 1982). Quartz flow cells with a path length of 0.2 cm were used. The effluent was discharged into 2% potassium iodide (KI) solution. Flushing the solution with helium removed detectable ozone and terminated all reactions. After the desired ozonation time, the sample was removed and 0.1 mg $Na_2S_2O_3$ was added as a free radical quenching agent. The ozonated samples were then kept in the dark and shaken at 250 rpm overnight in 250 ml glass bottles. The solution was then rotary evaporated to recover the solid byproduct.

HPLC Analysis

A Gilson HPLC unit and an Alltima C18 5 micron column with dimension 250 mm x 4.6 mm were used for HPLC Analysis. The effluent was monitored at two wavelengths (225 nm and 260 nm). The linear gradient for the separation of the ozonated samples consisted of 25%/75% acetonitrile/water at the time of injection and increased to 90%/10% acetonitrile/water over 15 minutes. The mobile phase was then held at 90%/10% acetonitrile/water for 3 minutes and then linearly decreased to 25%/75% acetonitrile/water over 2 minutes and held for an additional 5 minutes at this concentration. The total run time was 25 minutes.

Toxicology Studies

Sample Preparation

The dried ozonated product was dissolved in acetonitrile (99.8% purity, EM Science, Gibbstown, NJ). Acetonitrile was selected as the solvent because it has little

effect on the GJIC assay. The parent compound and the mixtures of ozonation byproducts treated at stoichiometric ratios of 1.75, 3, 4.25 and 5 mol ozone/mol as chrysene (mol O_3/mol as Chr) were analyzed for toxicity. The molecular weight of chrysene was used to calculate the stoichiometric rations because the true molecular weight of the mixture byproducts is unknown. Therefor all rations are reported as mol O_3/mol as Chr.

Cell Culture

WB-F344 rat epithelial cell lines were obtained from Dr. J.W. Grisham and M.S. Tsao of the University of North Carolina (Chapel Hill, NC). Cells were cultured in 25 ml of D medium (Formula No. 78-5470EG, GIBCO Laboratories, Grand Island, NY) containing 5% fetal bovine serum (FBS) (GIBCO Laboratories, Grand Island, NY) and 1 ml gentamicin. The cells were incubated at 37° C in a humidified atmosphere containing 5% CO_2 and 95% air. The cells were grown in 150 mm plastic flasks, and the culture was split and new medium was added every other day.

Bioassay for GJIC

Bioassays were conducted in 35 mm^2 Petri dishes with confluent cultures grown for 2 days in 2 ml of D medium supplemented with 5% fetal bovine serum. The procedure for the scrape loading/dye transfer (SL/DT) technique was adapted from the method used by El-Fouly et. al. All tests were run in triplicate and at noncytotoxic levels determined by the neutral red uptake assay kit (Sigma Chemical Co., St. Louis, MO).

After dosing the plates with the target compound for the desired time, the cells were washed with Ca^{2+}/Mg^{2+} phosphate buffered saline (Ca^{2+}/Mg^{2+} PBS). Lucifer yellow

was added to the plate and the cells were scraped using a surgical steel-blade. The cells were incubated for 3 minutes, washed using the Ca^{2+}/Mg^{2+} PBS to remove excess dye, and the dye fixed using 7 drops of 5% formalin solution.

In the GJIC assay, at the site of the scrape, the cells will absorb some of the Lucifer yellow dye and transfer dye to neighboring cells if communication is not inhibited. The distance the dye travels from the scrape is an indication of the intercellular communication. The cells were then photographed at 200-x magnification using a Nikon Diaphot-TMD epiflourescencse phase-contrast microscope (Nikon, Japan). Under fluorescent light, the Lucifer yellow dye will fluoresce to indicate the distance the dye travels from the scrape. This distance was measured and compared to a control group of cells exposed only to acetonitrile (vehicle controls), but assayed using the identical SL/DT method. Three photographs were taken for each concentration tested and the distance the dye traveled perpendicular to the scrape was measured. For each picture measurements were taken every 1 cm for a total of 10 cm. A total of 30 measurements (10 from each photo) were averaged together to obtain a representative fraction of control (*f*). All photographs were taken within 1 hour of experiment completion and developed by PhotoMart of Lansing, MI.

Bioassay for Cytotoxicity

Cytotoxicity was tested using the neutral red uptake assay according to the method of Borenfreund and Puerner (1985). WB-F344 cells were grown using the same method as the cells used for the GJIC assay. The neutral red dye was incubated in D-media with 5% FBS for 2 hours at 37°C and then centrifuged at 1200 rpm for 5 minutes

to remove any solid dye residue. The effluent was then filtered using a 0.22-μm Millipore syringe filter (Millipore Corp., New Bedford, MA) into D-media with 5% FBS to a concentration of 0.033%. Following treatment of the cells for the desired dosage and treatment times, the cells were washed three times with PBS and 2 ml of the dye solution added. After 1 hour of incubation with the dye at 37°C the cells were washed three times with PBS. The neutral red dye absorbed by the cells was lysed using 1 ml of neutral red solubilizer containing 1% acetic acid and 50% ethanol. After 15 minutes, the neutral red released by the cells was measured spectrophotometrically (Beckman Spectrophotometer) at a wavelength of 540 nm and a background absorbance measured at 630 nm. The cytotoxicity was evaluated based on a fraction of control exposed to only acetonitrile. A fraction of control value greater than 0.8 is considered non-cytotoxic.

CHAPTER 3

RESULTS AND DISCUSSION

RESULTS

HPLC. Figure 7 presents the HPLC chromatographs for chrysene, 0.41, 1.75, 3, and 5 mol O_3/mol chrysene. Pure chrysene has a retention time of 19 - 20 minutes. As the ozone concentration increases, chrysene quickly degrades and the byproducts formed increase in polarity. The HPLC method separated the more polar compounds first by starting at 25%/75% acetonitrile/water mobile phase at the time of injection. The production of polar compounds is evident with the detection of compounds with retention times of 2 - 3 minutes. As the mobile phase was linearly increased to 90%/10% acetonitrile/water, the less polar, more hydrophobic organic compounds could be separated and eluted from the column. From 3 – 16 minutes, the compounds detected increase with increasing ozone concentration. The peaks that elute after18.6 minutes decrease with increasing ozonation.

Toxicology

Interpretation of GJIC values. The following studies include dose response, time response, and time recovery, and cytotoxicity. For discussion, tables are presented for the observed data with standard deviation. Values presented without standard deviation were read from the plots of the data provided in the Appendix A. To determine the ozone concentration, mol O_3/mol as Chrysene is used as a standardization value because the true molecular weight of the mixture is unknown. GJIC fraction of Control value (f) is assessed by the decrease in communication of the cells exposed to the toxicant compared

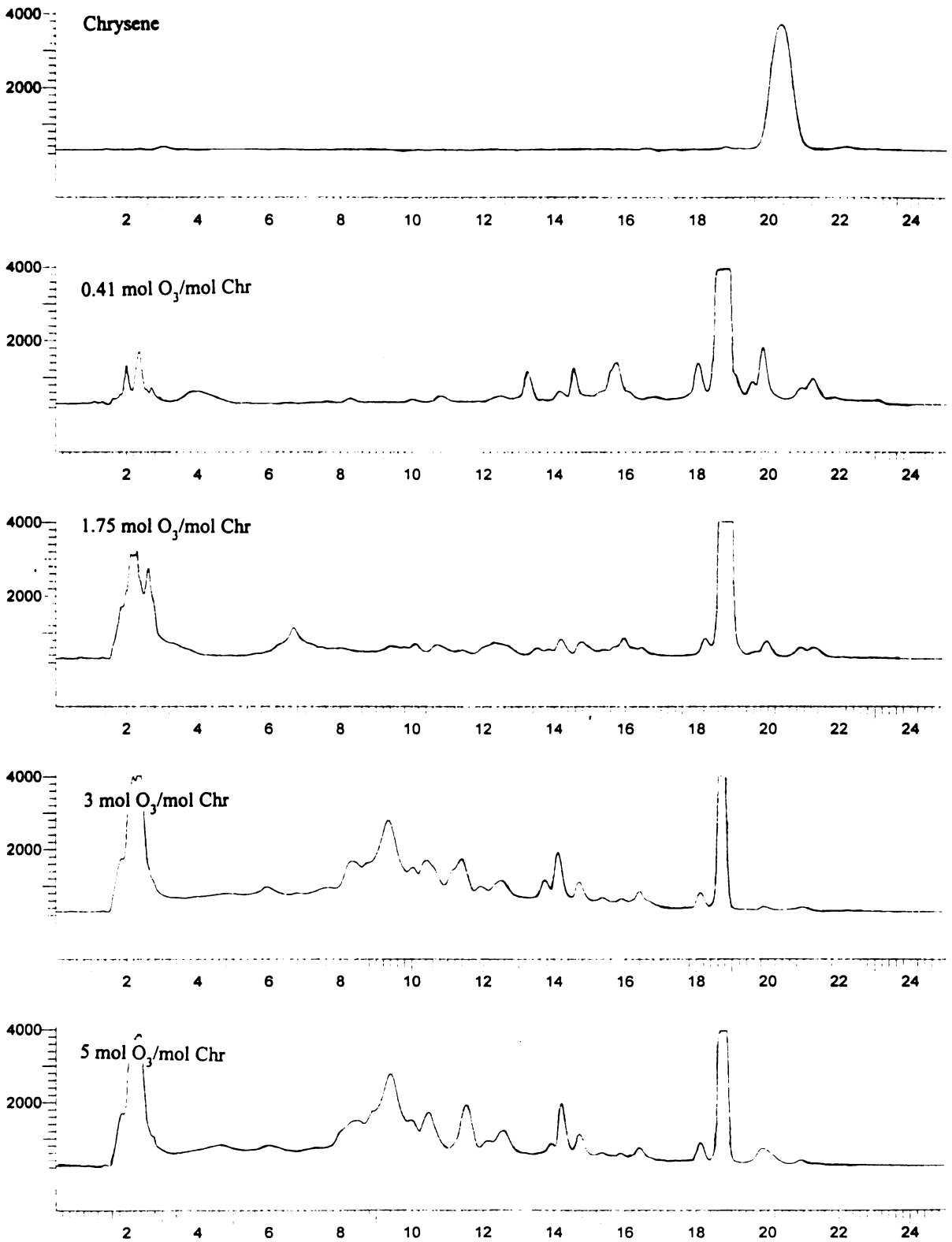


Figure 7. HPLC Chromatographs

to the control group (only acetonitrile). Normal Communication (i.e. no blockage of GJIC) is identified as 1.0 or 100% fractional value (denoted as $f = 1.0$). GJIC values less than $f = 0.5$ or 50% fractional value indicate a significant decrease in cellular communication. GJIC values between 0.0-0.2 are considered to have no cellular communication.

Dose Response. Dose response experiments evaluate the effect of different doses of the target compound on GJIC. Cells were exposed to the compound for 30 minutes. Table 1 is a comparison of the dose response curves for the mixtures tested.

Table 1. Representative *f* values for dose response experiments. Chrysene, 1.75, 3, 4.25 and 5 mol O_3/mol as Chr at 50 μM concentration.

	FOC (*f*)				
	10 μM	30 μM	50 μM	70 μm	90 μM
Compound					
Chrysene	0.93 ± 0.02	0.73 ± 0.1	0.62 ± 0.09	-	-
1.75	0.77 ± 0.08	0.68 ± 0.07	0.35 ± 0.02	0.15	-
3	0.92 ± 0.03	0.83 ± 0.03	0.67 ± 0.1	0.38 ± 0.1	0.24 ± 0.1
4.25	0.95 ± 0.04	0.99 ± 0.004	0.97 ± 0.06	0.77 ± 0.05	0.57 ± 0.04
5	1 ± 0.05	1 ± 0.03	1 ± 0.1	1	1

The results of the dose experiments show that chrysene moderately inhibits gap junctional communication. Due to the low solubility of chrysene, concentrations higher than 50 μM could not be tested. Inhibition of GJIC is highest for the 1.75 mol O_3/mol as Chr sample. The *f* values for the 3 mol O_3/mol as Chr were similar to chrysene. At this ozone concentration, according to the HPLC profile, the chrysene peak has completely disappeared and only byproduct peaks remain. The mixtures generated using higher concentrations of 4.25 and 5 mol O_3/mol as Chr have $f > 0.9$ at concentrations less than

50 μM indicating minimal or no inhibition of cellular communication. Inhibition for the 5 mol O_3/mol as Chr was not seen until concentrations of 150 - 240 μM were reached (Figures A-1 and A-2).

Time Response. Time response experiments were used to evaluate the cellular response to various periods of exposure to mixtures of ozonation byproducts. This experiment is used to help determine if the cells are capable maintaining cellular function over a longer exposure time. Table 2 summarizes the *f* values for Chrysene, 1.75, 3, 4.25 mol O_3/mol as Chr 50 μM concentration. The *f* value for 5 mol O_3/mol as Chr at 210 μM is reported below because no inhibition (*f* = 1) was seen for 5 mol O3/mol as Chr at 50 μM.

Table 2. Representative *f* values for Time Response experiments. Chrysene, 1.75, 3, 4.25 mol O_3/mol as Chr at 50 μM concentration and 5 mol O_3/mol as Chr at 210 μM.

	FOC (*f*)			
	15 min	30 min	1 hour	2 hours
Compound				
Chrysene	0.81 ± 0.03	0.67 ± 0.07	0.68 ± 0.03	0.64 ± 0.03
1.75	0.36 ± 0.07	0.094 ± 0.07	Cell death	Cell death
3	0.79 ± 0.05	0.73 ± 0.04	0.34 ± 0.04	0.18 ± 0.02
4.25	1 ± 0.04	0.67 ± 0.1	0.88 ± 0.02	0.82 ± 0.05
5 (210 μM)	0.73 ± 0.07	0.57 ± 0.08	0.55 ± 0.02	0.47 ± 0.07

The 1.75 mol O_3/mol as Chr mixture rapidly blocked communication. Total inhibition *f* = 0.1 for this compound occurs within 30 minutes of exposure. The 3 mol O_3/mol as Chr had a level of inhibition (*f* = 0.67) as chrysene after incubation for 30 minutes. Chrysene, however, maintains an average *f* = 0.66, while the 3 mol O_3/mol as Chr reaches *f* = 0.18 within the two hours. This result may indicate that the 3 mol O3/mol

as Chr sample may contain a toxicant that slowly is metabolized by the cells into a carcinogenic or mutagenic compound, while pure chrysene metabolism may lead to a less toxic metabolite. The 4.25 mol O_3/mol as Chr mixture has a drop in communication during the first 30 minutes, but the cells appear to be capable of recovering to f = 0.8-0.9 within 1 hour. The time response curve for 5 mol O_3/mol as Chr was evaluated at 210 μM. Inhibition at this concentration drops to f = 0.47 after 2 hours of exposure (Figures A-3 – A-7).

Time Recovery. In the time recovery experiments, the cells are exposed to the toxicant for 30 minutes and then rinsed to remove the contaminant. Fresh media is added to the plates and the cells are incubated at 37°C. At various times, the plates were removed from the incubator and assayed to determine the level of communication recovery. Table 3 summarizes the time recovery for chrysene, 1.75, 3, and 4.25 mol O_3/mol as Chr at a concentration of 50 μM concentration. The f value for 5 mol O_3/mol as Chr at 210 μM is reported below because no inhibition (f = 1) was seen for 5 mol O_3/mol as Chr at 50 μM.

Table 3. Representative f values for time recovery experiments. Chrysene, 1.75, 3, 4.25 mol O_3/mol as Chr at 50 μM concentration.

	FOC (*f*)					
	0	30 min	1 hour	2 hours	4 hours	5.5 hours
Compound						
Chrysene	0.56 ± 0.01	0.47	0.6	0.83 ± 0.04	0.88 ± 0.03	0.88 ± 0.03
1.75	0.24 ± 0.02	0.17 ± 0.04	0.13 ± 0.01	0.22 ± 0.01	0.39 ± 0.03	0.52 ± 0.08
3	0.55 ± 0.01	0.28 ± 0.06	0.35 ± 0.01	0.46 ± 0.03	0.6 ± 0.08	0.68 ± 0.05
4.25	1 ± 0.02	0.53 ± 0.05	0.65 ± 0.03	0.92 ± 0.07	0.99 ± 0.06	0.96 ± 0.04
5 (210 μM)	0.39 ± 0.03	0.52 ± 0.01	0.7 ± 0.05	0.8 ± 0.02	0.8 ± 0.1	0.9 ± 0.1

For chrysene, f = 0.56 at time zero, communication decreased slightly to f = 0.47,

and then increases to f = 0.88 after 330 minutes (5.5 hours). The 1.75 mol O_3/mol as Chr starts at f = 0.24, decreases to f = 0.17, and recovers to f = 0.52 after 5.5 hours. The 3 mol O_3/mol as Chr starts at f = 0.55, drops to f = 0.28, then increases to f = 0.68. The 4.25 mol O_3/mol as Chr compound initially dropped to f = 0.53 μM before recovering to f = 0.96. This is similar to the results seen in the time response data for the same concentration where the communication initially drops and later recovers. For 5 mol O_3/mol as Chr at 210 μM, communication recovers to f = 0.9 in 330 minutes. Figures A-8 and A-9 in the Appendix are the plots for time recovery experiments.

24 hour Time Response and Time Recovery. For these experiments, three different exposure times were tested. The first experiment evaluated cells exposed to the compound for 30 minutes. The second experiment evaluated cells exposed to the compounds for 24 hours uninterrupted. The third experiment evaluated cells exposed for 30 minutes, rinsed and new media added, and then incubated for 24 hours. Table 4 presents the 24 hour exposure and time recovery for chrysene, 1.75, 3, and 4.25 mixtures at 50 μM concentration. The f value for 5 mol O_3/mol as Chr at 150 μM is reported below because no inhibition (f=1) was seen for 5 mol O_3/mol as Chr at 50 μM.

Table 4. f values for 24 hour exposure and time recovery experiments. Chrysene, 1.75, 3, 4.25, and 5 mol O_3/mol as Chr at 50 μM concentration.

	FOC (f)		
	30 min exposure	24 hour exposure	24 hour recovery
Compound			
Chrysene	0.73 ± 0.07	0.68 ± 0.02	0.68 ± 0.06
1.75	0.16 ± 0.01	cell death	0.74 ± 0.03
3	0.54 ± 0.04	cell death	0.8 ± 0.02
4.25	0.86 ± 0.04	0.95 ± 0.1	0.95 ± 0.04
5 (150 μM)	1 ± 0.01	0.95 ± 0.05	0.93 ± 0.01

For chrysene, 30 minutes of exposure yields an f = 0.73 and after 24 hours of exposure f = 0.68. The replacement of new media for 24 hours had little effect on improving the communication and f = 0.68. For the 1.75 mol O_3/mol as Chr mixture, the 30 minute exposure resulted in f = 0.16 and 24 hour exposure resulted in cell death. The recovery experiment however demonstrates that after the 30 minute exposure and media replacement, the cells can recover to f = 0.74 within 24 hours. The 3 mol O_3/mol as Chr mixture after 30 minutes exposure had an f = 0.54, but 24 hour exposure killed the cells. The 24 hour recovery resulted in f = 0.8. Both 4.25 and 5 mol O_3/mol as Chr mixtures were not significantly inhibited and f ranged between 0.86 – 1.0 (Figure A-10).

Cytotoxicity. All doses tested at non-cytotoxic levels (Figures A-11 – A-15). Cytotoxicity experiments mimic dose response experiments for the doses tested and the exposure time. A cytotoxicity experiment was also performed to determine the time length for the 1.75 mol O_3/mol as Chr and 3 mol O_3/mol as Chr compounds to become cytotoxic. Figures A-16 and A-17 respectively are the results of these assays.

DISCUSSION

For low ozone concentrations like 1.75 mol O_3/mol as chrysene, the ozonated byproducts inhibit cellular communication more than the parent compound chrysene. This initial increase in toxicity at low ozonation dosages is consistent with the results observed by Upham et. al. (1994) for ozonated pyrene byproducts at low ozone doses. At higher ozone concentrations and longer ozonation times, the GJIC value returned to 0.5 – 1.0 FOC. GJIC values for the 3 mol O_3/mol as Chr were similar to the parent compound.

At this ozone concentration, according to HPLC analysis, chrysene has completely reacted with ozone leaving only the byproducts.

In Yao et. al. (1999), a 1.4 mol O_3/mol as chrysene solution was fractionated in an attempt to separate byproducts. Three of the byproducts identified in the fractions were 2-(2'-formyl) phenyl-1-naphthaldehyde, 2-(2'-formyl) phenyl-1-naphthoic acid, and 2-2 carboxyphenyl-1-naptholic acid. The GJIC assay indicated that 2-(2'-formyl) phenyl-1-naphthaldehyde is inhibitory. This compound may be an early precursor with its highest concentrations occurring during early ozonation (less than 2 mol O_3/mol as Chrysene). This could support the increase in GJIC inhibition seen in the 1.75 mol O_3/mol as Chr samples. Yao et. al. (1999) also presents results indicating 2-2 carboxyphenyl-1-naptholic acid appears not to inhibit GJIC. This compound may be more prevalent at ozone concentrations greater than 3 mol O_3/mol as Chr, thus returning FOC values to non-inhibitory levels. More HPLC ad GC/MS analyses, however, is required to verify these hypotheses.

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

Ozonation appears to be effective in aqueous systems when ozone is supplied at a sufficiently high dosage to degrade the parent compound and the early toxic precursors. To summarize the results from this study:

- An increase in cellular communication blockage is seen at 1.75 mol O_3/mol as Chr and results indicate irreversible damage can be done to GJIC.
- At higher ozone doses, blockage of cellular communication decreased with increasing ozone concentration.
- The 3 mol O_3/mol as Chr byproducts inhibit communication at the same level as chrysene.
- The 4.25 mol O_3/mol as Chr initially inhibits communication; however, the cells are able to return to normal communication levels.
- The 5 mol O_3/mol as Chr sample showed little to no inhibition of communication at levels less than 100 μM. Concentrations ranging between 150 –210 μM were required to see inhibition for this sample.

In the environment, PAH compounds naturally occur as unknown mixtures that vary in composition and concentration. For successful engineering application, the interactive effect of the potential byproducts produced in the field is more important than individual toxicity of a specific compound found within the matrix. However because this

is a new area of investigation and all PAH waste mixtures are site specific, a good start is investigating individual compounds. Studying individual compounds leads to understanding PAH ozonation and byproduct toxicity. Aqueous ozonation appears to be effective when ozone is supplied at a sufficiently high dosage to degrade the parent compound and the early toxic precursors. One major concern with PAH remediation is that a mixture of PAHs will exist in real engineering applications. This thesis investigated only one PAH for ozonation, but in actuality the mixture of PAHs and other substances present in contaminated wastewater will compete for ozone. Therefore, longer ozonation times and higher ozone dosages will be required to reduce the carcinogenic potential of PAH mixtures formed. Important questions such as the interactive toxicity of the byproduct mixtures following ozonation and identification of the byproducts produced must be addressed before implementation.

Ozone has proven to be beneficial in remediating PAHs in both wastewater and soil systems. The production of byproducts in soil could present new challenges for PAH remediation such as byproduct sorption onto soil or the potential for mobility of the byproducts due to their increased solubility into water. For future studies, a comparison of aqueous and soil ozonation would be interesting. These experiments are recommended to determine if similar byproducts or toxicology results are seen in both aqueous ozonation and soil ozonation. Engineering application for ozone use in aqueous and soil PAH remediation could lead to a viable means of contaminant removal; however, more evaluation of the process is necessary.

APPENDIX

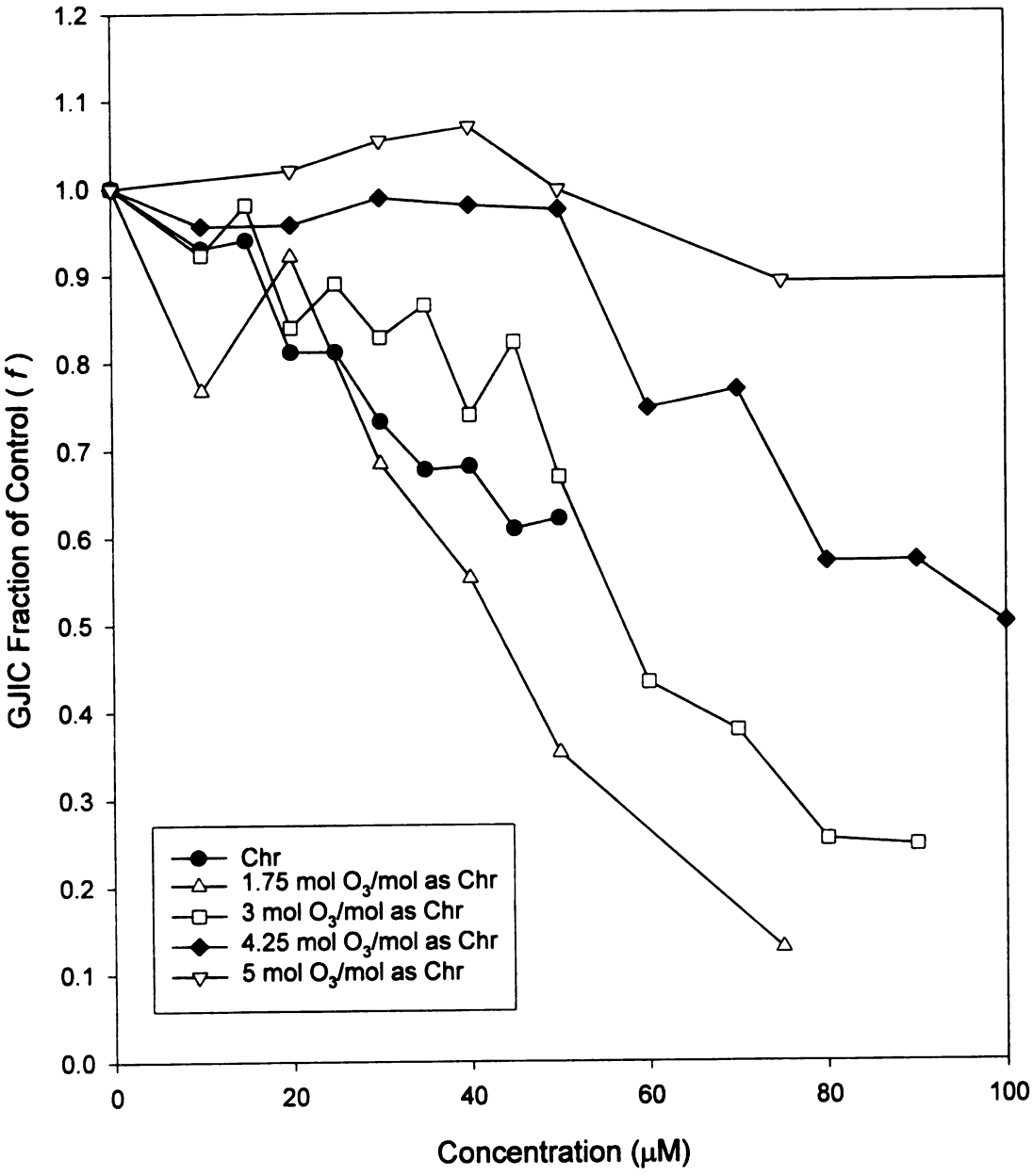
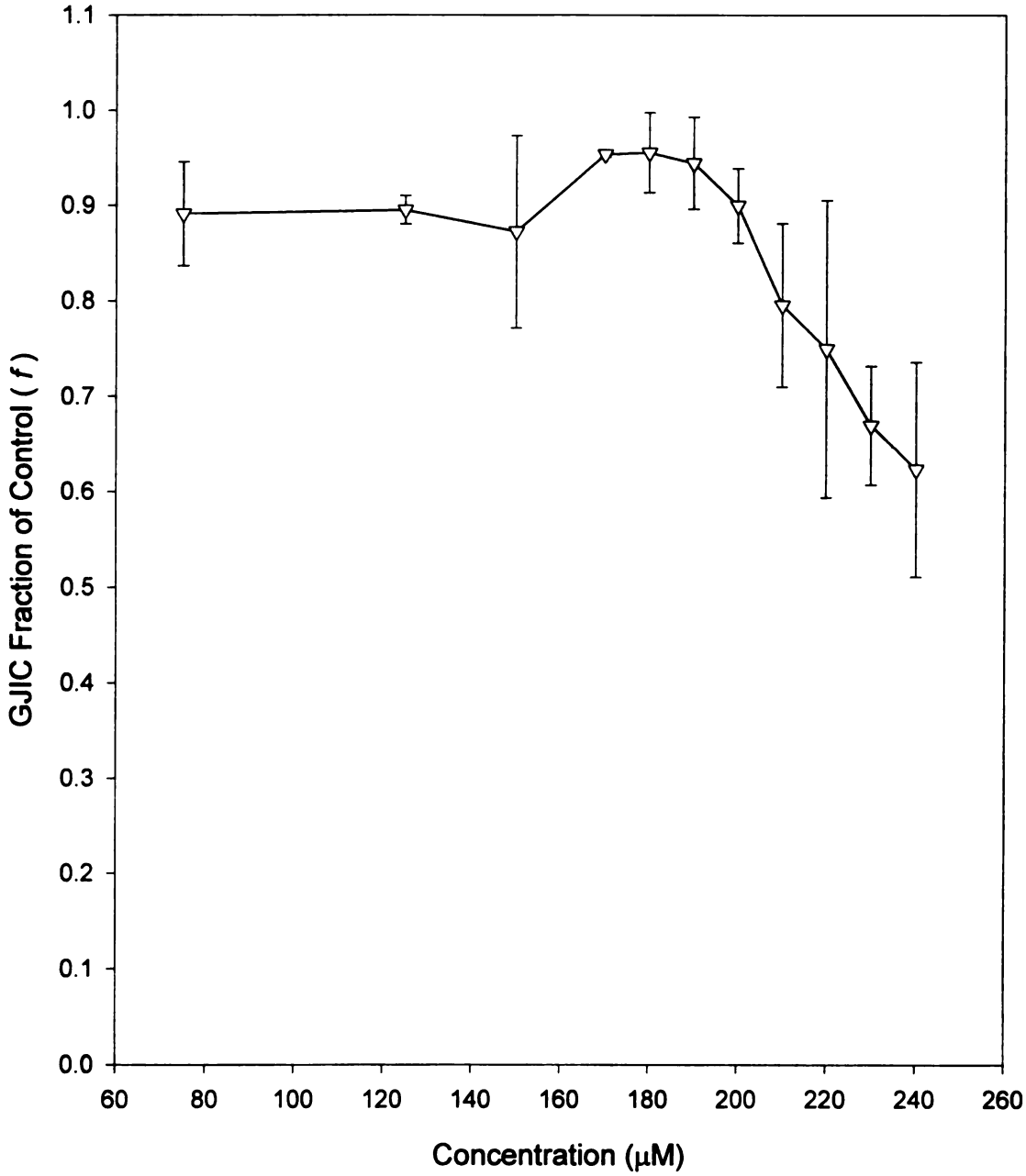


Figure A-1. Comparison of Dose Response Curves: Chrysene, 1.75, 3, 4.25, and 5 mol O_3/mol as Chrysene



FigureA-2. Extended Dose Response Curve: 5 mol O_3/mol as Chrysene

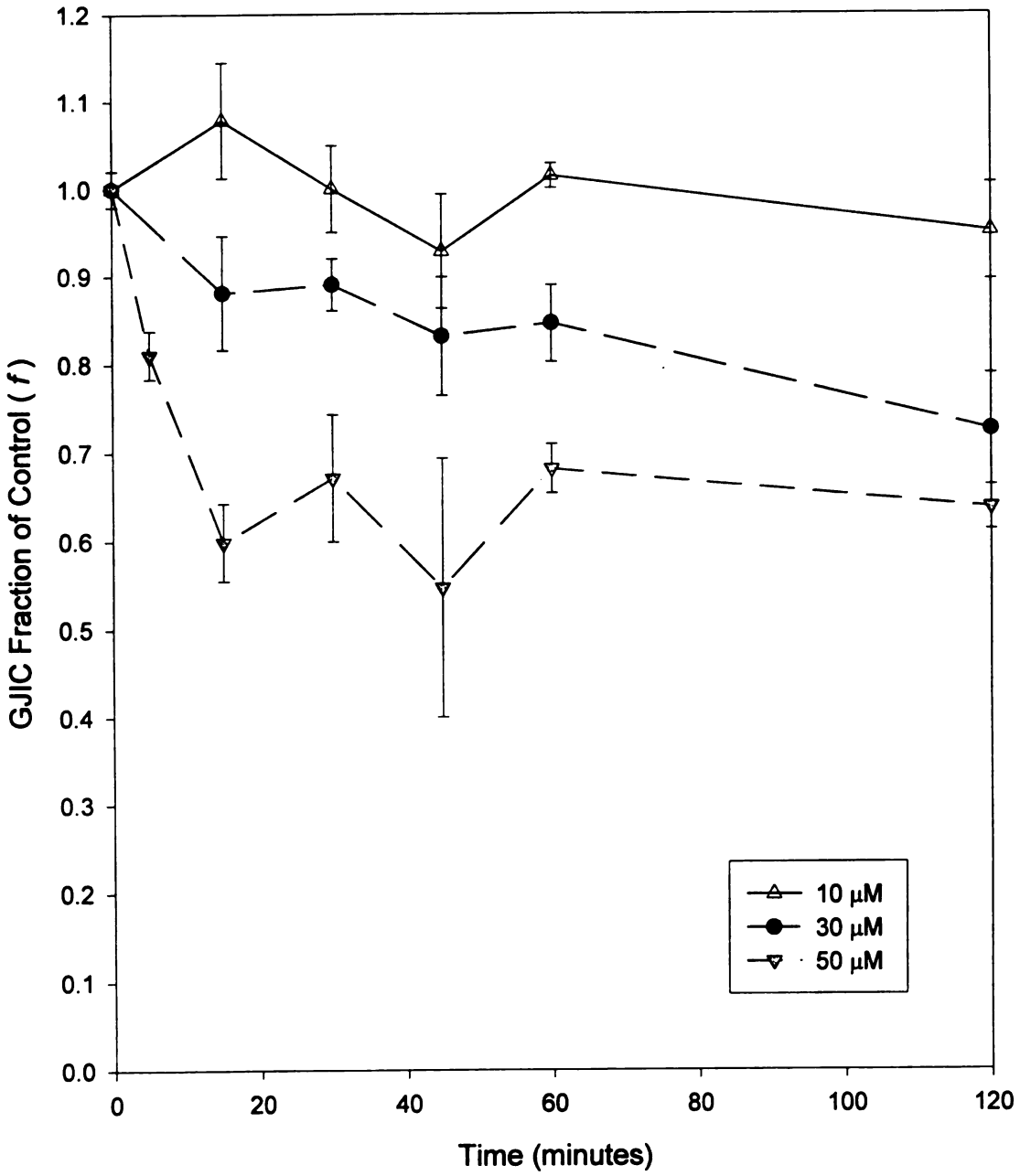


Figure A-3. Time Response Curve: Chrysene

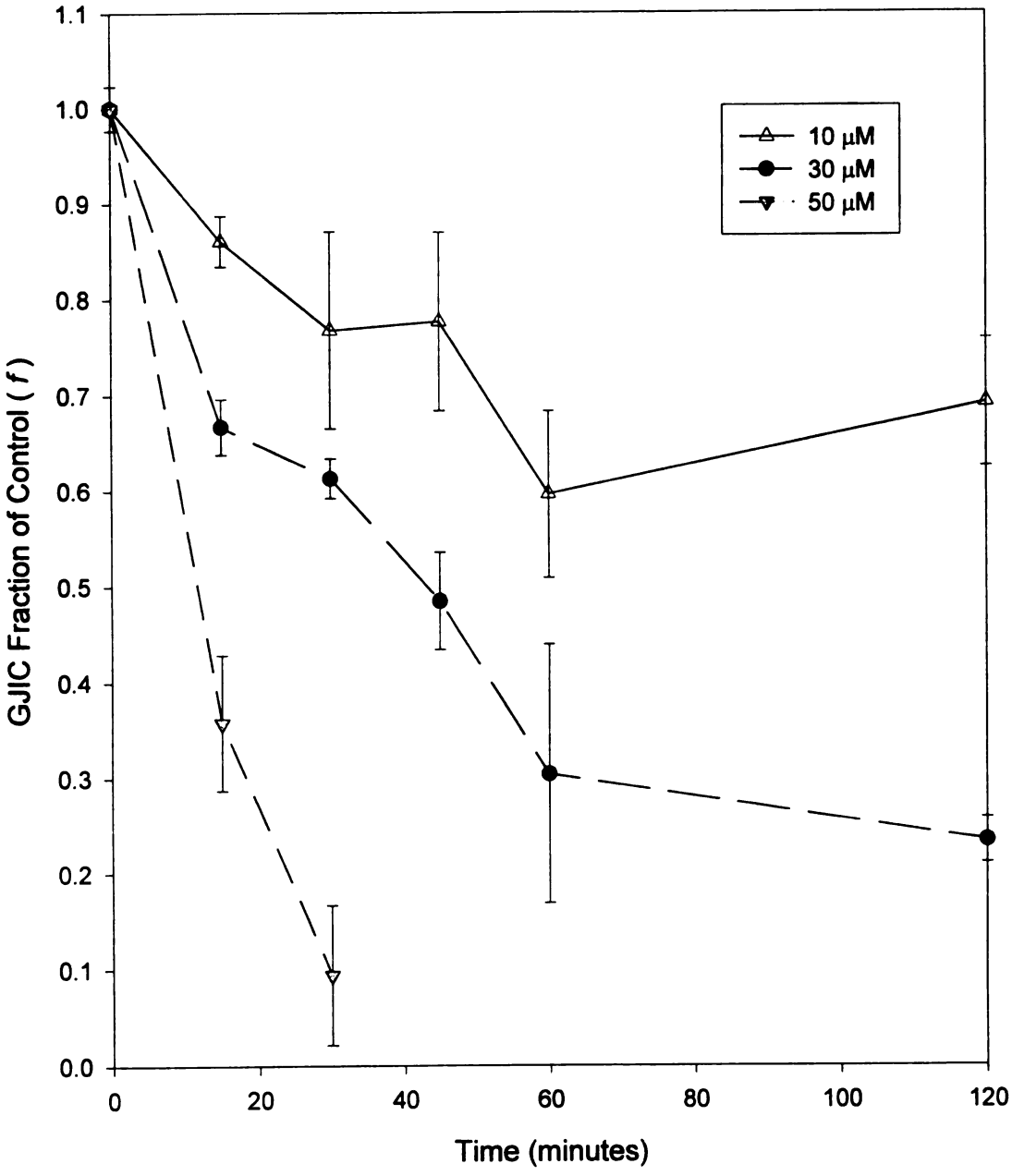


Figure A-4. Time Response Curve: 1.75 mol O_3/mol as Chrysene

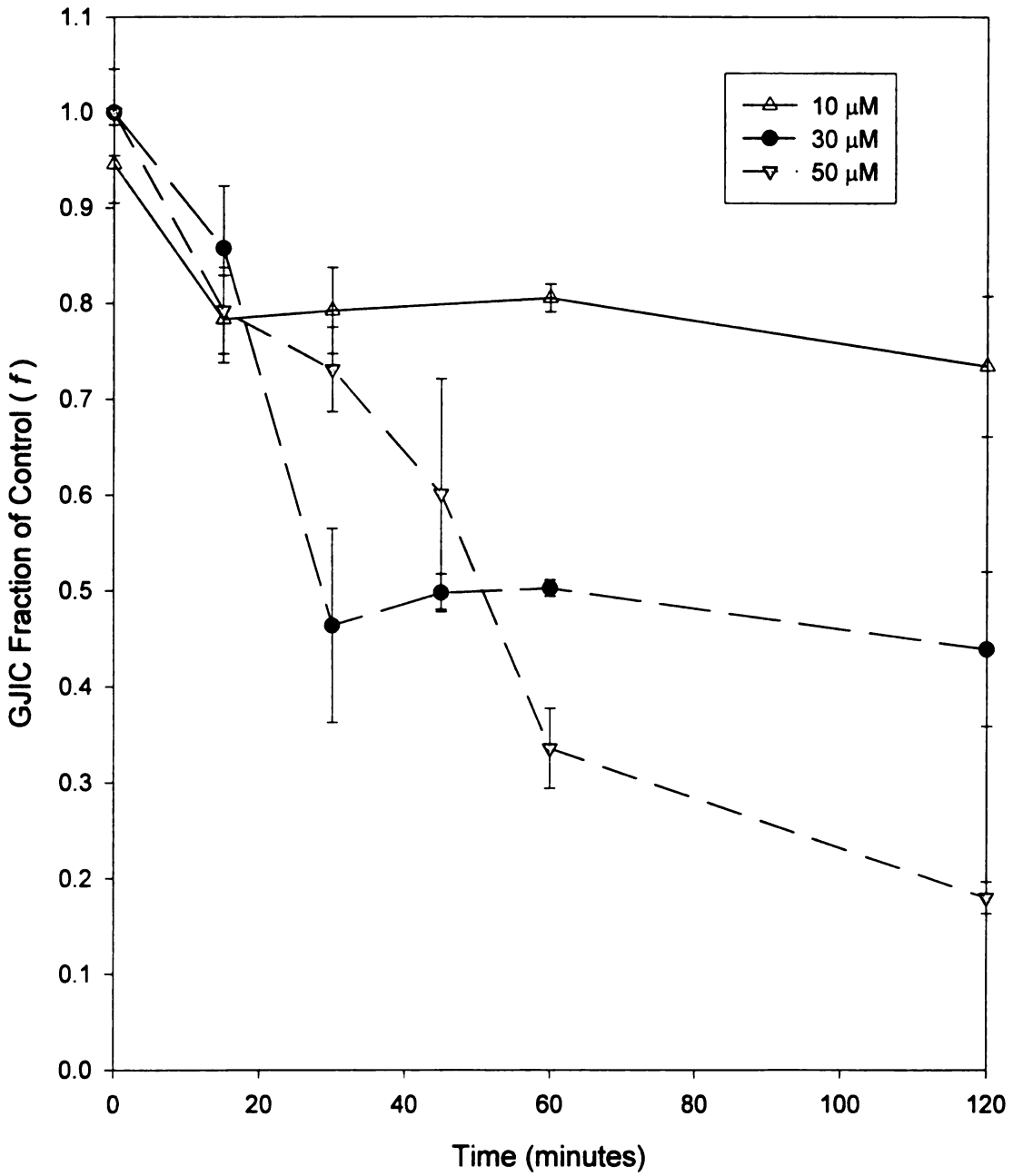


Figure A-5. Time Response Curve: 3 mol O_3/mol as Chrysene

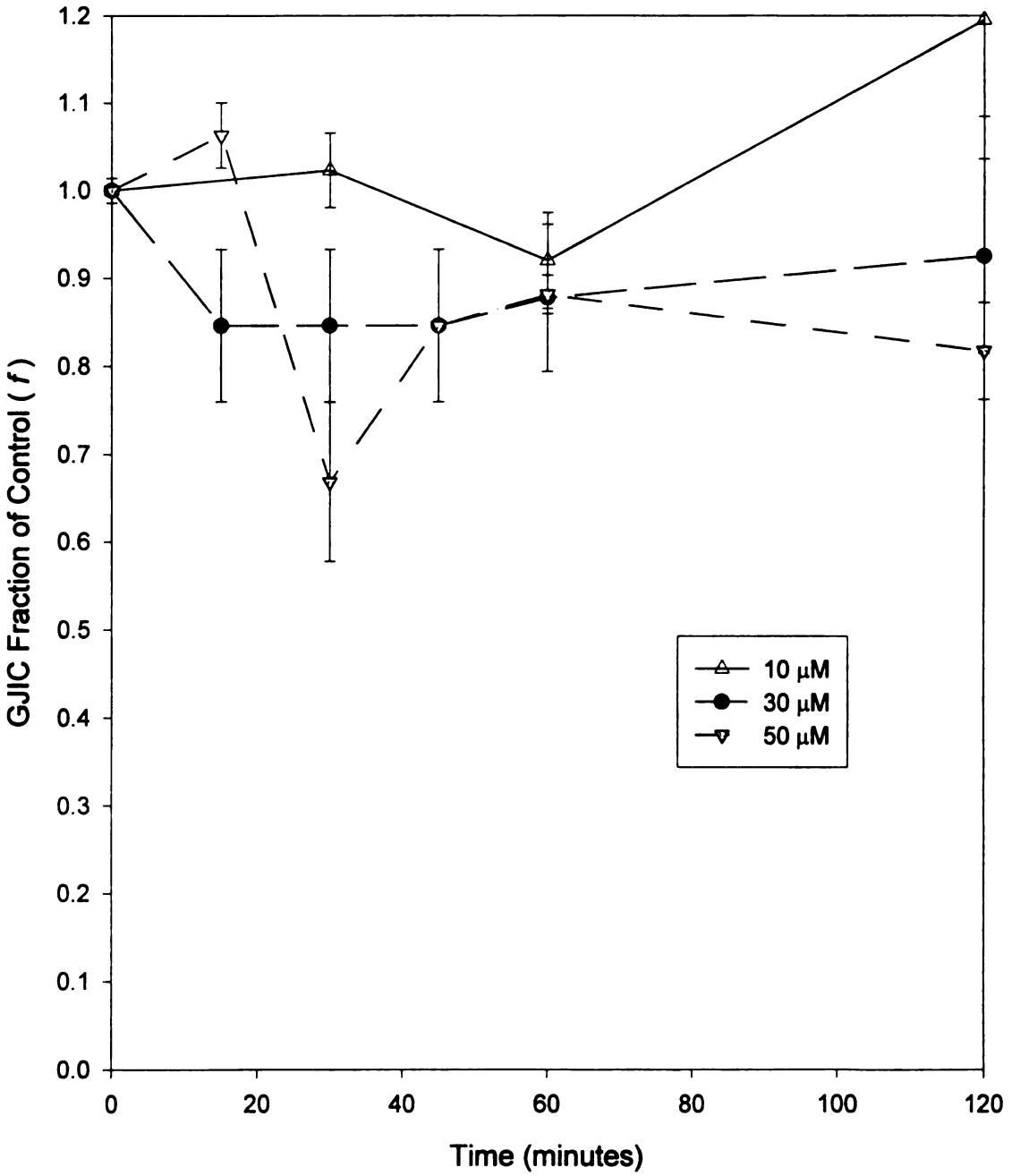


Figure A-6. Time Response Curve: 4.25 mol O_3/mol as Chrysene

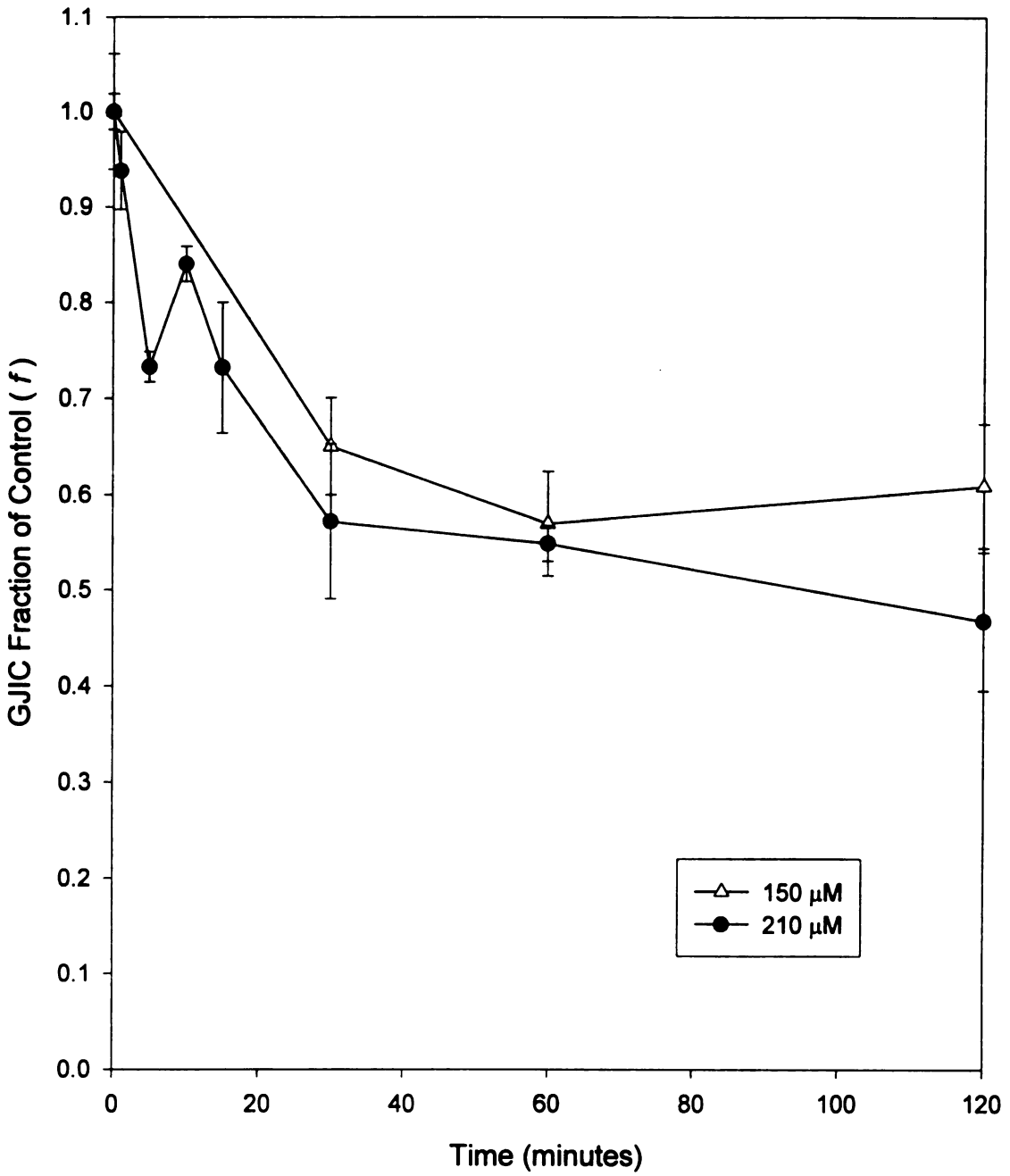


Figure A-7. Time Response Curve: 5 mol O_3/mol as Chrysene

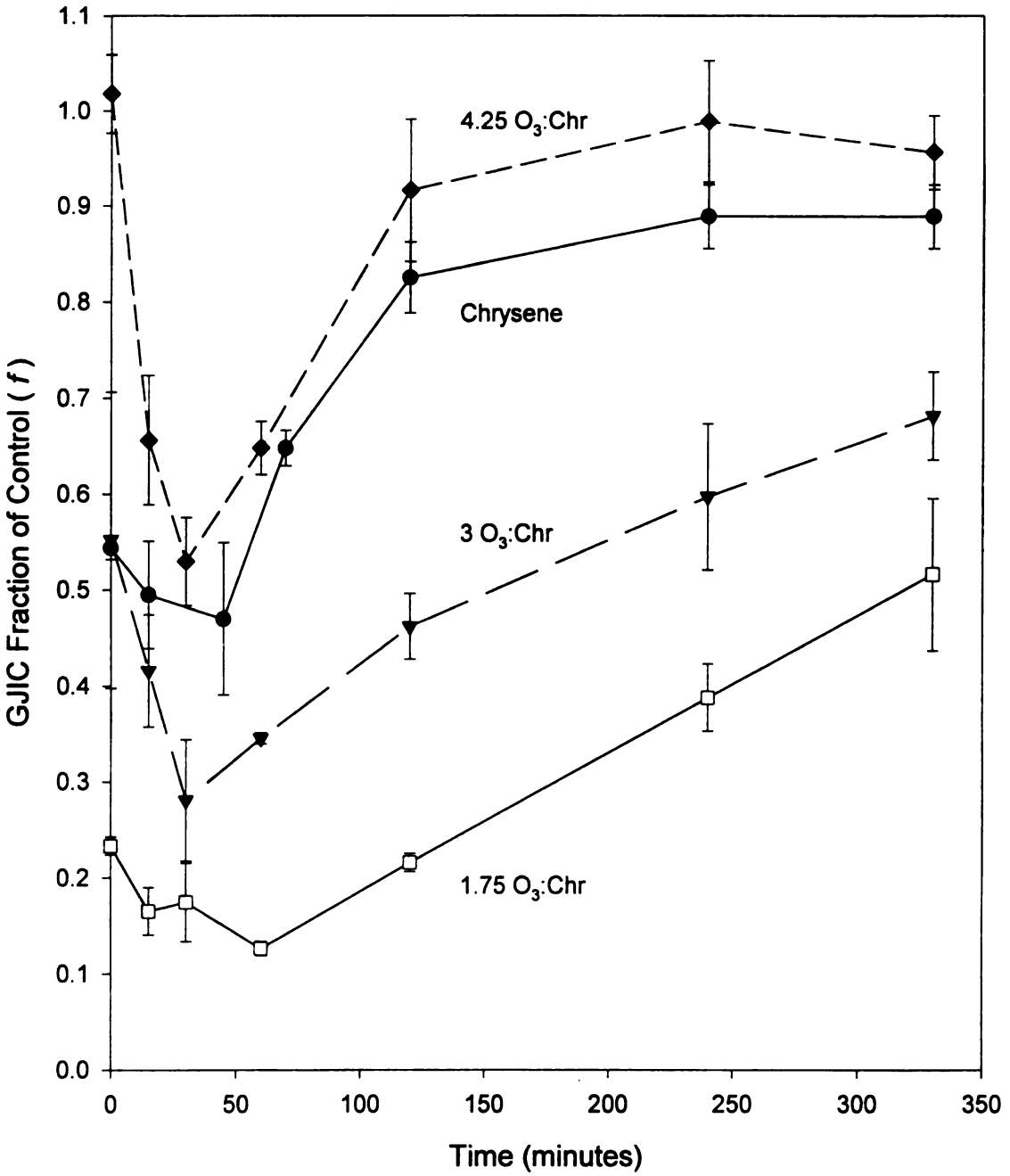


Figure A-8. Comparison of Time Recovery Curves: Chrysene, 1.75, 3, and 4.25 mol O_3/mol as Chrysene at 50 μM concentration

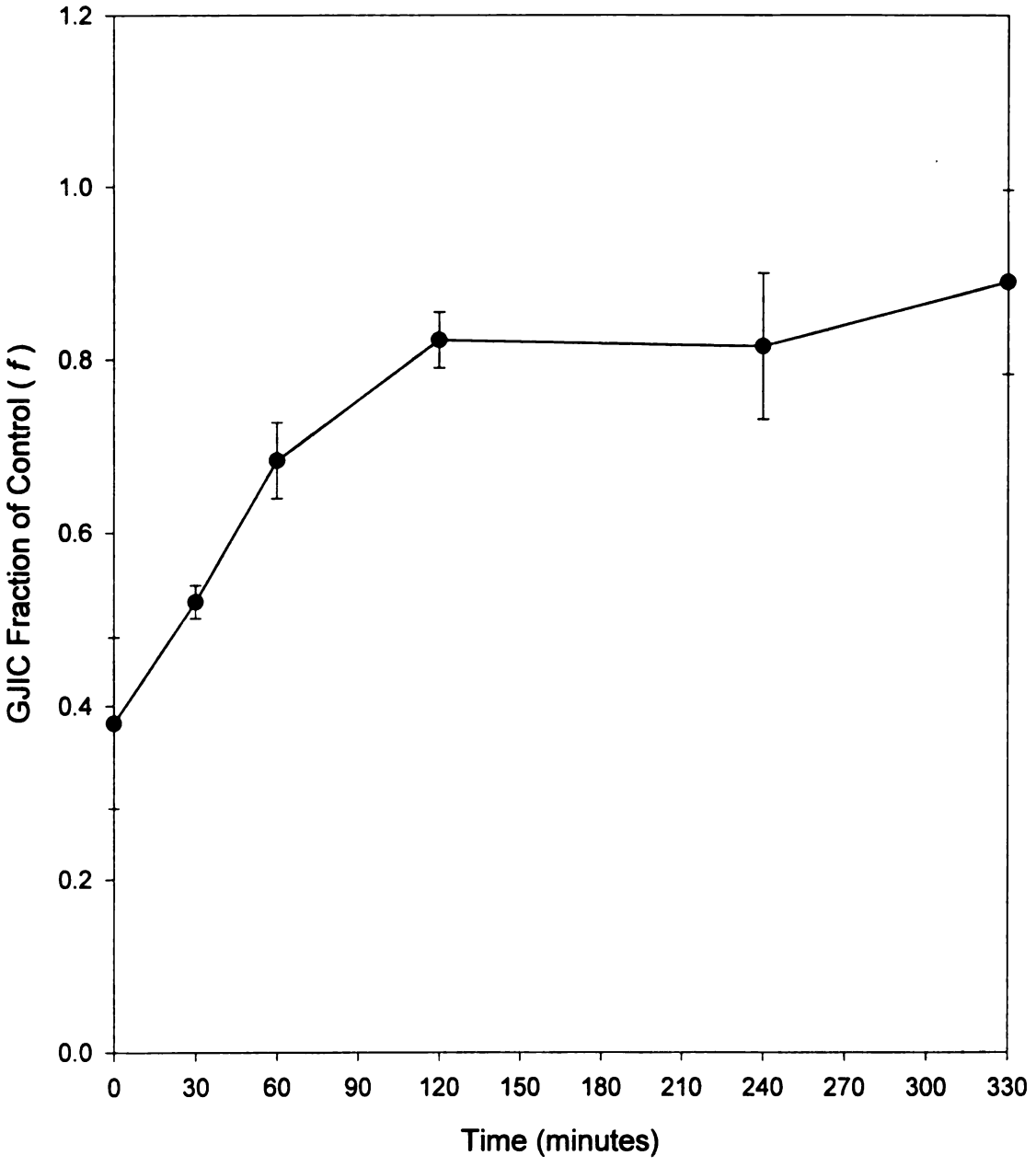


Figure A-9. Time Recovery: 5 mol O_3/mol as Chrysene at 210 μM concentration

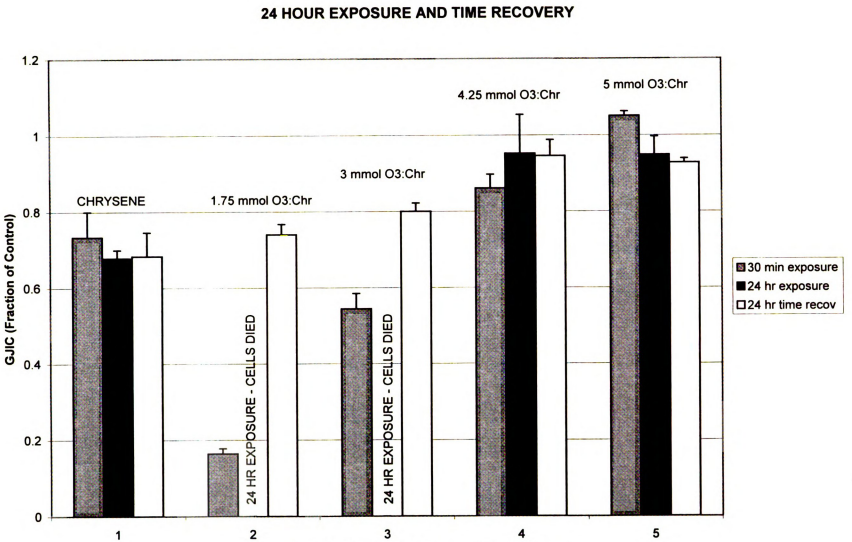


Figure A-10. 24 Hour Exposure and Time Recovery

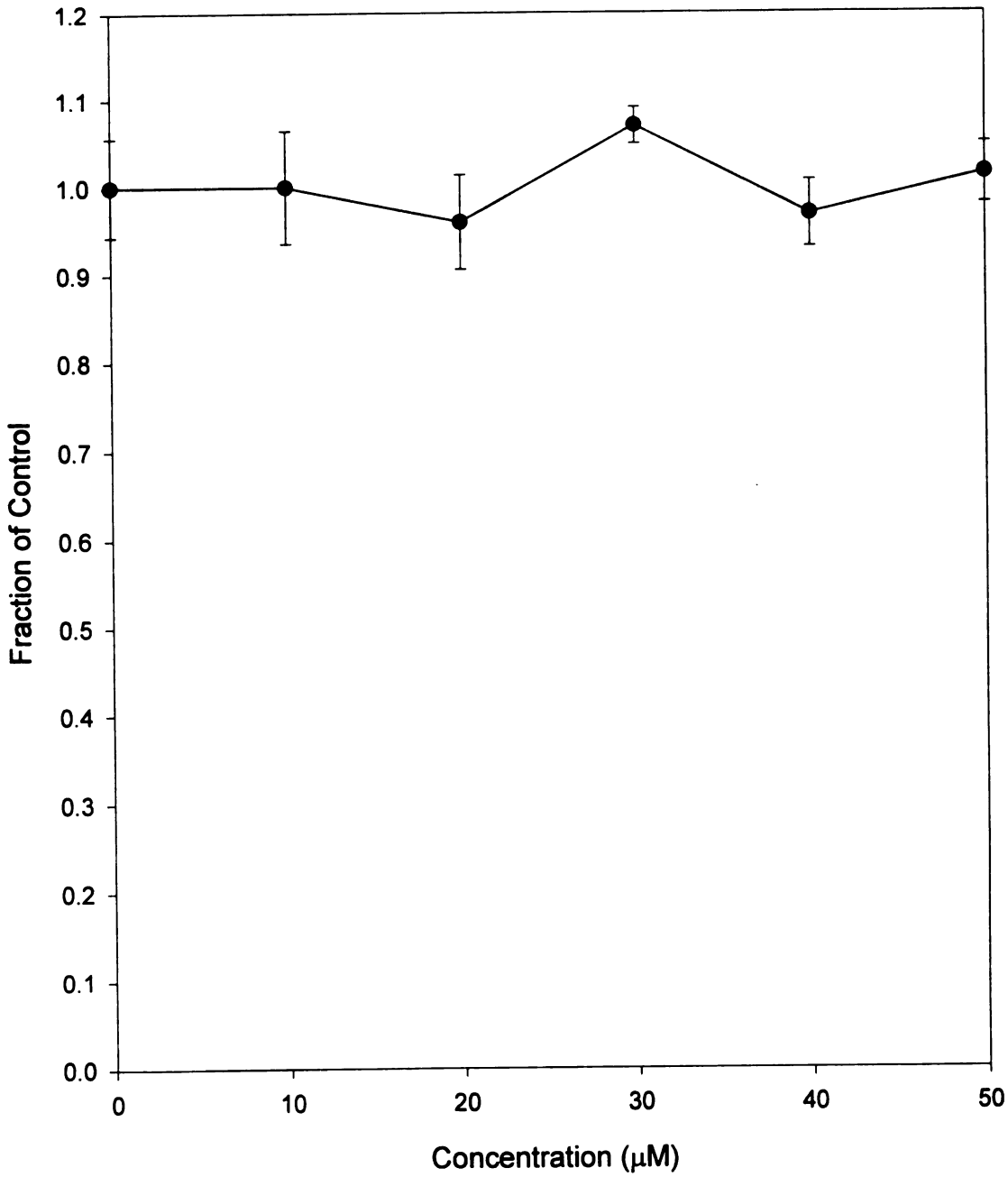


Figure A-11. Cytotoxicity: Chrysene

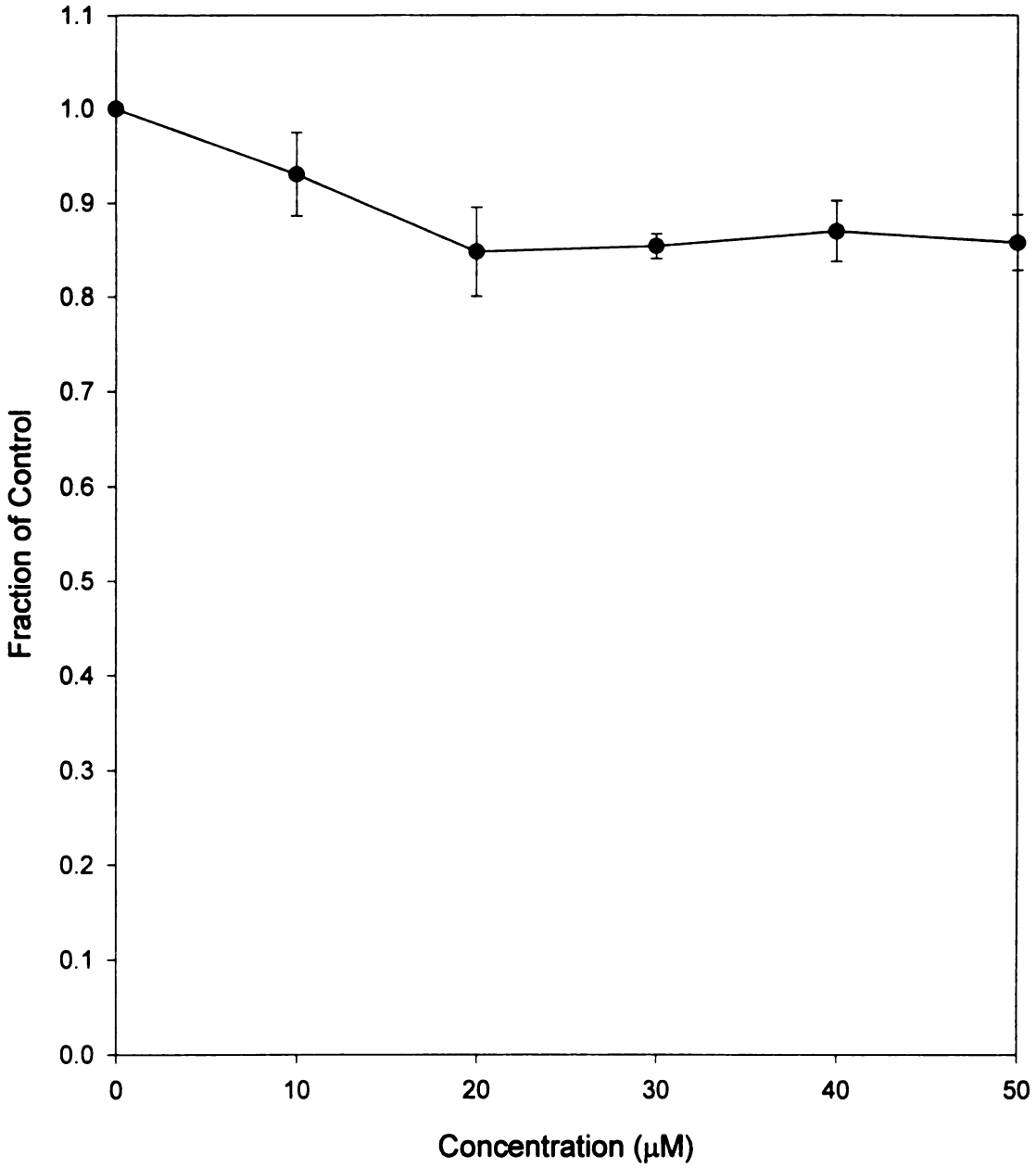


Figure A-12. Cytotoxicity: 1.75 mol O_3 /mol as Chrysene

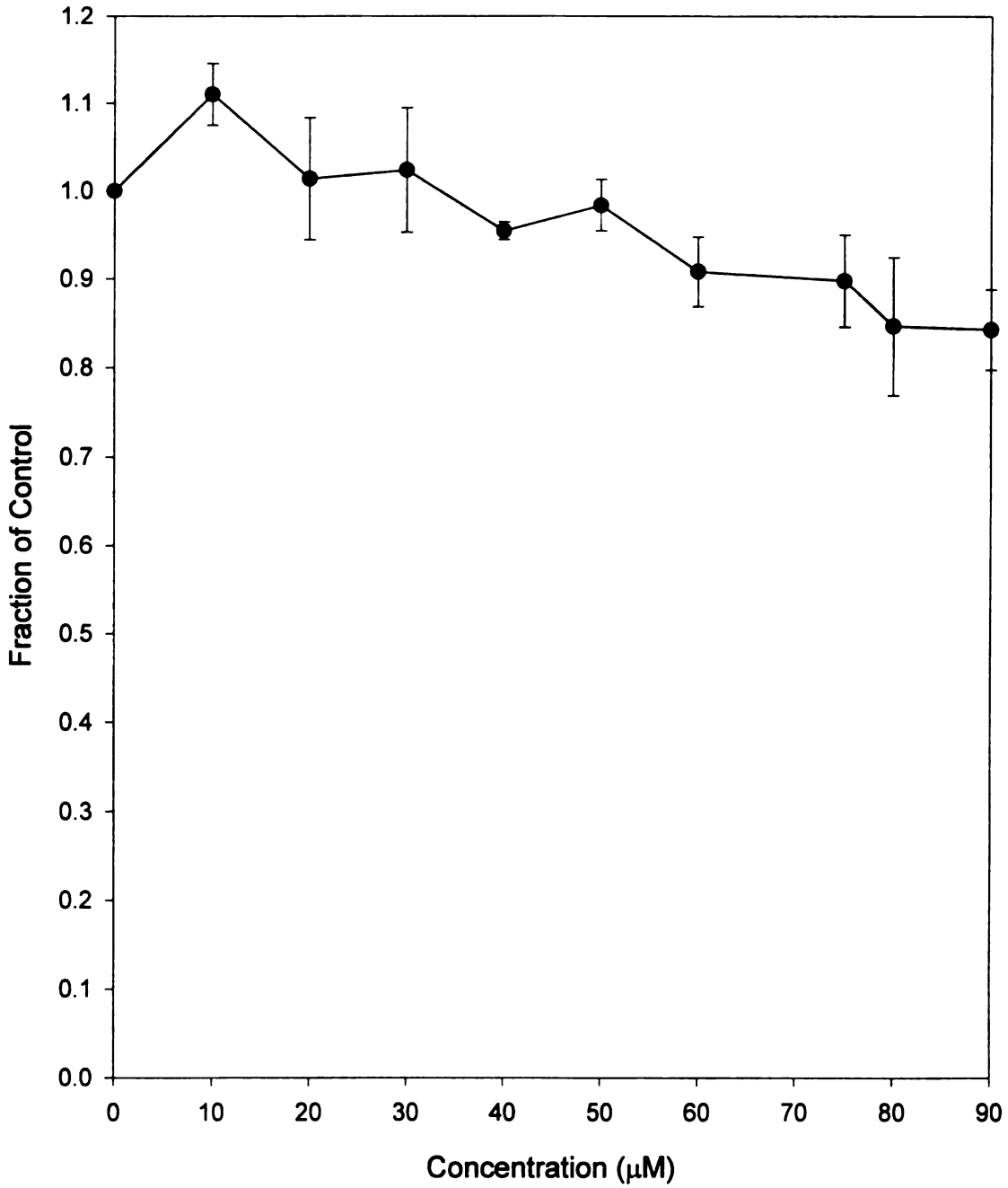


Figure A-13. Cytotoxicity: 3 mol O_3/mol as Chrysene

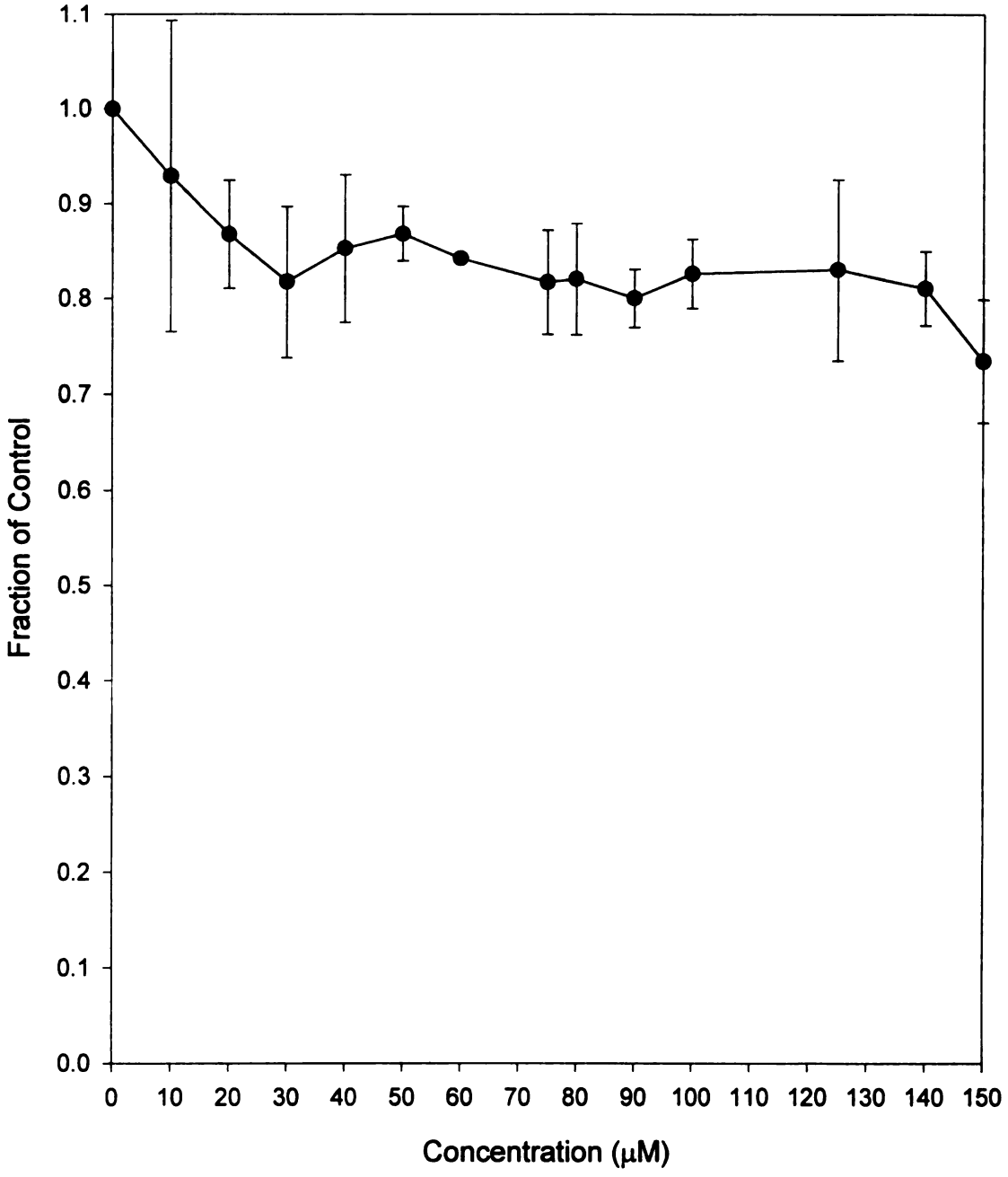


Figure A-14. Cytotoxicity: 4.25 mol O_3/mol as Chrysene

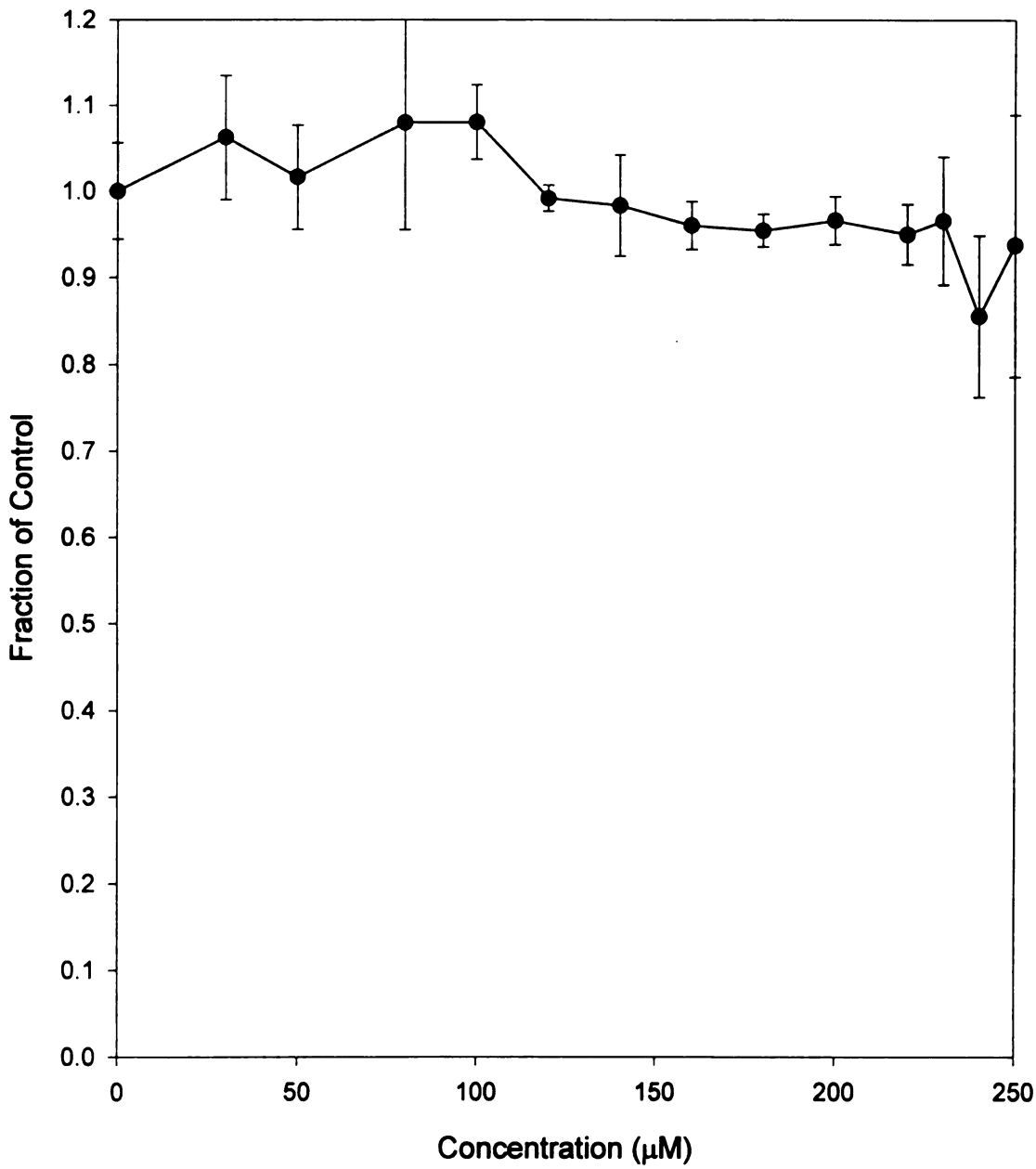


Figure A-15. Cytotoxicity: 5 mol O_3/mol as Chrysene

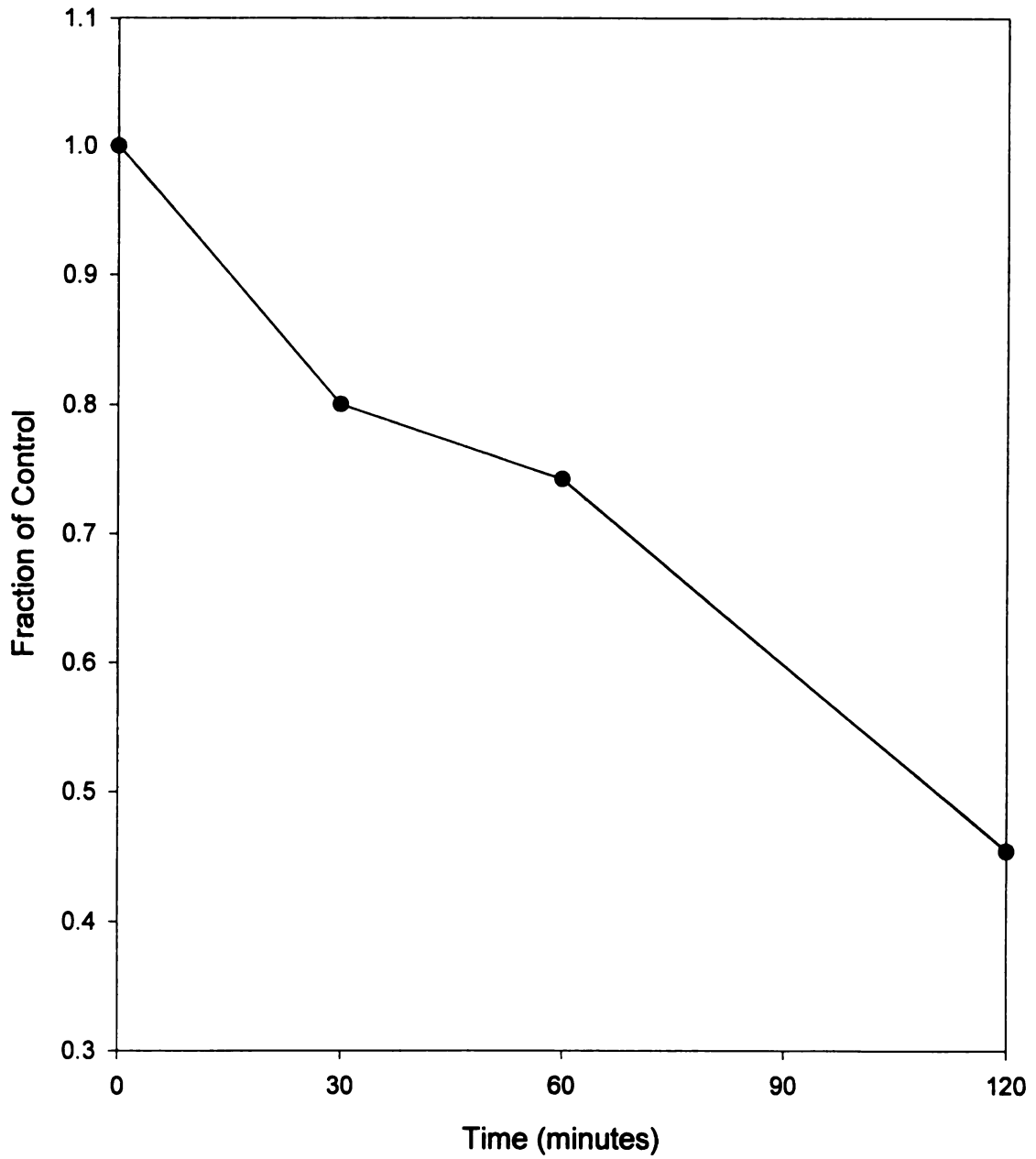


Figure A-16. Time Response for Cytotoxicity: 1.75 mol O_3/mol as Chrysene 50 μM concentration

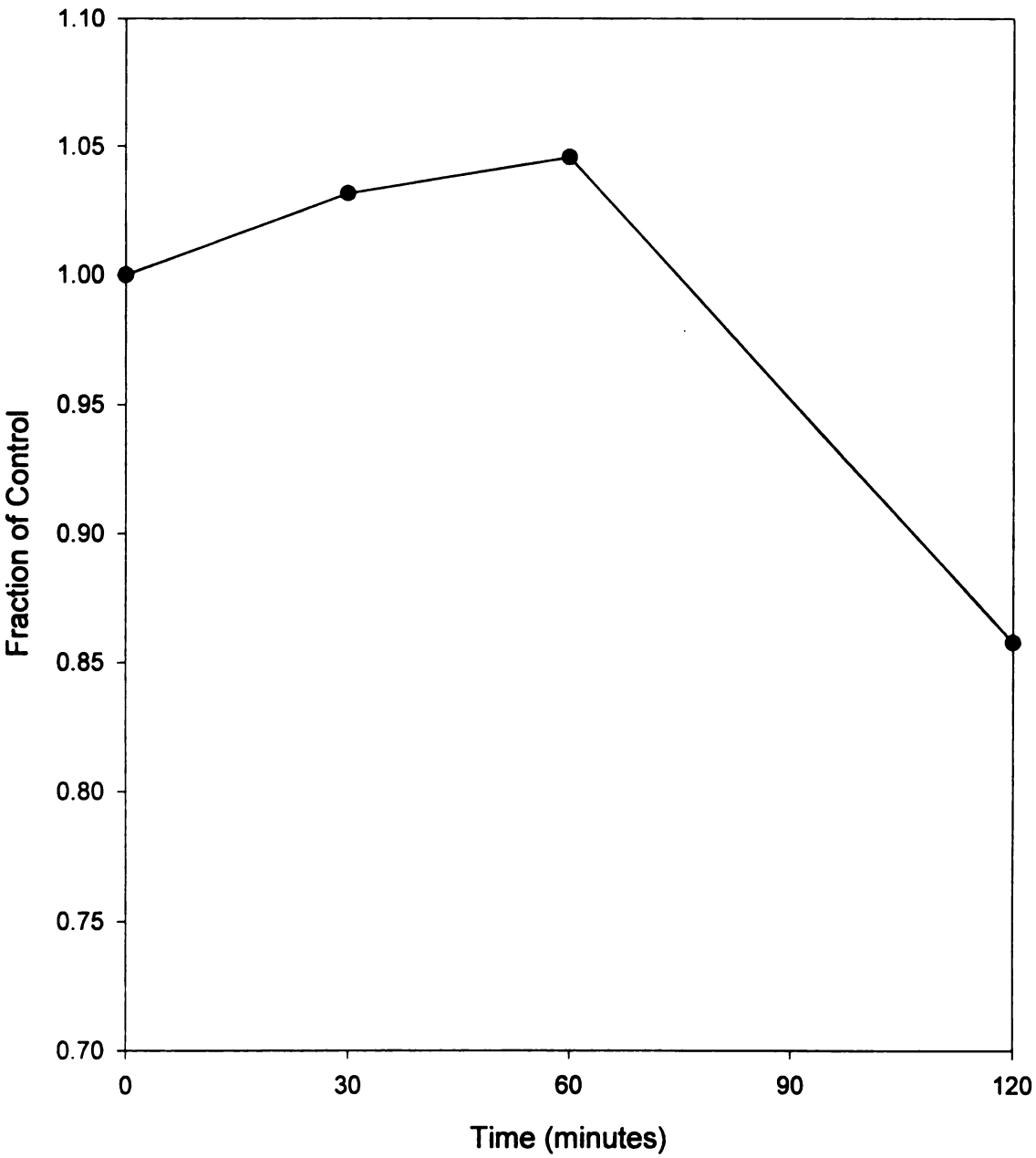


Figure A-17. Time Response for Cytotoxicity: 3 mol O_3/mol as Chrysene 50 μM concentration

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