EFFECTS OF RELATIVELY LOW MOLECULAR WEIGHT AROMATIC HYDROCARBONS ON FROG SKIN NA+ TRANSPORT

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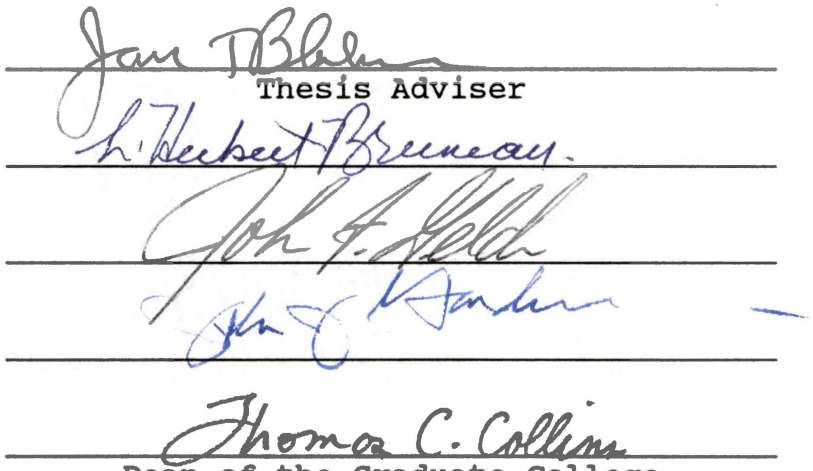

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CHAPTER I

INTRODUCTION

Background of the Problem

Amphibians

Until recently worldwide reduction of amphibians has primarily been recognized by workers in the field (Freda and Dunson 1985). Although the cause for the reduction in population has not been determined, a chief suspect is contaminants in the aquatic environment and the problems associated with such contaminants. It is probable that amphibians are early bioindicators that detect environmental stress from contaminants. In an earlier century canaries were used as bioindicators for air quality in the mining industry. Closely dependent upon the aquatic environment for successful reproduction, amphibians may be bioindicators of aquatic environmental contamination.

Amphibian embryonic development and larval development take place in an aquatic environment. In the aquatic environment frogs are constantly exposed to pond water containing lower concentrations of salts than are present in their cells, consequently salts are constantly lost from the amphibian's skin cells to the pond environment. Lost salts

must be recovered from the pond environment for proper maintenance of the frog's cells. One of the principal routes of salt recovery is through the frog skin. Ionic permeability is an important influence on the rate of sodium ion transport (Ussing 1949a).

Frogs in a stressed environment demonstrate behavioral changes increasing the amount of abdominal skin contact to the aqueous environment. Marine organisms may exhibit disruption in normal feeding sequences, sex attraction, and intricate social interactions. Such behavioral changes may be caused by the presence of sublethal concentrations of hydrocarbons (Boylan and Tripp 1971).

Aromatic Hydrocarbons

Many of the organic contaminants present in amphibians' aquatic environments are aromatic hydrocarbons. Many of these compounds have been identified as the relatively low molecular weight fractions that are produced during petroleum processing and that are present in petroleum wastes. They have been identified as acutely toxic (Anderson et al. 1974). Chlorinated polyaromatic hydrocarbons are also found to be in the environment from petroleum sources (Eklund and Stromberg 1983).

Aromatic hydrocarbons can be absorbed through the epithelial cells of frog skin. It has recently been demonstrated that naphthalene, a low molecular weight aromatic hydrocarbon, affects sodium active transport in

frog skin (Blankemeyer and Hefler 1990). Other organic toxicants, such as the other relatively low molecular weight aromatic hydrocarbons, may also effect active transport in frog skin.

Cell membranes

Living organisms are composed of cells, and cells are bounded by a cell membrane. Materials such as nutrients and oxygen must enter cells and materials such as wastes and carbon dioxide must leave the cells. Although many substances enter or leave the cell by simple diffusion, others require carrier-mediated transport such as facilitated diffusion and active transport. The sodium-potassium pump is perhaps the most important pump involved in the type of energy requiring carrier-mediated transport known as active transport.

Large negatively charged ions cannot penetrate the cell membrane. They remain inside the cell and attract small positively charged ions which are able to diffuse through pores in the membrane. As the cell membrane is more permeable to potassium than to any other positive ion, potassium ion accumulates inside the cell. Sodium ion is expelled from the cell interior and accumulates outside the cell. The constant activity of sodium-potassium pumps moves the ions against their diffusion gradients. A steep artificial ionic gradient of Na+ and K+ is created across the cell membrane that exhibits an electrical potential

difference (Fox 1990).

Statement of Hypothesis

Nagel suggested that the frog skin outer membrane is rate limiting for sodium entry and thus for transport. Under increased permeability conditions of the outer membrane, this resistance may be exceeded by the resistance of the interior membranes. The electrochemical gradient across the outer membrane may then significantly influence the effective response of the frog skin (Nagel 1978).

Exposing the frog skin to an organic toxicant may change the resistance of the outer membrane. What effect will low molecular weight aromatic hydrocarbons have on frog skin Na+ transport?

Purpose of the Study

There has been a growing awareness by the public that the environment is rather fragile and needs to be protected. Environmental contamination is effecting organisms in ways that are just now being discovered. Relatively low molecular weight aromatic hydrocarbons have been identified as acutely toxic (Anderson et al. 1974).

Assumptions of the Study

Abdominal frog skin is similar to human first trimester fetal skin. In vitro frog skin Na+ transport is similar to in vivo frog skin Na+ transport. Short-circuit current

mirrors Na+ transport.

Limitations of the Study

To set conditions for measuring short-circuit current across isolated frog skin, frog Ringer's was placed on both sides of the skin in the Ussing chamber. In the in vivo aqueous environment pond water would bathe the outside of the skin and physiological saline (frog Ringer's) the inside of the skin. A relatively small number of frogs were killed and used in the study. Each frog skin responds individually to short-circuiting. Seasonal differences occur in frog skin response. Successive shipments of frogs developed "red leg" necessitating obtaining frogs from an alternative source.

CHAPTER II

REVIEW OF THE LITERATURE

Epithelial Tissue

Epithelial tissue covers external and internal body surfaces and determines which substances can enter the body and which substances cannot. External body surfaces are covered with developmental derivatives of ectoderm. The alimentary canal and gut are covered with endodermal derivatives and the body cavity is covered with mesodermal derivatives. Although derived from the various embryonic germ layers, epithelial cells have been found to be broadly similar in form and function.

Vertebrate

Vertebrate epithelial tissue functions in many varied ways. Epithelial tissue provides sense organs, secretes materials, protects underlying tissues, and provides a selectively permeable barrier for the underlying tissues (Raven and Johnson 1991). Epithelial cells are arranged in membranes in one or more layers. The membrane surface adjacent to the lumen is referred to as the apical surface, the surface adjacent to the basal lamina as the basal surface, and the surfaces between adjacent cells as the

lateral surfaces (Leeson, Leeson, and Paparo 1985). Covering every external or internal body surface, epithelium facilitates or impedes the passage of every substance that enters or leaves the vertebrate body (Raven and Johnson 1991).

Epithelial cells are joined to each other by gap junctions. Gap junctions occur where the plasma membrane has a connexon, a doughnut-shaped patch of proteins, that connects across the intercellular space to another connexon in the membrane of a neighboring cell. The gap junction proteins protrude from the membrane leaving a gap of 2-4 nm. Gap junctions are specialized for transfering low molecular weight substances between adjacent cells (Campbell 1990).

Human

Stratified squamous epithelial cells are found in the skin. Human skin consists of five layers: stratum corneum (outermost layer), stratum lucidum, stratum granulosum, stratum spinosum, and stratum germinativum (Leeson, Leeson, and Paparo 1985). First trimester embryonic human skin is similar to amphibian abdominal skin in its ability to transport Na+. After the first trimester human skin function diverges to that of the adult.

Frog

Frog skin epithelium consists of four layers: stratum corneum (outermost layer), stratum granulosum, stratum

spinosum, and stratum germinativum (Farquhar and Palade 1964). Isolated frog skin is the best epithelial tissue for detailed study of ion active transport. As frogs are readily available and their skins are loosely attached to their musculature, frog skin can be handled and prepared easily with minimal damage to the epithelial tissue. As the frog is a poikilothermic animal, its epithelial tissues are able to retain their physiological activity under in vitro conditions (Kidder 1973).

Epithelial tissue of frogs has been shown to possess the property of Na+ active transport (Ussing 1949c). For decades researchers have utilized the properties of frog skin epithelium to study the processes of ion transport across cell membranes (Ussing 1949a; Koefed-Johnsen and Ussing 1958; Keynes 1969; Erlij and Ussing 1978; Rick et al. 1984; Blankemeyer and Hefler 1990).

Early Work by Ussing

Transport, or active transport, refers to the transfer of a substance against a chemical gradient. Active transport of ions involves the transfer of the ions from a place of lower electrochemical potential to a place of higher electrochemical potential. Active transport refers to the work done to transfer an ion across a membrane to overcome a difference in chemical concentration or to overcome a potential difference. Such a definition distinguishes between active transport and the passive

diffusion of ions. Specific active transport of at least some ions has been assumed to explain the net transfer of ions across living membranes.

The flux of free ions under the influence of chemical concentration and potential difference gradients has been investigated. Ussing found that the potential difference associated with short-circuiting the current of the isolated frog skin was due primarily to the active transport of sodium ions from the outside of the skin to the inside of the frog skin. He discovered that the potential difference depended upon the rate at which sodium ions were transported and the resistance to diffusion of passive ions which tended to short out the potential difference. High sodium ion active transport was found to be associated with high potential difference values (Ussing 1949a; Ussing 1949b).

Radioactive iodine tracer studies with iodine 131 demonstrated that the radioactive tracer iodine diffused inwards faster than it diffused outwards. No active transport of the diffusing radioactive iodine ion occurred as the potential difference across the frog skin was higher than that required to explain the difference in diffusion rate. High potential difference values were found only when the radioactive tracer iodine ion permeability was low. This finding confirmed the correlation between the potential difference and the active transport of sodium ions (Ussing 1949b).

Sodium Channels

Replacing external sodium with other cations demonstrated cationic selectivity of the sodium entry mechanism in the outer membrane of the bullfrog Rana catesbeiana. Results of tracer uptake experiments have demonstrated that only sodium and lithium are actively transported through the epithelium. Impermeable cations have been shown to be competitive inhibitors of sodium entry in a sequence corresponding to a high field strength site with tetrahedral symmetry (Benos, Mandel, and Simon 1980).

Epidermal cells of the frog skin were described as bound by two membranes. An outward facing membrane was shown to be highly permeable to sodium and chloride ions but practically impermeable to potassium ions. An inward facing membrane was shown to be permeable to potassium ions and chloride ions and slightly permeable to sodium ions. Coupled sodium and potassium pumps were described as maintaining low concentrations of extracellular sodium and high concentrations of intracellular potassium (Koefoed-Johnsen and Ussing 1958). The inward facing membrane has been described as the stratum granulosum (Rick et al. 1978). The outward facing membrane has been described as the superficial membrane of the stratum corneum (Curran, Herrera, and Flanigan 1963).

Sodium penetrates first into the epithelial cells of the outer barrier membranes of frog skin epithelium. Active transport moves sodium from within the cells into the

solution of the extracellular space (Ussing 1960). It has been suggested that overall sodium transport involves successive layers of cells with cells deeper in the epithelium having progressively higher concentrations of sodium. This multi-compartment model suggested transport from one cell layer to the next (Biber, Chez, and Curran 1966). Sodium uptake was not considered to be a linear function of sodium concentration. One compartment appeared to be a saturating one and the other compartment appeared to vary linearly with concentration (Biber and Curran 1970).

Sodium Paracellular Shunt Pathway

Two pathways for sodium movement across frog skin epithelium have been proposed, the classic active transport path and a paracellular shunt pathway for passive ion movement. Potential differences have been measured utilizing microelectrodes. A model attempted to explain the discontinuous electrical gradient observed in frog skin epithelium as a series arrangement of cell layers. Sodium ion movement from the outside of the skin to the inside was suggested to be linked with observed jumps in potential difference. Numerous mechanical cell-to-cell attachments, desmosomes, provide points of cell contact for adjacent epithelial cells. It has been suggested that the desmosome membranes might have a higher permeability to sodium than the membranes of the epithelial cells themselves. A selective pathway for the movement of sodium ions would

allow sodium to be transferred to pumping sites in the innermost epithelial membranes. A general shunt pathway, partly between cells, has been suggested for all passive ions (Ussing and Windhager 1964).

Microprobe analysis has been utilized to determine the electrolyte concentrations within single cells of the frog skin epithelium to determine the epithelial layer directly involved in transcellular sodium transport. Sodium free Ringer's solution and amiloride were the test solutions chosen, and both showed the same influences on epithelial transcellular transport. The stratum corneum was found to be in equilibrium with extracellular space. Sodium concentration in the stratum granulosum was decreased almost to zero. Sodium was not found in either of the two layers of the stratum spinosum. Sodium concentration in the stratum germinativum not changed. Ussing had ascribed sodium active transport to the stratum germinativum (Ussing 1949d). The stratum granulosum was demonstrated to be the sodium transport compartment (Dorge et al. 1973).

Potential Difference

Salt concentration outside the membrane and pH conditions inside the membrane were found to affect the potential difference and the sodium ion influx uniformly. The rate at which sodium ion is allowed to enter the cells was described as a very important factor in determining the rate of active transport and thus the potential produced

(Ussing 1949d). A potential difference, due to the Goldman equation, was said to be present between solutions that are separated by a membrane at equilibrium (Donnan 1911).

Mounting an excised abdominal frog skin in a Ussing chamber has enabled researchers to study the electrical parameters of frog skin epithelium. Epithelial membranes that are permeable to one ion (with a concentration difference across the membrane) in excess of permeability to another ion exhibit an electrical potential difference caused by the concentration difference. An electrogenic pump that pumps ions either unidirectionally or bidirectionally has been suggested as another possible source of current that could generate a potential (Kidder 1973).

The outer border might behave as a simple voltage independent resistor with an electromotive force of zero (Helman 1979). The net ion flux across the outer border approaches zero when the potential difference across the membrane is reduced to zero, in spite of the existing chemical potential gradient for the sodium ion (Nagel 1978). When current is increased from a situation of open current towards a state of zero potential, a "short-circuit" is said to exist (Ussing and Windhager 1964).

Short-circuit Current

Excised frog skin, bathed on both sides with frog Ringer's solution, maintains a potential difference between

the inside of the isolated frog skin and the outside. The inside of the skin has been shown to be more than 100 mV positive relative to the outside as sodium ions are actively transported to the inside. The internal positive charge attracts negative chloride ions inward resulting in net inward passive transport of sodium chloride. Some exchange of external sodium and internal potassium also occurrs.

Short-circuiting the skin potential difference places both sides of the skin at the same potential, zero mV. If both sides of the bathing solution are identical, no net transfer of passive ions takes place. Ions that were subject to active transport continue to flow in the same direction. Current can be drawn from the totally short-circuited frog skin. Current running through the short-circuited skin demonstrates all of the net transport processes. Short-circuit current and sodium flux have been found to agree almost completely, but the sodium-electromotive force has been found to vary considerably from one frog skin to another (Ussing and Zerahn 1951). Frog skin active transport experiments have been carried out under short-circuit conditions because under these conditions the electrical potential in the epithelium of the frog skin is not affected by changes in the sodium concentration of the outside solution (Cereijido and Curran 1965).

Schoen and Erlij (1985) used microelectrodes to determine current-voltage relations of both the apical and

basolateral membranes of the frog skin. They found that when cell current was plotted as a function of the apical membrane potential, experimental points differed from those predicted by the Goldman equation by less than experimental noise (Schoen and Erlij 1985).

Effect on Active Transport

The sodium ion transport pathway, rate-limiting for the transport of Na+ across the entire epithelium, is specifically inhibited by the diuretic drug amiloride (Benos, Hyde, and Latorre 1983). When sodium ion entry across the outer border of frog skin epithelium was blocked with the addition of amiloride, quantitatively important leaks were absent. Amiloride was assumed to affect outer membrane resistance only (Nagel 1978).

Application of combined amiloride and naphthalene produced a rapid decrease in short-circuit current. As amiloride alone blocked the short-circuit current, the entry path enhanced by naphthalene was surmised to be the same pathway through which sodium normally enters the skin (Blankemeyer and Hefler 1990). Stimulation of transepithelial Na+ transport by arginine vasopressin resulted in a marked increase in the sodium concentration and a reciprocal drop in potassium concentration in all epithelial layers. The effects of arginine vasopressin were cancelled by the addition of amiloride. It was concluded that the primary mechanism by which arginine vasopressin

stimulated transepithelial Na+ transport was by an increase in the Na+ permeability of the apical membrane (Rick et al. 1984).

Low Molecular Weight Aromatic Hydrocarbons

Given a general idea of the toxicity index of oil and oil fractions in polluted environments, naphthalene and naphthalene-type compounds were the most toxic components identified in seawater extracts of crude oil and crude oil fractions (Boylan and Tripp 1971). Water-soluble fractions of crude oils studied had higher total oil-hydrocarbon concentrations and were richer in light aliphatics and single-ring aromatics than were water-soluble fractions of refined oils (Anderson et al. 1974).

The relatively low molecular weight aromatic hydrocarbon benzene is common in petroleum wastes as are several slightly water soluble polynuclear aromatic hydrocarbons (Beach 1989). Chlorinated aromatics are also found in the environment from petroleum sources (Eklund and Stromberg 1983). Existing municipal sewage treatment technology does not prevent waste petroleum from entering receiving bodies of water (Tancredi 1990).

CHAPTER III

MATERIALS AND METHODS

Selected Toxicants

The effect of organic toxicants on frog skin transport was investigated. Relatively low molecular weight aromatic hydrocarbons were selected for study. Benzene represented the simplest ringed structure, a single-ringed aromatic hydrocarbon. A substituted benzene, 1-chlorobenzene or monochlorobenzene, was selected to represent halogenated single-ringed aromatic hydrocarbons. Another substituted benzene, phenol, was selected to represent hydroxyl-substituted aromatic hydrocarbons and was the only hydroxyl-substituted compound studied.

The simplest double-ringed aromatic hydrocarbon, naphthalene, had been observed to increase active sodium transport in isolated frog skin (Blankemeyer and Hefler 1990). A substituted naphthalene, 1-chloronaphthalene, was selected to represent halogenated double-ringed hydrocarbons. Triple-ringed hydrocarbons were represented by phenanthrene. As the selected aromatic hydrocarbons are not very water soluble, dimethylsulfoxide (DMSO) was used as a transport carrier for most of the hydrocarbons. Aromatic hydrocarbon concentrations were all given in terms of

amounts of hydrocarbon added to the water and not in terms of hydrocarbon concentration actually present in the aqueous phase. The concentration of most aromatic hydrocarbons was adjusted to 0.1%.

Effluents from four separate locations in Oklahoma were selected for possible correlation with chronic assays on fathead minnows and Cerriodaphnia Water Quality and Research Lab results.

Sample Preparation

Two methods of benzene preparation were used in this study. Either one mL of benzene was added to 9 mL of DMSO and enough frog Ringer's was added to prepare a volume of one liter or one mL of benzene was added directly to frog Ringer's to prepare a volume of one liter. The flask containing the sample was placed on a magnetic stirrer and allowed to continue stirring until time to add to the pond-side of the frog skin in the Ussing chamber. One mL of monochlorobenzene was added to DMSO and frog Ringer's was added to one liter volume with stirring. Phenol was added directly to frog Ringer's to make a 1% solution.

One mL 1-chloronaphthalene was added to 9 mL DMSO and warmed gently. When the solution was added to room temperature frog Ringer's, an emulsion formed immediately. The flask containing the emulsion was placed on a magnetic stirrer and allowed to continue stirring until time to add to the pond-side of the Ussing chamber.

Two methods of sample preparation were attempted for phenanthrene. Solid phenanthrene (0.1 g) was dissolved in 1 mL DMSO and added directly to frog Ringer's to one liter. Phenathrene (0.1 g) was also dissolved in 0.2 g Pluronic F-127, added to 1 mL DMSO and heated gently until it went into solution. When the solution was added to room temperature frog Ringer's, an emulsion formed immediately. The flask containing the phenanthrene emulsion was placed on a magnetic stirrer and allowed to stir until use.

As the oil field effluent samples included in this study were preliminary experiments for future studies, aromatic hydrocarbon concentrations were not determined. Effluents were tested after substitution for the water in frog Ringer's.

Control Frog Ringer's Solutions

Control frog Ringer's solutions were prepared by adding 110 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$ 2.5 mM TRIS buffer adjusted to pH 8.3 and water to one liter. If toxicants were dissolved first in carrier DMSO, the same concentration of DMSO was added to the frog Ringer's bathing and rinse solution.

Frog Ringer's solution was selected as the control solution as it contains the major ionic constituents of frog blood. The serosal or blood side of the frog epithelial tissue (right compartment of the Ussing chamber) was physiologically compatible with the living tissue. The

mucosal or pond-side of the frog epithelial tissue (left compartment of the Ussing chamber) was not compatible physiologically either with the pond water in which the frog was living in the wild or with the tap water in which the frog was living in the laboratory.

Adding frog Ringer's to both sides of the chamber was necessary to set the conditions for measuring the short-circuit current. Solutions on both sides of the frog skin must be the same. Since frog Ringer's is required to match the physiological requirements of the inside of the skin, frog Ringer's was used in the pond-side compartment bathing the outside of the skin (Kidder 1973).

Frogs

Grass frogs, Rana pipiens, were obtained from William Lemberger, (Oshkosh, Wisconsin) and from the Carolina Biological Supply Company, (Burlington, North Carolina) and kept unfed until used. The frogs were rinsed daily with tap water. Before use a frog was anesthetized with 10% urethane in the dorsal lymph sac, the head was removed, and the skin was excised. The isolated skin was placed in frog Ringer's solution. All animal handling was performed in accordance with recommendations of the 1986 AVMA Council on Euthenasia.

Excised frog skin is able to maintain physiological activity for many hours under in vitro conditions. As frog skin has been shown to possess the property of active transport of sodium across its epithelial tissue and is

readily available, it was selected as the tissue of choice in early studies (Ussing 1949a).

Epithelial membranes that are permable to one ion in excess of permeablity to another ion exhibit an electrical potential difference caused by the difference in concentration (Kidder 1973). Short-circuiting the skin, with frog Ringer's as the bathing solution in both compartments of the Ussing chamber, puts both sides of the skin at the same potential. Electrical current running through the short-circuited skin has demonstrated agreement between the flow of sodium ions and the short-circuited current (Ussing and Zerahn 1951).

Apparatus

A glass Ussing chamber (Figure. 1) was the apparatus selected to measure the short-circuit current. The excised abdominal frog skin was mounted as a flat, vertical, membraneous sheet between two frog Ringer's-filled compartments. The fluid in the chamber overfilled the two compartments and extended into the paired gas lift pumps. Fluid levels in the lift pumps were equal, or horizontal, so the hydrostatic pressure difference across both sides of the frog skin was maintained at zero.

Air was injected via latex tubing into the gas lift pumps above the chamber permitting aeration and circulation of the chamber fluids. Adequate aeration and circulation of the bathing fluids of the frog skin was possible as the

oxygen consumption demands of this epithelial tissue are low (Kidder 1973).

Two agar-KCl bridges made contact with two 3 M KCl-calomel reference electrodes. The electrical potential difference existing across the frog skin between the two bathing solutions was recorded by the agar bridges. Silver-silver chloride plates applied the current necessary to offset the electrical potential difference to zero millivolts. An automatic voltage clamp was used to pass enough current to maintain the potential difference at preset values.

A strip chart recorder (Schlumberger, Benton Harbor, Michigan) was used to record time-based records at five-minute intervals. Any unexpected deviations from the five-minute intervals were recorded individually from the voltage clamp to the chart paper. Drains connected to vacuum lines simultaneously drained the chamber compartments as they were filled (Ussing and Zerahn 1951; Kidder 1973; and Blankemeyer and Hefler 1990).

Procedures

An excised abdominal skin of Rana pipiens was mounted in a glass Ussing chamber and bathed with control frog Ringer's on both sides. Electrical current was applied opposing the electrical potential difference arising from active transport of sodium ions. When the electrical potential was zero, the skin was said to be short-circuited.

The short-circuited skin measured the net transport of sodium ions. A voltage clamp was used to maintain the potential difference.

The isolated frog skin was allowed to adjust to the frog Ringer's bathing solution for approximately one hour. New bathing solutions were added and old bathing solutions were removed simultaneously. An aliquot of 50 mL frog Ringer's was added to the pond-side of the frog skin in the chamber and short-circuit current was recorded at five-minute intervals for 15 minutes. An aliquot of 50 mL of the solution being investigated was added to the pond-side and short-circuit current was recorded at five-minute intervals for 15 minutes. An aliquot of 100 mL of frog Ringer's was added as a rinse and the current was recorded at five-minute intervals for 15 minutes.

"Sandwich" experiments were attempted for some toxicants. An aliquot of 50 mL frog Ringer's was added to the pond-side and the short-circuit current was recorded at five-minute intervals for 15 minutes. An aliquot of 50 mL of the sample was added and the short-circuit current was recorded at five-minute intervals for 15 minutes. An aliquot of 100 mL of frog Ringer's rinse was added and short-circuit current was recorded at five-minute intervals for 45 minutes. A second 50 mL aliquot of the toxicant was added and short-circuit current was recorded at five-minute intervals for 15 minutes. A final frog Ringer's rinse was added to the pond-side and the short-circuit current was

recorded at five-minute intervals for 15 minutes.

After the frog skin was removed from the Ussing chamber, the chamber was rinsed with frog Ringer's. If the odor of the organic toxicant lingered in the vicinity of the chamber, a rinse of 50:50 ethanol:frog Ringer's replaced the frog Ringer's and was allowed to remain overnight. The chamber was rinsed with frog Ringer's the next morning.

Software

Graphs of the effect of representative low molecular weight aromatic hydrocarbons on frog skin Na+ transport were plotted by Graphpad. Primary data spreadsheets were prepared by LOTUS 123. Statistical probabilities were calculated by t-ease by Graphpad. Layout and typing of the manuscript were facilitated by Word Perfect 5.1.

CHAPTER IV

RESULTS

Introduction

The following compounds were applied to the pond-side of the frog skin: benzene, phenol, chloronaphthalene, phenanthrene, and oil field effluents. Dose responses were studied for each of the following compounds: benzene, monochlorobenzene, and phenol.

Single-ringed Aromatic Hydrocarbons

Single-ringed aromatic hydrocarbons utilized in the study were benzene, monochlorobenzene, and phenol. The effects of the representative single-ringed aromatic hydrocarbons on the short-circuit current are listed in Tables I - XII. Benzene and the benzene-substituted aromatic compounds decreased the short-circuit current.

Benzene

Five minute interval short-circuit current readings in uA for 0.1% benzene in frog Ringer's are listed in Table I. Short-circuit current readings for frog Ringer's control bathing solutions were averaged and recorded as baseline averages. The baseline average before the addition of

benzene was recorded as -12.1 uA and the after average was recorded as -11.7 uA. Peak short-circuit current response for benzene was observed to occur between the five minute intervals and was recorded after the first minute as -10.8 uA. Calculated percent response is -10.7%. The p value was calculated as 0.0059, very significant.

Table II lists the timed short-circuit current readings for a second representative benzene experiment. The before baseline average was -61.1 uA and after baseline average was -60.9 uA. Benzene peaked at -43.7 uA and the peak response was calculated as -28.5%. The calculated p value was 0.0021, very significant. Figure 2 is a graphical representation of the effect of benzene on short-circuit current. Both applications of benzene are shown with control frog Ringer's before and after each application. Average for the control baseline before the second benzene application was -62.5 uA and the after average was -47.3 uA. The second benzene application peaked at -32.8 uA with a response of -47.5%. The p value was 0.0009 or extremely significant.

Summary comparisons of the effects on short-circuit current by two representative additions of benzene to the pond-side of frog skins are listed in Table III. Percent response of the short-circuit current to the first benzene application was -10.7%, with p value 0.0059, very significant. Percent response to the second sample was -28.5%, with p value 0.0021, very significant. Average

percent response was -19.6%, and average recovery was -1.8%.

Table IV reports a representative benzene dose response. Before baseline average of control frog Ringer's, containing the transport carrier DMSO, was -16.6 uA and the after average -12.5 uA. Short-circuit current peak for 0.01% benzene in DMSO transport carrier in frog Ringer's was -14.7 uA with percent response -11.4% and p value of 0.0021, very significant. Peak for 0.05% benzene was -12.5 uA, percent peak response of -24.7%, and p value 0.0005, extremely significant. Peak for 0.1% benzene was -11.2 uA, percent response of -32.5%, and p value 0.0003, extremely significant.

Figure 3 shows the plot for the first benzene dose response. Before baseline average was -16.6 uA and the after average -12.5 uA. Short-circuit current peak for 0.01% benzene in DMSO transport carrier in frog Ringer's was -14.7 uA with percent response -11.4% and p value of 0.0021, very significant. Peak for 0.05% benzene was -12.5 uA, percent peak response of -24.7%, and p value 0.0005, extremely significant. Peak for 0.1% benzene was -11.2 uA, percent response of -32.5%, and p value 0.0003, extremely significant.

The second benzene dose response is listed in Table V. Before baseline average of 0.1% DMSO in frog Ringer's was -106.7 uA and after average was -75.4 uA. Peak for 0.01% benzene was -87.6 uA, percent response -17.9 %, and p value 0.0292, significant. Peak for 0.05% benzene was -71.3%,

percent response -33.2%, and p value 0.0088, very significant. Peak for 0.1% benzene was -72.9 uA, percent response -31.7%, p value 0.0096, very significant.

Monochlorobenzene

Table VI lists a dose response for monochlorobenzene. Baseline control average before application of toxicant was -18.9 uA and after -13.2 uA. Peak for 0.01% monochlorobenzene was -15.7 uA, percent response -16.9%, and p value 0.003, extremely significant. Peak for 0.05% monochlorobenzene was -10.9 uA, percent response -42.3%, and p value <0.0001, extremely significant. Peak for 0.1% monochlorobenzene was -11.0 uA, percent response -41.8% and p value <0.0001, extremely significant.

Figure 4 shows the plot of a dose response for monochlorobenzene. Baseline control average before application of toxicant was -18.9 uA and after -13.2 uA. Peak for 0.01% monochlorobenzene was -15.7 uA, percent response -16.9%, and p value 0.003, extremely significant. Peak for 0.05% monochlorobenzene was -10.9 uA, percent response -42.3%, and p value <0.0001, extremely significant. Peak for 0.1% monochlorobenzene was -11.0 uA, percent response -41.8% and p value <0.0001, extremely significant.

Phenol

The results of the first representative phenol sample are recorded in Table VII. Frog Ringer's before baseline

average was -19.2 uA and after baseline average was -25.1 uA. Peak short-circuit current response to 0.1% phenol was -26.3 uA, percent response 37.0%, p value 0.0003, extremely significant.

Table VIII lists the timed short-circuit current recordings for the second representative phenol experiment. Baseline before average was -74.3 uA and after average was -49.1 uA. Peak phenol was recorded at -47.6 uA with percent response of -35.9%, and p value of 0.0016, very significant.

Figure 5 delineates graphically the same data as is present in Table VIII for the second representative phenol experiment. Baseline before average was -74.3 uA and after average was -49.1 uA. Peak phenol was recorded at -47.6 uA with percent response of -35.9%, and p value of 0.0016, very significant. A second addition of phenol to the pond-side of the frog skin produced a peak of -23.0 uA and peak percent response of -44.0% with p value of 0.0107 or significant.

Phenol sample number three is reported in Table IX. Before baseline average was -45.8 uA and after was -24.5 uA. Peak phenol was -27.0 uA with percent response of -41.0%, and p value 0.0011, extremely significant.

The fourth phenol sample is listed in Table X. The before control frog Ringer's baseline average was -23.2 uA and after was -19.0 uA. Peak phenol was -18.7 uA with percent response of -19.4% and p value of <0.0001, extremely

significant.

Summary phenol comparisons are grouped in Table XI. Percent responses were 37.0%, -35.9%, -41.0%, and -19.4%. Corresponding p values were 0.0003 or extremely significant for phenol I, 0.0016 or very significant for phenol II, 0.0011 or extremely significant, and <0.0001 or extremely significant for phenol III. Average percent response was -33.3%, and average recovery was -16.5%. Standard error was -33.3 ± 11.2.

A representative phenol dose response from greater to lesser concentration is summarized in Table XII. The before baseline average was -82.9 uA and after average was -46.2 uA. The 0.1% phenol peak was recorded at -39.4%. Percent response was -52.5% with p value of 0.0019, very significant. With before a baseline average of -46.2 uA and an after baseline average of -38.2 uA, 0.05% phenol peaked at -33.4 uA. Percent response was -59.0% with p value of 0.0015, very significant. With a baseline before average of -38.2 uA and an after baseline average of -49.8 uA, 0.025% phenol peaked at -40.7 uA. Percent response was -50.9% with p value of 0.0021 or very significant.

Double-ringed Aromatic Hydrocarbons

The double-ringed aromatic hydrocarbon increased the short-circuit current. Table XIII lists the results for the first 1-chloronaphthalene experiment. Control DMSO-containing frog Ringer's before baseline average was -54.1

uA and after average was -53.5 uA. Peak 1-chloronaphthalene was -56.5 uA and peak recent response was 4.4%. The p value was 0.0341, significant.

Figure 6 graphically describes the 1-chloronaphthalene information in Table XIII. Before baseline average was -54.1 uA and after average was -53.5 uA. Peak was -56.5 uA and peak percent response was 4.4%. The p value of 0.0341 was significant. The second before baseline average was -53.3 uA and after was -56.0 uA. Second peak was -57.4 uA and peak percent response was 7.7%%. The p value of 0.0006 was extremely significant.

Results of another representative 1-chloronaphthalene experiment are listed in Table XIV. The before control baseline average was -53.9 uA and after average was -54.8 uA. Peak was -55.7 uA and peak percent response was 3.3%. The p value was 0.0893, marginally significant

Table XV lists comparisons for 1-chloronaphthalene experiments shown in Tables XXIII and XIV. Peak percent response for the first 1-chloronaphthalene was 4.4% and 3.3% for the second. Corresponding p values were 0.0341 or significant and 0.0893 or marginally significant. Average % response was 3.9%, and average recovery was 0.3%. Standard error was 3.9 $\pm$ 3.5.

Triple-ringed Aromatic Hydrocarbons

The triple-ringed aromatic hydrocarbon demonstrated mixed effects, increasing the short-circuit current in some

frog skins and decreasing it in others. Phenanthrene sample I results are listed in Table XVI. Control DMSO frog Ringer's baseline average was -7.4 uA and after average baseline was -10.0 uA. Peak phenanthrene was recorded as -8.8 uA and peak percent response was 18.9%. The p value was calculated as 0.0038, very significant.

Phenanthrene sample II results are listed in Table XVII. Control DMSO frog Ringer's baseline average was -44.5 uA and after average baseline was -40.0 uA. Peak phenanthrene was recorded as -40.3 uA and peak percent response was -9.4%. The p value was calculated as 0.0371, significant.

Table XVIII lists comparisons for representative phenanthrene samples prepared in DMSO frog Ringer's. Peak percent response for the first phenanthrene was 18.9% and -9.4% for the second. Results of the t test indicated p values of 0.0038 or very significant for the first phenanthrene and 0.0371, or significant for the second. The average percent response was 9.5%, and the average recovery was 12.5.

The results of the first representative sample of phenanthrene (phenanthrene III) prepared in Pluronic F-127, dissolved in DMSO and frog Ringer's are listed in Table XIX. Control DMSO frog Ringer's before baseline average was -41.7 uA and control after average was -54.6 uA. Peak phenanthrene was recorded at -47.9 uA and peak percent response was 14.9%. The p value was 0.0037, very

significant.

The results of the second representative sample of phenanthrene (phenanthrene IV) prepared in Pluronic F-127, dissolved in DMSO and frog Ringer's are listed in Table XX. Control DMSO frog Ringer's before baseline average was -62.8 uA and control after average was -57.4 uA. Peak phenanthrene was recorded at -57.0 uA and peak percent response was -9.2%. The p value was 0.0668, marginally significant.

The results of the third representative sample of phenanthrene (phenanthrene V) prepared in Pluronic F-127, dissolved in DMSO and frog Ringer's are listed in Table XXI. Control DMSO frog Ringer's before baseline average was -38.9 uA and after average was -40.5 uA. Peak phenanthrene was recorded at -41.0 uA and peak percent response was 5.4%. The p value was 0.0068, very significant.

Figure 7 shows results from Table XXI graphically. Control DMSO frog Ringer's before baseline average was -38.9 uA and after average was -40.5 uA. Peak phenanthrene was recorded at -41.0 uA and peak percent response was -5.4%. The p value was 0.0068, very significant. The second before baseline average was -41.7 uA and the second after baseline average was -54.6 uA. The second peak was -47.9 uA and peak percent response was 14.9%. The p value was 0.0037 or very significant.

Table XXII lists comparative data for the three representative phenanthrene samples which were dissolved in

Pluronic F-127 before being dissolved in DMSO transport carrier. Peak percent responses were 14.9% for phenanthrene III, -9.2% for phenanthrene IV, and 5.4% for phenanthrene V. Calculated p values included respectively p of 0.0037 or very significant, 0.0668 or marginally significant, and 0.0068 or very significant. Average percent response was 3.7%, and average recovery was 8.9%. Standard error was 3.7 ± 20.5.

Effluents

Table XXIII lists experimental results for the first representative effluent. Control frog Ringer's before baseline average was -85.2 uA and after baseline average was -84.3 uA. Peak effluent was recorded as -75.1 uA and peak recent response was -11.9%. The p value was 0.0003, extremely significant.

The second effluent data are recorded in Table XXIV. Before baseline average was -40.3 uA and after the average was -42.5 uA. Effluent peak was -39.4 uA with peak percent response calculated as -2.2% The p value was 0.0440 or significant.

Table XXV lists the results of the third effluent. Frog Ringer's control baseline was -37.2 uA and the after average was -34.4 uA. Peak was -31.6 uA and effluent percent response was -15.1%. The p value was 0.0585 and was marginally significant.

Figure 8 demonstrates the third effluent data

graphically. The first frog Ringer's control baseline average was -37.2 uA and after average was -34.4 uA. Peak was -31.6 uA and percent response was -15.1%. The p value was 0.0585 and was marginally significant. The second control average baseline was -35.5 uA and after average was -32.9 uA. The second effluent peak was -28.5 uA and peak percent response was -19.7%. The p value was 0.0001, extremely significant.

The fourth effluent is reported in Table XXVI. Frog Ringer's baseline before was -32.3 uA and after was -25.7 uA. Effluent peak was -23.1 uA and peak percent response was -28.5%. The p value was <0.0001, extremely significant.

Table XXVII summarizes and compares effluent results. The peak response for the first effluent was -11.9%, the second -2.2 %, the third -15.1%, and the fourth -28.5%. The p values for the first was 0.0003 or extremely significant, the second 0.0440 or significant, the third 0.0585 or marginally significant, and the fourth <0.0001 or extremely significant. The average percent response was -14.4%, and the average recovery was -2.7. Standard error was -14.4 $\pm$ 12.8.

Table XXVIII lists effluent water test sample results. For the first effluent, Daphnia NOEC survival was >100%, fathead minnow survival >100%, and short-circuit current response -11.9%. For the second effluent, Daphnia NOEC survial was 95% and reproduction 52%, fathead minnow NOEC survival 63% and reproduction 10%, short-circuit current

response -2.2%. For the third effluent, Daphnia NOEC survival was 100% and reproduction 100%, fathead minnow survival 100%, and short-circuit current response -15.1%. For the fourth effluent, Daphnia NOEC survival was 30% and reproduction 100%, fathead minnow NOEC survival 100%, and short-circuit current response -28.5%.

CHAPTER V

DISCUSSION

Introduction

Excised abdominal frog skin, bathed on both sides with frog Rınger's solution, maintains a potential difference between the serosal-side and the pond-side of the skin. Short-circuiting the skin places both sides of the skin at the same potential or at a zero potential difference. Short-circuit current, applied to the frog skin, correlates with sodium ion flow (Ussing and Zerahn 1951).

Single-ringed Aromatic Hydrocarbons

Single-ringed aromatic compounds utilized in the study were benzene and the benzene-substituted compounds, monochlorobenzene and phenol. Benzene and the benzene-substituted compounds decreased the short-circuit current. Decreasing the short-circuit current on the isolated frog skin may decrease Na+ ion transport in the isolated frog skin and may decrease Na+ transport in the living frog.

Benzene

Applıcation of benzene to the pond-side of the excised frog skin decreased the short-circuit current an average

of -19.6% within one minute. Generally the short-circuit current began to recover to pre-application values within five minutes after maximum decrease. Average baseline carryover was -1.8%, a return to near baseline values.

Benzene dose responses indicated that the short-circuit current also began decreasing after application. Lower concentrations of benzene (0.01% and 0.05%) demonstrated maximum response 15 to 30 minutes after application of toxicant. At concentrations identical to that of the single application studies (0.1%) the short-circuit current showed a delayed response, maximizing at 10 to 15 minutes after application. Benzene-altered baselines demonstrated an average -27.0% carryover response by the short-circuit current with 15 minutes of rinsing with frog Ringer's.

Monochlorobenzene

The monochlorobenzene study reported was that of a dose response. After application of 0.01% monochlorobenzene, short-circuit current response decreased -15.9% at five minutes, remained at that response, then decreased one percent more to -16.9% at 15 minutes. Application of 0.05% monochlorobenzene permitted further decrease in the current with maximum response of -42.3% at 15 minutes. The highest dose studied was 0.1% which showed maximum percent response of -41.8% five minutes after application. The short-circuit current did not recover after rinsing with frog Ringer's indicating an average carryover response of -30.2% with 15

minutes of rinsing.

Phenol

Pond-side application of 0.1% phenol demonstrated a decrease of -33.3% in average response 23 minutes after application. Phenol altered baselines showed an average carryover response of 16.5% with 15 minutes of frog Ringer's rinsing. All doses of phenol in the representative dose response decreased the short-circuit current. The highest concentration of 0.1% decreased the short-circuit current -52.5% and after rinsing there was a carryover response of -44.3%. An intermediate concentration of 0.05% decreased the short-circuit current -59.7% and after rinsing there was a carryover response of -53.9%. The lowest concentration of 0.01% decreased the baseline short-circuit current -50.9% and after rinsing a carryover response of -39.9% was observed. Standard error was -33.3 $\pm$.

Double-ringed Aromatic Hydrocarbons

Blankemeyer and Hefler (1990) showed that naphthalene increases Na+ active transport in the frog skin as indicated by the increase in short-circuit current. Probable site of action was determined to be at the pond-side membrane of the frog skin. Application of 0.1% 1-chloronaphthalene to the pond-side of the isolated frog skin increased the short-circuit current an average of 3.9%. Baseline carryover after rinsing averaged 0.3% indicating a return to baseline.

Standard error was 3.9 ± 3.5.

Triple-ringed Aromatic Hydrocarbons

Phenanthrene applications to the pond side of the isolated frog skin gave inconsistent effects on the short-circuit current, increasing the short-circuit current on some frog skins and decreasing short-circuit current on other frog skins. In an attempt to ascertain whether phenanthrene had actually entered the aqueous phase of the solution, some studies were run after first dissolving the solid phenanthrene in Pluronic F-127, a loading medium, before adding it to DMSO. Application of 0.1% phenanthrene in DMSO produced both an increased response in short-circuit current of 18.9% and a decreased response of -9.4% with representative samples for an average 9.5% response increase in short-circuit current. After rinsing with DMSO frog Ringer's baseline carryover response was 35.1% and -10.1% respectively with an average of 12.5%.

Three representative studies of phenanthrene in Pluronic F-127 were included. One indicated an decrease of -9.2% response by the short-circuit current. Average increase in the short-circuit current response was 10.2%, and total average response was 3.7%. Phenanthrene altered baseline carryover average was 8.9%. Standard error was 3.7 ± 20.5.

Effluents

Representative effluent studies indicated a decreased response by the short-circuit current maximizing 10 to 15 minutes after application to the pond-side of the isolated frog skin. Percent response averaged -14.4% and baseline carryover averaged an increase of 3.3% for two of the studies and -8.7% for two others. Total average was -2.7%.

Correlation with Microtox and Ceriodaphnia Water Testing Lab data was attempted. Effluents were available for comparison testing when unused test water remained after biomonitoring tests were completed.

Ceriodaphnia NOEC survival and fathead minnow survival were greater than 100% for the first effluent. Short-circuit response by the frog skin to the same effluent one day later was -11.9%, p value 0.0003, extremely significant. A more sensitive biomonitoring assay is suggested by the short-circuit response.

NOEC survival by Ceriodaphnia for the second effluent was 95% and reproduction was 52%. NOEC survival by fathead minnow was 63% and reproduction was 10%. Twelve days later short-circuit response by the frog skin was -2.2%, p value 0.0440, significant. It is assumed that the apparent greater sensitivity by the Ceriodaphnia and fathead minnow assays was caused by the twelve day delay in short-circuiting the isolated frog skin.

The third effluent tested indicated 100% survival and reproduction by Ceridaphnia and 100% survival by fathead

minnow. Short-circuit response by the isolated frog skin was -15.1%, p value 0.0585, marginally significant three days later. A more sensitive biomonitoring assay is suggested by the short-circuit response.

Ceriodaphnia NOEC survival for the fourth effluent was 30% and reproduction was 100%. Fathead NOEC minnow survival was 100%. Short-circuit response by the isolated frog skin was -28.5%, p value <0.001, extremely significant, three days later. A more sensitive biomonitoring assay is suggested by the short-circuit response. Average percent response was -14.4%, and averge recovery was -2.7%. Standard error was -14.4 $\pm$ 12.8.

Effect on Transport

Current passing through the short-circuited skin correlates with sodium ion flow (Ussing and Zerahn 1951). Cyanide poisoning reduces sodium ion influx 5 - 25% of the original value (Ussing 1949a). Sodium ion transport in the isolated frog skin appears to be decreased by exposure to the single-ringed aromatic hydrocarbons benzene, monochlorobenzene, and phenol. Sodium ion transport in the isolated frog skin appears to be increased by exposure to the double-ringed naphthalene (Blankemeyer and Hefler 1990) and to 1-chloronaphthalene. Sodium ion transport appears to be both increased and decreased in the isolated frog skin by exposure to phenanthrene. Cyanide poisoning reduces Na+ influx 5 - 25% of the original value (Ussing 1949a).

Disruption of sodium ion transport could effect the environmental fitness of frogs rendering them unfit to compete favorably in their aquatic environment.

Questions for Future Studies

All single-ringed aromatic hydrocarbons studied demonstrated a decrease in short-circuit current in the isolated frog skin. Would other substitutions demonstrate a similar effect? Is the single ring responsible for the decrease in short-circuit current? Both double-ringed aromatic hydrocarbons, either studied or referred to (Blankemeyer and Hefler 1990), demonstrated an increase in short-circuit current. Would a hydroxylated naphthalene or other substituted naphthalenes decrease the short-circuit? Is the double ring responsible for the increase in short-circuit current?

Phenanthrene demonstrated a decrease in short-circuit current in some isolated frog skins and an increase in short-circuit current in other frog skins. Is the triple ring of the structure of phenanthrene responsible for the lack of consistency in membrane permeability by phenanthrene? Is the triple-ringed phenanthrene molecule turned to simulate a single-ringed benzene molecule when it decreases the short-circuit current? Is it turned to simulate a double-ringed naphthalene molecule when it increases the short-circuit current. Would a substituted phenanthrene, such as a halogenated phenanthrene or a

hydroxylated phenanthrene, demonstrate a single effect on the short-circuit of the isolated frog skin?

Phenanthrene showed no change on the permeability of cytochrome c-loaded liposomes to ascorbic acid in Chironomus attenuatus (Darville et al. 1983). Is it possible that the cause for the mixed effects on sodium ion transport by phenanthrene in the isolated frog skin is similar to the lack of change in permeability by phenanthrene in the liposome membrane of the freshwater dipteran?

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APPENDIXES

TABLES

TABLE I

REPRESENTATIVE BENZENE SAMPLE I

SOLUTION	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)	
0.1% FROG RINGER'S			
	130	-11.9	
	135	-12.2	
	140	-12.2	
BASELINE AVERAGE		-12.1	
0.1% BENZENE			
	141	-10.8	PEAK
	145	-12.7	
	150	-12.8	
	155	-11.7	
-10.7% RESPONSE			
p = 0.0059, very significant			
0.1% FROG RINGER'S			
	160	-11.7	
	165	-11.7	
	170	-11.8	
BASELINE AVERAGE		-11.7	

TABLE II

REPRESENTATIVE BENZENE SAMPLE II

SOLUTION	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)
0.1% FROG RINGER'S		
	100	-59.6
	105	-61.4
	110	-62.3
BASELINE AVERAGE		-61.1
0.1% BENZENE		
	113	-43.7 PEAK
	115	-46.4
	120	-56.3
	125	-62.9
-28.5% RESPONSE p = 0.0021, very significant		
0.1% FROG RINGER'S		
	130	-62.4
	135	-60.4
	140	-60.0
BASELINE AVERAGE		-60.9

TABLE III

SUMMARY BENZENE COMPARISONS

EXPERIMENT #	BENZENE I		BENZENE II
BEFORE 1 (uA)	-11.9		-59.6
BEFORE 2 (uA)	-12.2		-61.4
BEFORE 3 (uA)	-12.2		-62.3
BASELINE AVG (uA)	-12.1		-61.1
PEAK (uA)	-10.8		-43.7
% RESPONSE	-10.7%		-28.5%
p VALUE	0.0059		0.0021
SIGNIFICANCE LEVEL	very		very
AVERAGE % RESPONSE		-19.6%	
AFTER 1 (uA)	-11.7		-62.4
AFTER 2 (uA)	-11.7		-60.4
AFTER 3 (uA)	-11.8		-60.0
BASELINE AVG (uA)	-11.7		-60.9
BASELINE RECOVERY	-3.3%		-0.3%
AVERAGE RECOVERY		-1.8%	

TABLE IV

REPRESENTATIVE BENZENE DOSE RESPONSE I

SOLUTION	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)	
0.1% DMSO FROG RINGER'S			
	60	-16.6	
	65	-16.8	
	70	-16.5	
BASELINE AVERAGE		-16.6	
0.01% BENZENE			
	75	-17.0	
	80	-17.2	
	85	-17.2	
	90	-16.4	
	95	-15.3	
	100	-14.7	PEAK
-11.4% RESPONSE			
p = 0.0021, very significant			
0.05% BENZENE			
	105	-13.3	
	110	-13.2	
	115	-12.9	
	120	-12.5	PEAK
-24.7% RESPONSE			
p = 0.0005, extremely significant			
0.1% BENZENE			
	125	-11.7	
	130	-11.2	PEAK
	135	-11.4	
	140	-11.5	
-32.5% RESPONSE			
p = 0.0003, extremely significant			
0.1% DMSO FROG RINGER'S			
	145	-12.3	
	150	-12.8	
	155	-12.5	
	160	-12.5	
BASELINE AVERAGE		-12.5	

TABLE V

REPRESENTATIVE BENZENE DOSE RESPONSE II

SOLUTION	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)
0.1% DMSO FROG RINGER'S		
	100	-100
	105	-110
	110	-110
BASELINE AVERAGE		-106.7
0.01% BENZENE		
	115	-93.6
	120	-88.9
	125	-87.6 PEAK
-17.9% RESPONSE		
p = 0.0292, significant		
0.05% BENZENE		
	130	-76.3
	135	-74.8
	140	-71.3 PEAK
-33.2% RESPONSE		
p = 0.0088, very significant		
0.1% BENZENE		
	145	-81.3
	150	-76.3
	155	-72.9 PEAK
-31.7% RESPONSE		
p = 0.0096, very significant		
0.1% DMSO FROG RINGER'S		
	160	-78.3
	165	-76.8
	170	-71.1
BASELINE AVERAGE		-75.4

TABLE VI

REPRESENTATIVE MONOCHLOROBENZENE DOSE RESPONSE

SOULTION	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)
0.1% DMSO FROG RINGER'S		
	115	-18.9
	120	-18.9
	125	-18.9
BASELINE AVERAGE		-18.9
0.01% MONOCHLOROBENZENE		
	130	-15.9
	135	-15.9
	140	-15.7 PEAK
-16.9% RESPONSE		
p = 0.0003, extremely significant		
0.05% MONOCHLOROBENZENE		
	145	-15.3
	150	-12.4
	155	-10.9 PEAK
-42.3% RESPONSE		
p = <0.0001, extremely significant		
0.1% MONOCHLOROBENZENE		
	160	-11.0 PEAK
	165	-11.4
	170	-12.7
-41.8% RESPONSE		
p = <0.0001, extremely significant		
0.1% DMSO FROG RINGER'S		
	175	-13.0
	180	-13.4
	185	-13.1
BASELINE AVERAGE		-13.2

TABLE VII

REPRESENTATIVE PHENOL SAMPLE I

SOLUTION	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)
0.1% FROG RINGER'S		
	90	-19.0
	95	-19.2
	100	-19.4
BASELINE AVERAGE		-19.2
0.1% PHENOL		
	105	-20.7
	110	-24.5
	115	-26.3 PEAK
	120	-26.3
	125	-25.3
	130	-24.4
	131	-24.4
	135	-22.9
	140	-22.3
	145	-22.4
	150	-23.2
37.0% RESPONSE		
p = 0.0003, extremely significant		
0.1% FROG RINGER'S		
	155	-24.3
	160	-25.1
	165	-25.8
BASELINE AVERAGE		-25.1

TABLE VIII

REPRESENTATIVE PHENOL SAMPLE II

SOLUTIONS	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)	
0.1% FROG RINGER'S			
	100	-76.2	
	105	-74.1	
	110	-72.5	
BASELINE AVERAGE		-74.3	
0.1% PHENOL			
	111	-63.3	
	115	-53.7	
	120	-53.8	
	125	-51.9	
	130	-47.6	PEAK
-35.9% RESPONSE			
p = 0.0016, very significant			
0.1% FROG RINGER'S			
	131	-49.6	
	135	-51.9	
	140	-45.7	
BASELINE AVERAGE		-49.1	

TABLE IX

REPRESENTATIVE PHENOL SAMPLE III

SOLUTION	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)
0.1% FROG RINGER'S		
	215	-38.0
	220	-43.9
	225	-47.7
	230	-48.9
	235	-50.3
BASELINE AVERAGE		-45.8
0.1% PHENOL		
	236	-51.9
	240	-52.3
	245	-45.0
	250	-35.2
	255	-27.0 PEAK
-41.0% RESPONSE		
p = 0.0011, extremely significant		
0.1% FROG RINGER'S		
	256	-25.9
	260	-24.7
	265	-25.6
BASELINE AVERAGE		-25.4

TABLE X

REPRESENTATIVE PHENOL SAMPLE IV

SOLUTION	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)
0.1% FROG RINGER'S		
	190	-23.8
	195	-23.4
	200	-23.3
	205	-23.1
	210	-22.6
BASELINE AVERAGE		-23.2
0.1% PHENOL		
	211	-21.4
	215	-21.6
	220	-21.5
	225	-20.9
	230	-18.7 PEAK
-19.4% RESPONSE		
P = <0.0001, extremely significant		
0.1% FROG RINGER'S		
	231	-19.3
	235	-18.7
	240	-19.0
BASELINE AVERAGE		-19.0

TABLE XI

SUMMARY PHENOL COMPARISONS

EXPERIMENT #	PHENOL I	PHENOL II	PHENOL III	PHENOL IV
BEFORE 1 (uA)	-19.0	-76.2	-38.0	-23.8
BEFORE 2 (uA)	-19.2	-74.1	-43.9	-23.4
BEFORE 3 (uA)	-19.4	-72.5	-47.7	-23.3
BASELINE AVG	-19.2	-74.3	-45.8	-23.2
PEAK (uA)	-26.3	-47.6	-27.0	-18.7
% RESPONSE	-37.0%	-35.9%	-41.0%	-19.4%
p VALUE	0.0003	0.0016	0.0011	<0.0001
SIGNIFICANCE	extremely	very	extremely	extremely
AVERAGE % RESPONSE		-33.3%		
AFTER 1 (uA)	-24.3	-49.6	-25.9	-19.3
AFTER 2 (uA)	-25.1	-51.9	-24.7	-18.7
AFTER 3 (uA)	-25.8	-45.7	-25.6	-19.0
BASELINE AVG	-25.1	-49.1	-25.4	-19.0
RECOVERY	30.7%	-33.9%	-44.5%	-18.1%
AVERAGE RECOVERY		-16.5%		

TABLE XII

REPRESENTATIVE PHENOL DOSE RESPONSE

SOLUTION	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)
0.1% FROG RINGER'S	85	-86.0
	90	-83.3
	95	-79.4
BASELINE AVERAGE		-82.9
0.1% PHENOL		
	96	64.0
	100	-47.0
	105	-44.2
	110	-39.4 PEAK
-52.5% RESPONSE		
p = 0.0019, very significant		
0.1% FROG RINGER'S	115	-52.6
	120	-43.0
	125	-43.0
BASELINE AVERAGE		-46.2
0.05% PHENOL		
	130	-40.9
	135	-36.4
	140	-34.8
	145	-33.4 PEAK
-59.7% RESPONSE		
0.0015, very significant		
0.1% FROG RINGER'S	150	-34.6
	155	-36.9
	160	-38.7
	165	-40.7
BASELINE AVERAGE		-38.2
0.025% PHENOL		
	170	-40.7 PEAK
	175	-41.0
	180	-43.6
	185	-45.6
	190	-47.0
-50.9% RESPONSE		
p = 0.0021, very significant		
0.1% FROG RINGER'S	195	-48.1
	200	-51.6
BASELINE AVERAGE		-49.8

TABLE XIII

REPRESENTATIVE 1-CHLORONAPHTHALENE SAMPLE I

SOLUTION	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)
0.1% DMSO FROG RINGER'S		
	65	-55.0
	70	-53.9
	75	-53.5
BASELINE AVERAGE		-54.1
0.1% 1-CHLORONAPHTHALENE		
	80	-55.7
	85	-56.5 PEAK
4.4% RESPONSE		
p = 0.0341, significant		
0.1 % DMSO FROG RINGER'S		
	90	-54.1
	95	-53.3
	100	-53.1
BASELINE AVERAGE		-53.5

TABLE XIV

REPRESENTATIVE 1-CHLORONAPHTHALENE SAMPLE II

SOLUTION	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)
0.1% DMSO FROG RINGER'S		
	50	-54.9
	55	-53.9
	60	-52.9
BASELINE AVERAGE		-53.9
0.1% 1-CHLORONAPHTHALENE		
	65	-53.8
	70	-55.5
	75	-55.7 PEAK
3.3% RESPONSE		
p = 0.0893, marginally significant		
0.1% DMSO FROG RINGER'S		
	80	-55.4
	85	-54.7
	90	-54.3
BASELINE AVERAGE		-54.8

TABLE XV

SUMMARY 1-CHLORONAPHTHALENE COMPARISONS

EXPERIMENT #	CHLORONAPHTHALENE I		CHLORONAPHTHALENE II
BEFORE 1 (uA)	-55.0		-54.9
BEFORE 2 (uA)	-53.9		-53.9
BEFORE 3 (uA)	-53.5		-52.9
AVERAGE (uA)	-54.1		-53.9
PEAK	-56.5		-55.7
% RESPONSE	4.4%		3.3%
p VALUE	0.0341		0.0893
SIGNIFICANCE LEVEL	significant		marginally
AVERAGE % RESPONSE		3.9%	
AFTER 1 (uA)	-54.1		-55.4
AFTER 2 (uA)	-53.3		-54.7
AFTER 3 (uA)	-53.1		-54.3
BASELINE AVG (uA)	-53.5		-54.8
BASELINE RECOVERY	-1.1		1.7
AVERAGE RECOVERY		0.3%	

TABLE XVI

REPRESENTATIVE PHENANTHRENE SAMPLE I

SOLUTIONS	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)
0.1% DMSO FROG RINGER'S		
	65	-7.2
	70	-7.4
	75	-7.5
BASELINE AVERAGE		-7.4
0.1% PHENANTHRENE		
	80	-8.1
	85	-8.4
	90	-8.8 PEAK
18.9% RESPONSE		
P = 0.0038, very significant		
0.1% DMSO FROG RINGER'S		
	95	-9.4
	100	-9.9
	105	-10.6
BASELINE AVERAGE		-10.0

TABLE XVII

REPRESENTATIVE PHENANTHRENE SAMPLE II

SOLUTION	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)
0.1% DMSO FROG RINGER'S		
	65	-45.7
	70	-44.8
	75	-42.9
BASELINE AVERAGE		-44.5
0.1% PHENANTHRENE		
	80	-41.5
	85	-40.8
	90	-40.3 PEAK
-9.4% RESPONSE		
p = 0.0371, significant		
0.1% DMSO FROG RINGER'S		
	100	-39.8
	105	-39.9
	110	-40.4
BASELINE AVERAGE		-40.0

TABLE XVIII

SUMMARY PHENANTHRENE COMPARISONS I

EXPERIMENT #	PHENANTHRENE I		PHENANTHRENE II
BEFORE 1 (uA)	-7.2		-45.7
BEFORE 2 (uA)	-7.4		-44.8
BEFORE 3 (uA)	-7.5		-42.9
BASELINE AVERAGE (uA)	-7.4		-44.5
PEAK (uA)	-8.8		-40.3
% RESPONSE	18.9%		-9.4%
p VALUE	0.0038		0.0371
SIGNIFICANCE LEVEL	very		significant
AVERAGE % RESPONSE		9.5%	
AFTER 1 (uA)	-9.4		-39.8
AFTER 2 (uA)	-9.9		-39.9
AFTER 3 (uA)	-10.6		-40.4
BASELINE AVERAGE (uA)	-10.0		-40.0
BASELINE RECOVERY	35.1%		-10.1%
BASELINE AVERAGE		12.5%	

TABLE XIX

REPRESENTATIVE PHENANTHRENE SAMPLE III

SOLUTIONS	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)
0.1% P-F127 DMSO FROG RINGER'S		
	185	-41.1
	190	-41.7
	195	-42.4
BASELINE AVERAGE		-41.7
0.1% PHENANTHRENE		
	200	-43.6
	205	-45.2
	210	-47.9 PEAK
14.9% RESPONSE		
p = 0.0037, very significant		
0.1% P-F127 DMSO FROG RINGER'S		
	215	-50.6
	220	-54.3
	225	-59.0
BASELINE AVERAGE		-54.6

TABLE XX

REPRESENTATIVE PHENANTHRENE SAMPLE IV

SOLUTION	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)
0.1% P-F127 DMSO FROG RINGER'S		
	60	-65.6
	65	-62.8
	70	-60.1
BASELINE AVERAGE		-62.8
0.1% PHENANTHRENE		
	75	-58.3
	80	-57.8
	85	-57.0 PEAK
-9.2% RESPONSE		
p = 0.0668, marginally significant		
0.1% P-F127 DMSO FROG RINGER'S		
	90	-58.2
	95	-57.6
	100	-56.3
BASELINE AVERAGE		-57.4

TABLE XXI

REPRESENTATIVE PHENANTHRENE SAMPLE V

SOLUTION	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)
0.1% P-F127 DMSO FROG RINGER'S		
	125	-39.2
	130	-38.8
	135	-38.6
BASELINE AVERAGE		-38.9
0.1% PHENANTHRENE		
	140	-40.0
	145	-40.5
	150	-41.0 PEAK
5.4% RESPONSE		
p = 0.0068, very significant		
0.1% P-F127 DMSO FROG RINGER'S		
	155	-40.6
	160	-40.4
	165	-40.5
BASELINE AVERAGE		-40.5

TABLE XXII

SUMMARY PHENANTHRENE COMPARISONS II

EXPERIMENT #	PHENANTHRENE III	PHENANTHRENE IV	PHENANTHRENE V
BEFORE 1 (uA)	-41.1	-65.6	-39.2
BEFORE 2 (uA)	-40.2	-62.8	-38.8
BEFORE 3 (uA)	-40.7	-60.1	-38.6
BASELINE AVG (uA)	-40.7	-62.8	-38.9
PEAK (uA)	-47.9	-57.0	-41.0
% RESPONSE	14.9%	-9.2%	5.4%
p VALUE	0.0037	0.0668	0.0068
SIGNIFICANCE	very	marginally	very
AVERAGE % RESPONSE		3.7%	
AFTER 1 (uA)	-50.6	-58.2	-40.6
AFTER 2 (uA)	-54.3	-57.6	-40.4
AFTER 3 (uA)	-59.0	-56.3	-40.5
BASELINE AVG (uA)	-54.6	-57.4	-40.5
BASELINE RECOVERY	34.2%	-8.6%	-1.2%
AVERAGE RECOVERY		8.9%	

TABLE XXIII

REPRESENTATIVE EFFLUENT SAMPLE I

SOLUTION	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)
FROG RINGER'S		
	75	-85.6
	80	-85.0
	85	-85.1
BASELINE AVERAGE		-85.2
EFFLUENT		
	90	
	95	-75.1 PEAK
	100	-75.5
-11.9 % RESPONSE		
p = 0.00003, extremely significant		
FROG RINGER'S		
	105	-81.8
	110	-84.8
	115	-86.4
BASELINE AVERAGE		-84.3

TABLE XXIV

REPRESENTATIVE EFFLUENT SAMPLE II

SOLUTION	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)
FROG RINGER'S		
	50	-40.3
	55	-40.0
	60	-40.7
BASELINE AVERAGE		-40.3
EFFLUENT II		
	65	-39.5
	70	-39.4 PEAK
	75	-39.7
-2.2% RESPONSE p = 0.0440, significant		
FROG RINGER'S		
	80	-40.3
	85	-43.1
	90	-44.0
BASELINE AVERAGE		-42.5

TABLE XXV

REPRESENTATIVE EFFLUENT SAMPLE III

SOLUTION	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)
FROG RINGER'S		
	85	-35.1
	90	-36.6
	95	-39.9
BASELINE AVERAGE		-37.2
EFFLUENT		
	100	-35.0
	105	-33.9
	110	-31.6 PEAK
-15.1% RESPONSE		
p = 0.0585, marginally significant		
FROG RINGER'S		
	115	-33.7
	120	-34.9
	125	-34.5
BASELINE AVERAGE		-34.4

TABLE XXVI

REPRESENTATIVE EFFLUENT SAMPLE IV

SOLUTION	TIME (MINUTES)	SHORT-CIRCUIT CURRENT (uA)
FROG RINGER'S		
	255	-32.3
	260	-32.4
	265	-32.3
BASELINE AVERAGE		-32.3
EFFLUENT	270	-28.2
	275	-25.1
	280	-23.1 PEAK
-28.5% RESPONSE		
p = <0.0001, extremely significant		
FROG RINGER'S		
	285	-24.2
	290	-26.0
	295	-26.9
BASELINE		-25.7

TABLE XXVII

SUMMARY EFFLUENT COMPARISONS

EXPERIMENT #	EFFLUENT 1	EFFLUENT 2	EFFLUENT 3	EFFLUENT 4
BEFORE 1 (uA)	-85.6	-40.3	-35.1	-32.3
BEFORE 2 (uA)	-85.0	-40.0	-36.6	-32.4
BEFORE 3 (uA)	-85.1	-40.7	-39.9	-32.3
BASELINE AVG	-85.2	-40.3	-37.2	-32.3
PEAK	-75.1	-39.4	-31.6	-23.1
% RESPONSE	-11.9%	-2.2%	-15.1%	-28.5%
p VALUES	0.0003	0.0440	0.0585	<0.0001
SIGNIFICANCE	extremely	significant	marginally extremely	
AVERAGE % RESPONSE		-14.4%		
AFTER 1 (uA)	-81.8	-40.3	-33.7	-24.2
AFTER 2 (uA)	-84.8	-43.1	-34.9	-26.0
AFTER 3 (uA)	-86.4	-44.0	-34.5	-26.9
BASELINE AVG	-84.3	-42.5	-34.4	-25.7
RECOVERY	1.1%	5.5%	-7.5%	-9.8%
AVERAGE RECOVERY		-2.7%		

TABLE XXVIII

EFFLUENT TEST SAMPLE RESULTS

EXPERIMENT #	EFFL 1	EFFL 2	EFFL 3	EFFL 4
Daphnia				
NOEC				
Survival	>100%	95%	100%	30%
Reproduction		52%	100%	100%
Fathead minnow				
NOEC				
Survival	>100%	63%	100%	100%
Reproduction		10%		
Frog skin				
Response	-11.9%	-2.2%	-15.1%	-28.5%

FIGURES

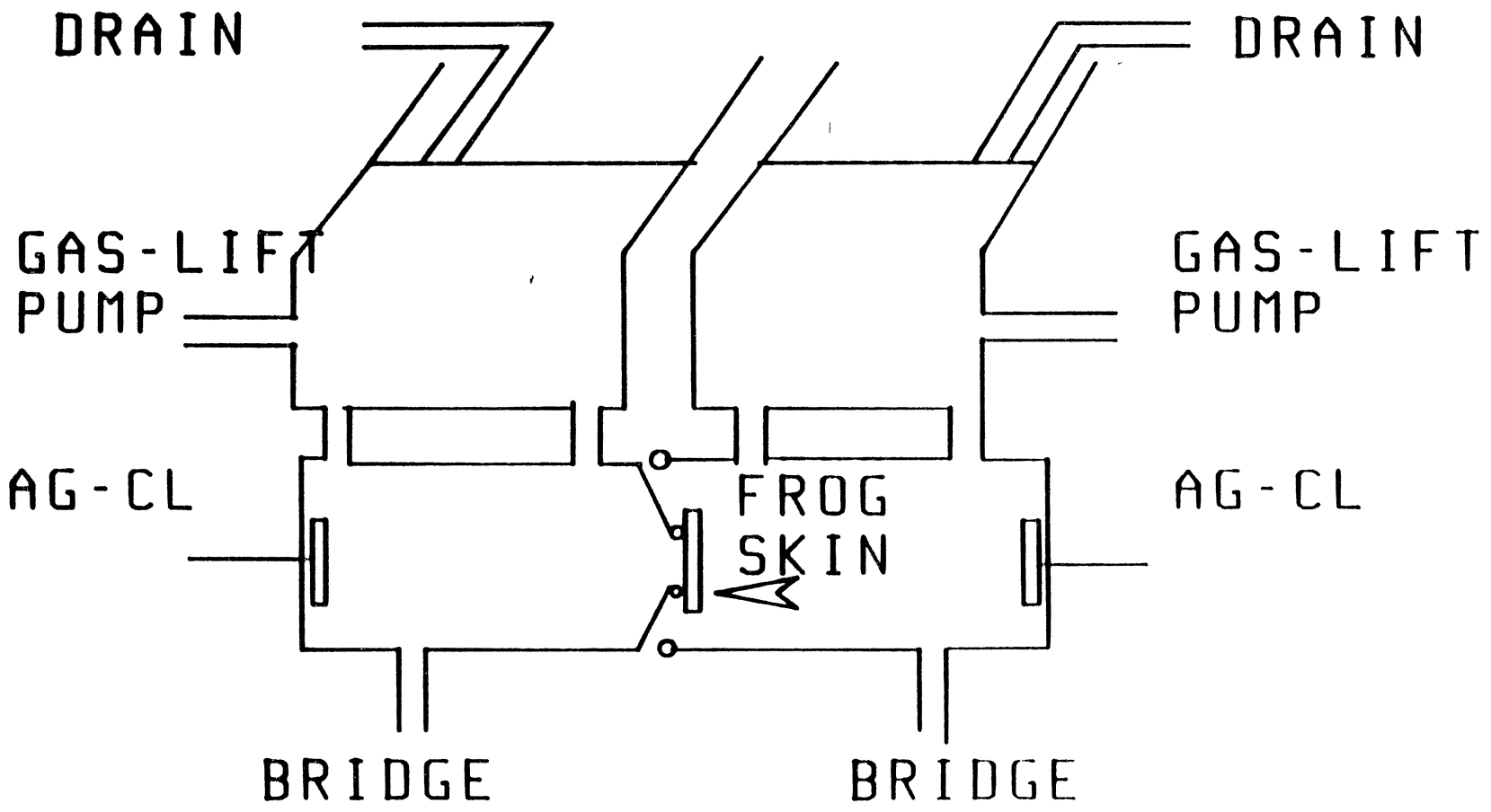

Figure 1. Ussing chamber

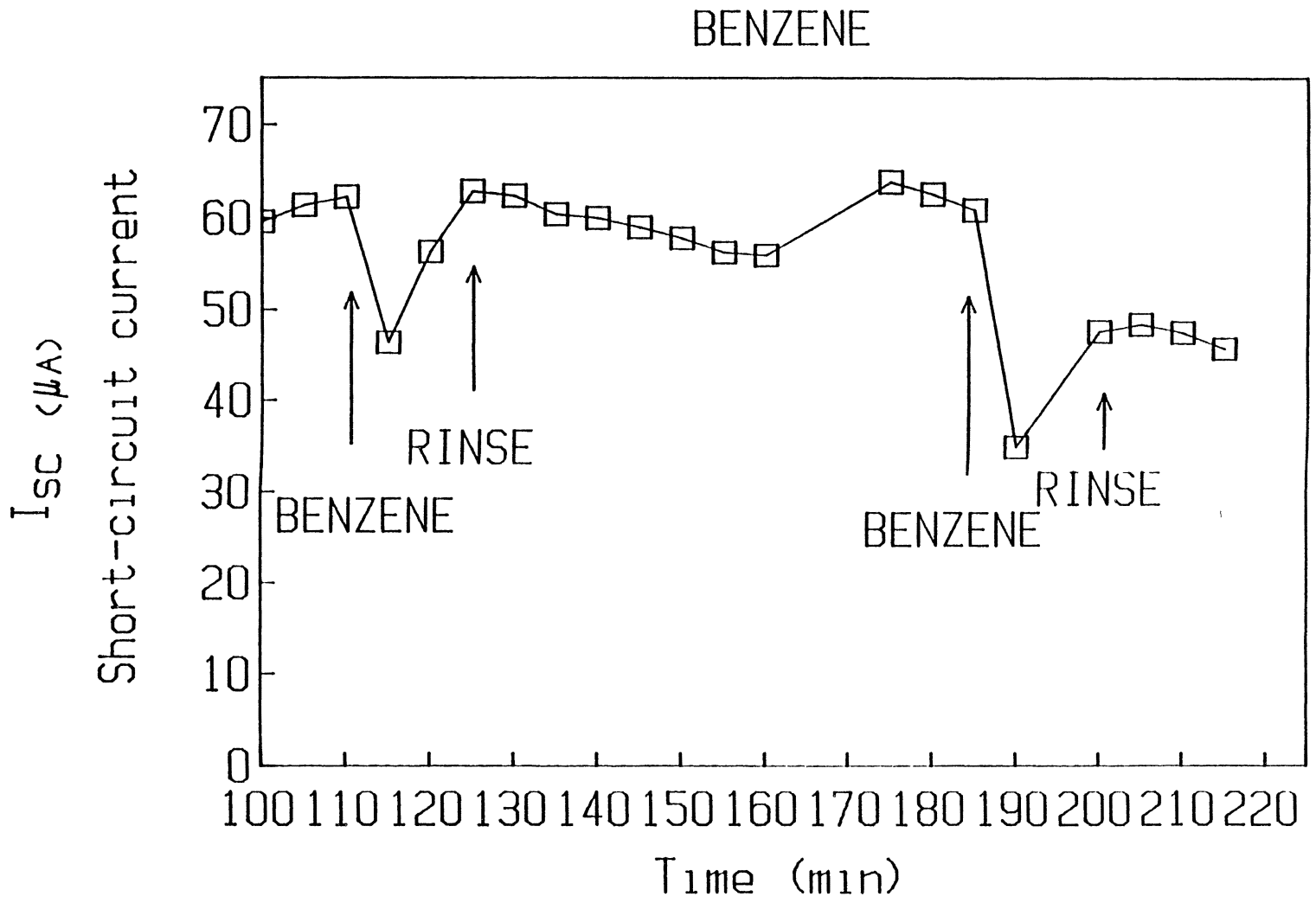

Figure 2. Representative benzene sample

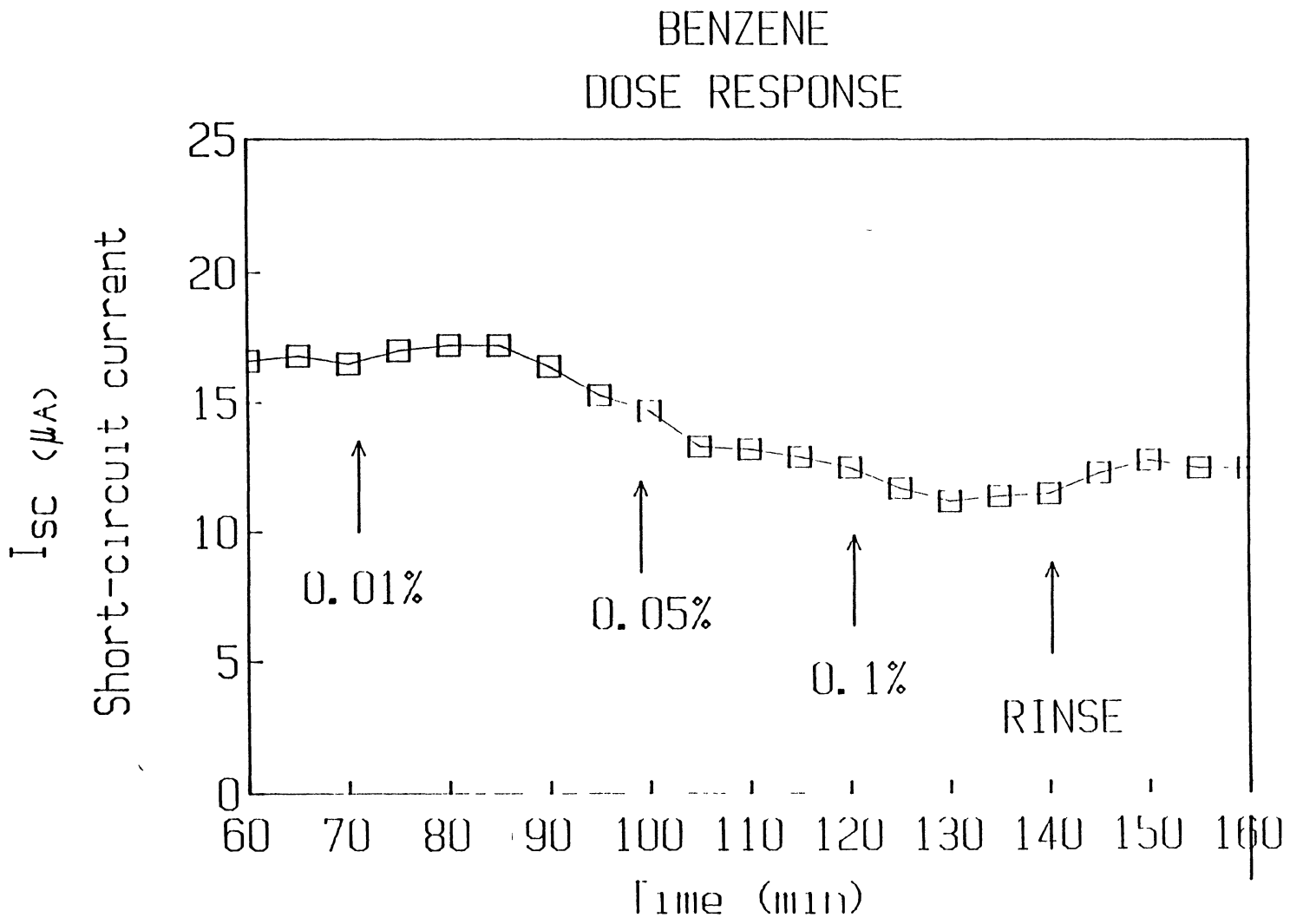

Figure 3. Representative benzene dose response

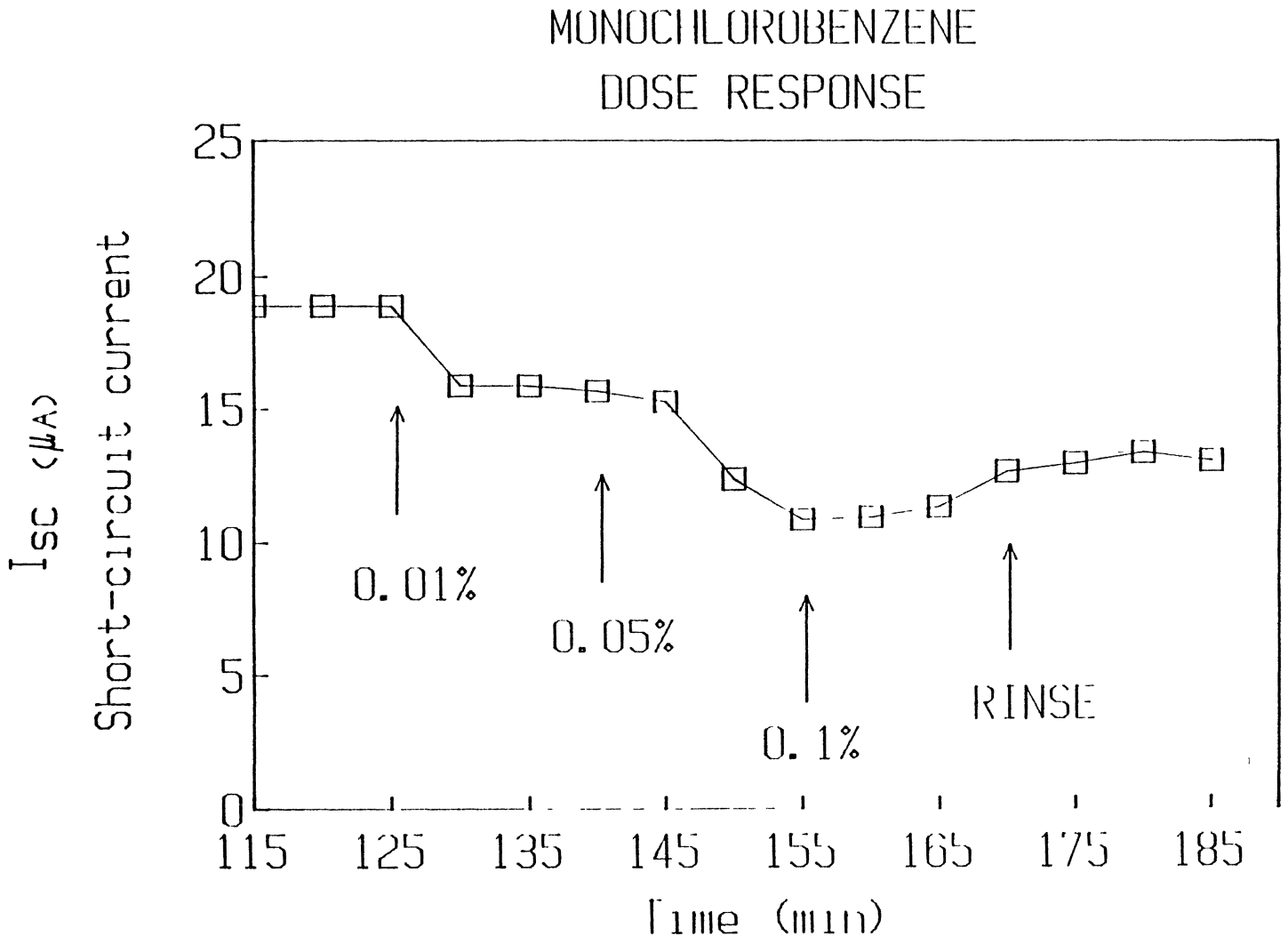

Figure 4. Representative monochlorobenzene dose response

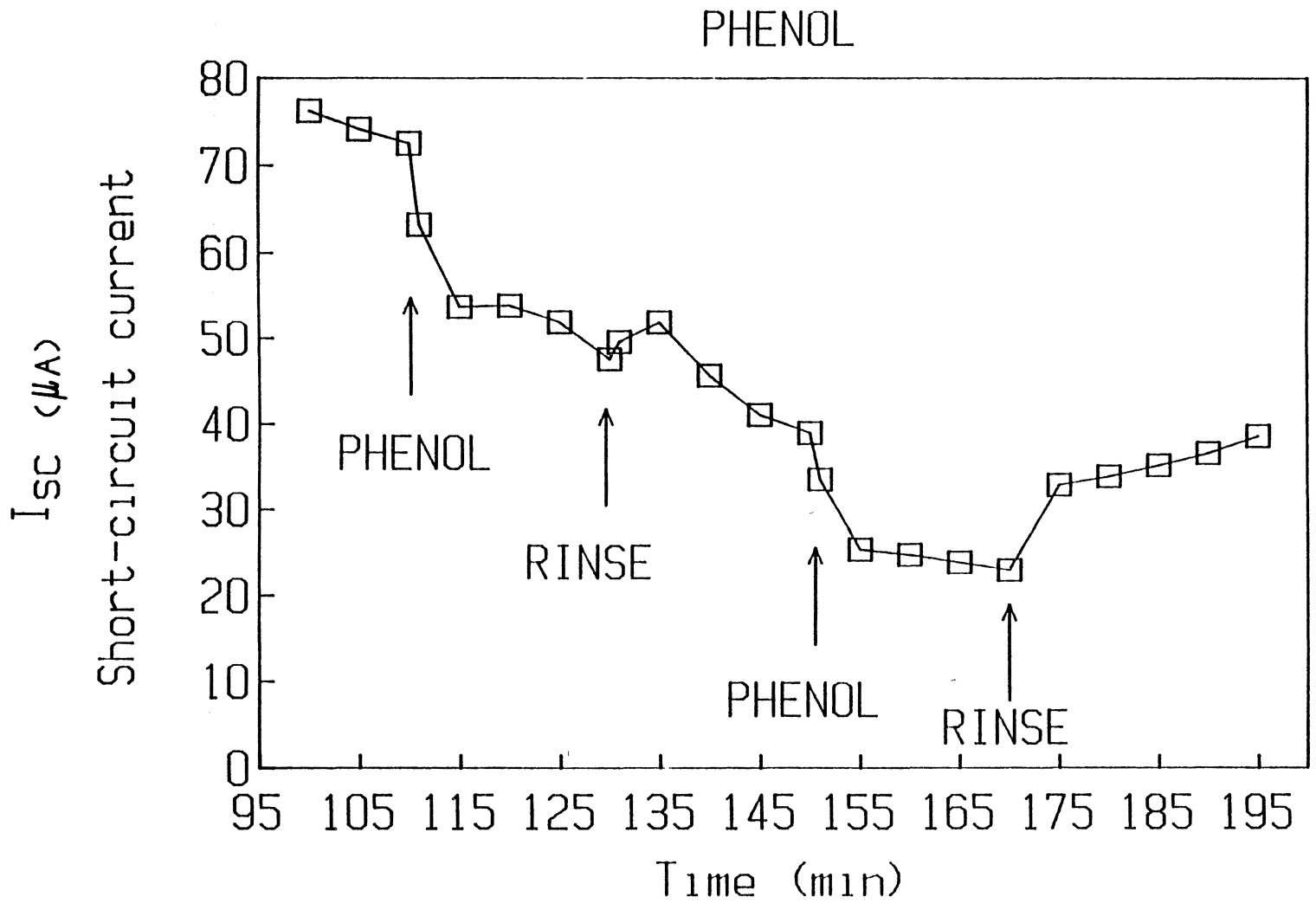

Figure 5. Representative phenol sample

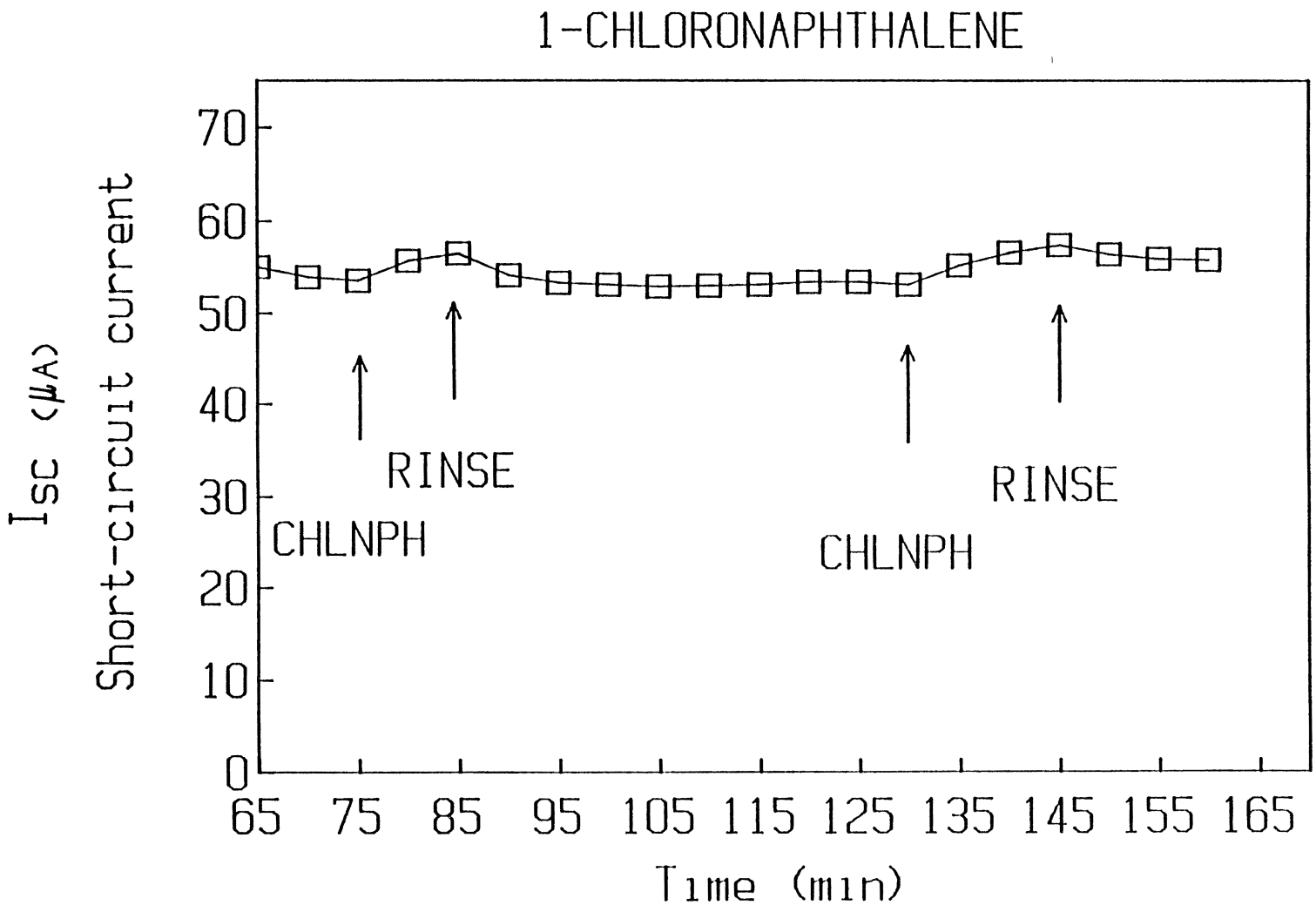

Figure 6. Representative 1-chloronaphthalene sample

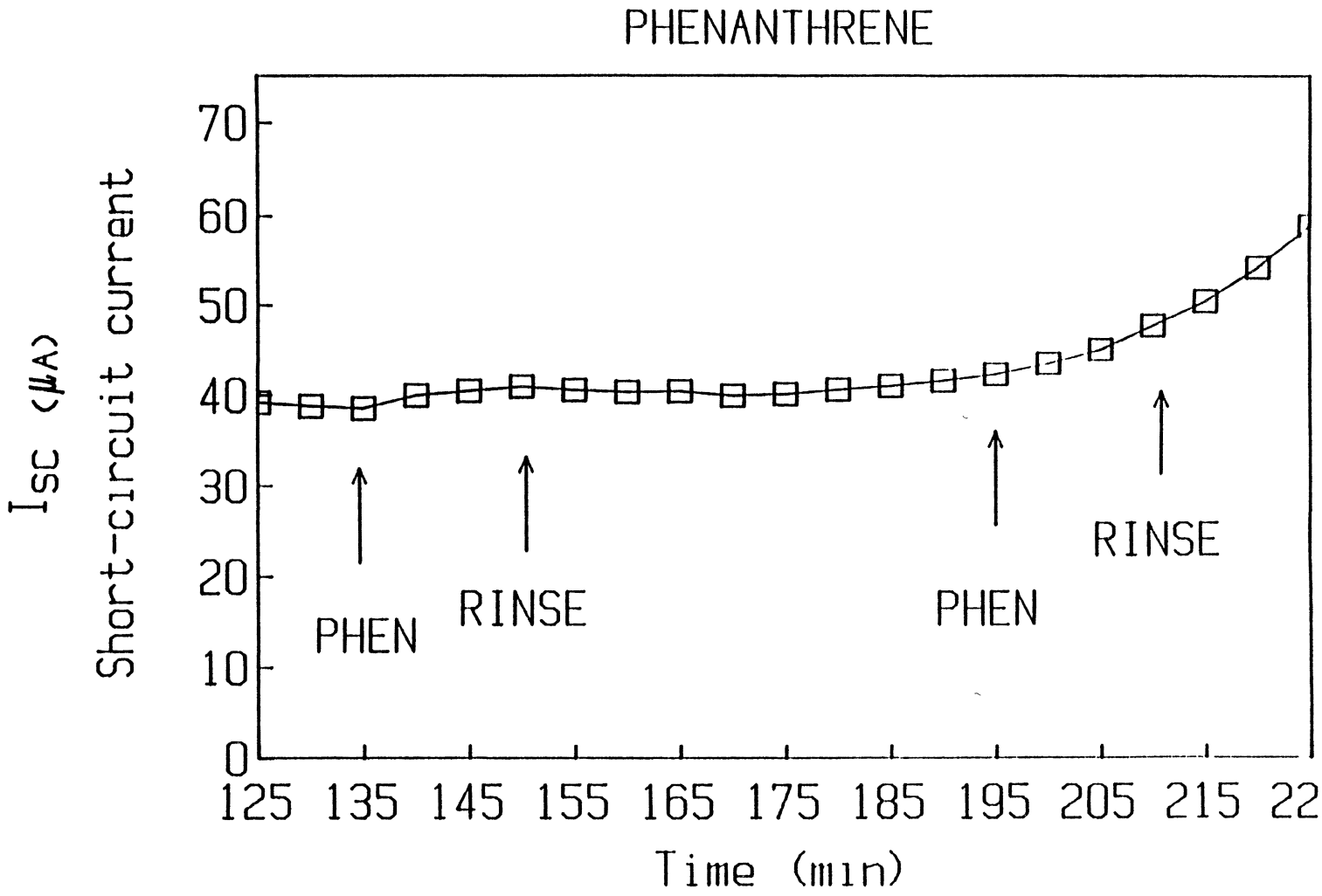

Figure 7. Representative phenanthrene sample

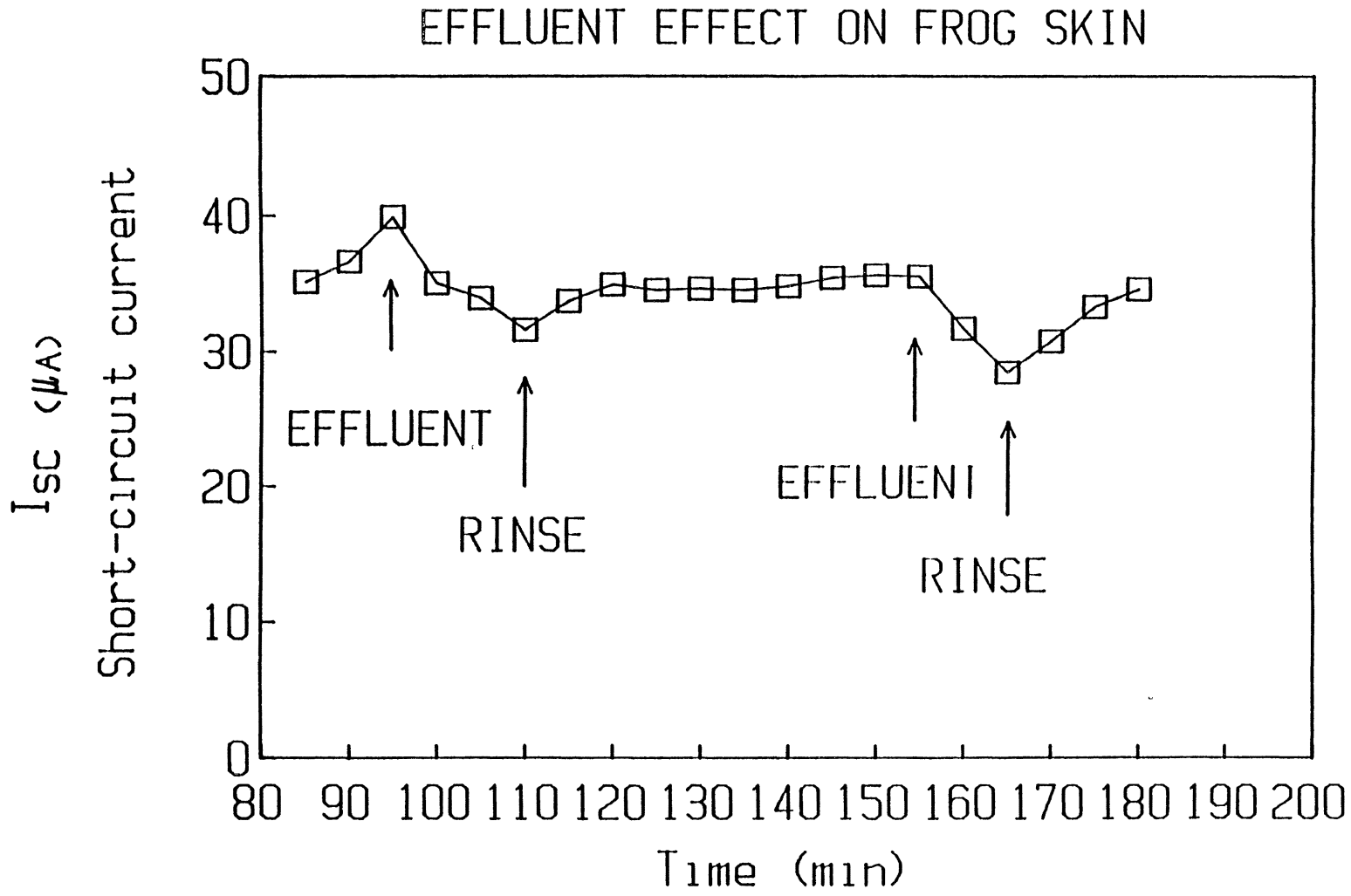

Figure 8. Representative effluent sample

VITA

Mary C. Bowerman

Candidate for the Degree of

Doctor of Education

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