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thesis entitled

DETERMINING A CORRELATION BETWEEN STRUCTURAL ELEMENTS OF POLYCYCLIC AROMATIC HYDROCARBONS AND INHIBITION OF GAP JUNCTIONAL INTERCELLULAR COMMUNICATION

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Major professor

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DETERMINING A CORRELATION BETWEEN STRUCTURAL ELEMENTS OF POLYCYCLIC AROMATIC HYDROCARBONS AND INHIBITION OF GAP JUNCTIONAL INTERCELLULAR COMMUNICATION

By

Liliane Marie Weis

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1996

ABSTRACT

DETERMINING A CORRELATION BETWEEN STRUCTURAL ELEMENTS OF POLYCYCLIC AROMATIC HYDROCARBONS AND INHIBITION OF GAP JUNCTIONAL INTERCELLULAR COMMUNICATION

By

Liliane Marie Weis

Ten polycyclic aromatic hydrocarbons (PAHs) were tested for their efficacy as tumor promoters by assessing their effect on gap junctional intercellular communication (GJIC) in rat liver epithelial cells. The PAHs used in this study were naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, anthracene, 1-methylanthracene, 2-methylanthracene, 9-methylanthracene, 9,10-dimethylanthracene, phenanthrene, and benz(a)anthracene. These structurally similar PAHs were chosen in order to determine if a correlation exists between chemical structure and inhibition of GJIC.

The results of this study suggest that PAHs whose chemical structures possess a bay region or bay-like regions are more potent inhibitors of GJIC when compared to PAHs (with similar chemical structures) whose structures do not possess a bay or bay-like region. However, the existence of this structural element does not necessarily mean that the compound will inhibit GJIC.

To my fiancé, Jamie, and my family.
I couldn't have made it without you.

ACKNOWLEDGMENTS

I would like to thank the National Institute of Environmental Health and Safety (NIEHS) Superfund grant #ES04911-066 and the National Food Safety and Toxicology Center at Michigan State University (MSU) in East Lansing, Michigan for funding this research. I would like to thank my advisor, Dr. Susan Masten (Department of Civil and Environmental Engineering, MSU) and the other members of my graduate committee, Dr. Simon Davies (Department of Civil and Environmental Engineering, MSU), Dr. Brad Upham (Department of Pediatrics and Human Development, MSU), and Dr. James Trosko (Department of Pediatrics and Human Development, MSU) for their guidance and input throughout this project.

I would especially like to thank Dr. Upham for his patience while training me in the technical aspects of this research and Alisa Rummel, an undergraduate research assistant (Food Safety and Toxicology Center, MSU), for her technical assistance.

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs), which are derived from the incomplete combustion of organic materials, are common industrial pollutants and are contained in cigarette smoke and coal tar in high concentrations. Many PAHs are known carcinogens and a considerable amount of research has been devoted to predicting the tumor initiating potential of PAHs based on chemical structure. However, there has been little research into the tumor promoting effects of these types of compounds and no structural correlation made thereof.

Ten PAHs were tested for their efficacy as tumor promoters by assessing their effect on gap junctional intercellular communication (GJIC) in rat liver epithelial cells. The PAHs used in this study were naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, anthracene, 1-methylanthracene, 2-methylanthracene, 9-methylanthracene, 9,10-dimethylanthracene, phenanthrene, and benz(a)anthracene. These structurally similar PAHs were chosen in order to determine if a correlation exists between chemical structure and inhibition of GJIC. Results show that neither anthracene nor 2-methylanthracene caused inhibition of GJIC up to 350 μM. Naphthalene and benz(a)anthracene partially inhibited GJIC below 350 μM. 1-Methynaphthalene and 2-methylnaphthalene

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were inhibitory to GJIC at 225 μM and 350 μM, respectively; however, both of these methylated naphthalenes were also cytotoxic at slightly higher concentrations. The following compounds completely inhibited GJIC at 70 μM: 1-methylanthracene, 9-methylanthracene, and 9,10-dimethylanthracene. Phenanthrene completely inhibited GJIC at 60 μM. It was found that the higher the aqueous solubility of the compound, the more quickly GJIC was inhibited.

Inhibition of GJIC was not due to cytotoxicity for most of these compounds. In the case of 1- and 2-methylnaphthalene, cytotoxicity may be the cause of inhibition of GJIC. For all of the PAHs tested, inhibition of GJIC was completely reversible, much like the non-cytotoxic, reversible process of tumor promotion *in vivo*. These results suggest that PAHs with structures containing a bay region or bay-like regions are more potent inhibitors of GJIC when compared to PAHs (with similar structures) which do not possess a bay or bay-like region.

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Chapter 1

OBJECTIVE

Background

Cancer accounts for approximately 20% of the 11 million deaths reported by industrialized nations to the World Health Organization (Vile and Morris, 1992). In the United States alone, about 1 million new cases of cancer are diagnosed each year and about 500,000 deaths are reported each year (Cooper, 1992). Lung cancer alone accounts for 28% of these deaths and has one of the lowest five-year survival rates compared to other types of cancer (Cooper, 1992). Smoking cigarettes has been linked to lung cancer and several studies have established the carcinogenicity of polycyclic aromatic hydrocarbons (PAHs) found in tobacco smoke (Hoffman and Wynder, 1972 and Severson *et al.*, 1976).

PAHs are probably one of the most widespread of all chemical environmental contaminants (Jones and Lever, 1979). They are formed from the incomplete combustion of organic materials (Manahan, 1990) and are generated to a large extent by industrial processes such as coal tar production, coal gasification/liquefaction, aluminum production, and municipal trash incineration. PAHs are also found in such substances as cigarette smoke (US Dept. of Health, 1993), automobile exhaust (Lee *et al.*, 1979) and asphalt residues

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(Manahan, 1990). A significant number of PAHs found in these substances are known to be carcinogenic (Freudenthal and Jones, 1976).

Since many PAHs are carcinogenic or can cause adverse health effects, it is important to protect society and minimize people's exposure to these types of harmful compounds. Environmental regulations such as the Clean Water Act of 1977 and the Clean Air Act of 1963 control the release of these types of compounds into the environment.

Both toxicology and risk assessment are significant in forming these types of environmental regulations. In toxicology, chemicals or environmental contaminants are studied for their harmful or toxic effects in animals. In risk assessment, toxicological research is evaluated in order to determine safe exposure levels for humans. Legislators use safe exposure levels to promulgate environmental regulations in order to prevent people's exposure to unsafe contaminant levels.

The primary focus of previous and current toxicological studies has been to determine and predict the carcinogenicity of various chemical agents (Trosko *et al.*, 1994). There has been substantial research to determine the tumor initiating potential of various PAHs such as chrysene (Rice *et al.*, 1988), benz(a)anthracene (Norpoth *et al.*, 1984), and benzo(a)pyrene (Iwata *et al.*, 1981), and PAH laden media such as cigarette smoke (Hoffman and Wynder, 1972 and Severson *et al.*, 1979) and coal tar (Bradley *et al.*, 1983). There has also been considerable focus on forming models, based on chemical structure, in order to

predict the cancer initiating potential of PAHs and PAH metabolites (Jerina *et al.*, 1978, Hoffman *et al.*, 1982, and Silverman and Lowe, 1982). Several theories such as the bay-region theory and the K-region theory have gained significant attention in the area of predictive modeling (Jerina *et al.*, 1978). Despite the numerous studies on the tumor initiating potential of PAHs, there has been little research into the tumor promoting potential of PAHs.

Carcinogenesis and GJIC

It has been well documented that the conversion of a normal cell into a malignant tumor does not occur in a single step (Vile and Morris, 1992). Rather the development of cancer is a multistep process consisting of three stages; tumor initiation, tumor promotion, and progression (Cooper, 1992). In tumor initiation, the gene of a cell is mutated in a way that will eventually lead to abnormal cell proliferation (Cooper, 1992 and Harris, 1991). Initiation is an irreversible event and is induced by certain chemicals known either as tumor initiators or carcinogens. Initiation is also induced by other physical stimuli such as ultraviolet radiation (Brock *et al.*, 1994). During the second stage of cancer, tumor promotion, the genetic expression of the initiated cell is altered and the cell begins to proliferate abnormally (Harris, 1991). Chemicals which induce tumor promotion are called tumor promoters and are required for the initial development of a population of proliferating tumor cells (Cooper, 1992). These compounds are usually not initiators in themselves, but induce abnormal

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proliferation of initiated cells (Franks, 1991). Tumor promotion, unlike tumor initiation, is a reversible process (Trosko *et al.*, 1993). The third stage of cancer, progression, involves additional mutational changes and continued unregulated growth of the tumorigenic cells (Cooper, 1992). Unlike normal cells, initiated cells lack the ability to terminally differentiate and the ability to control cell proliferation by contact inhibition (Trosko *et al.*, 1988 and Vile and Morris, 1992).

Intercellular communication is a process by which cells can transfer chemical information to each other (Trosko *et al.*, 1988). One form of intercellular communication takes place by the direct transfer of ions and small molecular weight molecules through a specialized cell membrane structure called a gap junction (Trosko *et al.*, 1988). A gap junction is a protein structure which forms an aqueous channel connecting the cytoplasm of two adjacent cells (Kumar and Gilula, 1996 and Trosko *et al.*, 1993). The basic structural unit of the gap junction is a membrane protein called a connexin (Trosko *et al.*, 1993). Six connexins come together to form a hexameric structure with a toroid appearance called a connexon (Kumar and Gilula, 1996 and Trosko *et al.*, 1993). The connexon of one cell associates with the connexon of a neighboring cell to form an aqueous channel called a gap junction (Kumar and Gilula, 1996). This channel allows for direct communication between cells through passive diffusion of ions and small molecular weight molecules (<1500 Daltons) (Caspar *et al.*, 1988 and Trosko *et al.*, 1993). This type of communication between cells through gap junctions is called gap junctional intercellular communication (GJIC).

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GJIC has been shown to be significant in tumor promotion. Normally an initiated cell is suppressed from proliferating as long as there is normal GJIC between the initiated cell and normal surrounding cells. If GJIC is inhibited (blocked) by a chemical, the initiated cell will proliferate and undergo tumor promotion (Trosko *et al.*, 1993). However, as long as an initiated cell is able to communicate with the normal cells surrounding it, there will be no proliferation of the initiated cell and therefore no formation of a tumor (Trosko *et al.*, 1993).

Research Objective

It has been shown that isomeric forms of PAHs affect GJIC differently. Upham *et al.* (1996) showed that both 1-methylanthracene and 9-methylanthracene are inhibitory to GJIC in rat liver epithelial cells, whereas 2-methylanthracene is not. These three methylated anthracenes are isomers, that is they have the same chemical formula but different chemical structures. This suggests that chemical structure may be linked to inhibition of GJIC.

The purpose of this research project was to determine if a correlation exists between the chemical structure of PAHs and inhibition of GJIC. The following two hypotheses were formed based on previous research by Upham *et al.* (1994 and 1996): regarding chemical structure of PAHs, (1) a bay region or the formation of a bay-like region by a methyl group indicates that the compound will inhibit GJIC and (2) increasing the number of benzene rings in a straight chain will either increase or decrease the potency of the chemical to inhibit GJIC

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(in chemical structures which contain a bay region or the formation of a bay-like region by a methyl group).

The term "bay region" has been commonly used in chemistry to describe the area of a PAH where three adjacent benzene rings form a semi-circle. "Bay-like region" is a term that has been developed to describe the chemical structure of a PAH which possesses a bay-type region not formed by three benzene rings. A bay-like region resembles a bay region except that a methyl group substitutes for one of the benzene rings in the semi-circle. The methyl group is positioned in such a way as to maintain a semi-circle. The difference between a bay region and a bay-like region is illustrated in Figure 1.

The following ten structurally similar PAHs were tested for their effect on GJIC *in vitro*: anthracene (ANT), 1-methylanthracene (1-meA), 2-methylanthracene (2-meA), 9-methylanthracene (9-meA), 9,10-dimethylanthracene (DMA), naphthalene (NAP), 1-methylnaphthalene (1-meN), 2-methylnaphthalene (2-meN), phenanthrene (PHE), and benz(a)anthracene (B(a)A). These PAHs, whose chemical structures are shown in Figure 2, were chosen in order to determine if a correlation exists between the chemical structure of a PAH and inhibition of GJIC.

No information was found on the tumor promoting effects of any of these compounds *in vivo* or *in vitro*. Two of these compounds, DMA and B(a)A have been shown to be weak initiators in mouse skin (Myers *et al.*, 1988 and Norpoth *et al.*, 1984). ANT (Iwata *et al.*, 1981), PHE (Wood, 1979), NAP (US Dept. of

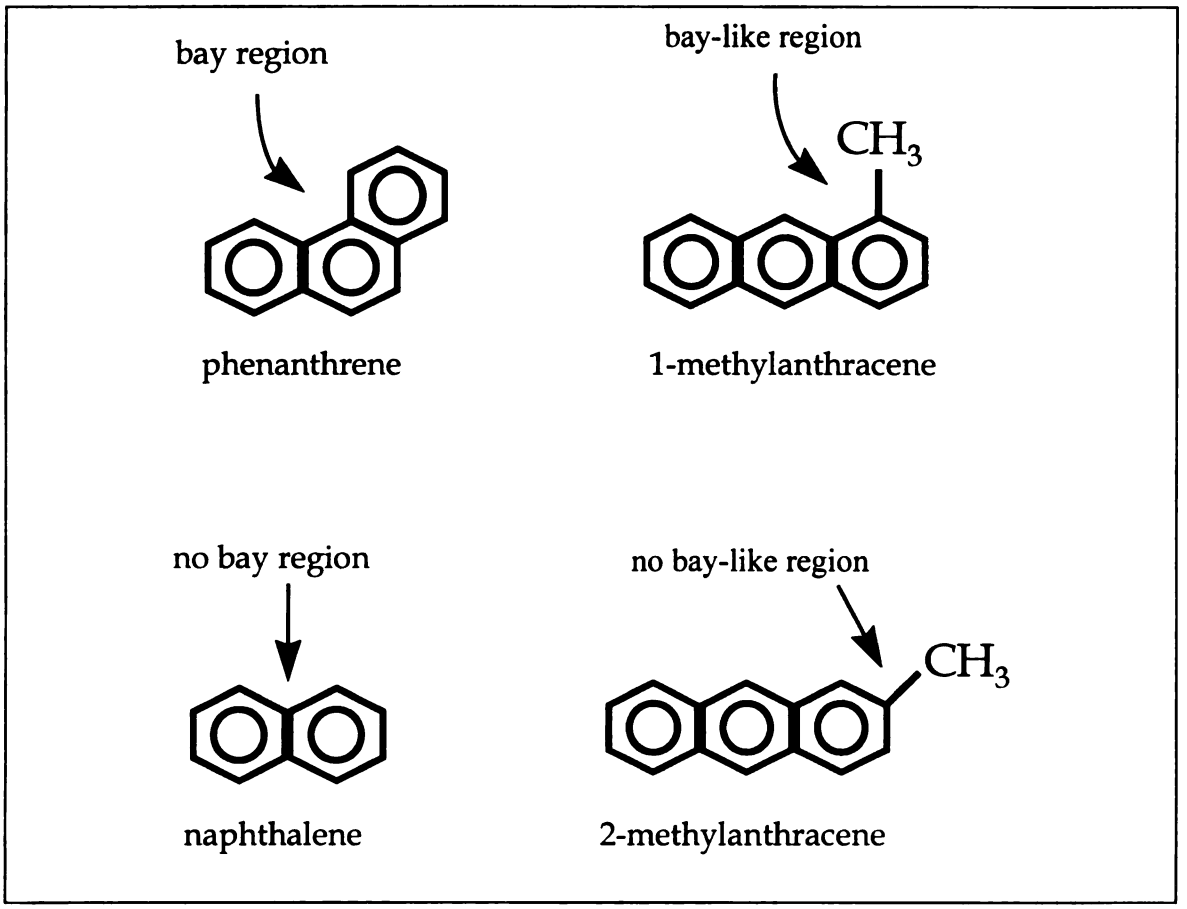

Figure 1. Illustration of a Bay Region and a Bay-like Region

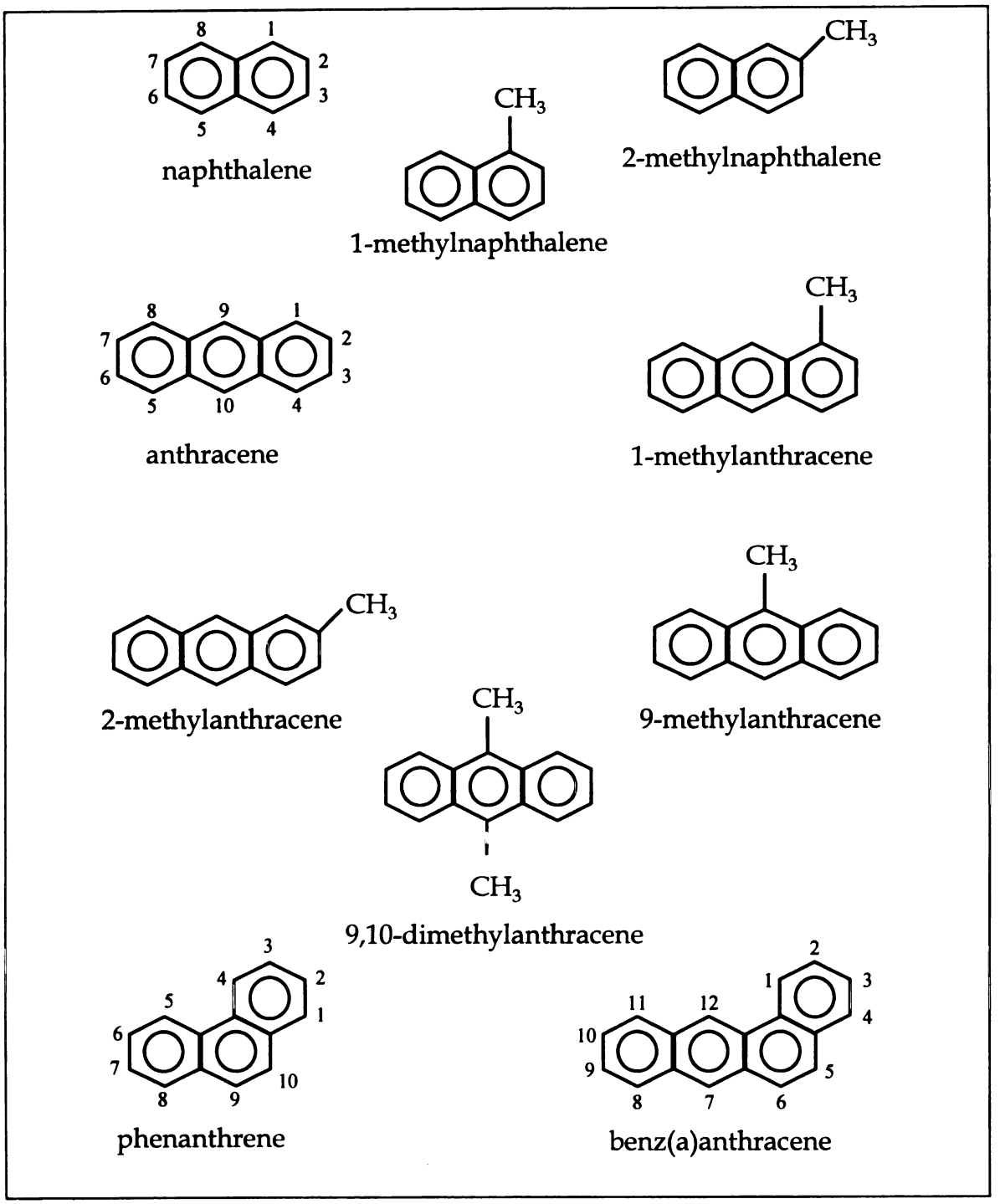

Figure 2. Chemical Structures of the Selected PAHs

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Health, 1994) and the monomethylated anthracenes (LaVoie *et al.*, 1985) have all been shown to be non-initiating in mouse skin bioassays. Although ANT has not been shown to be an initiator, this compound undergoes biomethylation in mice to form 9-meA and DMA, the latter of which is a weak initiator (Myers *et al.*, 1988). No information was found on the carcinogenicity of NAP, 1-meN and 2-meN; however, there is information which claims that naphthalenes cause irritation in the esophagus and lungs (US Dept. of Health, 1994).

As was mentioned previously, PAHs are ubiquitous environmental contaminants. The PAHs used in this study are common pollutants and are usually associated with certain industrial processes. Some of these PAHs are common in everyday surroundings suggesting that exposure to these compounds may be fairly high for some people. Listed in Table 1 are the PAHs used in this study and substances which have been shown to contain significant levels of these compounds.

Table 1. Predominant Sources of the Selected PAHs

Compound	Substance
ANT	cigarette smoke (Severson *et al.*, 1979), auto exhaust (Lee *et al.*, 1979), emissions from both coal-fired and oil-fired power plants (Bennett *et al.*, 1979), coal tar (Bradley *et al.*, 1983), emissions from wood burning stoves (Lao *et al.*, 1983), creosote (US Dept. of Health, 1993)
methylanthracenes (general)	cigarette smoke (Severson *et al.*, 1979), emissions from both coal-fired and oil-fired power plants (Bennett *et al.*, 1979)
1-meA and 2-meA	auto exhaust (Lee *et al.*, 1979)
9-meA	auto exhaust (Lee *et al.*, 1979), cigarette smoke (Severson *et al.*, 1979)
dimethylanthracenes (may include DMA)	cigarette smoke (Severson *et al.*, 1979)
NAP	coal tar (Bradley *et al.*, 1983), petroleum (US Dept. of Health, 1994), cigarette smoke (Severson *et al.*, 1979), creosote and moth balls (US Dept. of Health, 1993)
1-meN and 2-meN	coal tar (Bradley *et al.*, 1983), petroleum (US Dept. of Health, 1994), cigarette smoke (Severson *et al.*, 1979)
PHE	cigarette smoke (Severson *et al.*, 1979), ambient air during roofing and paving activities (Malaiyandi *et al.*, 1982), auto exhaust (Lee *et al.*, 1979), emissions from both coal-fired and oil-fired power plants (Bennett *et al.*, 1979), coal tar (Bradley *et al.*, 1983), emissions from wood burning stoves (Lao *et al.*, 1983), creosote (US Dept. of Health, 1993)
B(a)A	cigarette smoke (Severson *et al.*, 1979), ambient air during roofing and paving activities (Malaiyandi *et al.*, 1982), auto exhaust (Lee *et al.*, 1979), emissions from wood burning stoves (Lao *et al.*, 1983)

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Chapter 2

MATERIALS AND METHODS

Chemicals

ANT, 1-meA, 2-meA, 9-meA, DMA, NAP, 1-meN, 2-meN, B(a)A, and 37% formaldehyde were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). PHE, neutral red, and lucifer yellow CH were obtained from Sigma Chemical Co. (St. Louis, MO). Acetonitrile was obtained from EM Science (Gibbstown, NJ).

Cell Culture

WB-F344 rat liver epithelial cell lines were obtained from Drs. J. W. Grisham and M. S. Tsao of the University of North Carolina (Chapel Hill, NC) (Tsao *et al.*, 1984). Cells were cultured in 2 ml of D-medium (Formula No. 78-5470EG) and supplemented with 5% fetal bovine serum and 50 μg/ml gentamicin , all of which were obtained from GIBCO Laboratories (Grand Island, NY). The cells were grown in 35 mm^2 plastic petri dishes (Corning Glass Works, Corning, NY) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Bioassays were conducted on 95-100% confluent cultures that were obtained after 2 to 3 days of growth.

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Chemical Treatments

In the dose response experiments, plates of cells were exposed to different doses of the target compound for a set period of time. The doses applied to the cells ranged from a very low dose which did not cause inhibition of GJIC, to a higher dose which caused complete inhibition of GJIC. In the dose response experiments, even though the dose of target compound applied to the cells varied, the length of time to which the cells were exposed to the target compound did not. This experiment was performed to determine if the target compound causes inhibition of GJIC and if so, at what dose does inhibition of GJIC occur. Following chemical treatment, the cells were subjected to the scrape-loading/dye transfer (SL/DT) assay as described below in Bioassay of GJIC.

In the time response experiments, plates of cells were exposed to the target compound for various lengths of time at a dose that was known to cause inhibition of GJIC. Time periods ranged from a very short exposure time which did not cause inhibition of GJIC, to increasingly longer exposure times which caused complete inhibition of GJIC. In the time response experiments, even though the exposure time varied, the dose of target compound applied to the cells did not. This experiment was performed to determine how much time it takes for the inhibiting target compound to cause complete inhibition of GJIC. Following chemical treatment, the cells were subjected to the SL/DT assay.

In time recovery experiments, plates of cells were exposed to the target compound at a dose which causes inhibition of GJIC (determined by the dose

response experiment) for a length of time which causes inhibition of GJIC (determined by the time response experiment). Neither chemical dose nor exposure time were varied for a given target compound. After exposing the cells to the target compound for the specified time period, the cells were rinsed 5 times with phosphate buffered saline (PBS). Approximately 2 ml of fresh media were applied to the cells and the cells were incubated at various lengths of time in a humidified atmosphere containing 5% CO_2 and 95% air. The length of incubation time varied from zero minutes to 6 hours. This experiment was performed in order to determine if inhibition of GJIC by the target compound is a reversible process and if so, how much time is required for the cells to completely recover GJIC. Following incubation with fresh media, the cells were subjected to the SL/DT assay.

Bioassay of GJIC

GJIC was determined by using the SL/DT assay adapted from El-Fouly *et al.* (1987). The cells were rinsed 5 times with PBS. Approximately 1 ml of 0.05% lucifer yellow dye dissolved in PBS was applied to the cells. A surgical steel blade was used to make 8 to 10 scrapes on the cell culture. The lucifer yellow dye was allowed to incubate on the cells for 3 minutes at room temperature. The dye was removed from the plate of cells and the cells were rinsed 5 times with PBS. The cells were fixed with approximately 0.5 ml of 4% formalin.

Controls

Stock solutions of PAHs were prepared in 100% acetonitrile. Concentrations of stock solutions ranged from 5 to 30 mM, depending upon the solubility of the compound in acetonitrile. Stock solutions in volumes ranging from 2 to 35 µl were added directly to the culture medium in each plate. Vehicle controls were performed by exposing the cells to a volume of acetonitrile equivalent to the volume of PAH stock solution used. Upham *et al.* (1994) showed that acetonitrile is non-cytotoxic and non-inhibitory up to 2% by volume (40 µl of acetonitrile: 2 ml of D-medium). In these experiments, the maximum volume of stock solution applied to a plate of cells was 35 µl per 2 ml of D-media (1.8% by volume). Therefore, most of the PAHs were only tested up to 350 µM due to the solubility limits of the chemicals in acetonitrile. All experiments were done in triplicate.

Assessing GJIC

The migration of the dye in the cells was observed using a Nikon epifluorescene phase microscope, illuminated with an Osram HBO 200 W lamp. A 35mm camera, attached to the microscope, was used to take pictures at a magnification of 200x to record migration of the dye in the scraped cells. The distance the dye traveled in the cells was measured on photographs, perpendicular from the scrape line to the dye front. Ten equally spaced

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measurements (approximately 1 cm apart) were made on each photograph. The average distance of dye migration was determined by averaging these 10 measurements. GJIC was assessed by comparing the distance the dye traveled in the chemically treated cells to the distance the dye traveled in the controls. GJIC was reported as a fraction of the control (FOC). An FOC value of approximately 1.0 indicates normal GJIC and a distance of dye migration equivalent to the controls. An FOC value of approximately 0.3 to 0.8 indicates varying levels of partial inhibition of GJIC and a shorter distance of dye migration compared to the controls. An FOC value of approximately 0.0 to 0.3 indicates complete inhibition and virtually no dye migration compared to the controls. There is not an absolute FOC value for complete inhibition, since complete inhibition can only be verified by looking at the photographs to determine if dye migration occurred.

Blind Dose Response Study

Two areas have been noted in the SL/DT assay as being sources of experimenter bias: (1) taking photographs of dye migration in the cells and (2) measuring the distance of dye migration in the photographs. Based on the hypotheses formed for this research, certain dose response relationships were expected for each of the PAHs; therefore, a blind dose response study was conducted in order to minimize experimenter bias.

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In this blind study, the 35 mm^2 plastic petri dishes containing the treated cells were labeled in code by the person chemically treating the cells and performing the SL/DT assay on the cells. Another individual who did not know which chemical had been used to treat the cells, took pictures of the dye migration in the cells. A third party then labeled the photographs with a different code so that the person measuring dye migration was unaware of which chemical had been used to treat the cells.

Bioassay of Cytotoxicity

Cytotoxicity was determined by the neutral red uptake assay, developed by Borenfreund and Puerner (1985). Only viable cells will take up neutral red dye. A 0.033% solution of neutral red dye in D-medium was incubated at 37°C for approximately 2 hours. The neutral red solution was centrifuged at 1300 rpm and filtered through a 0.22 μm Millipore syringe filter (Millipore Corp., New Bedford, MA) to remove precipitates. Approximately 0.1 to 1.0 ml of 0.33% neutral red stock solution was added to the filtered neutral red solution in order to increase the absorbance of the solution. An absorbance of 0.8 to 1.5 at 540 nm is desirable. The final concentration of the neutral red solution was approximately 0.03%. Chemical treatments and vehicle controls were conducted in the same manner as were done for the dose response experiments. After chemical treatment, the cells were rinsed 5 times with PBS, and 2.0 ml of the 0.03% neutral red solution was applied to each plate of cells. The cells were

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incubated in the 0.3% neutral red solution for 1.5 hours at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. After incubation, the cells were rinsed 3 times with PBS. Two milliliters of neutral red solubilizer, an aqueous solution containing 1% acetic acid and 50% ethanol, was applied to each plate of cells. The cells were incubated in the solubilizer for a minimum of ten minutes in order to solubilize the dye in the cells. The solubilized dye was measured by a Beckman uv/vis spectrophotometer (model no. DU-7400) at a wavelength of 540 nm under visible light. Background absorbance was measured at 690 nm and subtracted from the absorbance measured at 540 nm. Cytotoxicity was reported as a fraction of the control (FOC). An FOC value of approximately 1.0 indicates neutral red uptake equivalent to the control and a non-cytotoxic response. An FOC value of less than approximately 0.8 indicates less neutral red uptake compared to the controls, and that the compound is cytotoxic.

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Chapter 3

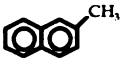
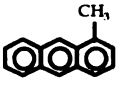
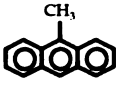
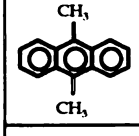
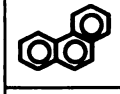
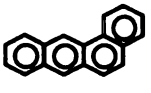
RESULTS, DISCUSSION AND CONCLUSIONS

The various PAHs were tested using the SL/DT assay to determine their effect on GJIC. The results of the dose response, time response, and time recovery experiments are summarized in Table 2. The PAHs were also tested for their cytotoxicity.

Dose Response Results

The results of the dose response experiments show that ANT and 2-meA did not cause inhibition of GJIC up to 350 μM as shown in Figure 3. B(a)A caused partial inhibition of GJIC up to 350 μM, also shown in Figure 3. ANT, 2-meA and B(a)A were not tested at doses higher than 350 μM in order to avoid cytotoxic effects caused by acetonitrile, as was discussed in Chapter 2. NAP, like B(a)A, caused partial inhibition of GJIC up to 350 μM, but did not completely inhibit GJIC as shown in Figure 4. 1-MeN and 2-meN caused complete inhibition of GJIC at 225 μM and 350 μM, respectively, also shown in Figure 4. PHE inhibited GJIC at 60 μM and the following compounds inhibited GJIC at 70 μM: 1-meA, 9-meA, and DMA (Figure 5). The results of the blind dose response study (Figures 6, 7, and 8) are similar to the results of the non-blind dose

Table 2. Summary of Experimental Results

Chemical Name and Structure	Inhibiting Dose (μM)	Inhibiting Response Time	Recovery Time	Effect on GJIC
naphthalene (NAP) 	200	not tested	not tested	Partial inhibition up to 350 μM
1-methylnaphthalene (1-meN) 	225	30 s	4 h	Complete inhibition
2-methylnaphthalene (2-meN) 	350	30 s	4 h	Complete inhibition
Anthracene (ANT) 	>350	not applicable	not applicable	No inhibition up to 350 μM
1-methylanthracene (1-meA) 	70	7 min	4.5 h	Complete inhibition
2-methylanthracene (2-meA) 	350	not applicable	not applicable	No inhibition up to 350 μM
9-methylanthracene (9-meA) 	70	7 min	3 h	Complete inhibition
9,10-dimethylanthracene (DMA) 	70	7 min	4 h	Complete inhibition
Phenanthrene (PHE) 	60	5 min	2 h	Complete inhibition
benz(a)anthracene (B(a)A) 	350	25 min	4 h	Partial inhibition up to 350 μM

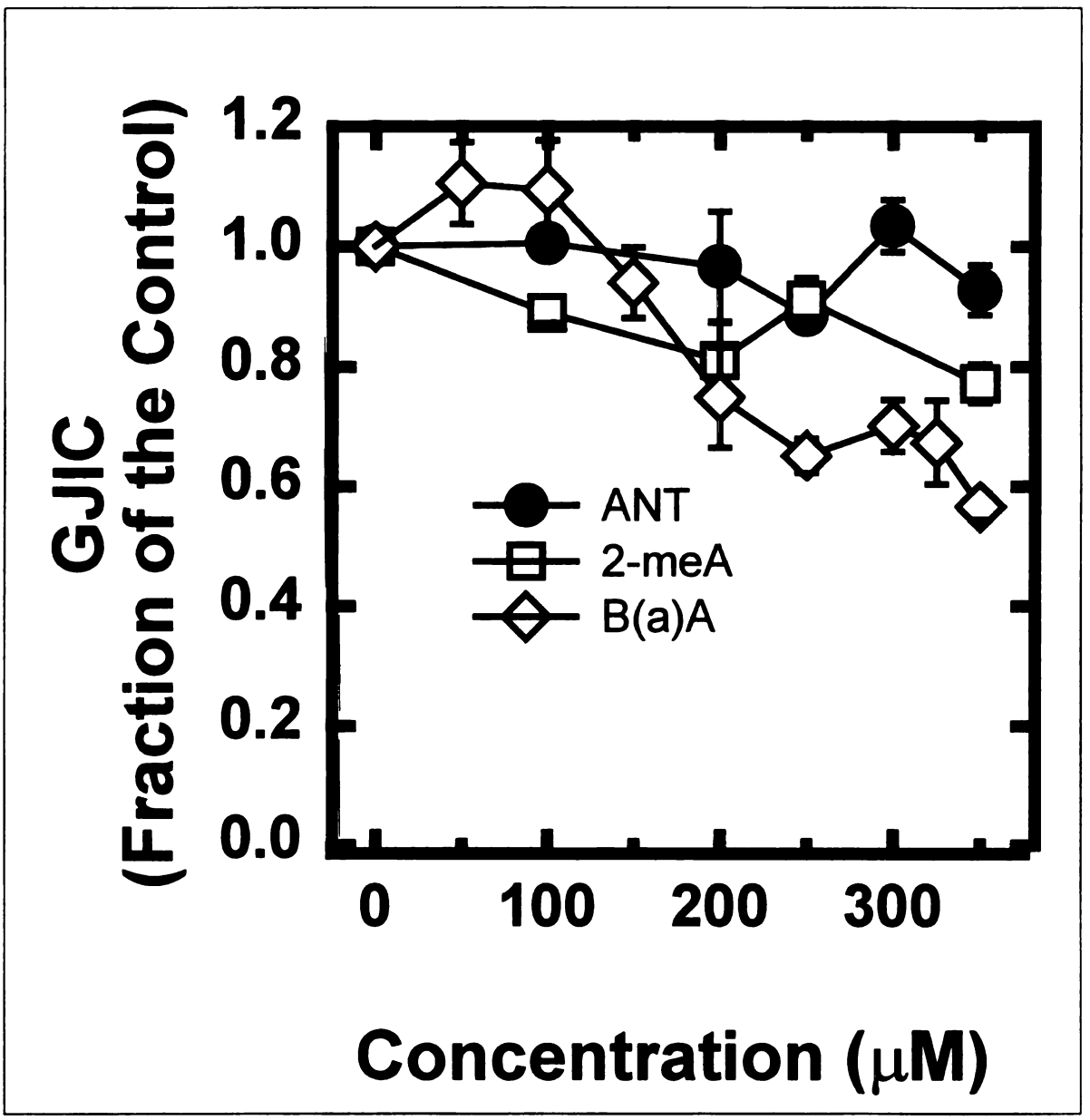


Figure 3. Dose Response Results of ANT, 2-meA and B(a)A

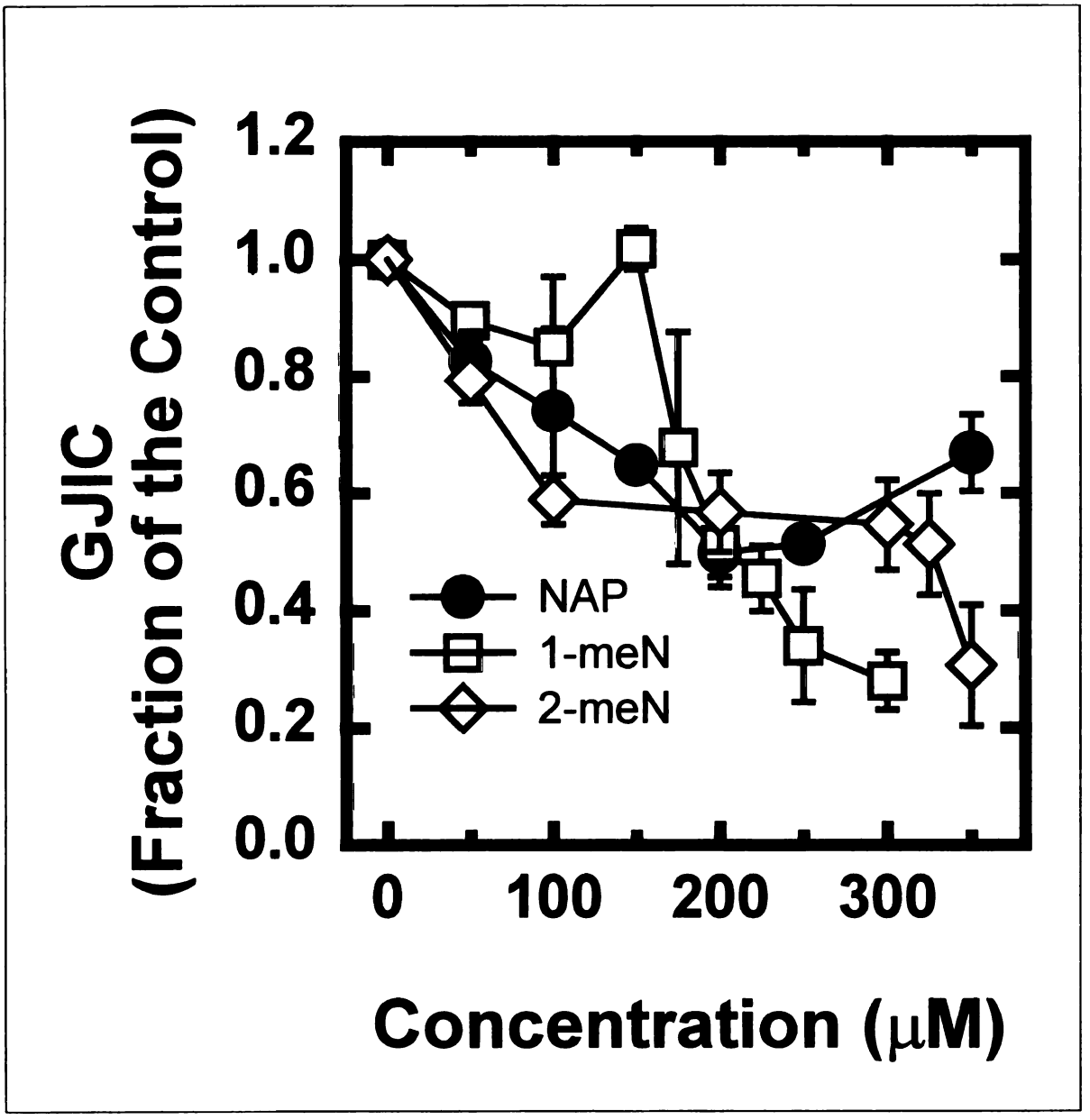


Figure 4. Dose Response Results of NAP, 1-meN and 2-meN


GJIC

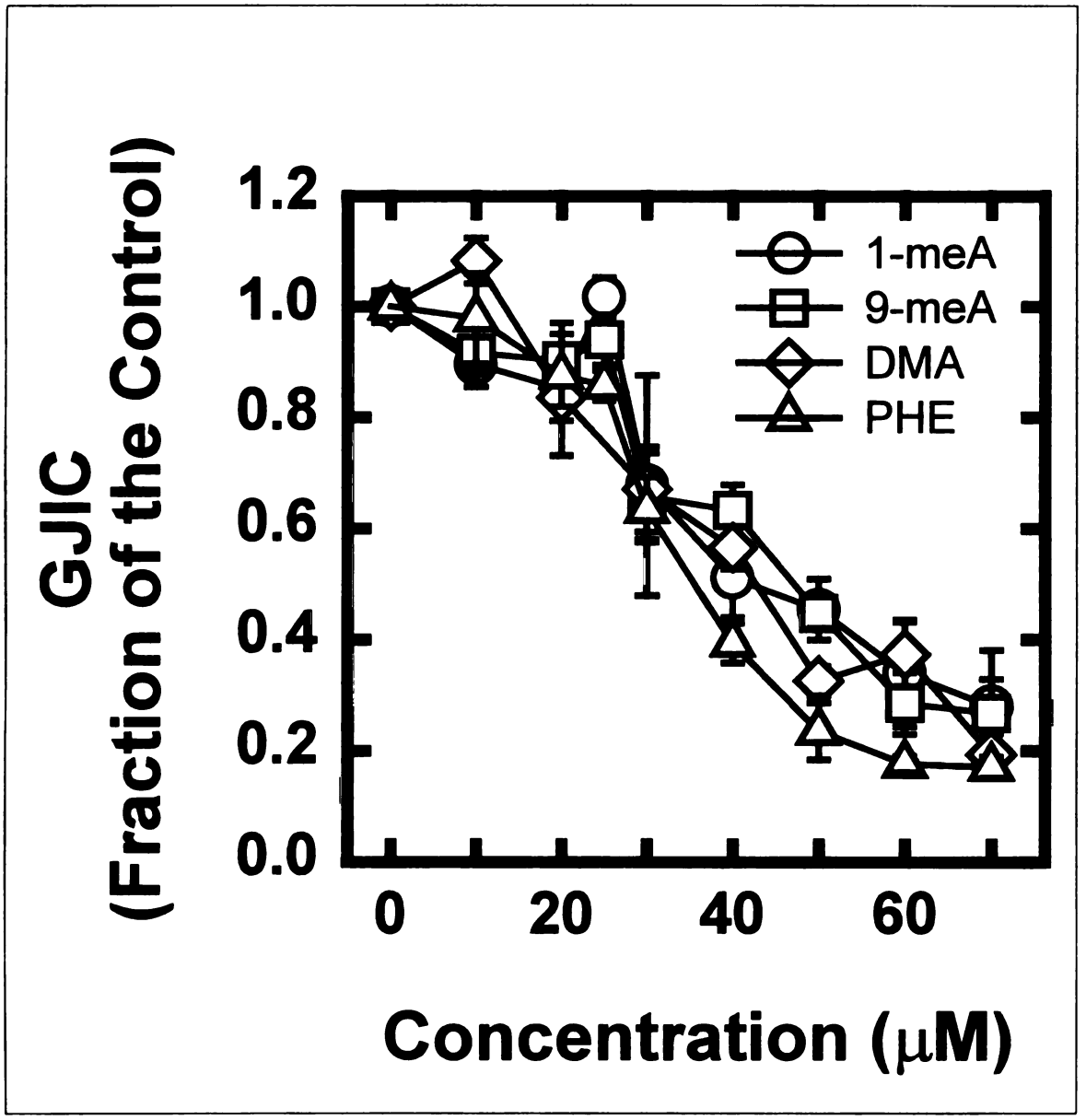


Figure 5. Dose Response Results of 1-meA, 9-meA, DMA and PHE

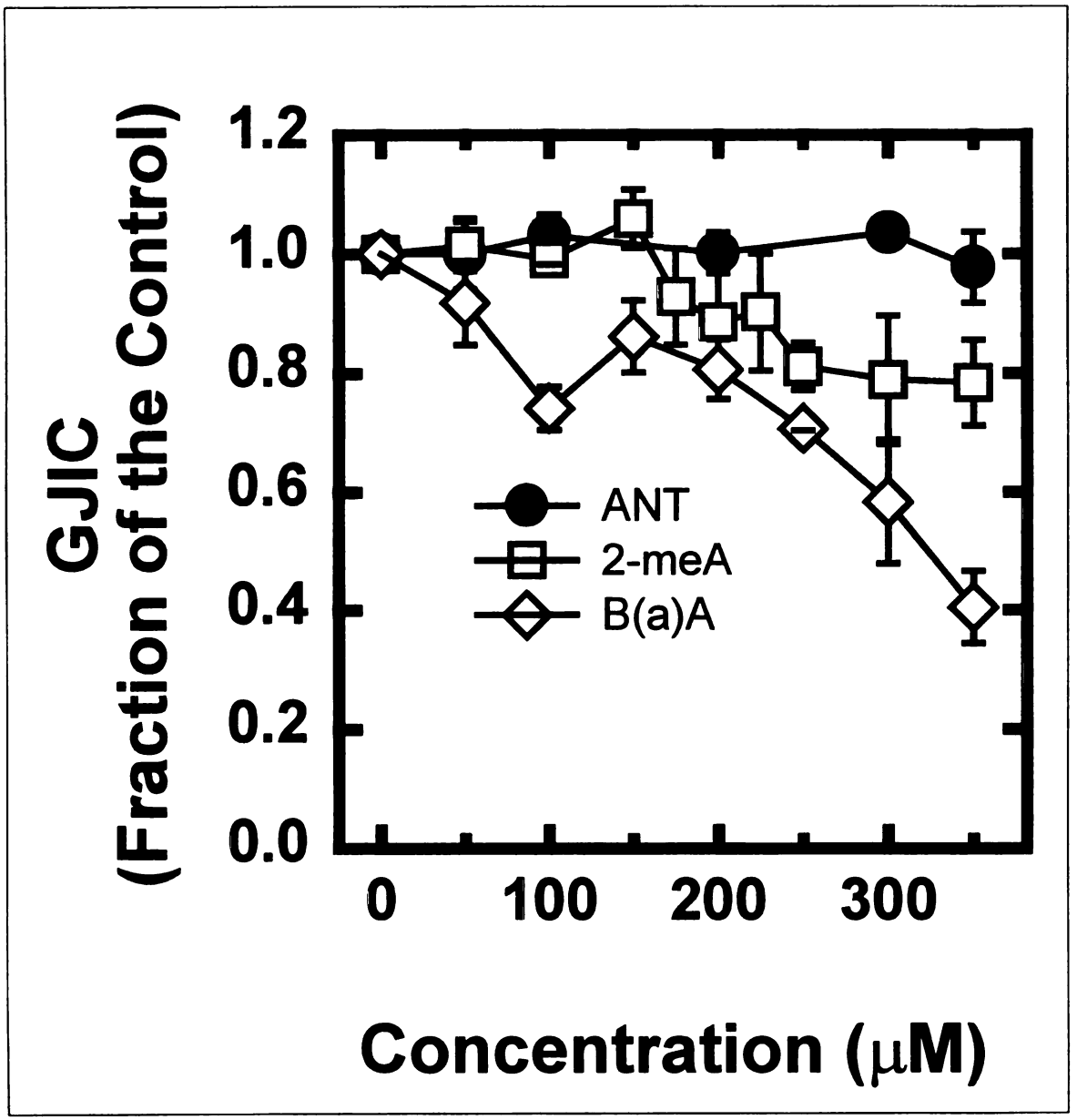


Figure 6. Blind Dose Response Results of ANT, 2-meA and B(a)A

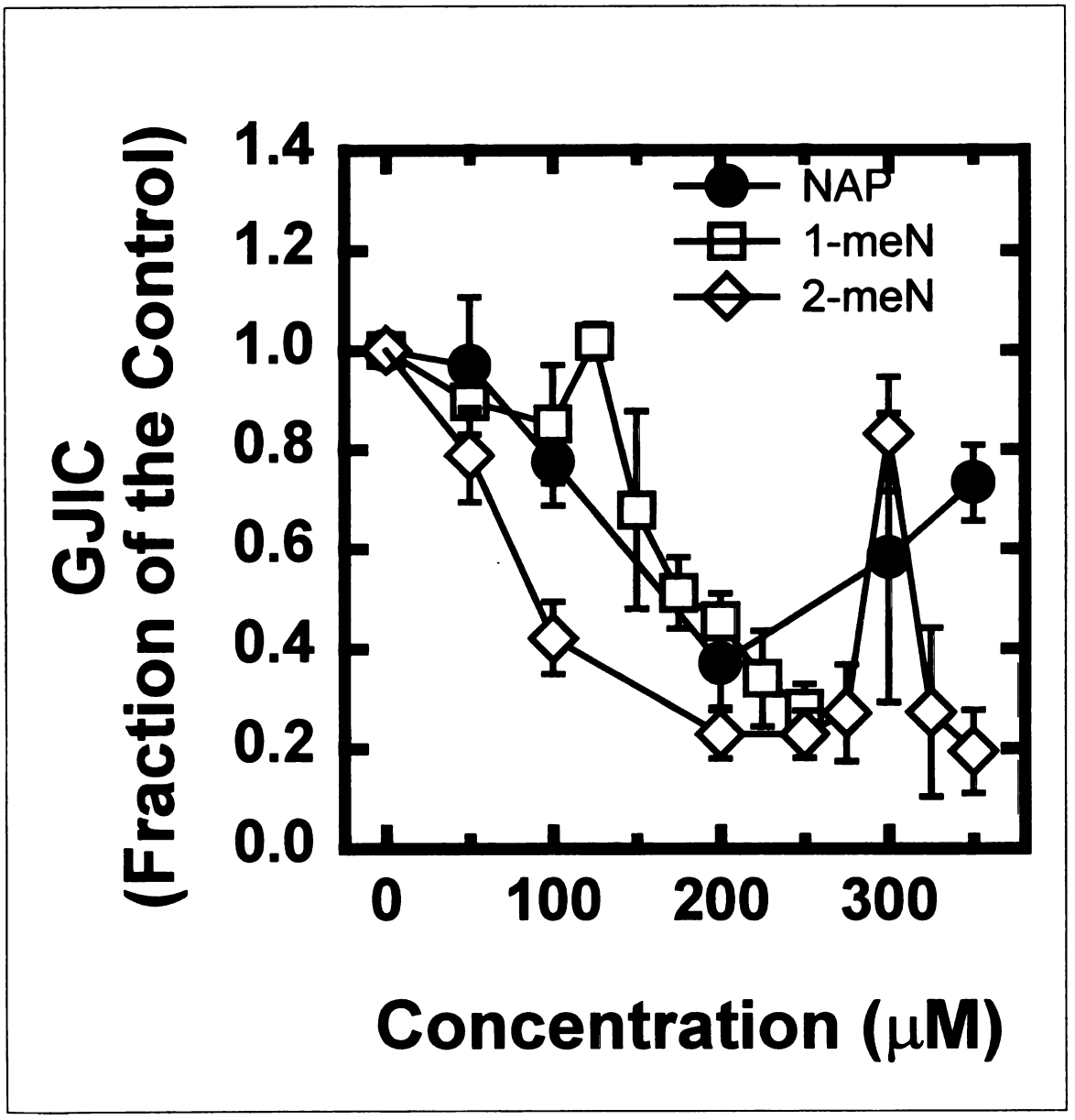


Figure 7. Blind Dose Response Results of NAP, 1-meN and 2-meN

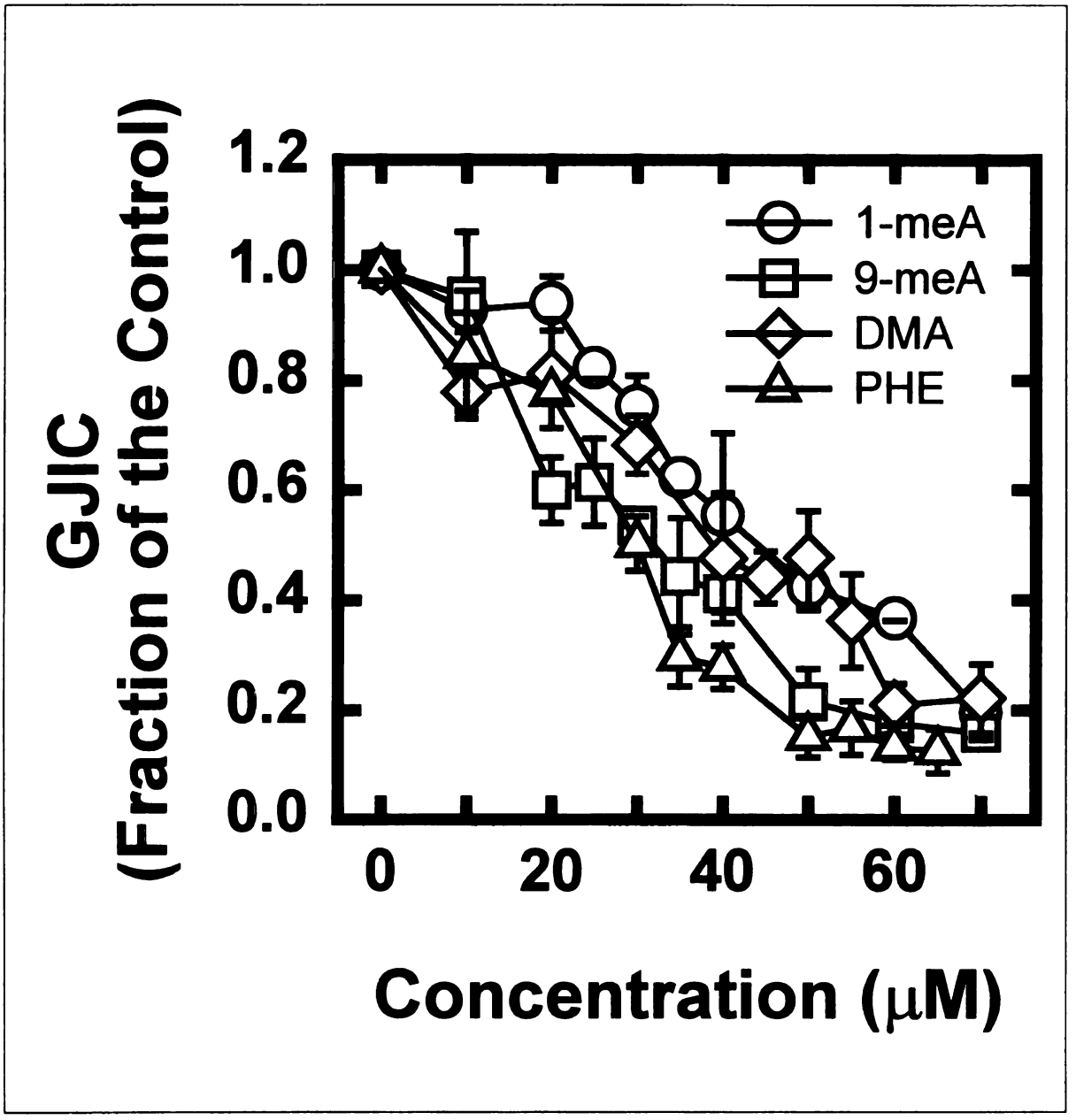


Figure 8. Blind Dose Response Results of 1-meA, 9-meA, DMA and PHE

response experiments. The values used to produce the graphs shown in Figures 3 through 8 are contained in Appendix A.

Time Response Results

The results of the time response experiments show that 1-meN and 2-meN caused inhibition of GJIC within the first 30 seconds of exposure to the target compound, as shown in Figure 9. Inhibition of GJIC in cells treated with PHE occurred within 5, minutes and cells treated with 1-meA, 9-meA and DMA showed inhibition of GJIC within 7 minutes, as shown in Figure 10. Cells treated with B(a)A showed maximum inhibition of GJIC between 25 and 40 minutes, as shown in Figure 11. The values used to produce the graphs shown in Figures 9 through 11 are contained in Appendix A.

Time Recovery Results

The results of the time recovery experiments are shown in Figure 12. These results show that inhibition of GJIC caused by the PAHs is reversible. When the cells were allowed to recover in fresh media, minus the target compound, GJIC was completely restored within 4 hours. Cells treated with B(a)A showed an increase in inhibition within the first 15 minutes, before beginning to recover. Recovery is similar for all of the compounds tested. The values used to produce the graphs shown in Figure 12 are contained in Appendix A.

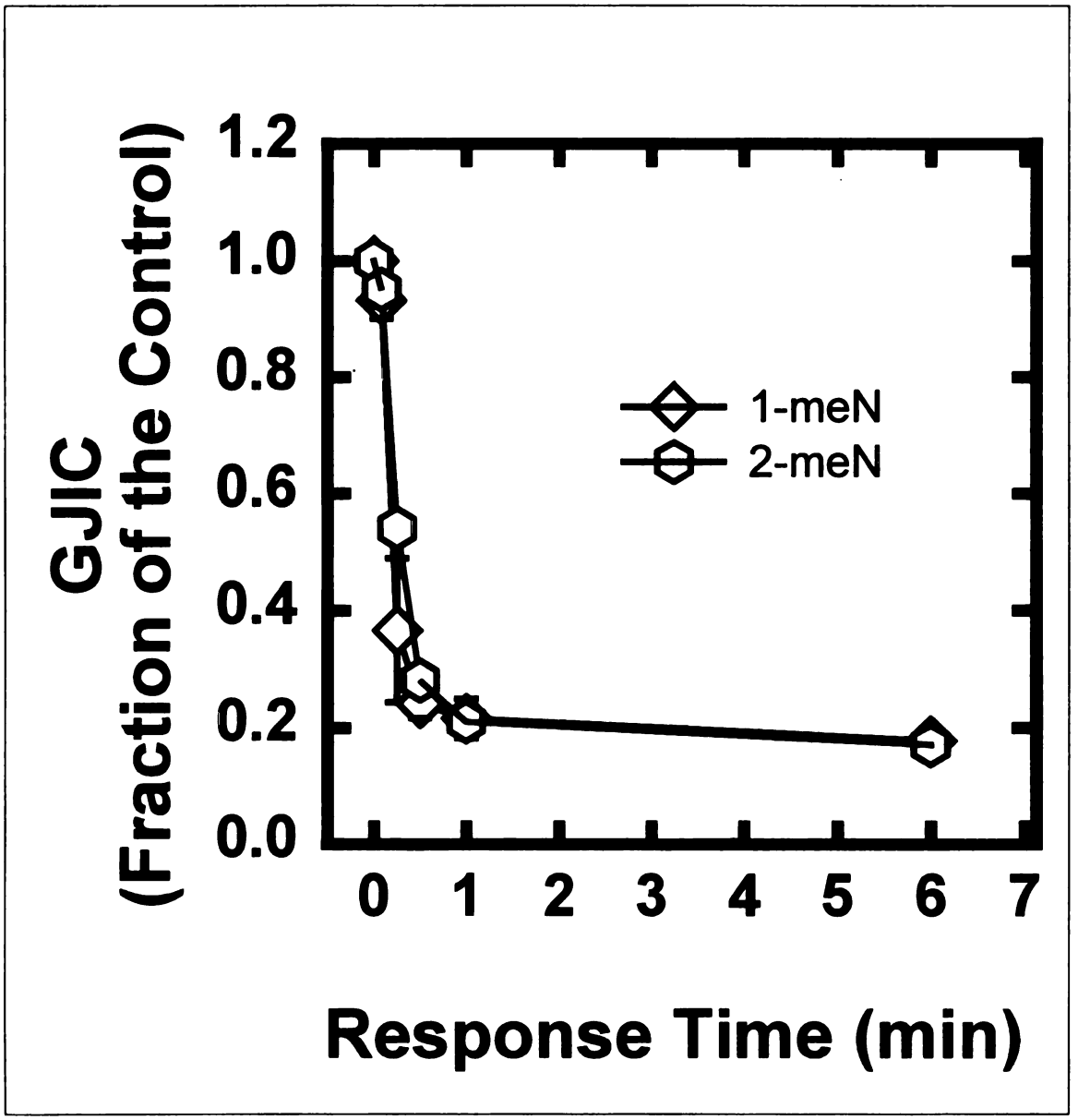


Figure 9. Time Response Results of 1-meN and 2-meN

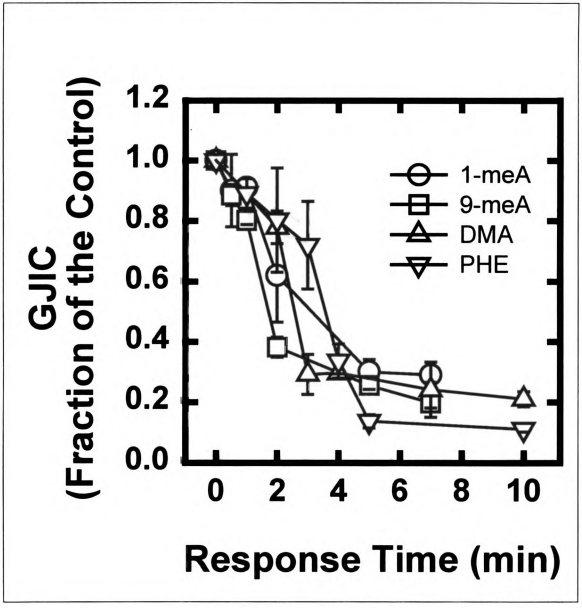


Figure 10. Time Response Results of 1-meA, 9-meA, DMA and PHE

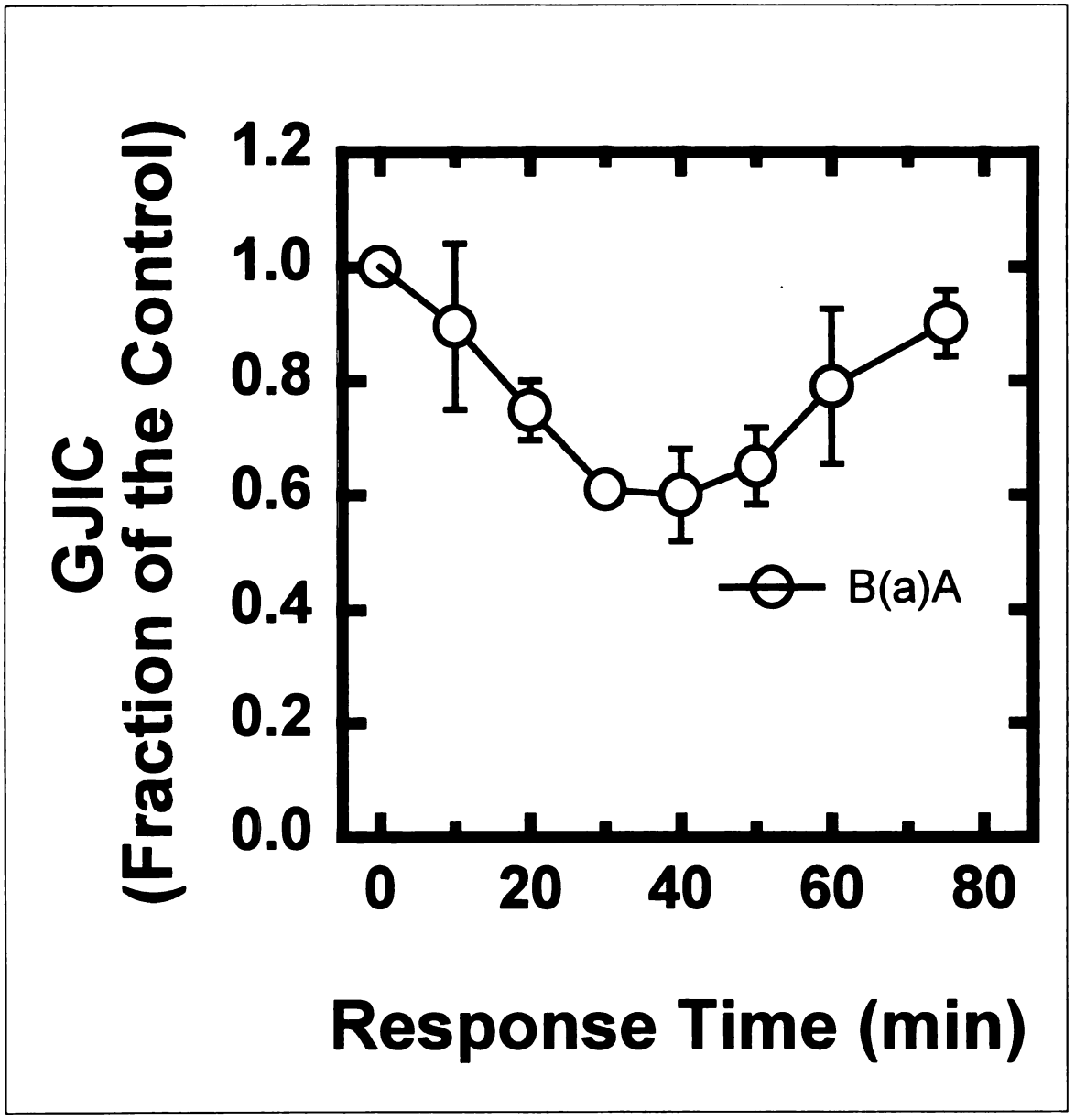


Figure 11. Time Response Results of B(a)A

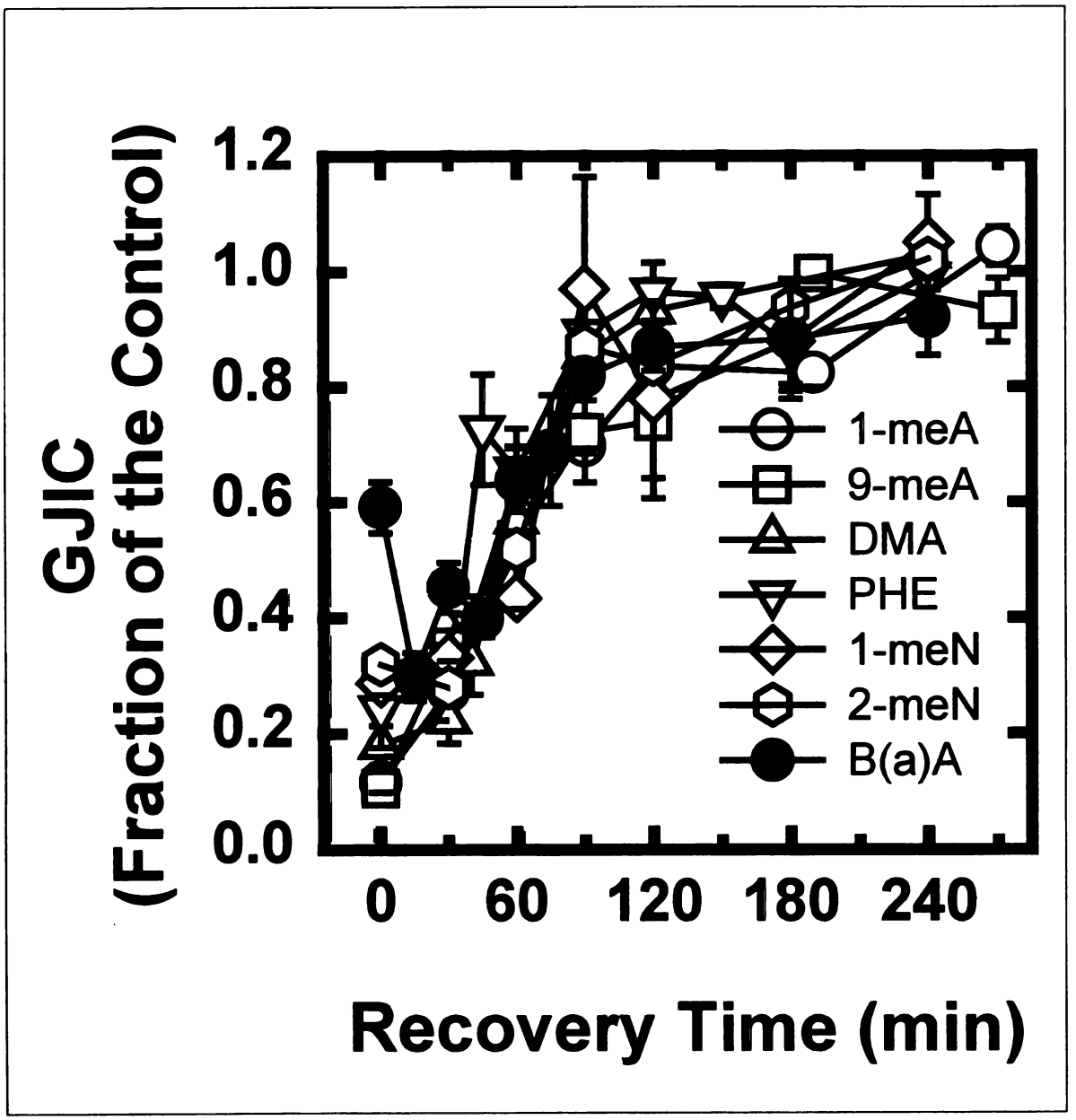


Figure 12. Time Recovery Results

Cytotoxicity Results

The results of the cytotoxicity experiments show that most of the PAHs do not induce cytotoxicity in the cells. 1-MeN was cytotoxic at concentrations above 250 μM, as shown in Figure 13, when treated with the target compound for 4 minutes. 2-MeN was somewhat cytotoxic to the cells at 450 μM, also shown in Figure 13, when treated with the target compound for 4 minutes. None of the other target compounds were cytotoxic to the cells within the range of doses tested (Figures 13, 14 and 15). The values used to produce the graphs shown in Figures 13 through 15 are contained in Appendix A.

Discussion

The results of the time recovery experiments show that inhibition of GJIC induced by these compounds is a reversible process in WB-F344 rat liver epithelial cells. Recovery time was shown to be similar for all of the compounds, suggesting that these compounds may inhibit GJIC by the same mechanism. Recovery of GJIC also shows that the cells were not killed by the target compound at the inhibiting dose. The reversible inhibition of GJIC induced by these chemicals is consistent with the reversible nature of tumor promotion *in vivo* (Trosko *et al.*, 1993).

All of the chemicals caused inhibition of GJIC in a relatively short period of time, within 1 to 25 minutes. This suggests that the mechanism of inhibition of GJIC is a post-translational modification of the gap junction proteins. Since

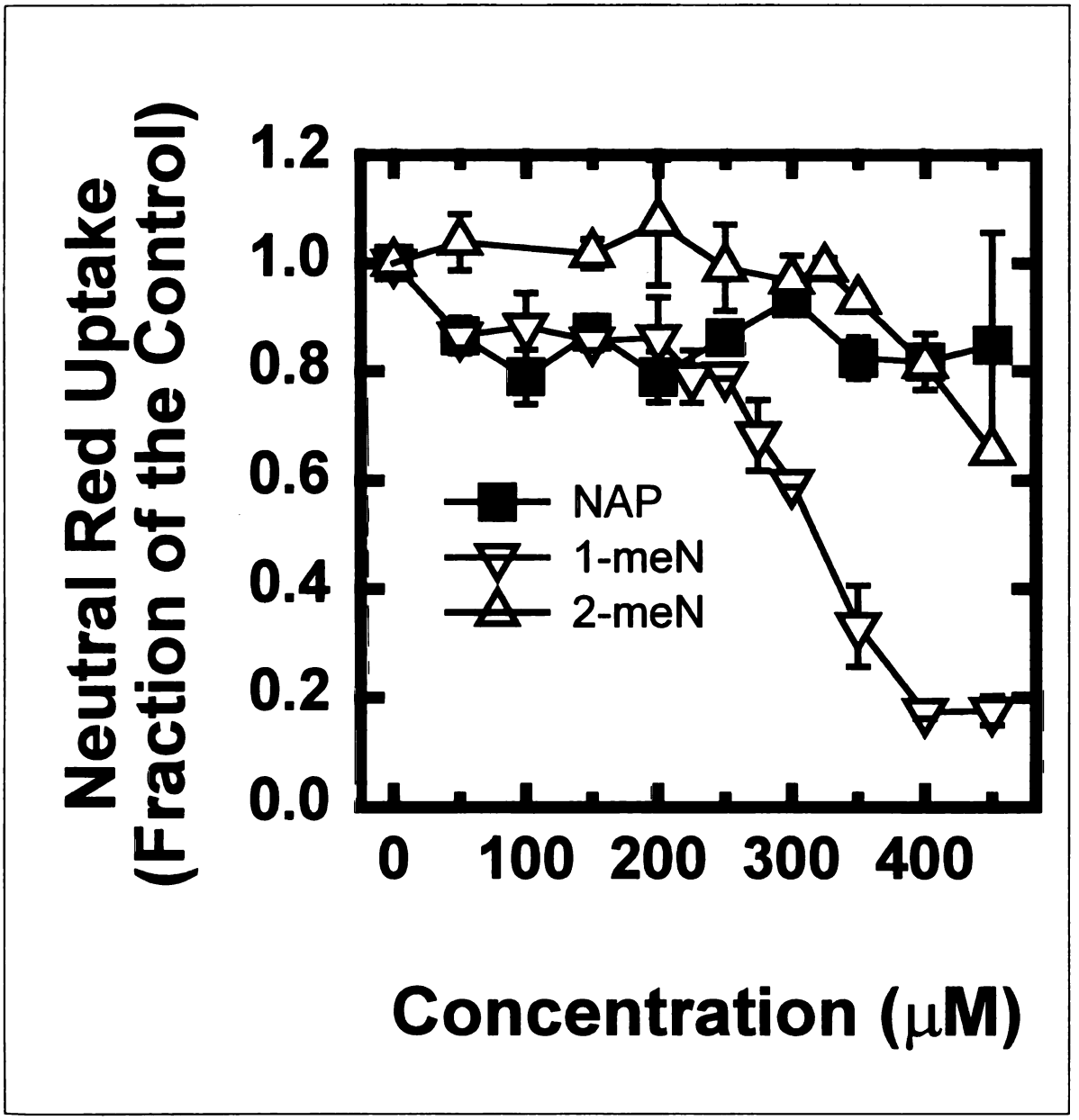


Figure 13. Cytotoxicity Results of NAP, 1-meN and 2-meN

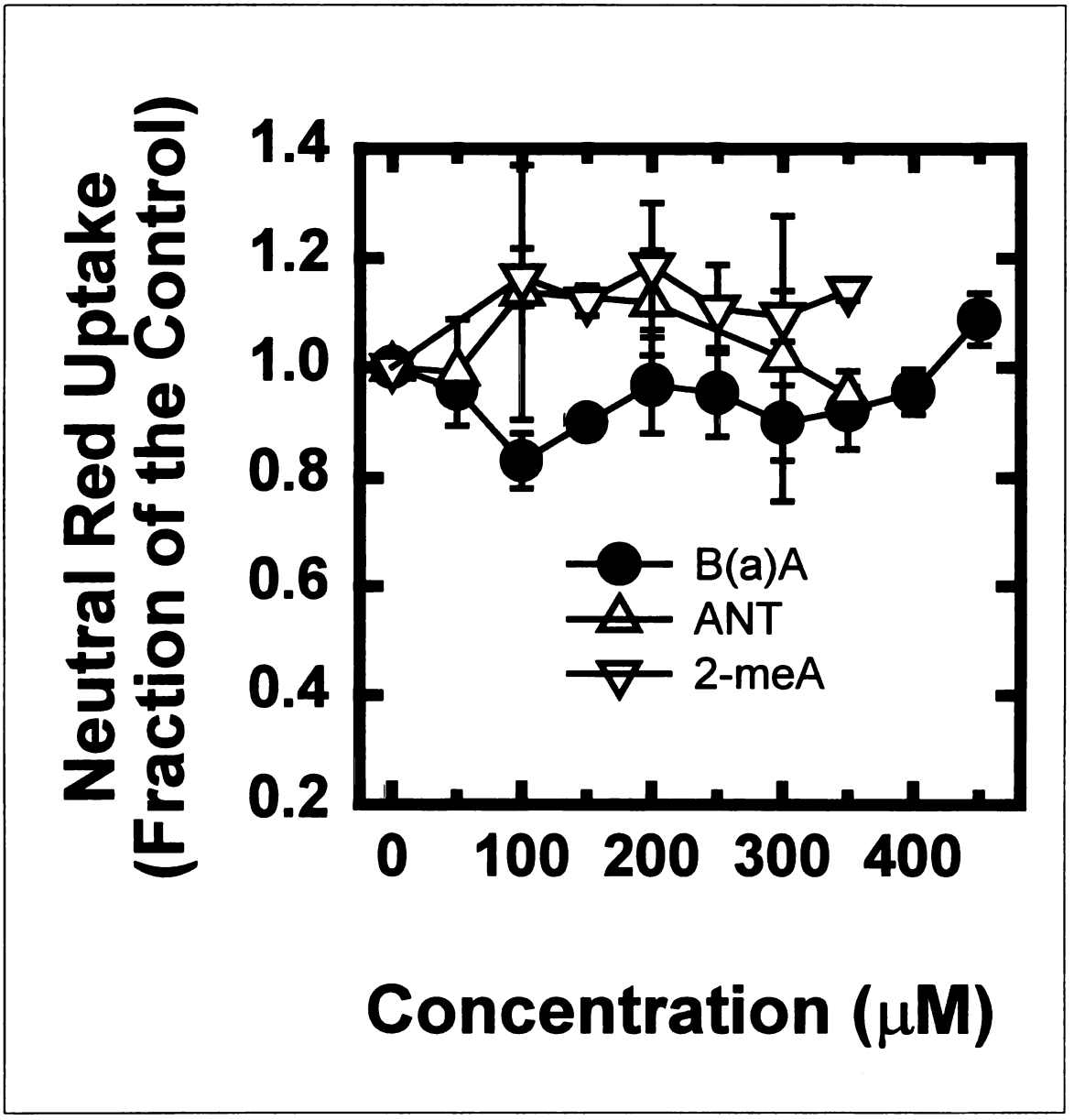


Figure 14. Cytotoxicity Results of B(a)A, ANT and 2-meA

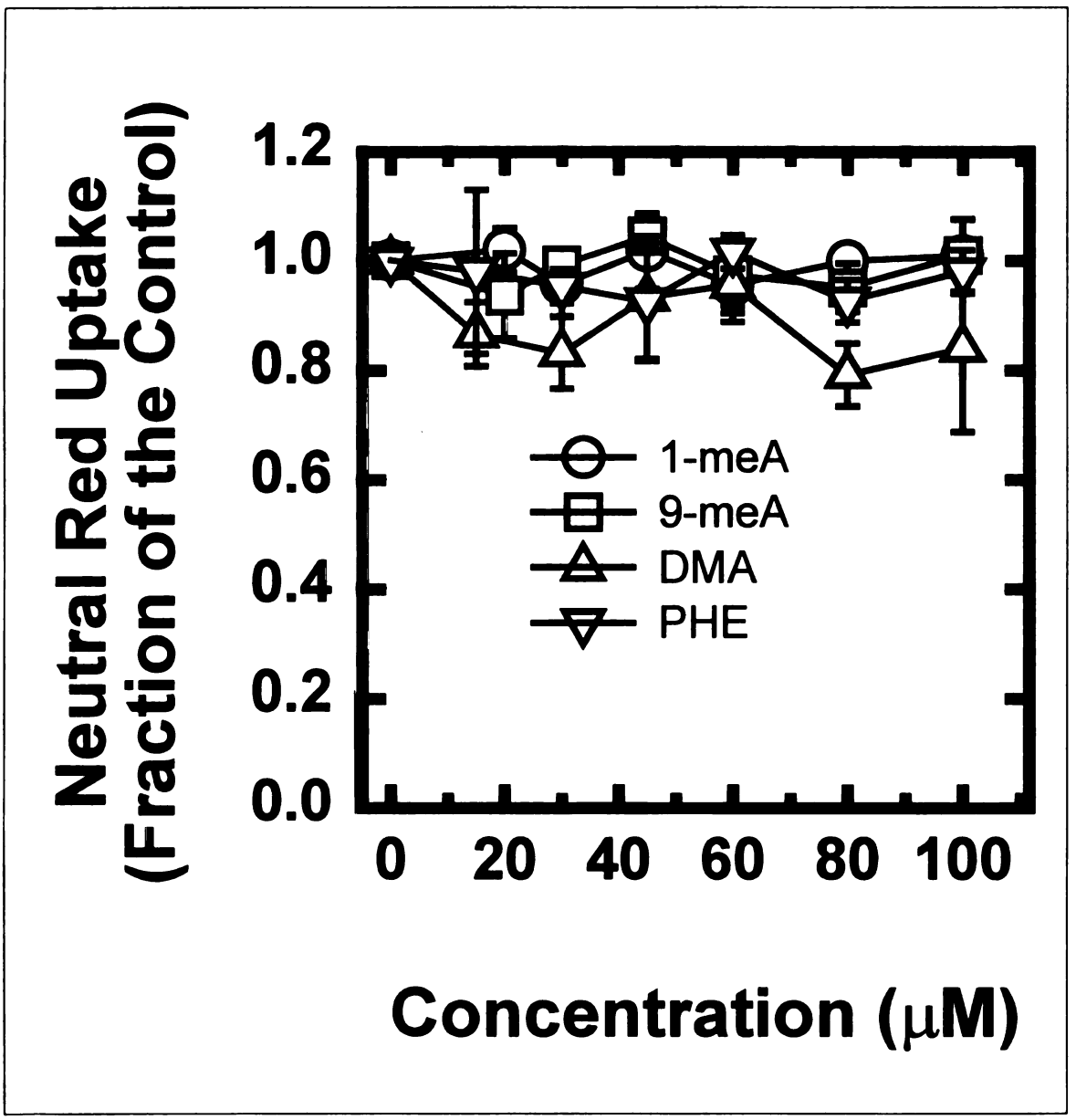


Figure 15. Cytotoxicity Results of 1-meA, 9-meA, DMA and PHE

WB-F344 rat liver epithelial cells have low mixed function oxidase activity, a quick response time also suggests that the metabolites of the compound are not involved in inhibition of GJIC (Upham *et al.*, 1996).

The results of the neutral red assay show that most of the PAHs do not cause cytotoxicity, which means that inhibition of GJIC is not due to cell death. Only 1-meN and 2-meN induce cytotoxicity; however, cytotoxicity occurs at concentrations much higher than that which causes inhibition.. It is uncertain whether or not inhibition of GJIC caused by the methylated naphthalenes is due to the cytotoxicity of the compounds. In both the dose response experiments and cytotoxicity experiments, the cells were exposed to the target compound for only 4 minutes, since inhibition of GJIC by 1-meN and 2-meN occurs within 30 seconds. A cytotoxic response induced by the methylated naphthalenes may not occur as quickly as inhibition of GJIC. Exposing the cells to the target compound for a longer period of time may cause cytotoxicity at lower doses. If these lower cytotoxic doses coincide with the inhibiting doses, it would indicate that inhibition of GJIC is a preliminary response to cell death. If no cytotoxicity is observed at lower doses over longer exposure times, then inhibition of GJIC is not due to cell death. Future experiments are being planned to determine if inhibition of GJIC by the methylated naphthalenes is due to cytotoxicity.

The results of the dose response experiments show that 1-meN, which contains a bay-like region, is a more potent inhibitor of GJIC than both 2-meN and NAP, neither of which possesses a bay-like region. In the case of the

methylated anthracenes, 1-meA, 9-meA and DMA all possess a bay-like region and are inhibitory to GJIC, whereas ANT and 2-meA are not inhibitory to GJIC and do not possess a bay-like region. These results suggest that the formation of a bay-like region by a methyl group enhances the potential of a PAH to inhibit GJIC, within a group of similar PAHs. It was also shown that 9-meA and DMA inhibit GJIC at the same concentration as 1-meA. Both 9-meA and DMA possess multiple bay-like regions, while 1-meA possesses only one bay-like region. This suggests that the presence of multiple bay-like regions does not significantly increase the potency of the compound to inhibit GJIC when compared to a compound which contains a single bay-like region.

Previous studies by Upham *et al.* (1996) showed 1-meA to be more inhibitory to GJIC than 9-meA in WB-F344 rat liver epithelial cells, and inhibition of GJIC occurred in a very narrow dose range. In this study, there were no noticeable differences between the inhibiting doses of 1-meA and 9-meA, and inhibition of GJIC occurred over a much wider dose range. The differences between the study by Upham *et al.* (1996) and this study suggest that the cell culture used in this study was more morphologically heterogeneous compared to the cell culture used in the study by Upham *et al.* These differences do not invalidate this study, they simply mean that a more morphologically heterogeneous cell culture may make it difficult to detect relatively small differences between inhibiting doses of 1-meA, 9-meA, DMA and PHE.

The methylated anthracenes are more potent inhibitors of GJIC than the methylated naphthalenes. The methylated anthracenes possess more benzene rings in a straight chain than the methylated naphthalenes. When comparing the doses at which the methylated anthracenes and the methylated naphthalenes inhibit GJIC, it would seem that increasing the number of benzene rings in a straight chain, increases the potency of the compound to inhibit GJIC. However, in the case of B(a)A and PHE, B(a)A contains more benzene rings in a straight chain than PHE, yet it is not as inhibitory to GJIC. In addition, 2-meA has more benzene rings in a straight chain than 2-meN, yet 2-meA does not inhibit GJIC whereas 2-meA does. These results seem to suggest that the number of benzene rings in a straight chain is not correlated with the potency of the compound to inhibit GJIC.

The following physicochemical properties of the PAHs were plotted against both the inhibiting response time and the inhibiting dose, in order to determine if a correlation exists; aqueous solubility (Sol), the octanol/water partition coefficient (K_{ow}), and the bioconcentration factor (BCF). The values for the inhibiting response time, inhibiting dose, log Sol, log K_{ow}, and log BCF of each compound are listed in Table 3. Some of the values for Sol, K_{ow}, and BCF were calculated using estimation methods by Lyman *et al.* (1990). Adescription of these methods is included in Appendix B.

Toraason *et al.* (1992) showed that the dose at which halogenated hydrocarbons inhibit communication in rat heart cells is linearly related to the

log K_{ow}. As shown in Figures 16 through 18, there seems to be a correlation between inhibiting dose and each of the physicochemical properties as indicated by the correlation coefficients for a linear regression. Inhibiting response time seems to be more strongly correlated to each of the physicochemical parameters as shown in Figures 19 through 21, and as indicated by the correlation coefficients for a linear regression. In each of these cases, more data points are needed in order to determine whether or not a definite correlation exists. Similar results were obtained for both the BCF and the K_{ow} which is not surprising since the BCF was calculated using the K_{ow} of the compound.

Table 3. Selected Physicochemical Properties of the PAHs

Compound	Inhibiting Dose (μM)	Response Time (min)	-log Sol (M)	log K_{ow}[c]	log BCF[d] (g/g)
NAP			3.61[b]	3.36[b]	2.09[a]
1-meN	225	0.5	3.55[a]	4.02[a]	2.59[a]
2-meN	350	0.5	3.64[a]	4.02[a]	2.59[a]
ANT			6.46[b]	4.54[b]	2.98[a]
1-meA	70	7	5.51[a]	5.20[a]	3.47[a]
2-meA			6.68[a]	5.20[a]	3.47[a]
9-meA	70	7	5.47[a]	5.20[a]	3.47[a]
DMA	70	7	6.87[a]	5.86[a]	3.47[a]
PHE	60	5	5.20[b]	4.57[b]	3.00[a]
B(a)A		25	7.31[b]	5.91[b]	4.01[a]

[a] Calculated values using method described by Lyman *et al.* (1990)
[b] Observed values reported by Schwarzenbach *et al.* (1993)
[c] Units of log K_{ow} are (mol/L octanol)·(mol/L water)$^{-1}$
[d] In species *Daphnia pulex* (invertebrate)

The aqueous solubility of the compounds may be a limitation in these aqueous-based *in vitro* assays, especially when testing inhibition of GJIC at

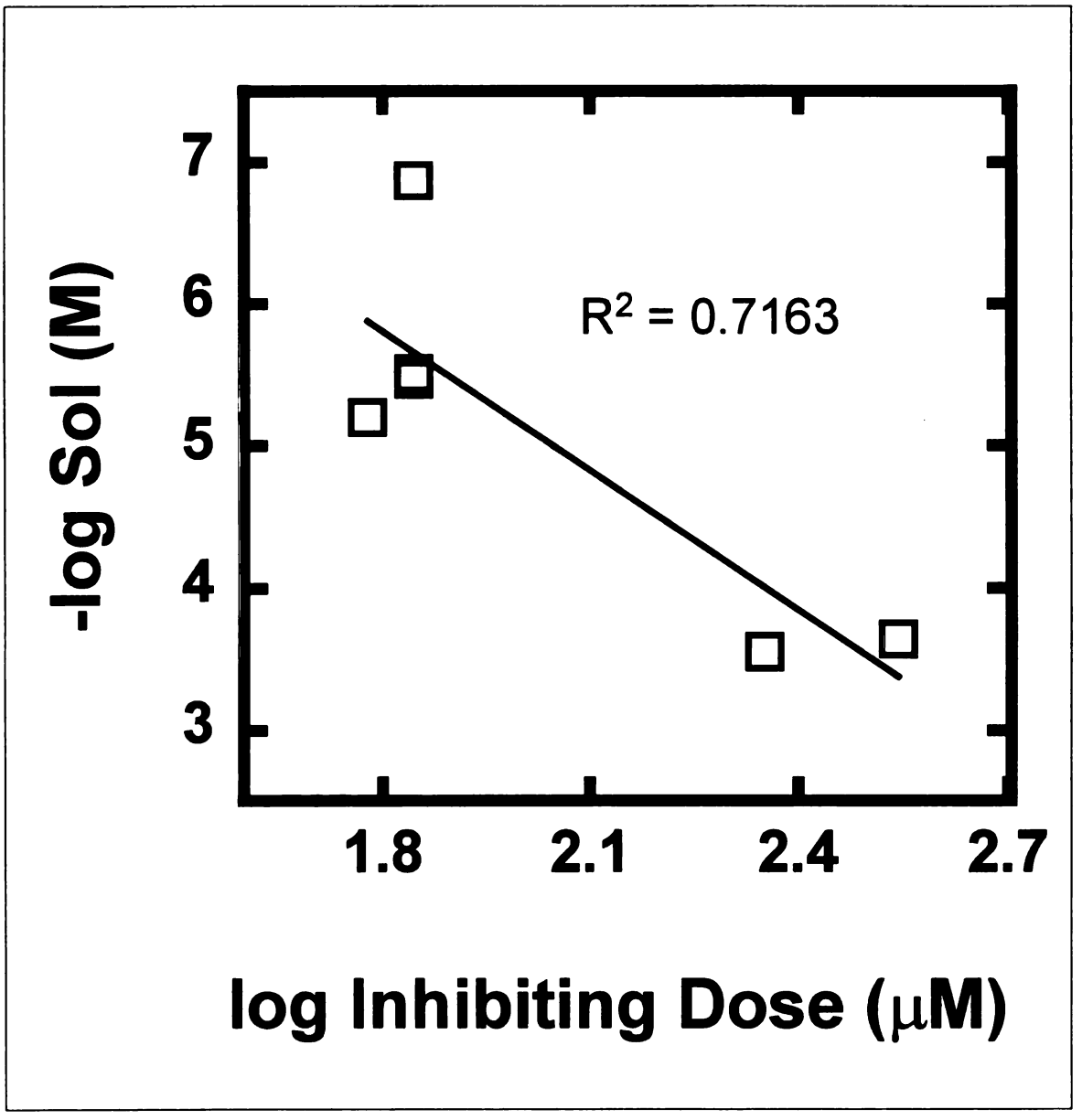


Figure 16. Correlation Between Solubility and Inhibiting Dose

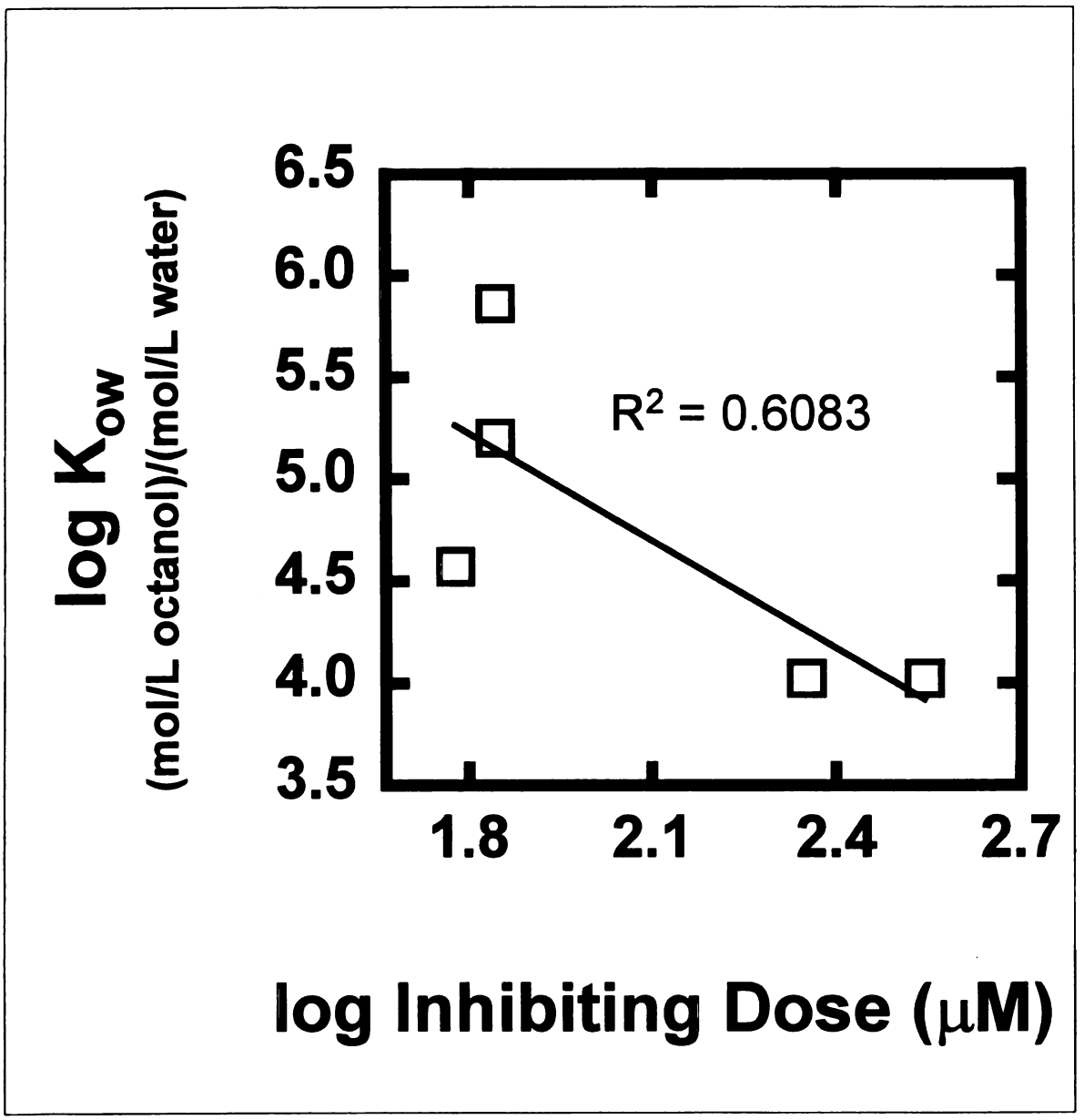


Figure 17. Correlation Between K_{ow} and Inhibiting Dose

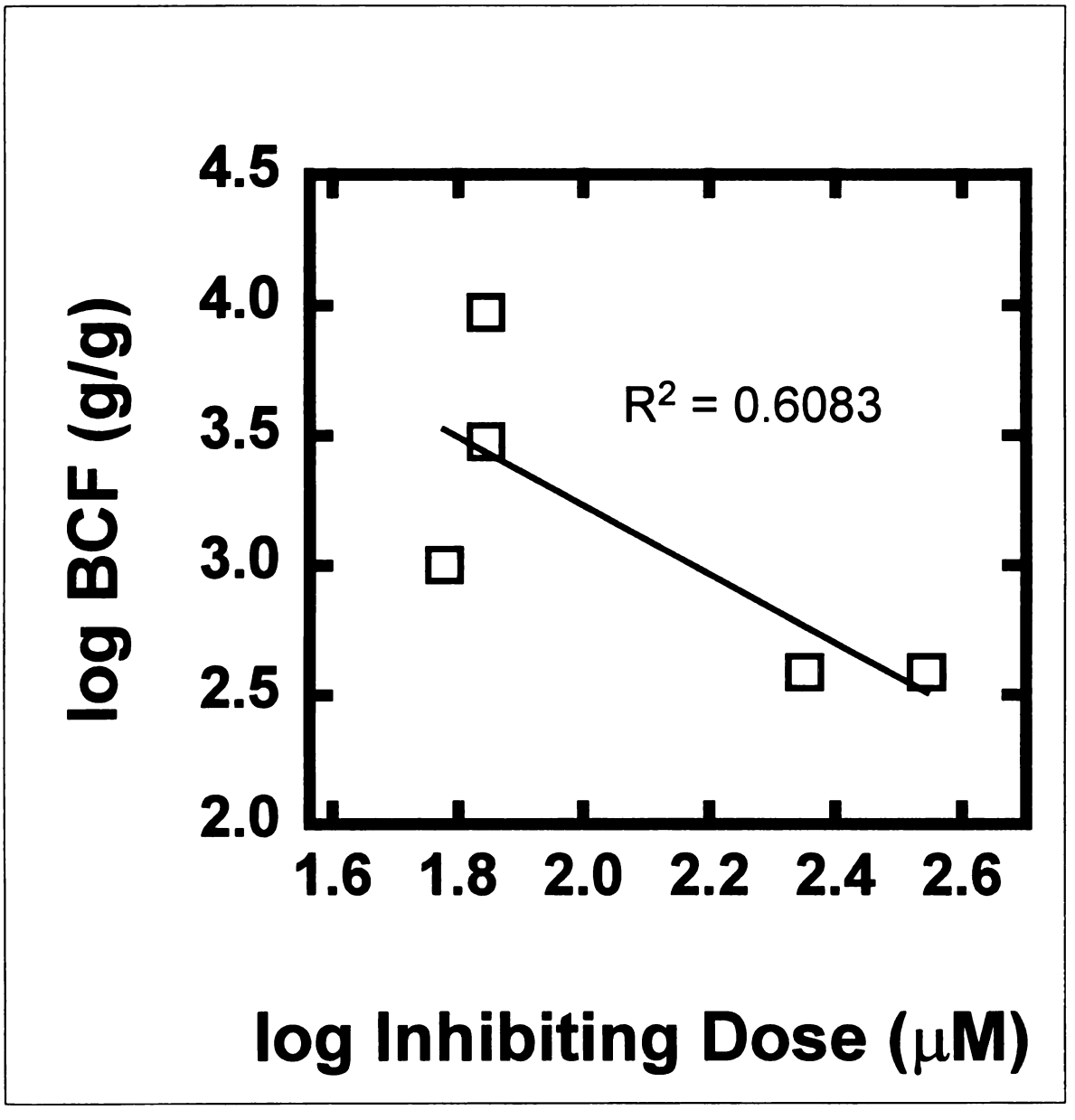


Figure 18. Correlation Between BCF and Inhibiting Dose

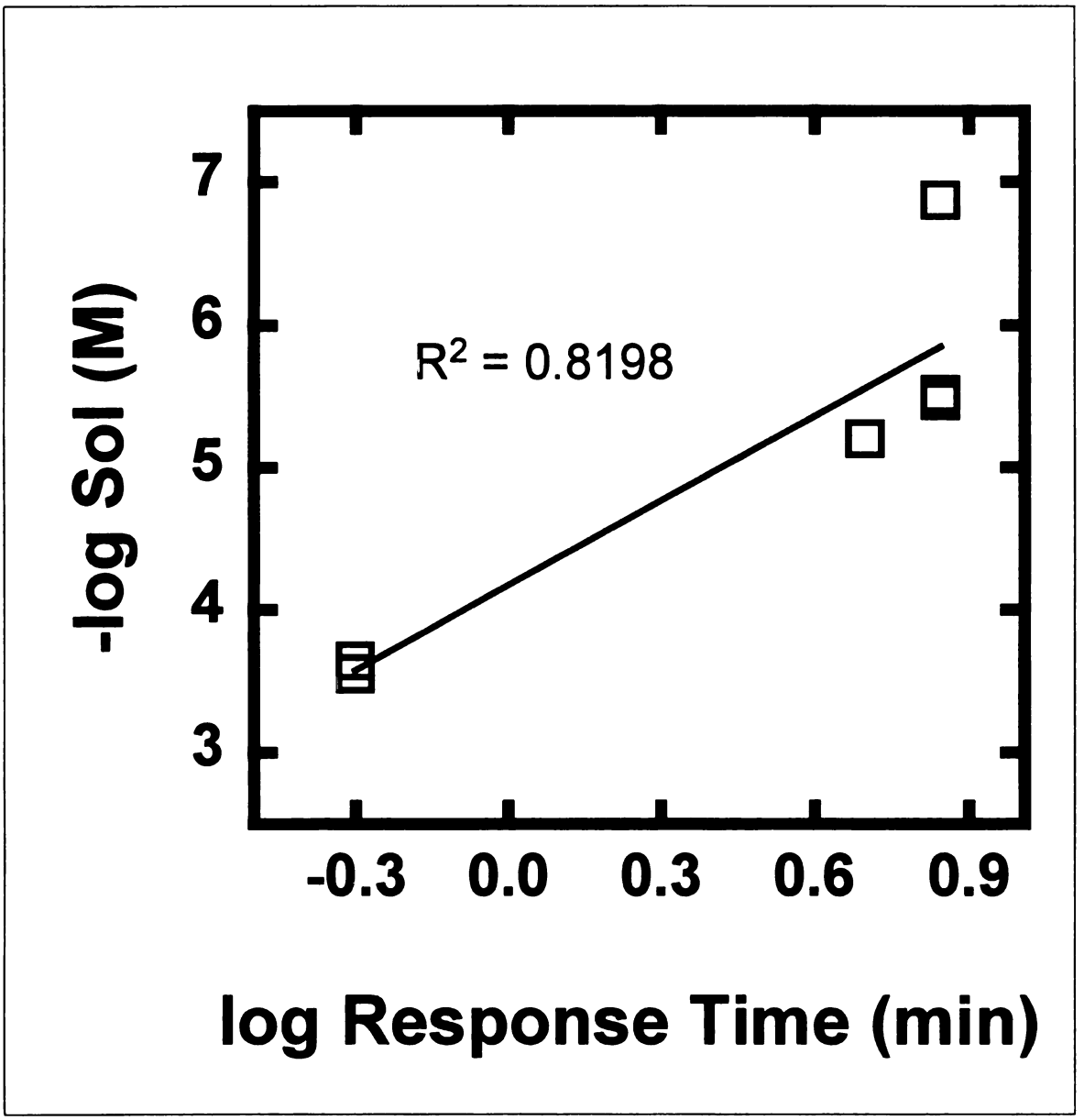


Figure 19. Correlation Between Solubility and Inhibiting Response Time

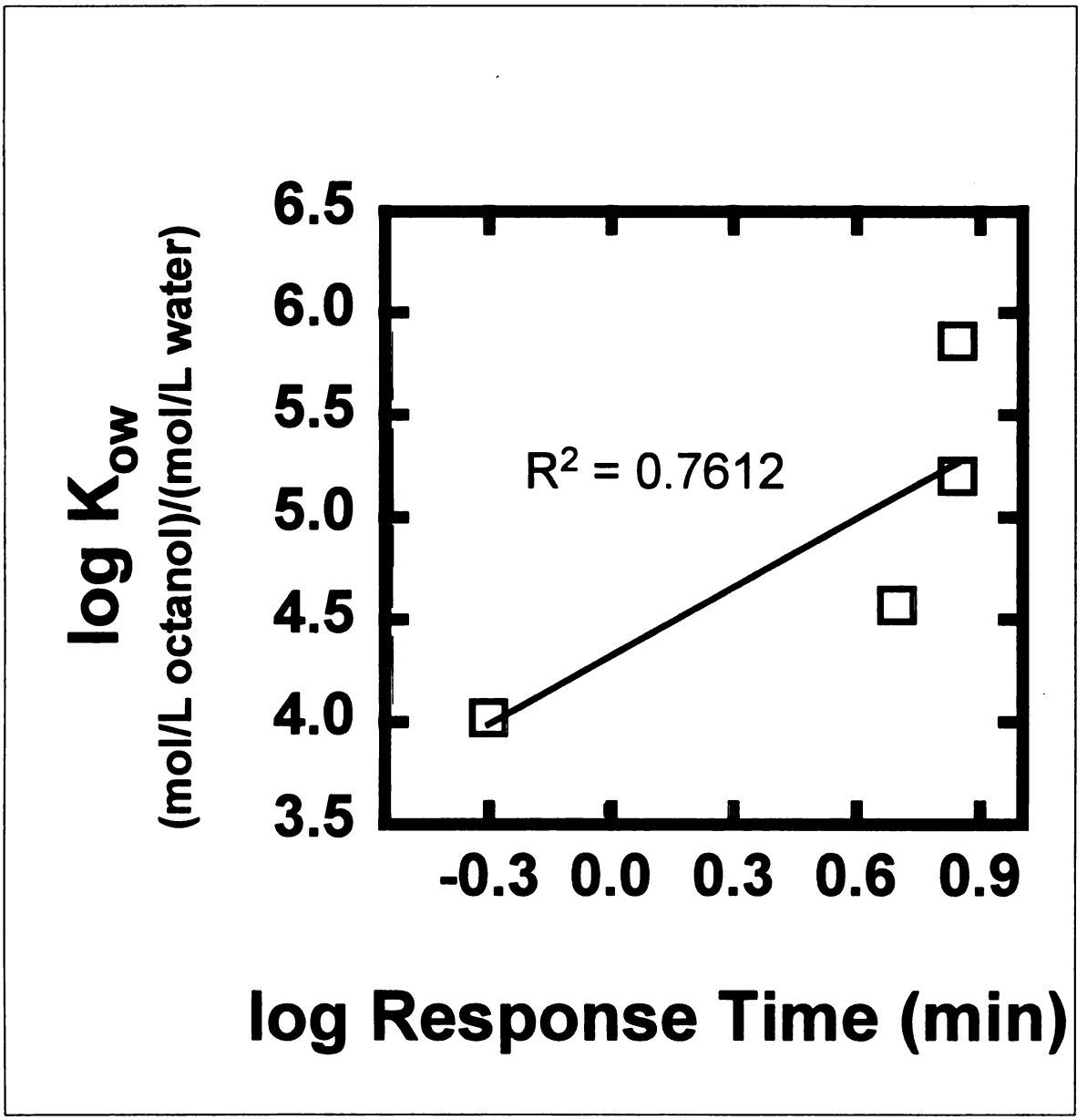


Figure 20. Correlation Between K_{ow} and Inhibiting Response Time

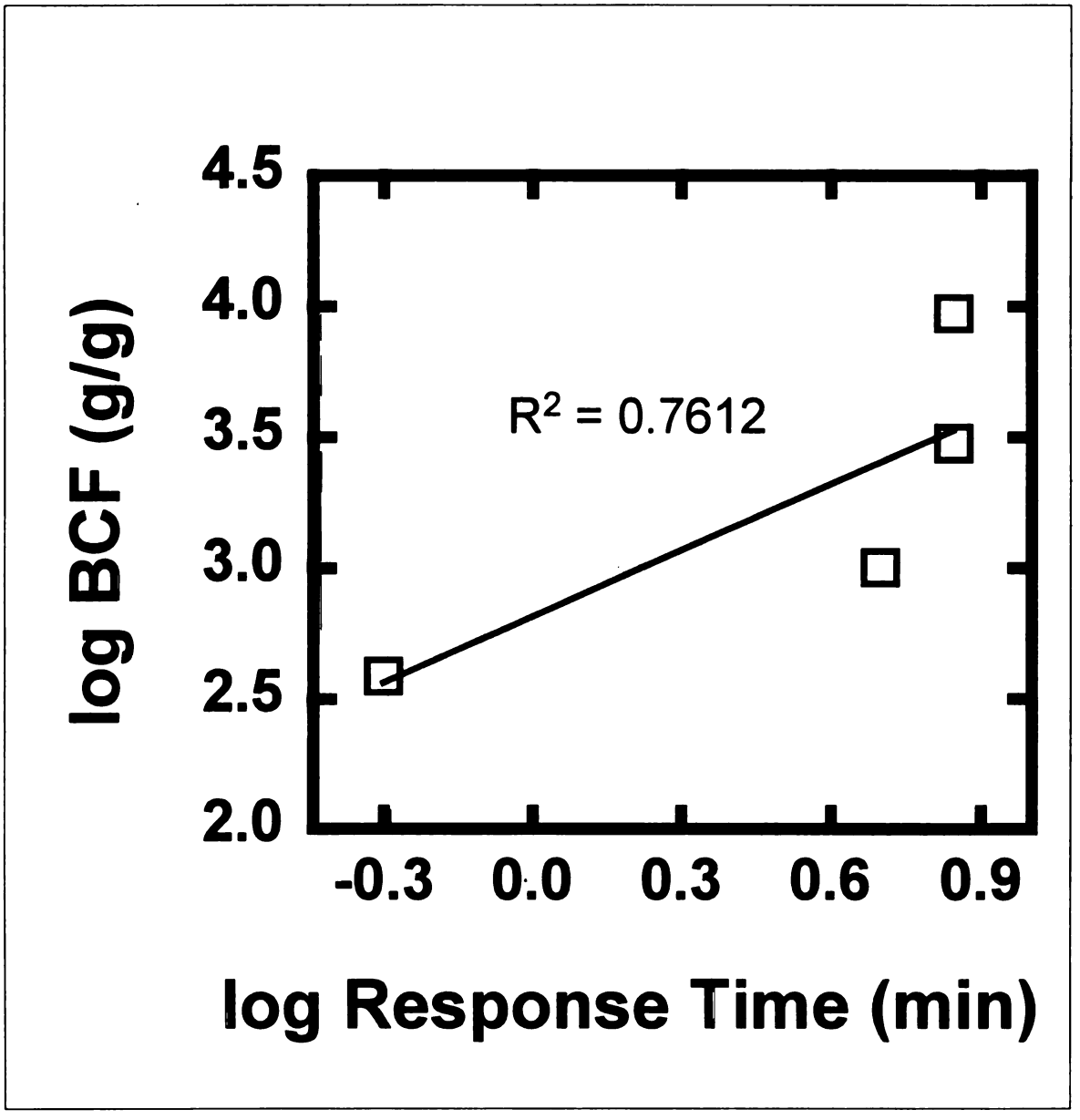


Figure 21. Correlation Between BCF and Inhibiting Response Time

higher concentrations. In general, PAHs are not very water soluble. The more benzene rings in the compound, the lower the aqueous solubility (Schwarzenbach *et al.*, 1993). Since these *in vitro* assays use an aqueous-based system, solubility of these relatively insoluble compounds could be a limiting factor in determining inhibiting potential. Precipitates in the media were noticeable when exposing the cells to higher concentrations of the target compound. At lower concentrations the precipitates were much less noticeable. The lower the aqueous solubility of the compound, the more noticeable were the precipitates for any given concentration.

When comparing compounds with similar solubility, those compounds whose structures possess a bay or bay-like region seem to be more potent in inhibiting GJIC. Comparing inhibiting doses between chemical groups such as the anthracenes and naphthalenes, did not reveal a correlation between structure and inhibition of GJIC; however, aqueous solubility may influence this comparison *in vitro*.

Conclusions

This research establishes an initial correlation *in vitro* between chemical structure and tumor promoting potential of PAHs from the same parent group. Of the compounds tested, a PAH possessing a bay-like region is a more potent inhibitor of GJIC than a similar PAH with similar solubility without a bay-like region. It is probable that solubility plays a significant role in these aqueous-

based *in vitro* assays. Determining a correlation between chemical structure and inhibition of GJIC may best be made between compounds with similar solubility. The results of this research also suggest that both inhibiting response time and inhibiting dose may be correlated to solubility, K_{ow} and BCF. The results of this study also suggest that chemical structure plays an important role in the inhibition of GJIC by PAHs and that structure-function relationships can be formed.

Future Research

Through this research study, an initial correlation has been formed between PAH structure and inhibition of GJIC. However, more experiments are needed in order to better understand the correlation. Future experiments should include the following: (1) evaluate inhibition of GJIC by PAHs which contain bay-like regions not formed by a methyl group, but by other groups such as halogens, alcohols, amino groups, etc., (2) determine if a correlation exists between the physicochemical parameters of various PAHs and inhibition of GJIC, (3) evaluate the three dimensional chemical structures of PAHs and determine if there is a correlation between the physical structure of the compound and inhibition of GJIC, (4) finally, determine if these inhibiting PAHs are tumor promoters *in vivo*. If those PAHs which inhibit GJIC are also tumor promoters, forming a predictive model will have tremendous significance in scientific research.

APPENDICES

APPENDIX A

APPENDIX A

TABLES OF EXPERIMENTAL RESULTS

Table A1. Results of the Dose Response Experiments

Chemical	Dose Applied to Cells (μM)	Exposure Time (min)	GJIC (FOC)	Standard Deviation (FOC)
anthracene	0	10	1.00	0.00
anthracene	100	10	1.01	0.02
anthracene	200	10	0.97	0.09
anthracene	250	10	0.89	0.02
anthracene	300	10	1.03	0.04
anthracene	350	10	0.93	0.04
1-methylanthracene	0	10	1.00	0.00
1-methylanthracene	10	10	0.90	0.02
1-methylanthracene	20	10	0.85	0.12
1-methylanthracene	25	10	1.02	0.04
1-methylanthracene	30	10	0.68	0.20
1-methylanthracene	40	10	0.51	0.07
1-methylanthracene	50	10	0.45	0.06
1-methylanthracene	60	10	0.34	0.10
1-methylanthracene	70	10	0.28	0.05
2-methylanthracene	0	10	1.00	0.00
2-methylanthracene	100	10	0.89	0.02
2-methylanthracene	200	10	0.81	0.06
2-methylanthracene	250	10	0.91	0.04
2-methylanthracene	350	10	0.77	0.03
9-methylanthracene	0	10	1.00	0.00
9-methylanthracene	10	10	0.92	0.06
9-methylanthracene	20	10	0.90	0.05
9-methylanthracene	25	10	0.94	0.05
9-methylanthracene	30	10	0.66	0.08
9-methylanthracene	40	10	0.63	0.05
9-methylanthracene	50	10	0.45	0.03
9-methylanthracene	60	10	0.29	0.06
9-methylanthracene	70	10	0.27	0.11
phenanthrene	0	10	1.00	0.00

APPENDIX A

Table A1 (cont'd)

phenanthrene	10	10	0.98	0.08
phenanthrene	20	10	0.88	0.06
phenanthrene	25	10	0.86	0.03
phenanthrene	30	10	0.63	0.06
phenanthrene	40	10	0.39	0.04
phenanthrene	50	10	0.23	0.05
phenanthrene	60	10	0.18	0.01
phenanthrene	70	10	0.17	0.02
9,10-dimethylanthracene	0	10	1.00	0.00
9,10-dimethylanthracene	10	10	1.08	0.04
9,10-dimethylanthracene	20	10	0.83	0.04
9,10-dimethylanthracene	30	10	0.67	0.08
9,10-dimethylanthracene	40	10	0.56	0.04
9,10-dimethylanthracene	50	10	0.33	0.03
9,10-dimethylanthracene	60	10	0.37	0.06
9,10-dimethylanthracene	70	10	0.19	0.02
naphthalene	0	4	1.00	0.00
naphthalene	50	4	0.83	0.03
naphthalene	100	4	0.74	0.14
naphthalene	150	4	0.65	0.01
naphthalene	200	4	0.50	0.04
naphthalene	250	4	0.51	0.01
naphthalene	350	4	0.67	0.07
naphthalene	450	4	0.72	0.08
1-methylnaphthalene	0	4	1.00	0.00
1-methylnaphthalene	50	4	0.70	0.05
1-methylnaphthalene	100	4	0.58	0.04
1-methylnaphthalene	150	4	0.77	0.01
1-methylnaphthalene	175	4	0.68	0.07
1-methylnaphthalene	200	4	0.47	0.07
1-methylnaphthalene	225	4	0.27	0.05
1-methylnaphthalene	250	4	0.30	0.02
1-methylnaphthalene	300	4	0.22	0.01
2-methylnaphthalene	0	4	1.00	0.00
2-methylnaphthalene	50	4	0.79	0.04
2-methylnaphthalene	100	4	0.59	0.04
2-methylnaphthalene	200	4	0.57	0.07
2-methylnaphthalene	300	4	0.55	0.08
2-methylnaphthalene	325	4	0.51	0.09
2-methylnaphthalene	350	4	0.31	0.10

APPENDIX A

Table A1 (cont'd)

benz(a)anthracene	0	30	1.00	0.00
benz(a)anthracene	50	30	1.11	0.07
benz(a)anthracene	100	30	1.09	0.08
benz(a)anthracene	150	30	0.94	0.06
benz(a)anthracene	200	30	0.75	0.08
benz(a)anthracene	250	30	0.65	0.03
benz(a)anthracene	300	30	0.70	0.04
benz(a)anthracene	325	30	0.67	0.07
benz(a)anthracene	350	30	0.57	0.02

APPENDIX A

Table A2. Results of the Blind Dose Response Experiments

Chemical	Dose Applied to Cells (μM)	Exposure Time (min)	GJIC (FOC)	Standard Deviation (FOC)
anthracene	0	10	1.00	0.00
anthracene	50	10	1.00	0.06
anthracene	100	10	1.03	0.04
anthracene	200	10	1.00	0.03
anthracene	300	10	1.04	0.02
anthracene	350	10	0.98	0.06
1-methylanthracene	0	10	1.00	0.00
1-methylanthracene	10	10	0.93	0.04
1-methylanthracene	20	10	0.94	0.05
1-methylanthracene	25	10	0.82	0.02
1-methylanthracene	30	10	0.75	0.06
1-methylanthracene	35	10	0.62	0.03
1-methylanthracene	40	10	0.55	0.15
1-methylanthracene	50	10	0.42	0.04
1-methylanthracene	60	10	0.37	0.00
1-methylanthracene	70	10	0.19	0.02
2-methylanthracene	0	10	1.00	0.00
2-methylanthracene	50	10	1.01	0.04
2-methylanthracene	100	10	0.99	0.01
2-methylanthracene	150	10	1.06	0.05
2-methylanthracene	175	10	0.93	0.08
2-methylanthracene	200	10	0.89	0.09
2-methylanthracene	225	10	0.90	0.10
2-methylanthracene	250	10	0.81	0.04
2-methylanthracene	300	10	0.79	0.11
2-methylanthracene	350	10	0.78	0.07
9-methylanthracene	0	10	1.00	0.00
9-methylanthracene	10	10	0.95	0.12
9-methylanthracene	20	10	0.60	0.06
9-methylanthracene	25	10	0.61	0.08
9-methylanthracene	30	10	0.54	0.03
9-methylanthracene	35	10	0.44	0.11
9-methylanthracene	40	10	0.41	0.03
9-methylanthracene	50	10	0.22	0.06
9-methylanthracene	60	10	0.18	0.04
9-methylanthracene	70	10	0.16	0.01

APPENDIX A

Table A2 (cont'd)

phenanthrene	0	10	1.00	0.00
phenanthrene	10	10	0.85	0.12
phenanthrene	20	10	0.78	0.01
phenanthrene	30	10	0.50	0.05
phenanthrene	35	10	0.30	0.05
phenanthrene	40	10	0.28	0.04
phenanthrene	50	10	0.15	0.04
phenanthrene	55	10	0.17	0.05
phenanthrene	60	10	0.13	0.02
phenanthrene	65	10	0.13	0.04
9,10-dimethylanthracene	0	10	1.00	0.00
9,10-dimethylanthracene	10	10	0.78	0.03
9,10-dimethylanthracene	20	10	0.81	0.10
9,10-dimethylanthracene	30	10	0.68	0.05
9,10-dimethylanthracene	40	10	0.48	0.12
9,10-dimethylanthracene	45	10	0.44	0.05
9,10-dimethylanthracene	50	10	0.48	0.09
9,10-dimethylanthracene	55	10	0.36	0.08
9,10-dimethylanthracene	60	10	0.21	0.04
9,10-dimethylanthracene	70	10	0.22	0.06
naphthalene	0	4	1.00	0.00
naphthalene	50	4	0.97	0.14
naphthalene	100	4	0.78	0.09
naphthalene	200	4	0.37	0.09
naphthalene	300	4	0.58	0.29
naphthalene	350	4	0.73	0.08
1-methylnaphthalene	0	4	1.00	0.00
1-methylnaphthalene	50	4	0.72	0.04
1-methylnaphthalene	100	4	0.65	0.10
1-methylnaphthalene	125	4	0.48	0.08
1-methylnaphthalene	150	4	0.55	0.19
1-methylnaphthalene	175	4	0.38	0.16
1-methylnaphthalene	200	4	0.76	0.07
1-methylnaphthalene	225	4	0.38	0.17
1-methylnaphthalene	250	4	0.83	0.06
1-methylnaphthalene	300	4	0.19	0.04
1-methylnaphthalene	350	4	0.18	0.01
2-methylnaphthalene	0	4	1.00	0.00
2-methylnaphthalene	50	4	0.79	0.09
2-methylnaphthalene	100	4	0.42	0.07

APPENDIX A

Table A2 (cont'd)

2-methylnaphthalene	200	4	0.23	0.05
2-methylnaphthalene	250	4	0.23	0.05
2-methylnaphthalene	275	4	0.27	0.10
2-methylnaphthalene	300	4	0.83	0.12
2-methylnaphthalene	325	4	0.27	0.17
2-methylnaphthalene	350	4	0.19	0.08
benz(a)anthracene	0	25	1.00	0.00
benz(a)anthracene	50	25	0.92	0.07
benz(a)anthracene	100	25	0.74	0.04
benz(a)anthracene	150	25	0.86	0.06
benz(a)anthracene	200	25	0.81	0.05
benz(a)anthracene	250	25	0.71	0.00
benz(a)anthracene	300	25	0.58	0.10
benz(a)anthracene	350	25	0.40	0.06

APPENDIX A

Table A3. Results of the Time Response Experiments

Chemical	Exposure Time (min)	Dose Applied to Cells (μM)	GJIC (FOC)	Standard Deviation (FOC)
1-methylanthracene	0	50	1.00	0.00
1-methylanthracene	0.5	50	0.90	0.12
1-methylanthracene	1	50	0.91	0.01
1-methylanthracene	2	50	0.62	0.15
1-methylanthracene	5	50	0.30	0.04
1-methylanthracene	7	50	0.29	0.02
9-methylanthracene	0	50	1.00	0.00
9-methylanthracene	0.5	50	0.88	0.04
9-methylanthracene	1	50	0.80	0.01
9-methylanthracene	2	50	0.38	0.03
9-methylanthracene	5	50	0.26	0.01
9-methylanthracene	7	50	0.20	0.02
9,10-dimethylanthracene	0	75	1.00	0.00
9,10-dimethylanthracene	2	75	0.78	0.05
9,10-dimethylanthracene	3	75	0.29	0.07
9,10-dimethylanthracene	4	75	0.30	0.00
9,10-dimethylanthracene	7	75	0.24	0.09
9,10-dimethylanthracene	10	75	0.21	0.02
9,10-dimethylanthracene	30	75	0.19	0.02
phenanthrene	0	50	1.00	0.00
phenanthrene	1	50	0.89	0.05
phenanthrene	2	50	0.80	0.17
phenanthrene	3	50	0.72	0.14
phenanthrene	4	50	0.34	0.06
phenanthrene	5	50	0.14	0.02
phenanthrene	10	50	0.11	0.01
1-methylnaphthalene	0	200	1.00	0.00
1-methylnaphthalene	0.083	200	0.93	0.03
1-methylnaphthalene	0.25	200	0.37	0.12
1-methylnaphthalene	0.5	200	0.24	0.02
1-methylnaphthalene	1	200	0.22	0.03
1-methylnaphthalene	6	200	0.18	0.02
2-methylnaphthalene	0	320	1.00	0.00
2-methylnaphthalene	0.083	320	0.95	0.05
2-methylnaphthalene	0.25	320	0.54	0.17
2-methylnaphthalene	0.5	320	0.28	0.10

APPENDIX A

Table A3 (cont'd)

2-methylnaphthalene	1	320	0.21	0.06
2-methylnaphthalene	6	320	0.17	0.01
benz(a)anthracene	0	350	1.00	0.00
benz(a)anthracene	10	350	0.90	0.15
benz(a)anthracene	20	350	0.75	0.05
benz(a)anthracene	30	350	0.61	0.01
benz(a)anthracene	40	350	0.60	0.08
benz(a)anthracene	50	350	0.65	0.07
benz(a)anthracene	60	350	0.79	0.14
benz(a)anthracene	75	350	0.90	0.06

APPENDIX A

Table A4. Results of the Time Recovery Experiments

Chemical	Recovery Time (min)	Exposure Time (min)	Dose Applied to Cells (μM)	GJIC (FOC)	Standard Deviation (FOC)
1-methylanthracene	0	20	60	0.12	0.01
1-methylanthracene	30	20	60	0.27	0.06
1-methylanthracene	60	20	60	0.64	0.06
1-methylanthracene	90	20	60	0.70	0.01
1-methylanthracene	120	20	60	0.84	0.03
1-methylanthracene	190	20	60	0.82	0.03
1-methylanthracene	270	20	60	1.04	0.03
9-methylanthracene	0	20	60	0.11	0.01
9-methylanthracene	30	20	60	0.35	0.01
9-methylanthracene	60	20	60	0.54	0.08
9-methylanthracene	90	20	60	0.72	0.09
9-methylanthracene	120	20	60	0.74	0.10
9-methylanthracene	190	20	60	1.00	0.01
9-methylanthracene	270	20	60	0.93	0.06
9-methylanthracene	420	20	60	1.13	0.01
9,10-dimethylanthracene	0	10	60	0.18	0.03
9,10-dimethylanthracene	30	10	60	0.22	0.04
9,10-dimethylanthracene	40	10	60	0.32	0.06
9,10-dimethylanthracene	60	10	60	0.57	0.13
9,10-dimethylanthracene	90	10	60	0.85	0.10
9,10-dimethylanthracene	120	10	60	0.93	0.05
9,10-dimethylanthracene	240	10	60	1.03	0.02
phenanthrene	0	10	60	0.24	0.07
phenanthrene	25	10	60	0.29	0.07
phenanthrene	35	10	60	0.42	0.01
phenanthrene	45	10	60	0.73	0.10
phenanthrene	60	10	60	0.66	0.07
phenanthrene	90	10	60	0.89	0.06
phenanthrene	120	10	60	0.96	0.05
phenanthrene	150	10	60	0.96	0.02
phenanthrene	180	10	60	0.87	0.07
phenanthrene	240	10	60	0.99	0.04
phenanthrene	360	10	60	1.03	0.03
1-methylnaphthalene	0	4	225	0.29	0.02
1-methylnaphthalene	30	4	225	0.33	0.01
1-methylnaphthalene	60	4	225	0.43	0.02

APPENDIX A

Table A4 (cont'd)

1-methylnaphthalene	90	4	225	0.97	0.19
1-methylnaphthalene	120	4	225	0.78	0.17
1-methylnaphthalene	180	4	225	0.88	0.07
1-methylnaphthalene	240	4	225	1.05	0.08
2-methylnaphthalene	0	4	325	0.32	0.03
2-methylnaphthalene	30	4	325	0.28	0.06
2-methylnaphthalene	60	4	325	0.52	0.05
2-methylnaphthalene	90	4	325	0.87	0.03
2-methylnaphthalene	120	4	325	0.84	0.08
2-methylnaphthalene	180	4	325	0.94	0.07
2-methylnaphthalene	240	4	325	1.02	0.14
benz(a)anthracene	0	25	350	0.59	0.04
benz(a)anthracene	15	25	350	0.30	0.04
benz(a)anthracene	30	25	350	0.45	0.04
benz(a)anthracene	45	25	350	0.40	0.03
benz(a)anthracene	60	25	350	0.64	0.04
benz(a)anthracene	75	25	350	0.69	0.10
benz(a)anthracene	90	25	350	0.82	0.01
benz(a)anthracene	120	25	350	0.87	0.04
benz(a)anthracene	180	25	350	0.88	0.10
benz(a)anthracene	240	25	350	0.92	0.07

APPENDIX A

Table A5. Results of the Cytotoxicity Experiments

Chemical	Dose Applied to Cells (μM)	Exposure Time (min)	Neutral Red Uptake (FOC)	Standard Deviation (FOC)
anthracene	0	10	1.00	0.00
anthracene	50	10	0.99	0.10
anthracene	100	10	1.14	0.23
anthracene	200	10	1.12	0.10
anthracene	300	10	1.02	0.26
anthracene	350	10	0.95	0.02
1-methylanthracene	0	10	1.00	0.00
1-methylanthracene	20	10	1.02	0.04
1-methylanthracene	30	10	0.96	0.06
1-methylanthracene	45	10	1.02	0.07
1-methylanthracene	60	10	0.96	0.05
1-methylanthracene	80	10	1.00	0.01
1-methylanthracene	100	10	1.01	0.07
2-methylanthracene	0	10	1.00	0.00
2-methylanthracene	100	10	1.16	0.05
2-methylanthracene	150	10	1.12	0.03
2-methylanthracene	200	10	1.18	0.12
2-methylanthracene	250	10	1.11	0.08
2-methylanthracene	300	10	1.09	0.05
2-methylanthracene	350	10	1.14	0.02
9-methylanthracene	0	10	1.00	0.00
9-methylanthracene	20	10	0.94	0.08
9-methylanthracene	30	10	0.99	0.03
9-methylanthracene	45	10	1.05	0.02
9-methylanthracene	60	10	0.97	0.06
9-methylanthracene	80	10	0.95	0.04
9-methylanthracene	100	10	1.01	0.02
9,10-dimethylanthracene	0	10	1.00	0.00
9,10-dimethylanthracene	15	10	0.87	0.06
9,10-dimethylanthracene	30	10	0.83	0.07
9,10-dimethylanthracene	45	10	0.93	0.11
9,10-dimethylanthracene	60	10	0.96	0.07
9,10-dimethylanthracene	80	10	0.79	0.06
9,10-dimethylanthracene	100	10	0.84	0.15
phenanthrene	0	10	1.00	0.00
phenanthrene	15	10	0.98	0.15

APPENDIX A

Table A5 (cont'd)

phenanthrene	30	10	0.95	0.03
phenanthrene	45	10	0.93	0.11
phenanthrene	60	10	1.02	0.03
phenanthrene	80	10	0.93	0.04
phenanthrene	100	10	0.98	0.04
naphthalene	0	4	1.00	0.00
naphthalene	50	4	0.87	0.03
naphthalene	100	4	0.79	0.05
naphthalene	150	4	0.87	0.02
naphthalene	200	4	0.79	0.05
naphthalene	250	4	0.86	0.02
naphthalene	300	4	0.93	0.03
naphthalene	350	4	0.82	0.04
naphthalene	400	4	0.82	0.05
naphthalene	450	4	0.85	0.21
1-methylnaphthalene	0	4	1.00	0.00
1-methylnaphthalene	50	4	0.86	0.02
1-methylnaphthalene	100	4	0.88	0.06
1-methylnaphthalene	150	4	0.86	0.02
1-methylnaphthalene	200	4	0.86	0.08
1-methylnaphthalene	225	4	0.79	0.05
1-methylnaphthalene	250	4	0.79	0.01
1-methylnaphthalene	275	4	0.68	0.06
1-methylnaphthalene	300	4	0.60	0.01
1-methylnaphthalene	350	4	0.33	0.07
1-methylnaphthalene	400	4	0.17	0.01
1-methylnaphthalene	450	4	0.18	0.03
2-methylnaphthalene	0	4	1.00	0.00
2-methylnaphthalene	50	4	1.04	0.08
2-methylnaphthalene	150	4	1.02	0.02
2-methylnaphthalene	200	4	1.07	0.00
2-methylnaphthalene	250	4	0.99	0.08
2-methylnaphthalene	300	4	0.97	0.02
2-methylnaphthalene	325	4	0.99	0.07
2-methylnaphthalene	350	4	0.93	0.04
2-methylnaphthalene	400	4	0.81	0.17
2-methylnaphthalene	450	4	0.65	0.05
benz(a)anthracene	0	25	1.00	0.00
benz(a)anthracene	50	25	0.96	0.01
benz(a)anthracene	100	25	0.83	0.05

APPENDIX A

Table A5 (cont'd)

benz(a)anthracene	150	25	0.90	0.01
benz(a)anthracene	200	25	0.97	0.09
benz(a)anthracene	250	25	0.95	0.08
benz(a)anthracene	300	25	0.90	0.07
benz(a)anthracene	350	25	0.92	0.07
benz(a)anthracene	400	25	0.96	0.04
benz(a)anthracene	450	25	1.09	0.05

APPENDIX B

APPENDIX B

CALCULATING PHYSICOCHEMICAL PARAMETERS

This section describes the methods used to calculate the values for the physicochemical parameters which appear in Table 3. All of the values were calculated using the methods by Lyman *et al.* (1990).

Aqueous Solubility

The following equation was used to determine the aqueous solubility (Sol) of the compound,

$$- \log Sol = -\log S + 0.0095*(T_m-25) \qquad \text{Equation 1}$$

where Sol is the aqueous solubility of the compound, S is solubility of the compound as a subcooled liquid, and T_m is the melting temperature of the compound. The value of S is determined by the chemical structure of the compound using the method of Lyman *et al.* (1990) where values of x, y, and z are needed.

The solubility was calculated for the following compounds; 1-meN, 2-meN, 1-meA, 2-meA, 9-meA and DMA. The assumption was made that there were special structural elements in each compound and that each methyl group represents aliphatic chain branching; therefore, a value of -.10 was assigned to z when calculating S.

APPENDIX B

Octanol-water Partition Coefficient

The octanol-water partition coefficient (K_{ow}) was calculated for the following compounds using the method of Lyman *et al.* (1990); 1-meN, 2-meN, 1-meA, 2-meA, 9-meA, and DMA. The K_{ow} of each compound was calculated using the reported K_{ow} value for the nonmethylated parent compound. For example, the K_{ow} for 1-meA was calculated using the reported K_{ow} value for ANT. This method is described in detain in Lyman *et al.* (1990).

Bioconcentration Factor

The bioconcentration factor (BCF) of each compound was calculated using the method of Lyman *et al.* (1990). The following equation was used to calculate BCF values in the species *Daphnia pulex* (invertebrate):

$$\log BCF = 0.7520 * \log K_{ow} - 0.4362 \qquad \text{Equation 2}$$

BCF values were calculated for each of the compounds using either calculated or observed K_{ow} values.

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