THE EFFECTS OF CADMIUM AND NAPHTHALENE ON OSMOREGULATORY TRANSPORT IN THE OPERCULAR EPITHELIUM OF THE CHANNEL CATFISH, *ICTALURUS PUNCTATUS*

By

LARRY LLOYD ZINN

Bachelor of Science
Oklahoma State University
Stillwater, Oklahoma
1974

Master of Science
Oklahoma State University
Stillwater, Oklahoma
1979

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Thesis Approved:

Dr. James E. Breazile
Thesis Adviser

George E. Burrows

Dr. L. L. Burks

W. R. Wade

Norman N. Durham
Dean of the Graduate College

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CHAPTER I

INTRODUCTION

The effects of toxic compounds on an organism are varied and complex. Historically, toxicity research has focused on the lethal and acute effects of toxicants. Chronically toxic effects are more difficult to determine because they may not appear until after long periods of exposure, usually longer than ninety days. Chronic toxicity is not necessarily a lesser degree of acute toxicity, but may represent an entirely different mechanism than the acute effects of the same compound (13).

Falling under the general grouping of chronic effects is a category of subtle sublethal effects. These sublethal, deleterious effects are changes in physiological parameters that are masked during more intense intoxication but are initiated at very low levels of toxicant exposure. Subtle effects have largely been ignored in the past, but much attention is now turning toward the development of test procedures to analyze for these changes. The necessary test techniques will be valuable in adding to knowledge of the toxic actions of particular compounds and, when integrated with a spectrum of existing tests may prove valuable as screening procedures for unknown toxicants. There is some

evidence to indicate that subtle toxic effects in a particular organism may be more sensitive monitors of toxic exposure than some currently accepted analytic procedures (54). Subtle toxic effects can be associated with either environmental stress or functional changes due to contact between a toxic compound and the organism. Environmental alterations such as; changes in temperature, salinity, water or soil pH, air quality, and light intensity are examples of stress factors that can cause subtle physiological changes in an organism. These kinds of factors are secondary to an external causative agent and, although easy to quantitate, are difficult to associate with their causative agents. Functional changes, such as decreased kidney or liver function, changes in rates of absorption, changes in hormone levels, and changes in active transport mechanisms are often linked directly to a causative chemical agent present in or on the organism. Sublethal levels of toxic agents may cause subtle deleterious physiological effects, which may not be compensated for by the organism. Most organisms are finely balanced within an environmental niche, and even slight alterations in basic functions can be reflected in the ability of that organism to compete. The consequence of continual low level exposure to a toxic compound is a decrease in the organisms survival potential.

The aquatic ecosystem is probably the most sensitive to agents producing subtle toxicity. Toxicants in aqueous environments continuously bathe aquatic organisms in a

myriad of trace contaminants. It should be possible to develop a very sensitive test to evaluate toxic effects from sublethal exposures to a variety of compounds by selecting a particularly sensitive physiological system with a common importance to a large variety of organisms.

The fish gill is a 'sentinal' tissue for response to toxic compounds. It is the first functional system to come in contact with aqueous environmental contaminants. In addition to gas exchange, the gill is the major tissue of osmoregulation, hydrogen ion balance, and nitrogenous waste excretion, all of which to some degree are active systems requiring metabolic energy in the form of adenosine triphosphate (ATP).

For this study, osmoregulation was chosen as the physiological system to be examined. Not only is osmoregulation a universally important process in vertebrates and aquatic invertebrates, but because it is totally dependant upon an active transport system, it models other active transport systems. It is the purpose of this study to use electrophysiological techniques to evaluate changes in osmoregulatory active transport caused by low levels of a heavy metal, cadmium, and a polynuclear aromatic hydrocarbon, naphthalene. The procedures used are modified from the methods of Karnaky and Kinter (32) and Degnan and Zadunaisky (10). The method is an in vitro study of epithelial tissue. Isolated in a lucite "Ussing" type chamber voltage clamp circuitry is used to measure active transport of ions

characterized by the electrical parameters of transepithelial potential difference and short circuit current.

The rationale for this research technique is that the results from this procedure are very sensitive and predictive (10). Since osmoregulation involves an active transport process, it is dependent on several properties of the epithelial cells, such as membrane permeability and transport protein structure. Disruption of normal function of any one of these properties will cause a noticeable change in overall osmoregulatory activity. It has been shown that several categories of toxic compounds can, under proper conditions, alter these cellular properties (10). It is apparent that with this delicately balanced system located in mucosal tissue that is constantly in direct contact with the external environment, the osmoregulatory capability of opercular epithelial tissue should serve as a good model for predicting effects of deleterious pollutants.

Prediction of toxic effects is a topic that is considered of paramount importance in the field of modern toxicology. It is important to develop the type of techniques that will allow not only prediction of toxic effect from one species to another, but prediction from one functional system to another and even more important from one compound to another similar compound. Changes in normal osmoregulatory active transport in intoxicated tissue is potentially predictive at all three of these levels. Virtually every physiological system in every higher organism incorporates

some principles of active transport. The possibilities for predictive evaluation of data from this experimental approach should prove positive. When incorporated into established testing protocols this procedure may add important information to our overall knowledge of the effects of toxic compounds on living systems.

CHAPTER II

REVIEW OF LITERATURE

Toxicity Evaluation

Paracelsus first described the relationship of dose to toxic effect some four hundred years ago (13). He also suggested that chemical "toxicons" vary in specificity. These ideas of dose and mechanism have been incorporated in most subsequent toxicity testing procedures. Douell (13) evaluates the purpose of toxicology experimentation as both descriptive and mechanistic in nature.

Toxicity tests are designed to evaluate the risk posed to man and the environment by exposure to specific chemicals. The predominant form of toxicity test is the bioassay. A bioassay is any test that uses the response of an organism to detect and measure the presence or effect of one or more substances or conditions.

There is a wide variety of bioassay procedures used to evaluate toxic effects at the acute, subacute, and chronic levels. Many currently used test procedures require considerable time and recent research has been concentrated on the development of more rapid procedures. The Ames rapid screening test was developed by B.N. Ames over a twelve year period. It is a carcingogenicity test that is widely used,

which is both rapid and highly sensitive. Four strains of Salmonella typhamuriun have been developed that are dependent on the amino acid histidine for growth and reproduction. The bacteria are placed in a histidine free medium with liver cell homogenates from rodents or man (to accomplish mammalian metabolism of toxicants). Back mutation to the native form of histidine independent bacteria initiates growth of colonies in the media around the dose spot. The number of mutated colonies are counted. This spot test is rapid and with some modification can be used to establish dose-response curves at nanogram sensitivity for many chemicals (1). The major limitation of this system is the toxicity of some test compounds to the micro-organism. Stimulated by the success of the Ames test, further developments of this and similar short term mutagen-carcinogen assays have been made and are summarized by De Bruin (9). Several toxicity tests are being developed that incorporate in vitro procedures associated with cell transformation and tissue culture (1).

Although much research still continues, aimed at the improvement of common tests and the introduction of new species into existing procedures, the most recent direction of toxicology research is toward rapid screening tests and in vitro studies of specific physiological systems. Private industry has seen the need for these newer procedures and is producing toxicity screening test kits such as the Microtox algal assay.

Osmoregulation in Fish

The concentration of electrolyte ions in fish blood is maintained at a constant level independent of the external environment. Osmotic work is required to maintain this independence of composition (50). The freshwater fish excretes a dilute urine. Sodium (Na) and chloride (Cl) ions may be lost by diffusion down their concentration gradient into the urine or across epithelial tissues exposed directly to the more dilute external medium. This loss is compensated for by the active uptake of these ions across the gill branchial or epithelia (42). Constant composition of electrolytes is a function of membrane phenomena, i.e., active transport of monovalent ions against their electrochemical gradient (67).

Saltwater or saltwater adapted fish appear to have a different system for controlling electrolyte concentrations, although, as it will be shown later, it is important to recognize that both systems contain the same operative components. Saltwater fish swallow large quantities of water which is absorbed by the gut (50). Seawater contains approximately 385 millimoles of sodium chloride, which is largely absorbed along with the water in the gut. Electrolyte balance is maintained by the excretion of mono-valent ions, again by active transport, across the gill epithelia (42).

The gill occupies a central position of singular importance in both fresh and saltwater fish. The fish gill has four major functions (42). The first is the obvious

function of gas exchange, allowing oxygen from the external environment to diffuse into the blood while waste carbon dioxide diffuses out of the blood. The three additional functions of the gill are active transport events, requiring either directly or indirectly the expenditure of metabolic energy in the form of ATP. These active functions are osmoregulation, acid-base balance, and nitrogenous waste excretion. It will be shown later in this review that a close interrelationship exists between these active gill functions.

Gill epithelium is composed of four basic cell types (50). The pavement cells are thin cells with a large surface area in direct contact with the external medium. These cells are believed to be the primary site of gas exchange across the gill (43). Also present, but fewer in number, are the mucous or goblet cells. These cells are located on all epithelial surfaces of the fish and function to secrete a protective mucous layer over the epithelial surfaces. The third cell type is described as a nondifferentiated cell of unknown function. The fourth cell type is a columnar shaped cell found in the gill epithelia in largest concentration between lamellae and around afferent vessels of the gill filament (42). It has a general columnar shape frequently interupted or displaced by other cells surrounding it. These cells may be isolated or surrounded by other cells of the same type.

This last type of cell was first identified by Keyes

and Willmer (36) in 1932 and was named the chloride cell because it was believed to be analagous to the hydrochloric acid secreting cells of the gastric mucosa (42). The chloride cells are believed to be the site of osmoregulatory transport (42, 50). One of the main reasons for the belief that the chloride cells are involved in osmoregulation is that they contain a very dense population of mitochondria, uniformly distributed throughout the cytoplasm (50). A dense network of agranular endoplasmic reticulum is closely associated with the mitochondria. These cells have a greatly increased basolateral cell surface characterized by invaginations and channels (30).

Chloride cells undergo cytological changes during alterations of external salinity (50, 30). Saltwater chloride cells have characteristic pits at the distal end of the cell (externally facing surface) which disappear when adapted to freshwater (42). Freshwater fish will develop these pits or apical crypts when adapted to more saline water (42). Saltwater chloride cells are more numerous and often larger than freshwater cells (64). There is evidence that most of the sodium-potassium activated adenosine triphosphate (Na,k-ATPase) in the gill is located in the chloride cells. Chloride cell rich fractions of gill epithelia exhibit much more Na,k-ATPase activity than gill fractions containing no chloride cells (34). Mitochondria increase in size and the endoplasmic reticulum channels swell during saltwater adaptation (50). Finally, Foskett

and Scheffey (22), using vibrating probe microtechniques, have located outward chloride fluxes at the apical crypt of the saltwater adapted chloride cell.

The hypothesis which locates osmoregulation in the chloride cell has been challenged by several authorities (59), however, there has been no experimental evidence presented to refute this function of the chloride cell (50). It will be accepted for the purpose of this study that the chloride cell is the site of active transport of ions across the gill epithelium.

Active transport is the movement of an ionic species across a cellular membrane, driven directly by energy yielding metabolic reactions occurring against a chemical, or for charged ions, an electrochemical gradient (30). This movement of ions involves a "pump" molecule which is accepted as being any of a number of integral membrane protein enzymes generally called transporting ATPases. All transporting ATPases catalyze the same overall reaction, the hydrolysis of ATP to ADP and inorganic phosphate, using the free energy of the reaction for the transport of ions against the gradient across the membrane of which they are an integral part (58).

The best characterized ATPase is the Na,K-activated ATPase or sodium-potassium "pump". This and another rate limiting enzyme, Carbonic anhydrase, are present in substantial quantities in the chloride cells of the branchial epithelium (42). The activities of both enzymes increase

during adaptation to saltwater. Na,K-ATPase is responsible for the reciprocal transfer of sodium and potassium across the plasma membrane of individual cells (56). The specific activity of this enzyme in the gills of freshwater and saltwater fish is proportional to the level of sodium transport demanded by the external environment (29). In freshwater, active transport of sodium by the gill is low, transport in a saltwater environment is much higher. The measured activity of Na,K-ATPase corresponds to these rates of transport. When chloride cells are treated with tritiated ouabain, autoradiography indicates high concentrations of Na,K-ATPase in the cell membranes (31).

Active transport can be measured by both direct and indirect methods. Measurement of ATPase biochemical activity is an indirect method which has been most used and is the most standardized. This measurement quantitates the activity of transport enzymes from the total tissue fraction but does not allow differentiation of ions transported and does not spatially locate the specific transport system (56, 58). Strong evidence exists that the same enzyme in a single cell transports different ions (net transport) depending on its location in the cell.

Ion flux measurement across intact or isolated tissues is the major direct method for determination of active transport (42, 12). Radioactive isotopes, sodium 24 and chloride 36, are used to determine the rates of unidirectional ion fluxes across transporting epithelia (42). In

intact fish a known quantity of labeled ion is administered by injection into the fish. Samples of the external medium are drawn at timed intervals. Isotopic radioactivity is measured with a scintillation counter (17). Inward flux may be measured in similar fashion but blood samples are more difficult to obtain.

In vitro flux measurements have the advantage of greater sample accessability. Both influx and efflux are often determined simultaneously with side by side matched tissues (43). Flux measurements display saturation kinetics and exhibit Michaelis-Menton characteristics (42), fitting the equation:

$$F\ in = Fmax[ion]\ ext/km+[ion]\ ext$$

The unidirectional fluxes of any ion across a transporting epithelia are correlated by the simple relationship;

$$Fnet = Fin-Fout$$

Although labeled flux measurement is a direct measure of active transport it is often a difficult undertaking. The handling of intact animals may be inconvenient and flux measurements are not descriptive enough for some studies.

A third type of measurement technique used in active transport research is an electrophysiological method of measuring transepithelial potential difference and short circuit current, which has been described by Zadunaiskyi and

Degnan (67) as one of the most powerful experimental techniques available to the physiology researcher. Since this technique was used in the research described in this study, it will be described in some detail.

Electrophysiological techniques for transport phenomena were popularized by the classic experimentation of Ussing and coworkers dating back to the 1940's (37, 63). The idea was not a new one. In 1848 the French scientist DuBois described an electrical potential across animal tissue (37). The work by Ussing simply used electrical parameters in animal tissue to study and describe physiologic function. They used the abdominal skin of the leopard frog Rana sp. isolated as a sheet of tissue on a test chamber, thus the technique is known also as 'flat sheet' electrophysiology (37).

It is possible to examine a great many aspects of active transport by studying the electrophysiology of transporting epithelial tissues. While all cells have the ability to maintain an intracellular steady state by the active transport of simple ions, epithelial tissues are specialized to conduct active transport across the tissue (37). The ideal epthelial tissue for study would contain a single layer of cells tightly joined together with a smooth membrane surface on each side. Although this ideal epithelium would be hard to find, each epithelium is similar to this basic model.

The early work by Ussing and his coworkers began with

the understanding that electrically charged ions move at different rates across the membrane barriers and that any separation of charge of dissimilar ions will cause a measureable difference in electrical potential across the tissue (63). The transepithelial potential difference (PD) is the result of the movement of all charged ions (63), both active and passive. It is easily measured by placing an electrode in solutions on both sides of a tissue. This is neither unique or original to flat sheet technology and can even be accomplished in intact animals (42, 44, 17). The truly elegant idea of electrophysiology measurement that was incorporated into the early work was Francis' (23) theory of short circuit current. If an epithelial tissue is artifically short circuited, that is, the potential is altered in such a way that both sides of the tissue are identical in electric charge, there will be no net passive movement of ions. Ions subject to active transport, facilitated by the expenditure of metabolic energy, will continue to move unidirectionally. The electrical current running through the circuit at this time is the result of all net active transport processess (63, 38).

Ussing and Zerahn (63) compared short circuit current (Isc) with radioactive flux studies, using labeled sodium in the frog skin, and were able to attribute the current to active transport of sodium. This was verified by Lund and Stapp (37) who reported Isc for the frog skin that approximated the flux studies of Ussing and Zerahn. The frog skin

model is summarized by the description - "The Spontaneous potential across the isolated frog skin develops as a result of active transport of sodium ions and the shunting effects of anions (chloride)" (63, p. 113). When impermeant anions (sulphate) are substituted for chloride ions, the outer cell membrane acts as a sodium electrode (selectively permeable to sodium) and the inner cell membrane acts as a potassium electrode (selectively permeable to potassium). Koefoed-Johnsen and Ussing (38) hypothesized that the active transport mechanism is located at the serosal cell membrane. This led to the frog skin model of cell transport in Figure 1 which was generally accepted as the basic cell model for many years (38).

The most important contribution of the early experiments is the 'Ussing' chamber apparatus that with slight alterations is still in use today (63). A schematic for the basic aparatus is shown in Figure 2. Transporting epithelia sheets are placed across an opening between two halves of a lucite chamber with fluid wells of equal capacity on both sides of the tissue. The tissue is continuously bathed with a Ringers solution on both sides. Agar bridges connect the chamber solutions, through a calomel electrode, to a high impedence voltmeter circuit (greater than 1 million ohms) which will not load the circuit (37). Potential difference is measured across this portion of the apparatus. Additional electrodes, usually silver coated by electrolysis with silver chloride, are mounted in the chamber at equal

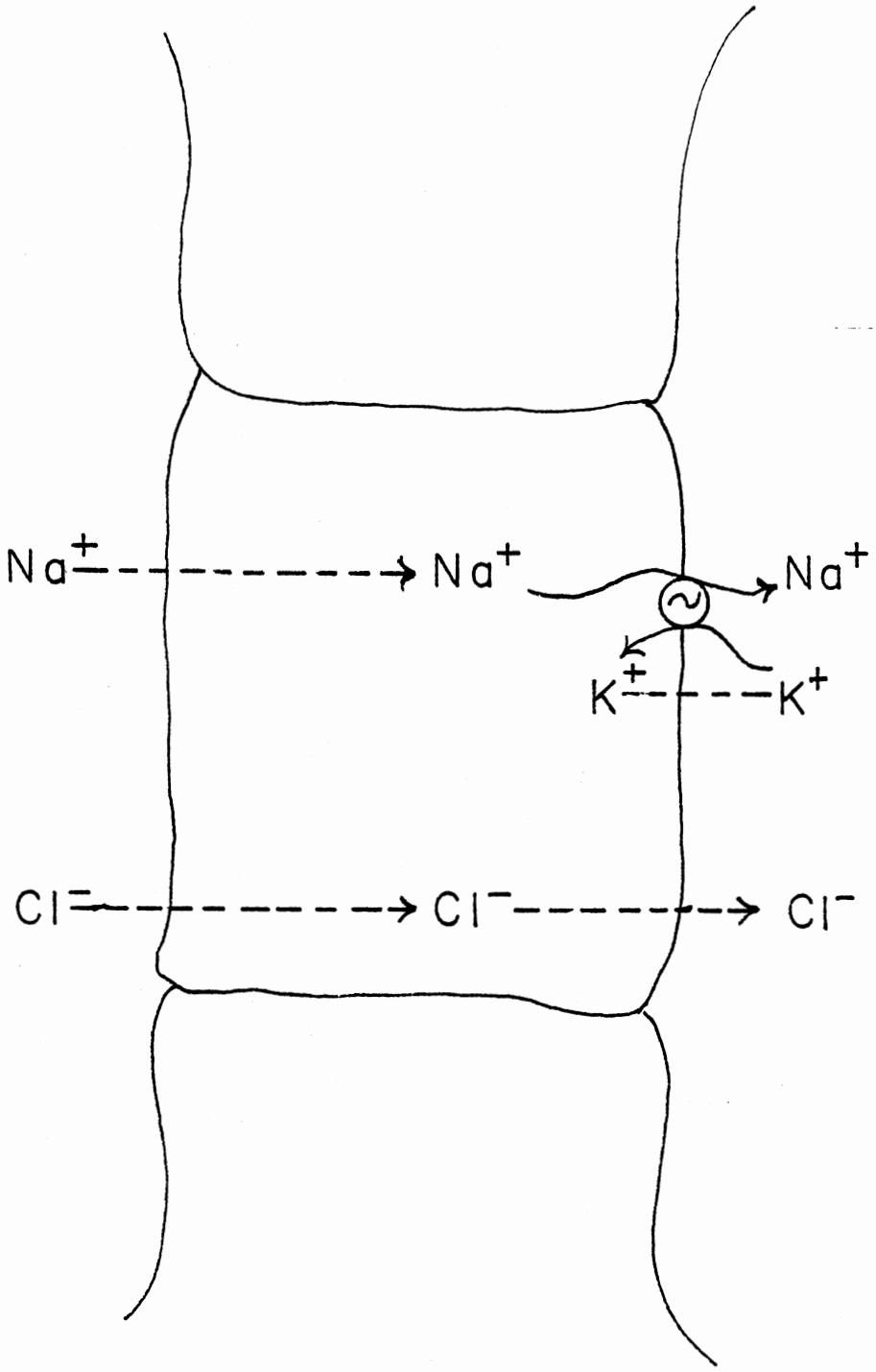

Figure 1. Basic Cell Model for Active Transport of Monovalent Ions

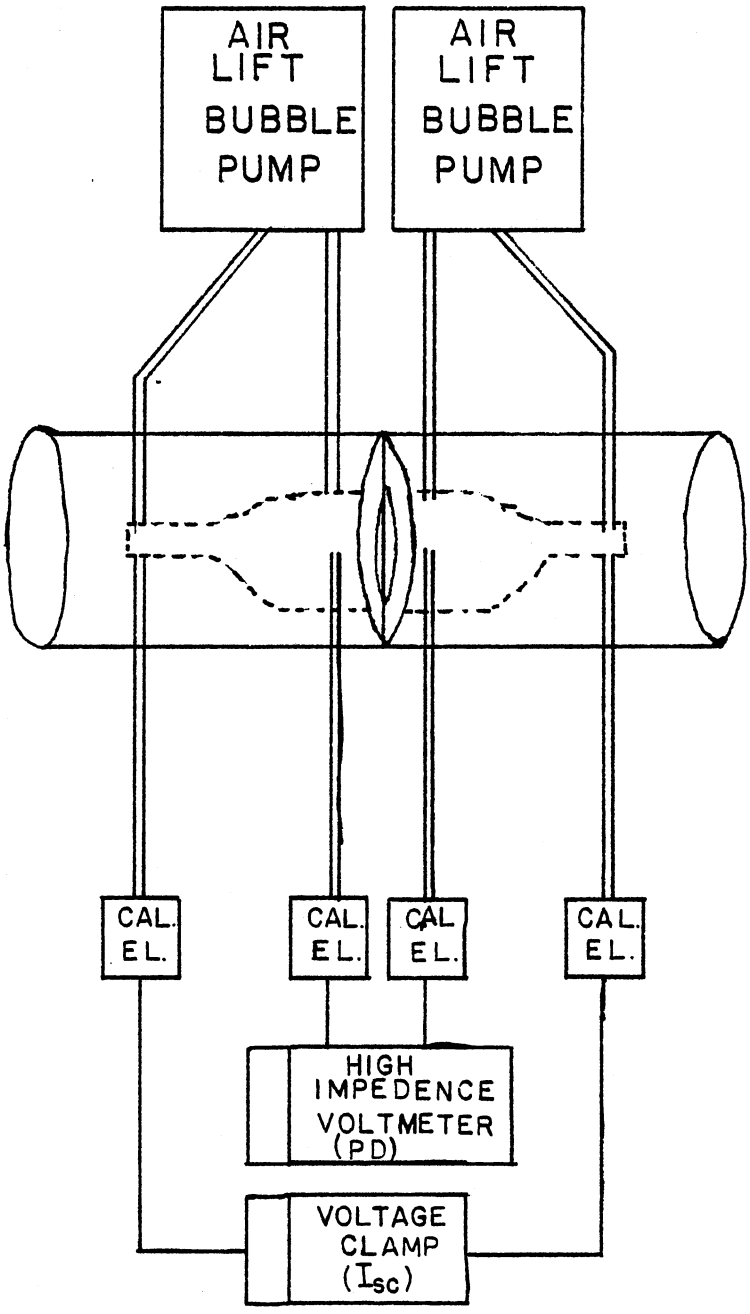

Figure 2. Schematic of the Basic 'Ussing' Apparatus

distances from the tissue. They, in turn, are connected to an adjustable voltage source or voltage clamp circuit. The solutions in both chamber halves are mixed and aerated with air lift bubble pumps. Specific tissues may require slight modifications to this design, such as components to maintain temperature in mammalian tissue or various types of apperture changes to prevent edge damage (37).

Many different tissues have been examined using the techniques outlined above. In the frog skin model the potential is composed of both a diffusion component and an active sodium-potassium pump component (due to the differential rate of ion exchange; 3 Na for 2K) (38). Many other tissues function in a similar manner, transporting sodium or chloride ions by means of variations of this electrogenic pump. An electrogenic pump transfers ions in such a way that it creates a separation of charge or PD (37).

Because of the early success of the frog skin experimenters, much work has been done with active sodium transport, relegating chloride to strictly a passive role. However, when Krogh first hypothesized osmoregulatory transport, he theorized both cationic and anionic mechanisms (41). Sodium transport is usually examined in systems with high potential differences. Tissues with lower potentials have been shown to exhibit chloride transport (67). In 1966 Martin and Curran (45) determined active transport of chloride in the frog skin and their findings were later

corroborated by Kristensen (39) and Watlington and Jessee (65). Since these studies in the frog skin, active transport of chloride has been described in intestinal mucosa, the kidney, fish gill, the opercular epithelium, retinal pigment epithelium, dogfish rectal gland, and the red blood cell (67). Through their work with the rabbit gall bladder, using microelectrodes to penetrate the cell, Frizzell, Field, and Schultz (26) showed a one to one coupling of sodium and chloride movement. They rejected the idea that these ions were transported by separate, electri-cally coupled pathways but were passed into the cell as a neutral NaCl complex. Frizzell and Duffey (25) accepted the basic model for sodium transport and described three modes of transepithelial chloride transport (Figure 3). The first type of chloride movement was described as the passive pathway of the frog skin model, by either transcellular or extracellular route. The second type of chloride transport is the neutral chloride-bicarbonate exchange as seen in the turtle urinary bladder, the mammalian colon, and pancreatic ducts. The third example of chloride transport was described as the coupled movement into the cell of the neutral NaCl complex in response to favorable gradients produced by apically located Na-K pumps. This scheme proposes that there is no primary active transport of chloride as would be reflected by the presence of a chloride sensitive ATPase. This idea is supported by the description of only one anion sensitive ATPase in epithelial tissues that is

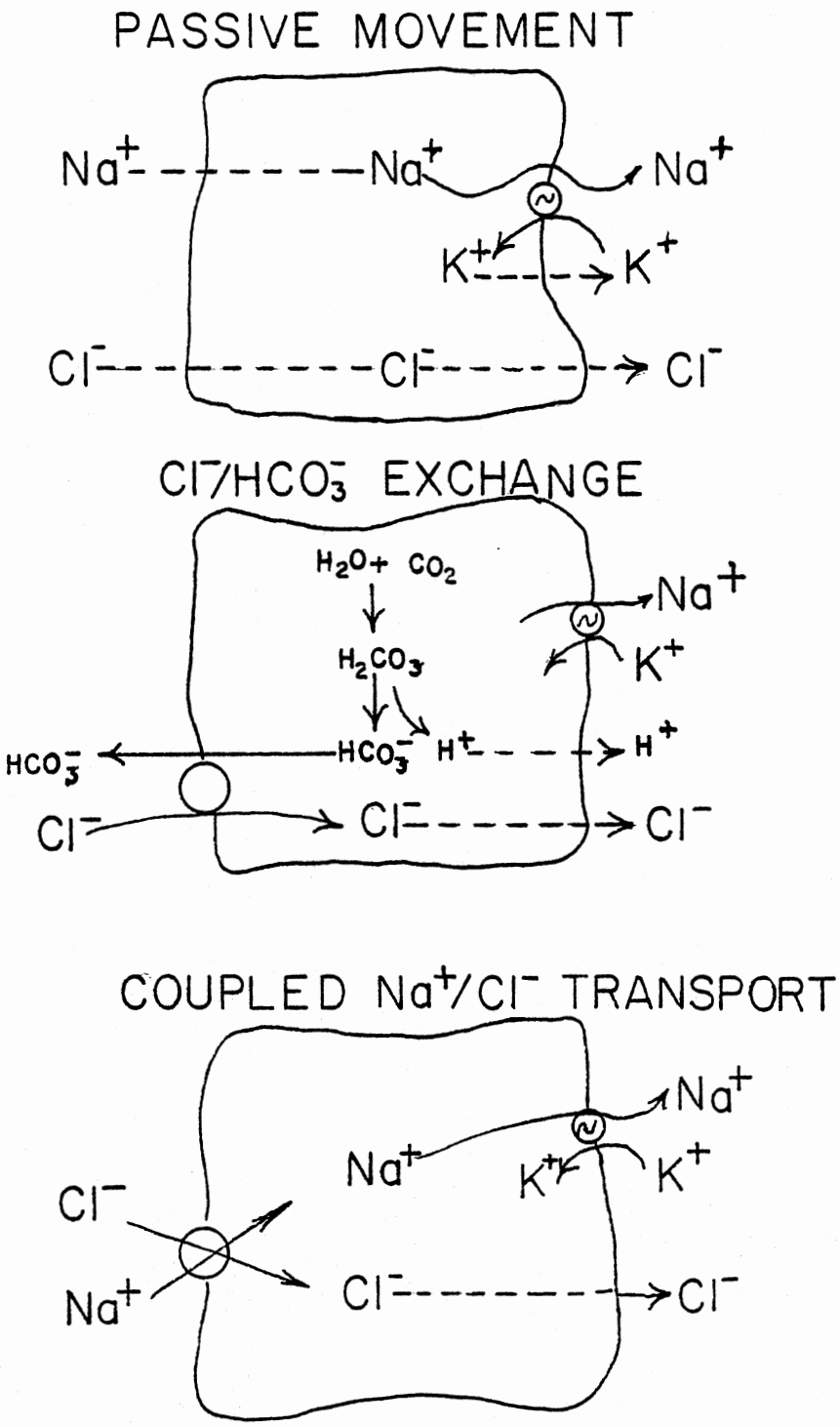

Figure 3. Modes of Transepithelial Chloride Movement

very sparse and has a low level of activity in the tissues in which it has been found (58). This protein, when present, may simply be an entry pathway with uphill transport being driven by another gradient such as the sodium gradient (24). The various theories of freshwater and saltwater osmoregulation are based on the above general models of sodium and chloride transport.

Before examining the specific osmoregulatory models in fish it may be helpful to briefly examine a few accepted models of other transport systems. The general model of monovalent ion transport in the frog skin has been discussed. Transport mechanisms in the mammalian intestine and the renal tubule have been well documented and are represented by the following cellular models.

The model for absorption and ion transport in the mammalian small intestine is summarized in Figure 4 (24). The outer cell membrane is freely permeable to sodium which moves into the cell down its concentration gradient. The gradient is maintained by a basolaterally located Na-K ATPase pump. The outer membrane also contains transport molecules for glucose, amino acids and phosphates that are coupled to sodium movement. Chloride moves passively down an electrochemical gradient created by sodium movement.

The renal tubule model in Figure 5 (61) operates in a similar manner to the gastric mucosa with a few notable exceptions. A coupled hydrogen ion sodium exchange mechanism located in the lumenal membrane allows for the

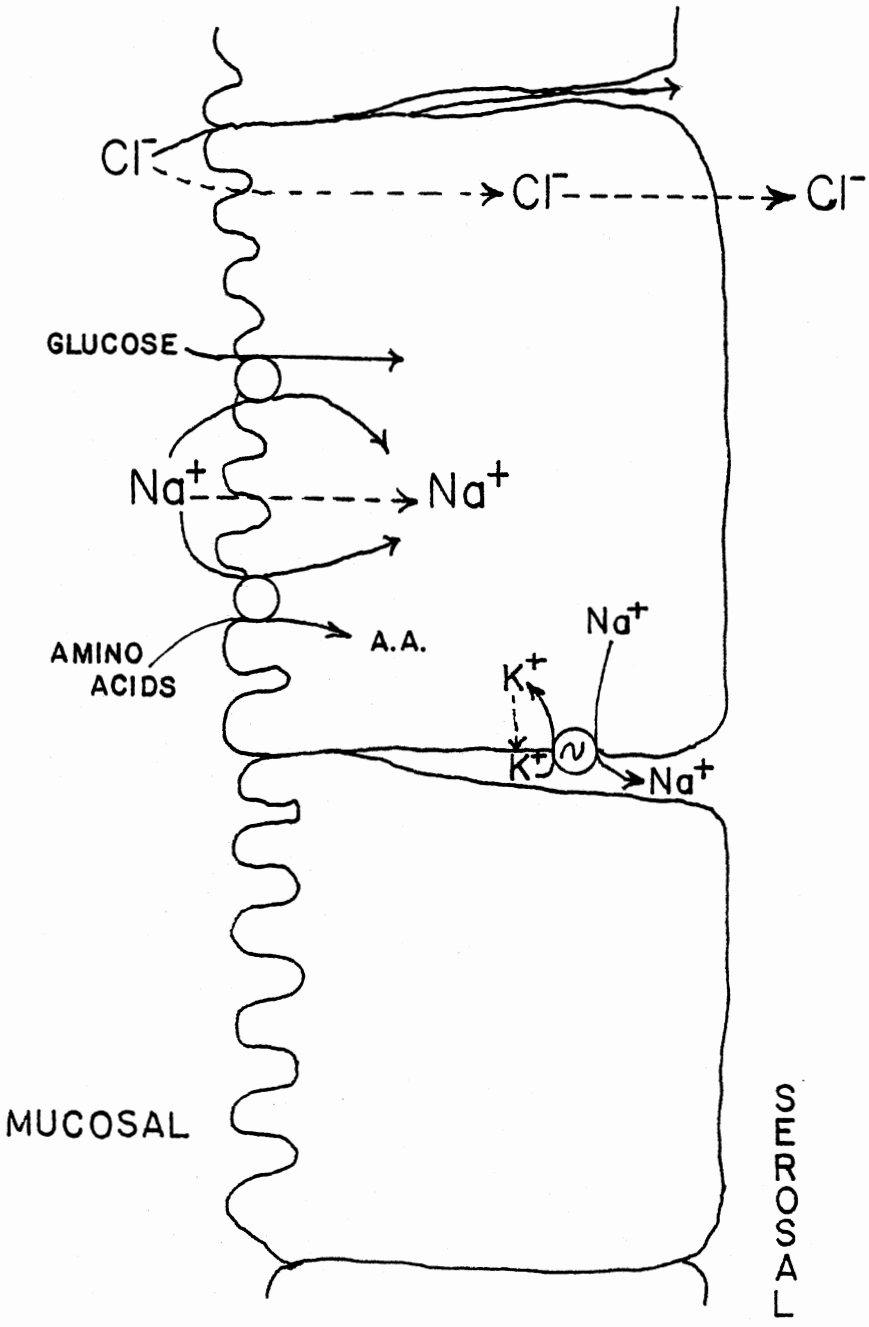

Figure 4. Transport by Intestinal Epithelial Cells

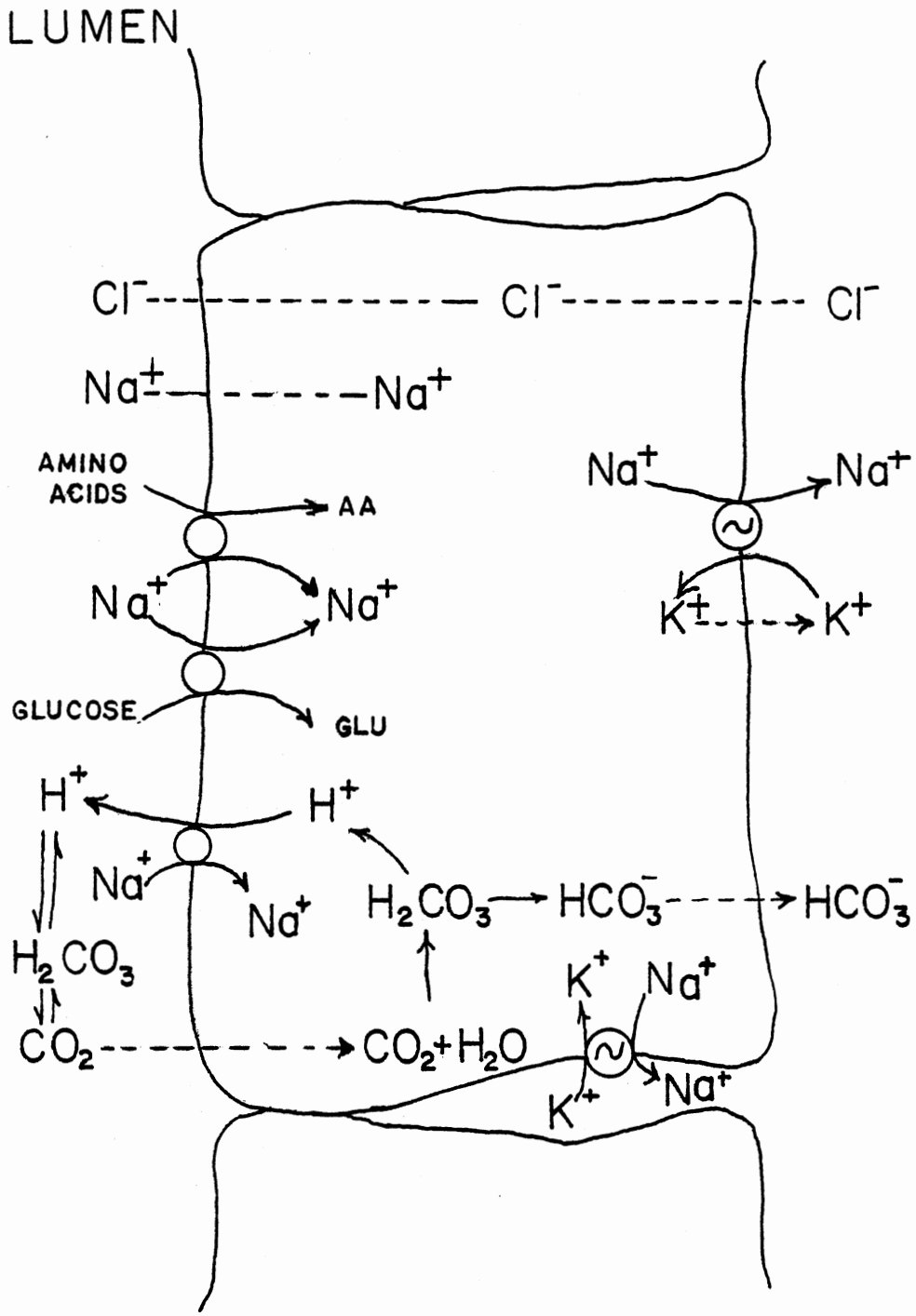

Figure 5. Renal Tubule Model of Ion Transport

conservation of bicarbonate. Again chloride moves down its electrochemical gradient in response to active sodium transport. In the distal tubule cells active chloride transport has been described but evidence suggests that this is by secondary active transport. A fact that becomes obvious after examining several models for active transport is the central role played by the Na-K ATPase pump in all modes of active monovalent transport except for the possibility of the ascending portion of the loop of Henle. The knowledge of these transport phenomena and observations of other ion fluxes led investigators to theorize models for gill osmoregulation in the freshwater and the saltwater fish. The theories reported here have been used in the development of a working model for the investigation of the toxic actions of cadmium and naph-thalene on osmoregulation.

The concepts for the basic model of freshwater osmoregulation were first discussed by Krogh (40). He hypothesized the movement of sodium and chloride ions into the cell to be coupled to the excretion of ammonia and bicarbonate. Maetz (42) used the goldfish Carassius auratus to show that sodium and chloride are absorbed at approximately the same rate from the external environment and used the substitution of impermeant ions, choline and sulphate to show that the transepithelial movement of these ions was not completely coupled (27). This led him to conclude that the law of electroneutrality predicts an exchange with endogenous ion of like charge. Kerstetter, Kirschner, and Rafuse

(35), using the rainbow trout Salmo gairdneri and labeled flux experimental methods, showed that a sodium-hydrogen ion exchange system exists that is independent of ammonium excretion. This was later confirmed in the goldfish (42). The model in Figure 6 summarizes the principles of fresh-water osmoregulatory transport.

Experimental evidence for this model has been provided by several researchers. Maetz (42) showed that ammonium ions in the external medium reversibly decreased sodium uptake in the goldfish. Intraperitoneal injection of ammonium salts increased sodium uptake. Both situations had no effect on chloride flux. This exchange of ammonium ions with sodium, however, was shown not to be obligatory by placing goldfish in deionized water. Ammonia excretion is not prevented although there was 60% increase in total plasma ammonia (12). Ammonia can be exchanged as the charged ammonium ion for sodium or excreted as the non-ionized gas depending on the prevailing external conditions.

Garcia-Romeau and Maetz (27) concluded that the major cation exchange mechanism is the sodium-hydrogen ion exchange. Small volume control correlates sodium influx with decreases in internal pH or the acidification of the external medium. It was subsequently determined that, in the goldfish, sodium uptake is best correlated with the sum of ammonium and hydrogen ion efflux (42, 17).

It was originally hypothesized that chloride was

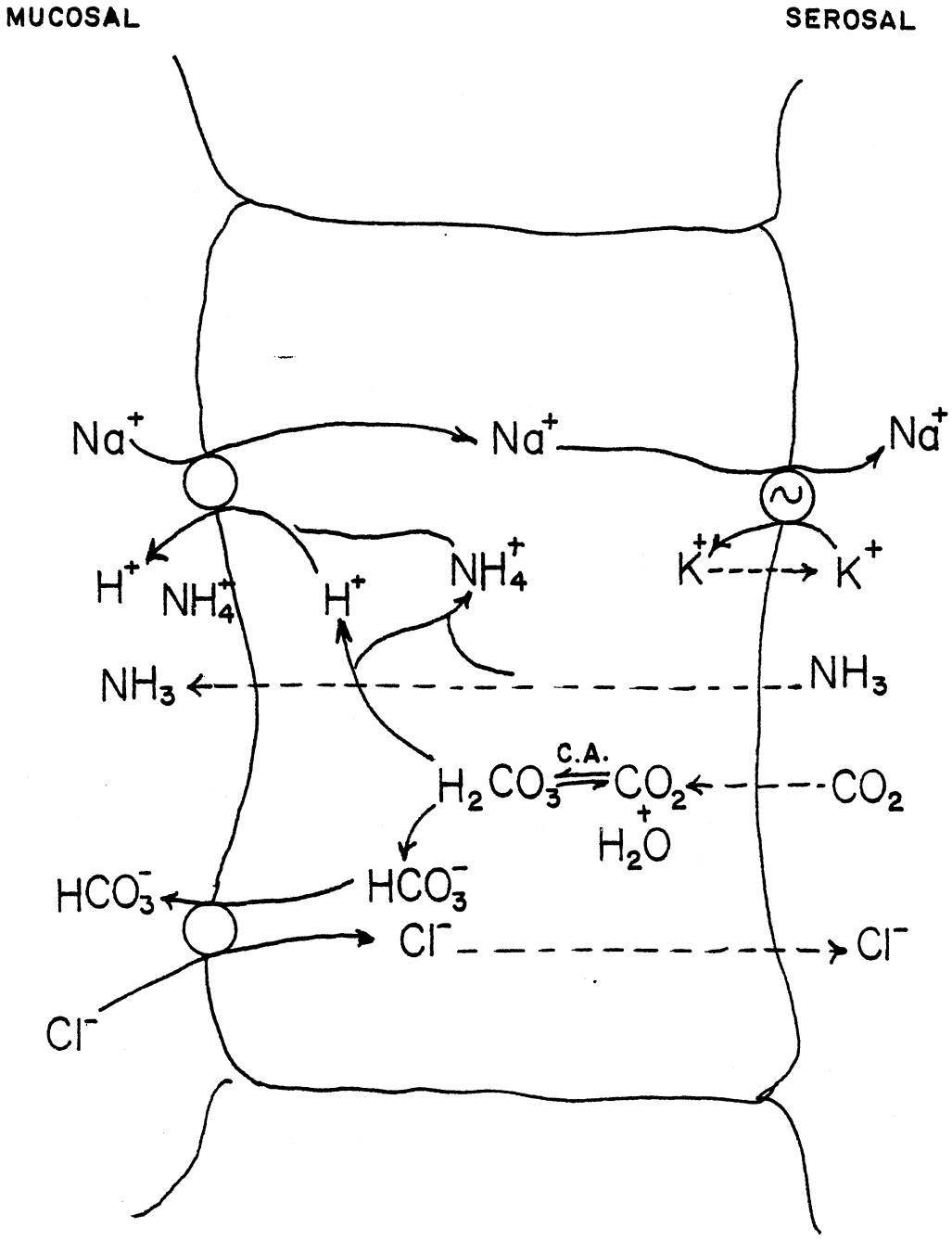

Figure 6. Ion Movement in the Freshwater Fish

exchanged with bicarbonate simply because it was the most prevalent endogenous ion that could be exchanged with chloride (41). Experimental evidence seems to confirm this theory. Garcia-Romeau and Maetz (27) showed that an increase in internal concentrations of bicarbonate stimulated chloride uptake while increased concentrations of bicarbonate in the external medium decreased uptake of labeled chloride. Garcia-Romeau and Maetz (27) found direct evidence of chloride-bicarbonate exchange in parallel with sodium-hydrogen ion exchange. By measuring base excretion (corrected for ammonia) it was determined that bicarbonate excretion closely correlated with chloride absorption. Fish kept in sodium free sulphate solutions have a significant alkalosis compared with tap water controls (12) and exhibit an accompanying increase in total plasma CO2. De Renzis and Maetz (12) investigated goldfish housed in a high sodium sulfate environment and found a sharp reduction in CO2 elimination. It was determined that the transepithelial movement of sodium and chloride were coupled, since it was not possible to deplete either ion in the internal medium by removing that ion from the external medium (12).

The coupling mechanism for sodium and chloride movement is the availability of endogenous ions produced by the carbonic anhydrase pathway. Inhibition of carbonic anhydrase reduces the available intracellular bicarbonate and hydrogen ions. Kerstetter, Kirschner, and Rafuse (35) reported that the carbonic anhydrase inhibitor, acetazolamide, reduces the

rate of sodium-hydrogen exchange. Maetz (42) showed the same effect on sodium-ammonium and chloride-bicarbonate exchange. Although internal sodium and chloride concentrations served as a feedback inhibition in freshwater ion transport, the stimulus of pH changes was more sensitive as internal control (17). As the freshwater model shows, the active transport component of this cell is the Na-K ATPase pump. The level of activity of this enzyme is under endocrine control. Hypophysectomy of Fundulus heteroclitus reduced ATPase activity (29). This activity was restored after the administration of cortisol. An important link between freshwater and saltwater osmoregulation is that this enzyme was also stimulated by ACTH and cortisol in the saltwater eel (29). When the salinity of the external medium changed significantly, the hormones cortisol and paralactin stimulated the differentiation of nondifferentiated cells into active chloride cells (42).

Osmoregulatory mechanisms of salt excretion in the marine teleost have recently been investigated in great detail using flat sheet electrophysiological techniques. The organism most often used was the euryhaline teleost, Fundulus heteroclitus. Euryhaline fish seem to have systems for osmoregulation that easily adapt to either fresh or saltwater environments (32). In saltwater orsmoregulation, the head region, predominatly the gill, plays a key role in the transport of monovalent ions (43). Most of the early work on saltwater osmoregulation was done using intact fish

or isolated perfused gill-head preparations (32).

A new tissue preparation described by Karnaky and Kinter (32) allows the use of flat sheet technology for gill transport examinations. The gross structure of the gill does not allow the use of flat sheet techniques directly. With the new tissue preparation the researcher can control the two most important thermodynamic parameters in epithelial transport; the chemical and electrical gradients across the epithelia (10).

This new tissue preparation is a continous sheet of the opercular epithelium, a thin epithelial tissue located on the inside of the bony operculum facing into the gill chamber. It is associated with an underlying layer of loose connective tissue which possesses an extensive vascular system and is made up of collagen fibers, pigment cells, and striated muscle fibers. The gill epithelium and the opercular epithelium have the same embryonic origin and share a common morphology. The four cell types of the gill are present in the opercular epithelium. In the opercular epithelium chloride cells extend to the external environment (32). They are abundant in both freshwater and saltwater fish. Small fish have a higher density population of chloride cells attributed to their proportionally greater problem of osmoregulation due to their larger surface to volume ratio.

The gill and the operculum have similar electrical and cytological characteristics (33). The transepithelial

potential difference (PD) and short circuit current (Isc) are respectively 24 millvolts and 190 microamps. The potential values correlate closely with in vivo potential measurements in saltwater fish (42). Comparing Isc with net chloride flux suggests that the most actively transported ion is chloride, from blood to seawater in the saltwater fish. The substitution of nonpermeant methylsulfate causes short circuit current and potential to decrease to near zero within thirty minutes (10). This effect was freely reversible when sodium and chloride were reintroduced. PD increased linearly with increased chloride concentration, the slope being approximately 28 millivolts per tenfold change in chloride concentration. The calculated Nernst slope was 58 millivolts which indicates a movement of additional ions (10). Replacing sodium with chloride as choline chloride causes a reversal in PD which slowly returns to normal as sodium is titrated back into the bathing solution. Degnan and Zadunaisky (11) interpreted this to mean that sodium is necessary to maintain Isc across the epithelium.

Degnan and Zadunaisky (10) showed the mean Isc accounted for 97.4% of the chloride flux. Active transport of chloride is electrogenic and responsible for the net movement of charge across the opercular epithelium. It was interpreted that this ruled out a neutral chloride-bicarbonate exchange across the entire epithelium but left the probability that a chloride-bicarbonate mechanism was present across one membrane (33). With no bicarbonate in

the bathing solution, bicarbonate added to the bathing solution increased Isc and the net chloride flux (10).

Ernst, Dodson, and Karnaky (16) found that ouabain sensitive Na-K pumps appeared to play a crucial role in the transepithelial excretion of salt. Using tritiated ouabain and autoradiography, Karnaky et al. (31) located Na-K ATPase on the tubular extensions of the basolateral cell membrane. Unexpectedly, this was similar orientation to that of chloride cells in freshwater absorptive epithelia. These facts lead to a model similar to the Field model (19) which is an adaptation from a more general Diamond hypothesis (25). Sodium is generated in intercellular spaces by Na-K pump located on the basolateral membrane. It enters the crypt space via the zona occludens or tight junctions in response to an electrical gradient derived from secondary transcellular active chloride transport. This model was predicted by the presence of high conductance cation selective shunt pathways across the zona occludens (16). Freeze fracture electron microscopy shows simple single strand occluding junctions between chloride cells, consistent with observations of low transmural resistance expected when junctions exhibit high conductivity for cations (16). Pavement cell-pavement cell junctions, on the other hand, are characterized by an anastomosing network of junctional strands on the p-fracture face and corresponding grooves on the e-fracture face. Degnan and Zadunaisky (11) demonstrated that bidirectional sodium fluxes across the

opercular epithelium can be blocked. Pd and Isc decreased to near zero. A stepwise increase in by triaminoprimadine (TAP), a compound that blocks cation selective shunt pathways in absorptive epithelia (11), was observed.

An extensive study by Degnan and Zadunaisky (10) evaluated the effects of a variety of known transport inhibitors on saltwater Fundulus. Tissue anoxia, produced by bubbling nitrogen instead of oxygen or room air into bathing solutions, decreased Isc and PD by 83% confirming that metabolic energy is required for salt excretion. The classical Na-K ATPase inhibitor ouabain, at .00001 molar concentration, inhibits transport reflected by a steady irreversible decrease in PD and Isc when it is administered to the serosal side of the tissue. Serosal furosemide, a specific chloride transport inhibitor, irreversibly decreases PD and Isc to zero at .001 molar concentration. Thioacyanate has no effect at .001 molar concentration but inhibits on both sides of the tissue at .01 molar concentration, showing a 50% decrease in Isc and PD within 10 minutes. Durbin (14) described this action as a competitive inhibition with chloride transport.

Amiloride, a potent sodium transport inhibitor showed only a slight decrease in Isc and PD. This was expected since the proposed mechanism of action of amiloride is to decrease membrane permeability to sodium. Diamox, a carbonic anhydrase inhibitor, when introduced at .001 molar concentration slightly increases Isc and PD. Ampotericin B,

which normally stimulates sodium transport, has no effect on the saltwater operculum. Theophylline normally stimulates chloride transport and does increase Isc and PD in the operculum by apparently lowering the resistance of the epithelium rather then altering chloride transport.

The injection of epinephrine into the intact saltwater fish caused an increase in plasma osmolality due to osmotic water loss and inhibition of ion transport (51). Epinephrine caused an increase in water permeability in the gill via beta receptors, probably located on the pavement cells. Alpha receptors, located on the chloride cells, were responsible for inhibition of chloride secretion (52). Epinephrine inhibited both sodium and chloride outflux by 40-60% in the saltwater mullet, Mugil capito (52). It increased cyclic AMP levels in the gill through beta receptors, as shown by the blocking of this effect by propanalol. Activation of alpha adrenergic receptors decreased chloride cell c-AMP and inhibited function of the neutral sodium chloride carrier molecule at the basal membrane causing a decreased rate of transepithelial salt secretion (10). Table I shows summarized comparisons of inhibitors in freshwater and saltwater fish.

Channel Catfish

The channel catfish Ictalurus punctatus is a commonly occurring fish native to North America (47). It is naturally distributed from the Rocky Mountains to the Appalachians but has been introduced throughout the continent. The channel catfish is found in most types of waters in streams, rivers,

TABLE I

INHIBITOR EFFECTS ON OSMOREGULATORY TRANSFER IN FRESH AND SALTWATER FISH

Inhibitor	Freshwater* Osmoregulation	Saltwater** Osmoregulation
Anoxia	Inhibition	Inhibition
Furosemide	Not Evaluated	Inhibition
Ouabain	Inhibition	Inhibition
Thiocyanate	Inhibition	Inhibition
Amiloride	Inhibition	Very Small Inhibition
Acetazolamide	Inhibition	No Effect
Diamox	Not Evaluated	Slight Stimulation
Ampotericin B	Not Evaluated	No Effect
Theophylline	Not Evaluated	Stimulation

*Determined by ion flux.

**Determined electrophysiologically.

lakes, and ponds, avoiding only the coldest of waters. It is a bottom dweller, usually sedentary during the day, actively feeding at night. The diet of the channel catfish is varied, since it has been reported to consume any organic material, living or dead, and may be described as a true aquatic omnivore (69). The life style of the catfish exposes it to many toxic compounds that concentrate in sediment and organic depositions. Lake Hefner, an Oklahoma City impoundment and drinking water supply, was found to be contaminated with the persistent insecticide Chlordane in 1981. The channel catfish was one of two species that had concentrated dangerous levels of this toxicant. The channel catfish is an important commercial and sport fish (69). In recent years catfish farming has become widespread in the southern United States. In 1981 catfish farming was the fastest growing industry in the state of Mississippi. This reason alone would make the channel catfish an important target organism and, therefore, a logical experimental species; however, the fact that it is a hardy fish that easily adapts to laboratory conditions and is easily obtainable makes it a promising experimental organism.

The organization of the channel catfish's respiratory and excretory systems fit the typical teleost models (69). There are four pairs of gills in the pharynx, covered by the operculum. Chloride cells are located at the base of the gill lamellae and in the troughs between lamellae. The blood supply from the efferent arterioles of the gill supply

the connective tissue underlying the opercular epithelium. Every indication is that this fish functions in a similar fashion to those species studied in the cited research.

Cadmium

Cadmium is a transition metal, appearing in group IIb as element 48 in the periodic table of elements (66). It has an atomic weight of 112.4. The specific gravity of cadmium is 8.65. The melting point is 320.9 C and the boiling point is 767 C. There are eight stable isotopes of cadmium. Because of the vapour pressure of cadmium (16mm at 500 C), any high temperature process involving cadmium as a component or contaminant tends to release cadmium vapour to the atmosphere (8).

Cadmium appears naturally in only the plus 2 valence state. It dissolves in weak and dilute acid. The cadmium compounds CdS, $CdCO_3$, CdO, and $Cd(OH)_2$ are insoluble in water while CdF, $CdCl_2$, CdBr, CdI_2, $CdNO_3$, and $CdSO_3$ are soluble in aqueous media. Cadmium forms a wide variety of soluble complexes with cyanides and amines. The basic difference between cadmium and the other heavy metals is that cadmium does not form highly toxic alkyl compounds (13). In fact, cadmium forms very few organic compounds at all.

Elemental cadmium occurs at an average concentration of .1 to .2 parts per million (PPM) in the earth's crust. It is principally concentrated in sulfide deposits in proximity with zinc (and occasionally lead and copper). Zinc ore may

contain from .1 to 5% cadmium impurity, and cadmium is always present as an impurity in zinc deposits. Coal contains from .25 to 5 ppm and oil as much as 16 ppm cadmium. Freshwater usually has a background level of 1-2 parts per billion (ppb) cadmium. Cadmium may concentrate to a level of from 5-430 ppm in freshwater sediments (8).

The commercial production of cadmium is a recent development. Seventy per cent of the world's production of cadmium had occurred since 1958 (8). It is used in the production of alloys and solders, as a component in metal plating, as a pigment and stabilizer in paint and plastic, and in the fabrication of nickel-cadmium batteries. Industrial and accidental release of cadmium into the environment from these sources is to be expected (13). More subtle sources of environmental exposure to cadmium are trash incineration, sewer sludge, zinc smelters, phosphate fertilizers, wear from automobile tires, combustion of coal and oil, and tobacco smoke (8).

Gastrointestinal absorption of cadmium is very low, seldom exceeding 12% of a total oral dose (13). Soluble cadmium compounds or ionic cadmium are readily absorbed across the mammilian lung and the fish gill. Cadmium binds readily to the metal seeking protein, metalothionein, which is responsible for its distribution throughout the organism. The high degree of tertiary sulphydryl bonding is believed to be the structural component responsible for metal ion affinity for metalothionein since heavy metals are

considered sulphydryl agents in terms of toxic action at the subcellular level. The cadmium-metalothionein complex is accumulated in the kidney and liver. Cadmium is highly cumulative with as much as 50% of total body content of cadmium located in these organs (13).

The toxic effect of cadmium depends upon the type of exposure. Respiratory intoxication and hypertension are related to inhalation of cadmium vapor or dust. Death by respiratory intoxication is due largely to pulmonary edema. The major target organ for sublethal doses of cadmium is the kidney. A proteinuria is the prominant sign of damage to either glomerular filtration or tubular reabsorption mechanisms.

Ionic cadmium in aqueous media has a distinct effect on epithelial cell membrane (3, 20, 60). Fleisher, Yorio, and Bentley (20) found that cadmium increases both PD and Isc when applied to the mucosal surface of the isolated frog skin at 0.001 molar concentration. In the isolated toad bladder, vasopressin increases the osmotic movement of water. Cadmium irreversibly reduced this effect by inhibiting the hydro-osmotic effect of c-AMP (3).

Pasow, Rothstein, and Clarkson (49) described the effect of cadmium as conforming to the Schultz-Arndt rule, which states that some toxicants may have a stimulatory effect at low concentrations and an inhibitory effect on the same system at higher concentrations. In the toad bladder, cadmium decreased both PD and Isc when exposed to the serosal

surface at 0.001 molar concentration, while it increased these parameters at 0.0001 molar concentration. The effect of cadmium in these transporting epithelia was due to a change in sodium permeability at the mucosal cell membrane, not to any action on the Na-K ATPase (60).

Naphthalene

Naphthalene is the simplest molecule in a series of polynuclear aromatic compounds. Its chemical formula is C10H8 formed into two benzene rings with a molecular weight of 128.16 (66). Naphthalene is volatile at room temperature and has a melting point of 80.2 degrees C. It is the most abundant component of coal tar, comprising approximately 11% of that compound. It is produced commercially from coal tar and is sold as scales, powder, balls, or cakes.

Naphthalene is widely used in dye making processes, in the synthesis of resins, celluloid, lampblack, and smokeless powder. It is a precursor of hydronaphthalenes that are used as solvents, in lubricants and internal combustion engine fuels. The primary use for naphthalene for many years was as a moth repellent and insecticide. This use has declined significantly in recent years in response to the developement of new and more effective chlorinated hydrocarbon insecticides (66). Medically, naphthlene is used as an antiseptic and anthelminthic. In veterinary medicine, naphthalene has historically been used as a dustable powder insecticide and an internal antiseptic and vermicide (66).

Naphthalene is virtually insoluble in water and highly soluble in organic solvents and animal tissue (9). Intoxication may occur from any or all of the three major routes of exposure: ingestion, respiration, and absorption. Acute symptoms in mammals include nausea, vomiting, diaphoresis, hematuria, hemolytic anemia, fever, hepatic necrosis, convulsions, and coma. The target organ of naphthalene is the liver; however, systemic toxicity has been observed and retinal damage and cataracts are common in naphthalene poisoning. The activating metabolic reaction occurs in the liver microsomes and is a dehydroxylation producing the active toxic metabolite, 1,2 dihydroxynaphthalene (9). This compound may be either eliminated by glucoronide conjugation or further metabolized to a primary epoxide. The epoxide produces the hemolytic action in red blood cells by the oxidation of sulphydryl groups in the hemoglobin molecule. The denatured globins from this reaction precipitate as Heinz bodies in the blood cells and can be identified by light microscopy.

In the respiratory epithelia of the fish, naphthalene has been reputed to disrupt the integrity of the cell membrane. The size of the naphthalene molecule and its lipid solubility are factors that predict alterations of cell membrane permeability to ions of osmoregulation. A primary metabolite of naphthalene, the epoxide, is a sulphydryl agent. Sulphydryl agents are very potent disruptors of active transport function, denaturing the ATPase protein pump molecules (13).

CHAPTER III

MATERIALS AND METHODS

The methods used in this study were adapted from procedures discussed in the literature review and are based on variations of the principles discussed there. A composite model of active osmoregulatory transport in the catfish gill and opercular epithelium was accepted for the purpose of the analysis of toxicant data.

A study of the catfish gill and opercular epithelium was performed by transmission electron microscopy to verify that the structure and organization of the components of the opercular epithelium of the catfish is similar to that described in other species (32).

Gill filaments and opercular epithelia were dissected from freshly pithed fish and placed directly into 2% gluteraldehyde in cacodylate buffer solution for two hours. Tissues were washed three times with cacodylate buffer for 20 minutes per wash, then postfixed in 2% osmium tetroxide in cacodylate buffer for two hours. Three more 20 minute washes in distilled water were followed with 0.5% uranyl acetate stain for 12 hours. The tissues were washed for 15 minutes in distilled water and dehydrated progressively with 50%, 70%, 90%, 95% and three 100% solutions of ethanol in 20

minute cycles. The fixed tissue was then washed three times with propylene oxide for 20 minutes per wash, infiltrated with 1:1 propylene oxide and Poly-bed, capped for 12 hours, and uncapped for 8. The final embedding medium was 100% Poly-bed, kept for 48 hours in a vacuum oven at 60 degrees C. Sections were cut with a diamond knife (DuPont) on an MT-2 Sorvall microtome. Electron microscopic examinations were made with, and photographs were taken through a Phillips EM-200 transmission electron microscope.

Solutions

A catfish Ringers solution was prepared to be used as a bathing solution for tissues mounted on the experimental chamber. The solution was prepared by matching the blood electrolytes measured in the channel catfish. Blood was drawn from unanesthesized fish by cardiac puncture with a heparinized syringe. Cardiac puncture was accomplished by inserting the needle at the base of the sternum on the ventral midline. Blood was centrifuged for 15 minutes and the serum was drawn off. The determination of serum electrolytes was made with a Beckman klinaflame photometer for sodium and potassium, a Corning model 940 calcium titrator for calcium, a Varian 1200 atomic absorption spectrophotmeter for magnesium, and a Buchler digital Chloridometer for the chloride anion. The values measured for serum electrolytes are listed in Table II.

TABLE II

BLOOD ELECTROYTE LEVELS IN THE CHANNEL CATFISH

	Electrolyte Ions				
	Na^+	K^+	Ca^{++}	Mg^{++}	Cl^-
$\frac{\text{Milliequivalents}}{\text{Liter}}$	146	13.4	19.3	4.3	111

A fish Ringers solution was made up with the chloride salts of the electrolyte cations. Sodium bicarbonate was added to some of the solution since both bicarbonate containing and bicarbonate free Ringers were used.

Calomel electrodes were maintained in and agar bridges were prepared from a three molar solution of potassium chloride.

Experimental Apparatus

The apparatus used in this project was a variation of the one described by Ussing and Zerahn (63). The central component was a lucite test chamber obtained from E. W. Wright, of Guilford, Connecticut, machined to the general specifications. Each chamber half was connected to its own air lift bubble pump which aerated and continually mixed the bathing solutions surrounding the tissue. Two agar bridges made of Intramedic polyethlylene tubing (PE-160) and filled

tubing (PE-160) and filled with 1% agar noble in three molar potassium chloride were connected to each half of the chamber by means of glass micro-pipettes. The other end of each bridge was submerged in a three molar potassium chloride bath with a Thomas Calomel electrode (no. 4092-F15). One calomel electrode from each chamber half was connected to a high impedence voltmeter. The second calomel electrode from each chamber half was connected to a variable voltage clamp circuit.

Experimental Method

The catfish opercular epithelium is continuous with the gill arches and is a thin layer of tissue lining the gill cavity, terminating on the margin of the bony operculum. The fish were decapitated well behind the gill arches and the head was pithed with a long hypodermic needle. The head was opened ventrally and gills were removed. The epithelium was teased away from the operculum and its connective tissue. Care was taken to keep the epithelium overlaying the dense vascular bed in the approximate center of the tissue being removed for analysis.

The opercular epithelium was an intact transparent sheet of tissue at this point. It was centered between two rubber gaskets with acrylic glue, taking care to keep glue free of the test surface located in the gasket aperture. A thin layer of petroleum jelly was spread on both surfaces of the chamber halves and the gasketed tissue was placed

between them with the tissue orientation carefully noted. Both sides of the chamber were simultaneously filled with solution through the two air lift pumps. Twenty-five milliliters of solution was routinely used on each side of the tissue. Aeration was accomplished through a common line to the source gas (either oxygen or 95:5 oxygen-carbon dioxide mixture) from the air lift pumps. Each experiment was begun using the operational protocol from Table III.

TABLE III

OPERATIONAL PROTOCOL

1)	With chamber assembled and full of solution, adjust P.D. to zero with offset knob.
2)	Turn on test switch, zero P.D. with chamber resistance knob, then turn off test switch.
3)	Place tissue across chamber and refill with solution.
4)	Read tissue P.D.
5)	Turn clamp switch to on position, selector switch to clamp voltage and adjust P.D. to zero with clamp potential knob.
6)	Read short circuit current.

Cadmium

The maximum allowable level for cadmium is approximately 10 micrograms per liter in the freshwater fish (Environmental Protection Agency #440/9-76-023). Four test groups of fish opercular epithelial tissue were exposed to one of four dilutions of this concentration. Opercular epithelia across the test chamber were thus exposed to 0.01, 0.1, 1.0, or 10 micrograms per liter (PPB) of cadmium in the external bathing solution. Each fish functioned as its own control. PD and Isc measurements were taken prior to dosing and continuously for 30 minutes after each dose.

Naphthalene

The solubility of naphthalene in water is approximately 30 milligrams per liter. Stock solutions of naphthalene were made fresh for each days experiments to avoid alteration of the compound by ultraviolet light. The same methods of dilution for exposure were used with naphthalene as with cadmium. The test groups were exposed to nominal concentrations of 0.03, 0.3, 3.0, or 30 micrograms per liter naphthalene in the external bathing solution. Results were expressed as mean plus or minus standard error. A t test for significant variation was performed on each group of tissues. This t test determined statistically different values from the control or tested the null hypothesis that the treatment values were the same as control values.

CHAPTER IV

EXPERIMENTAL RESULTS

Electron microscopic evaluation of the opercular epithelium was compared with that of catfish gill filaments. Both were examined for similarity to gill and opercular tissue previously discussed by Karnaky Degnan, and Zadunaisky (33). Figure 7 shows the organization of cell types in the gill filament. Four cell types are present in the catfish gill epithelium. The pavement cells (P) line the filament and are easily indentified by their externally oriented microvillae. Choride cells (CC) are more sparcely distributed on each filament and are concentrated at the base of or between filaments. Nondifferentiated cells (D) surround the chloride cells in the epithelial matrix. Goblet or mucous cells (G) are sparcely distributed along the mucosal surface of the gill tissue.

Figure 8 is a gill filament chloride cell of typical structure for channel catfish. The apical portion of the cell extends through the pavement cell layer to the external medium. As is characteristic of the chloride cell of other species of fish, mitochondria are numerous and prominent. There is an extensive smooth endoplasmic reticulum.

Chloride cells were also found in the opercular

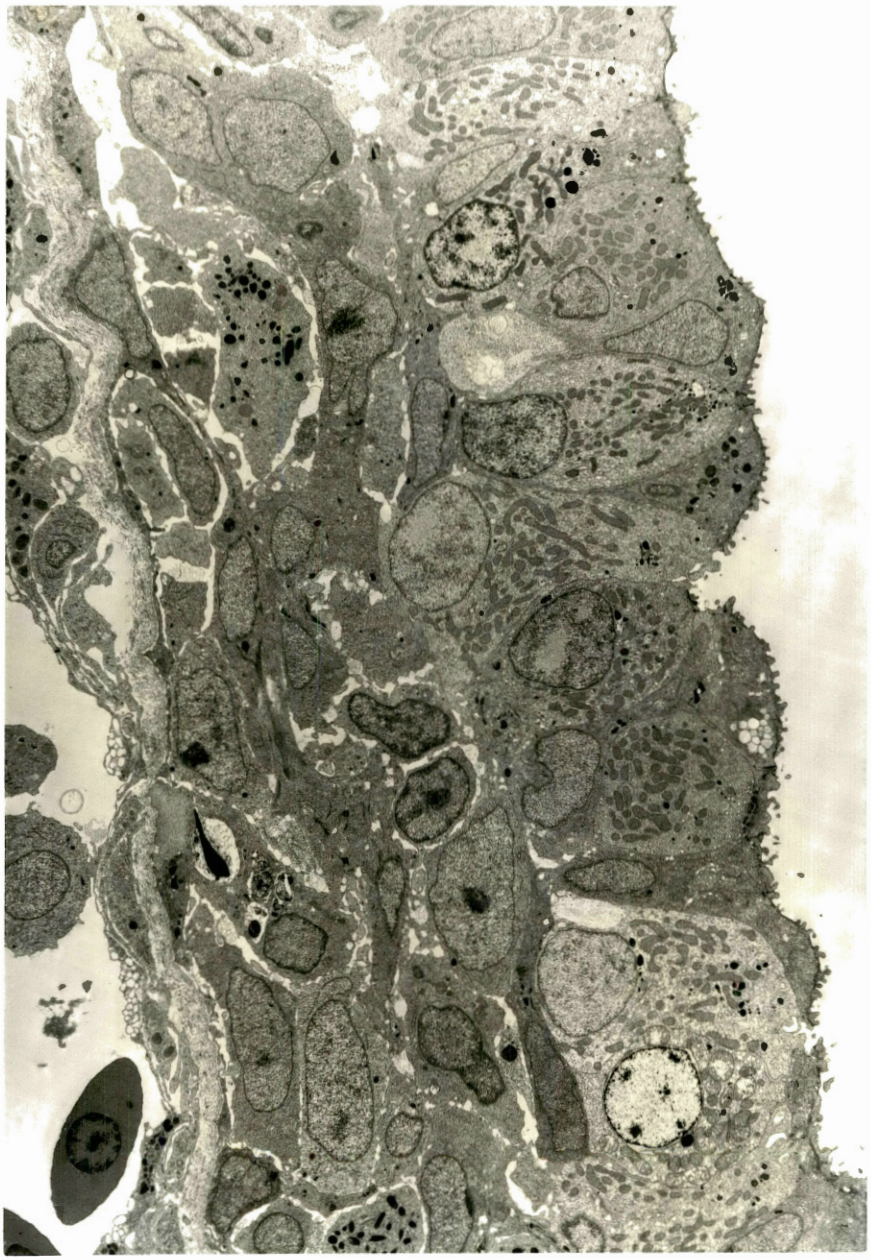

Figure 7. A Transmission Electron Micrograph (TEM) of Cell Types in the Catfish Gill Filament

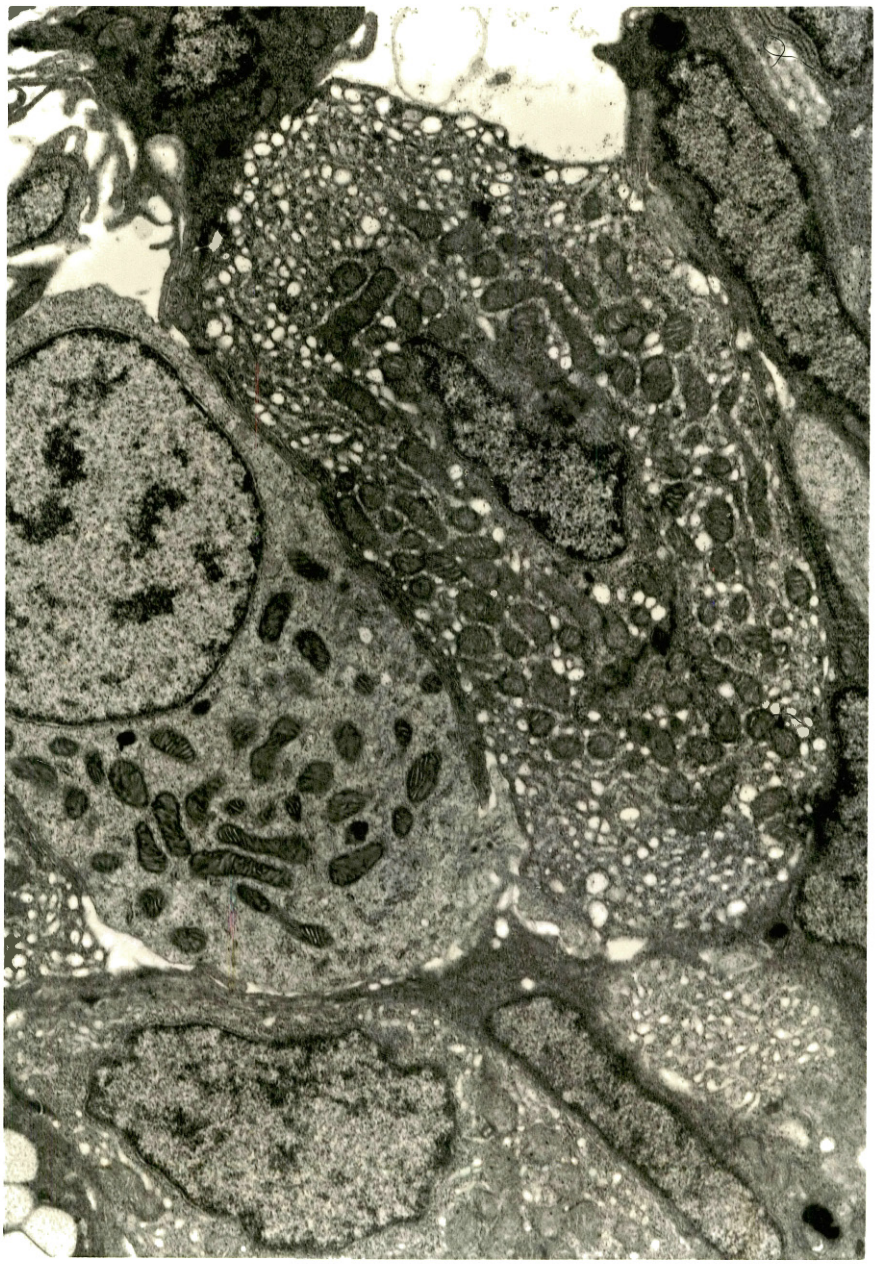

Figure 8. Typical Gill Filament Chloride Cell

epithelium. An opercular epithelium chloride cell in Figure 9 exhibits typical chloride cell characteristics. Unlike the dense population reported in euryhaline species by Karnaky, Degnan, and Zadunaisky (33), these cells were sparsely distributed throughout the opercular epithelium of channel catfish. Other cell types of the opercular epithelium were typical of respiratory epithelium for other fish.

Saltwater Adaptation

Fish were adapted to increased salinity in gradual steps of 7.7 meq sodium/l per day with a goal of 50% seawater. Fish acclimated well to each increase of salinity until the concentration of sodium chloride reached the level of 130-140 meq sodium/liter. At this level of salinity, 100% mortality was experienced in each of three experimental groups. At each addition of saline to the tank, environment fish showed immediate distress with coughing and avoidance behavior. Within one hour, all reactions to salinity changes ceased.

Electrical Characteristics

The test apparatus was regularly evaluated for instrumental drift. When fully assembled with catfish Ringer's solution filling the chamber, the apparatus was checked for electronic stability with potential difference variation of ±0.2 millivolts over a 24 hour period and short circuit current variation of ±1 microamp.

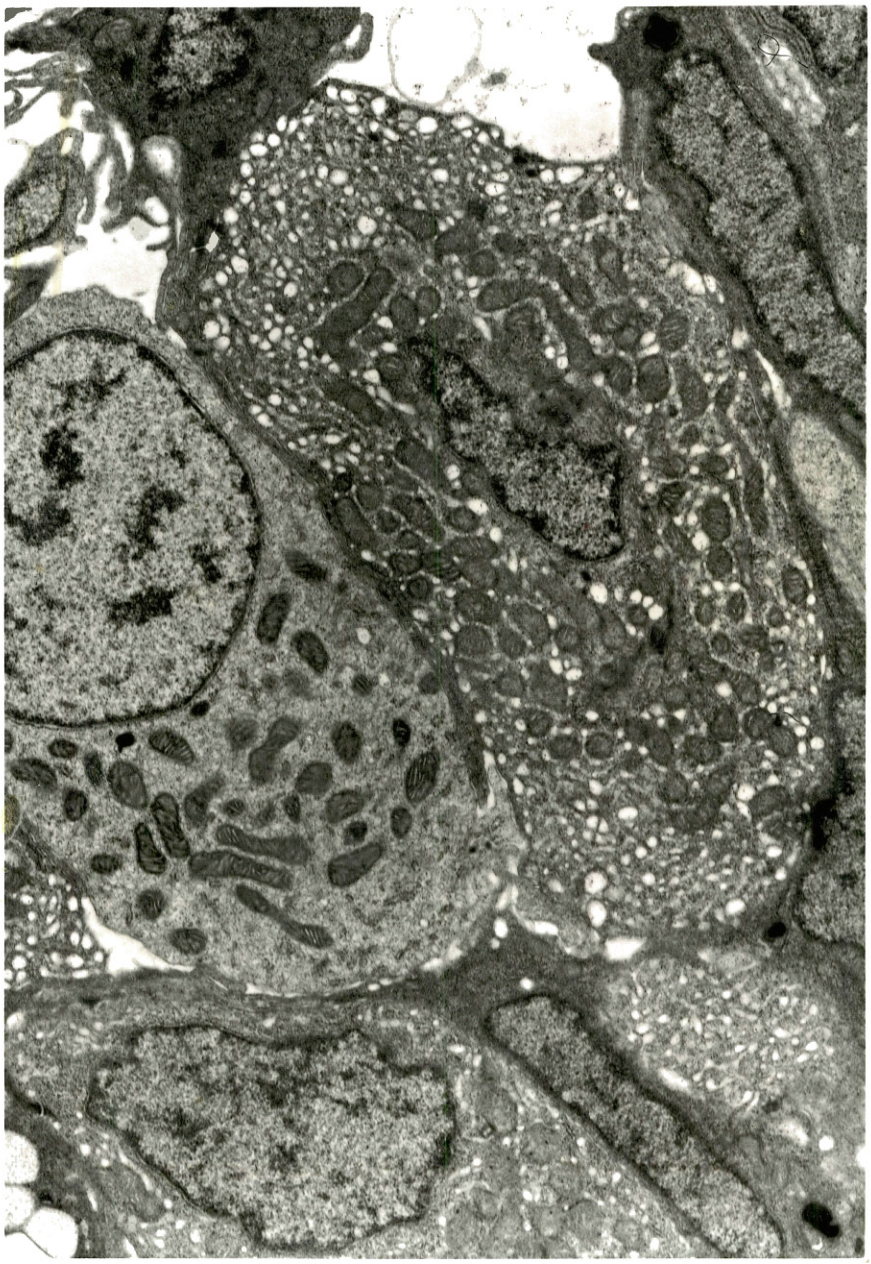

Figure 9. Chloride Cell from the Opercular Epithelium of the Channel Catfish, Ictalurus Punctatus

A preliminary study of epithelial life indicated that stable normal levels of potential difference and short circuit current were established after two hours on the test chamber and that tissue death occurs after 24 hours of isolation on the test chamber. Values of potential difference and short circuit current varied less than 5% between 2 hours and 24 hours on the chamber, maintained in Ringer's aerated with 95%, oxygen and 5% carbon dioxide.

Normal values of electrical parameters varied greatly from one fish to another and from season to season. Values of normal electrical parameters plus or minus standard error are:

Grouping	PDmv ± SE	Isc ± SE	R_{mean}
Overall mean n=42	3.05±0.344	32.6±2.94	93.6Ω
Fall Fish n=14	4.36±0.478	31.2±6.09	139.7Ω
Spring Fish n=28	2.38±0.397	33.2±3.13	71.7Ω

The potential difference range was from 0.5 millivolts to 11.0 millivolts and that of the short circuit current was 6.5 microamps to 86 microamps. These values of electrical parameters represent only the mean values for tissues actually used in experiments. Many tissues placed upon the chamber did not equilibrate above 0.5 millivolts potential and were not used in the study. Due to these wide variations, average normal values were not used as a basis for measuring toxic effects. Effects were recorded as per cent change in potential and short circuit current.

Fish maintained in low concentration saltwater (90 meq/l as sodium) for 60 days showed no significant change in PD or Isc from normal freshwater fish tissue.

Inhibitors

The effects of metabolic inhibitors upon the osmoregulatory function of both fresh- and saltwater fish were reported in Table I. Since freshwater values have only been measured as flux changes, it was necessary to establish the values measured in the opercular epithelium as approximations of the same system. Figures 10-13 represent test groups treated with inhibitors. Furosemide had no significant effect upon either short circuit current or potential difference when exposed to serosal and mucosal sides of isolated epithelium. Mucosal treatment with ouabain produced no effect on either parameter, however, ouabain in serosal solution at 0.01 molar concentration caused a rapid irreversible decrease in both potential difference and short circuit current. These effects were predicted from the model of freshwater osmoregulation selected for this experimental method.

Cadmium

Data from experimental groups exposed to mucosal cadmium are summarized in Figures 14-18 and statistically evaluated in Tables IV and V. Significant stimulation of both PD and Isc are the initial effects of cadmium. Increases in

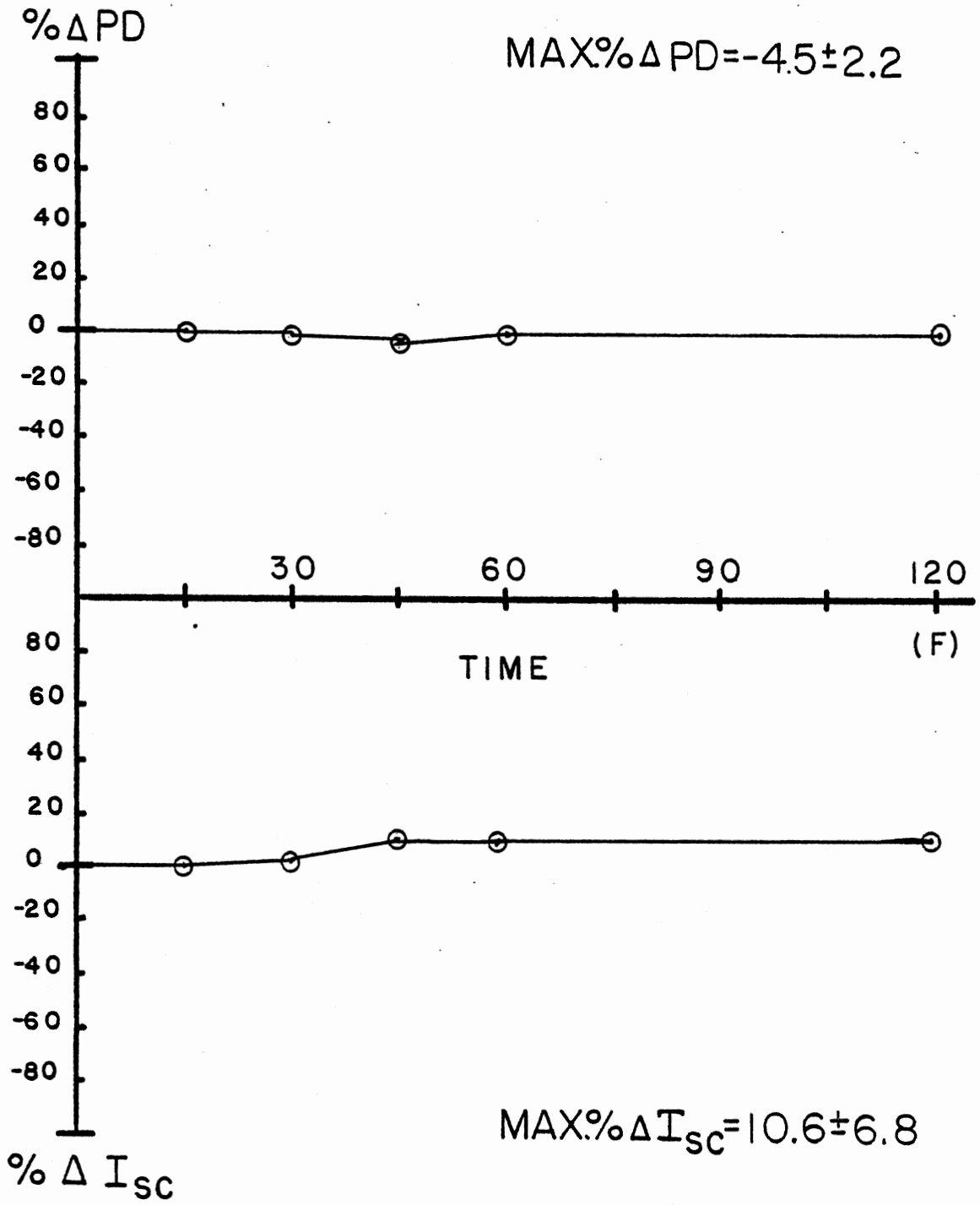

Figure 10. Electrical Response of Opercular Epithelium to Mucosal Furosemide

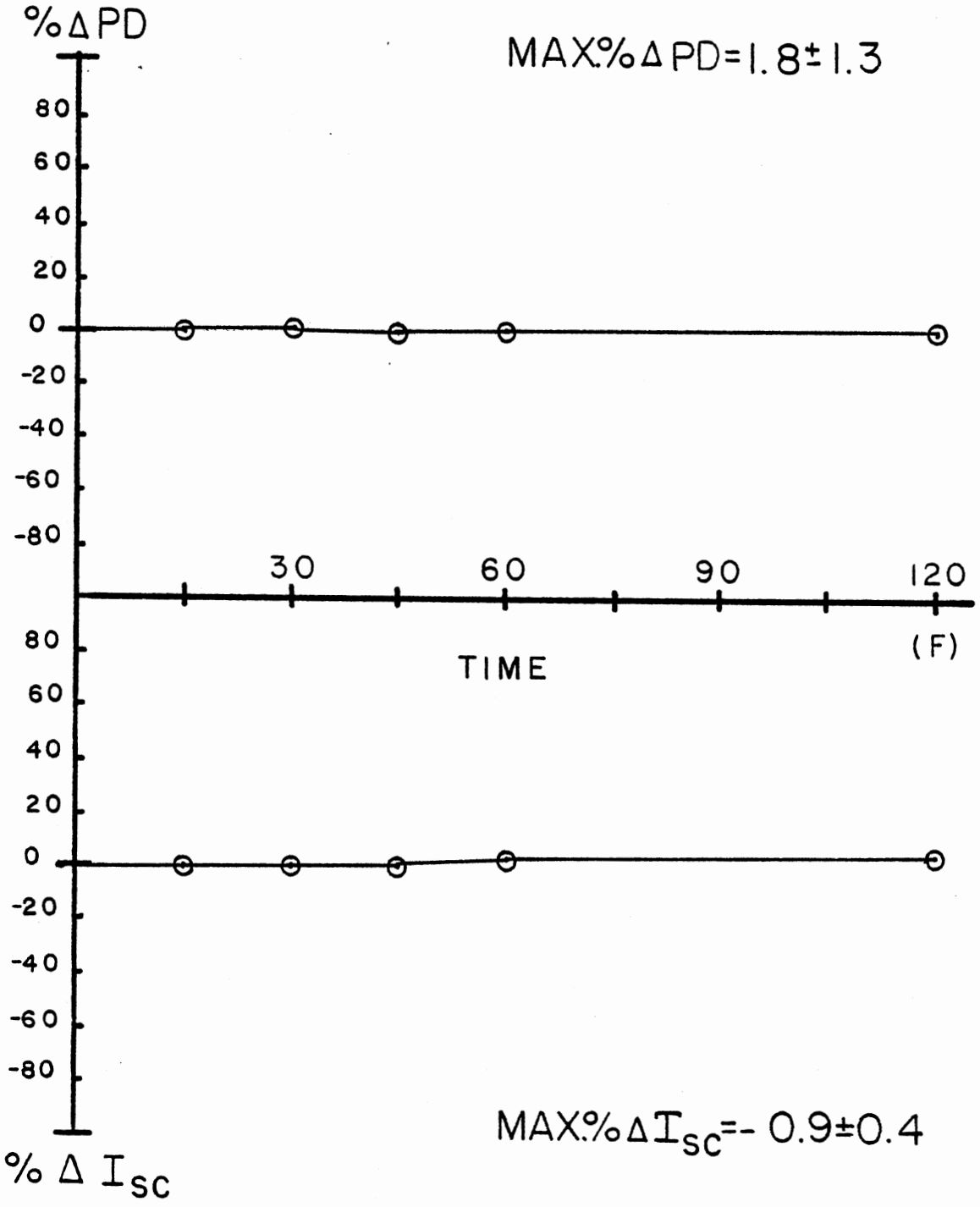

Figure 11. Electrical Response of Opercular Epithelium to Serosal Furosemide

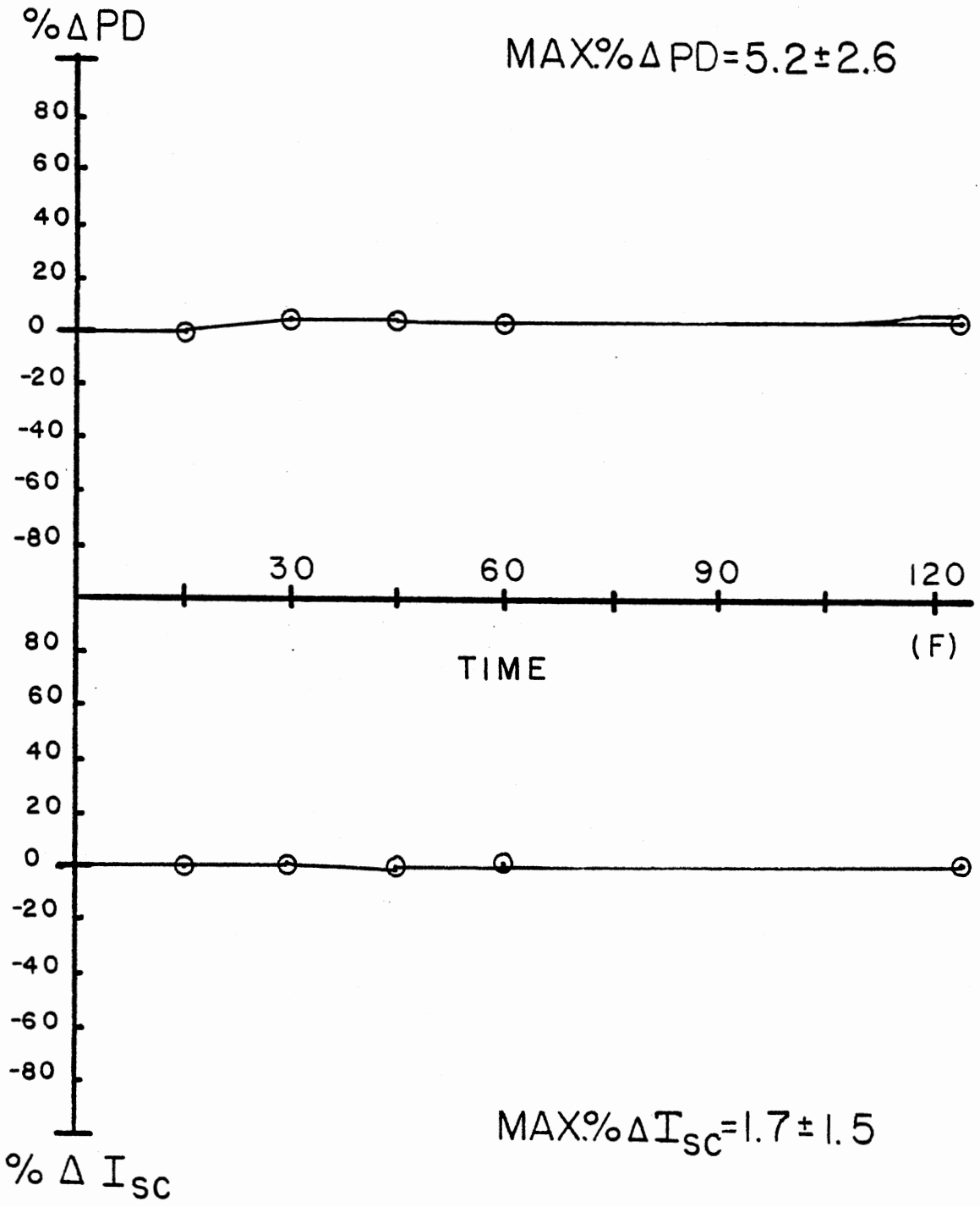

Figure 12. Electrical Response of Opercular Epithelium to Mucosal Ouabain

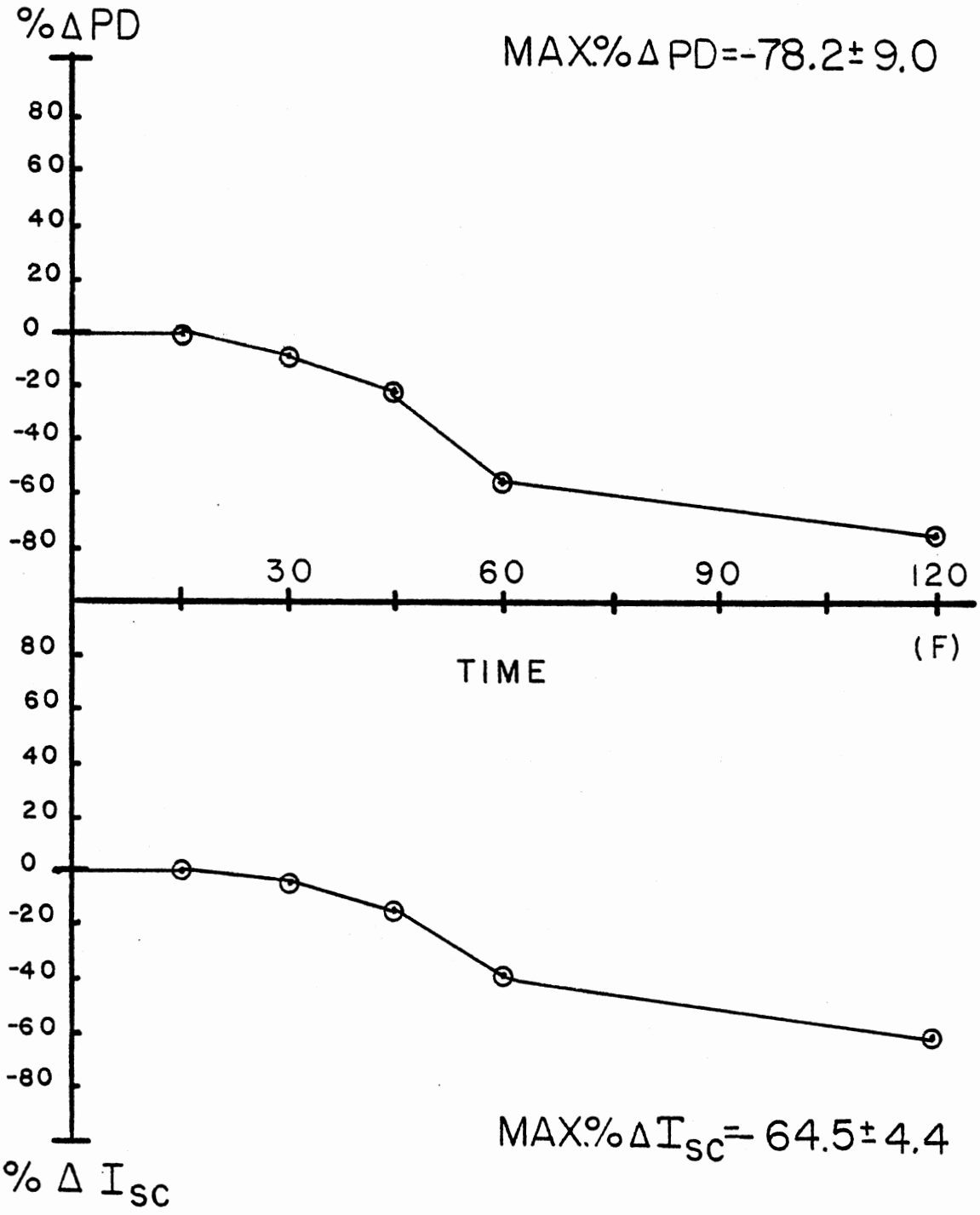

Figure 13. Electrical Response of Opercular Epithelium to Serosal Ouabain

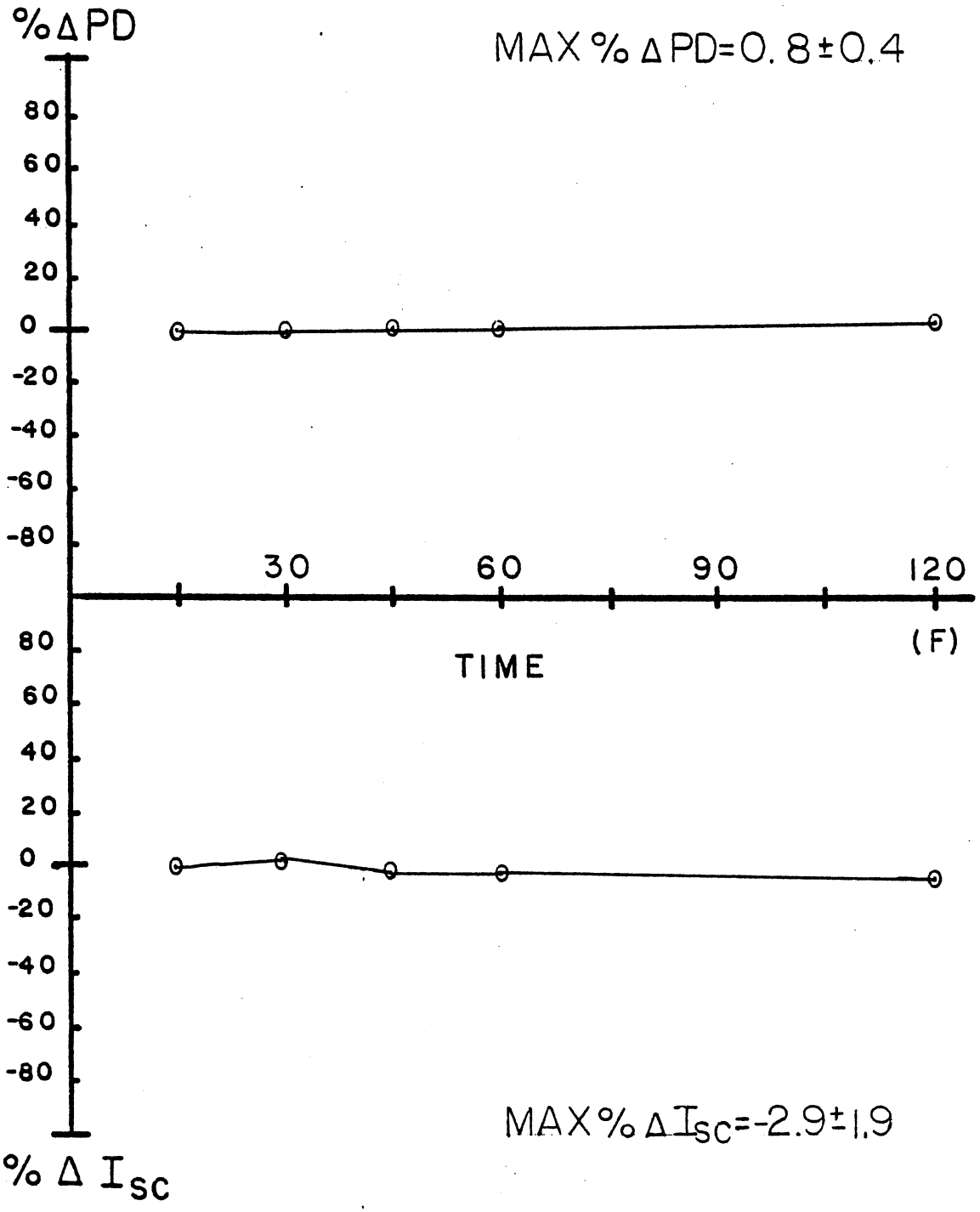

Figure 14. Electrical Response to 0.01 Micrograms Per Liter (PPB) Cadmium

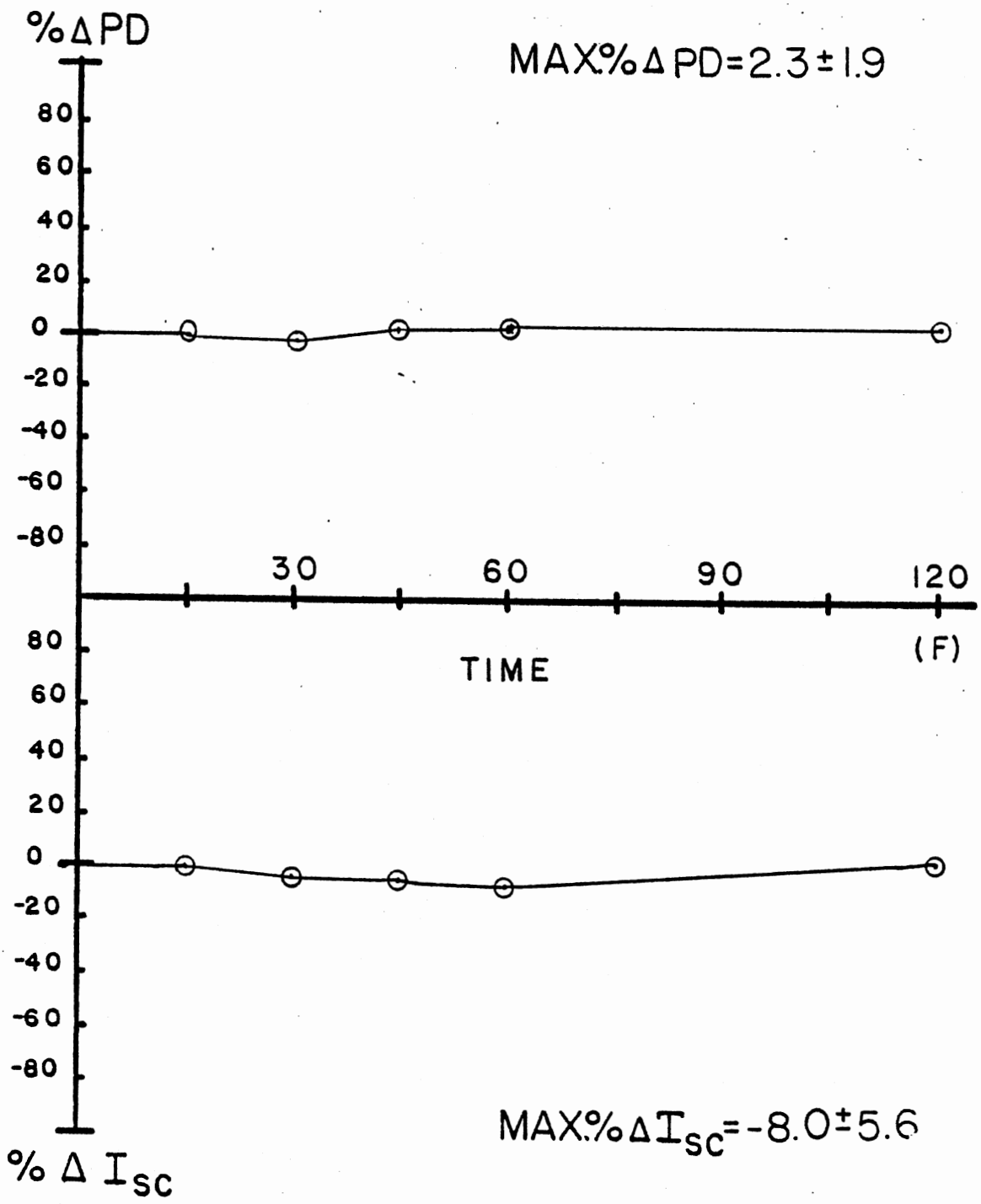

Figure 15. Electrical Response to 0.1 Microgram/Liter (PPB) Cadmium

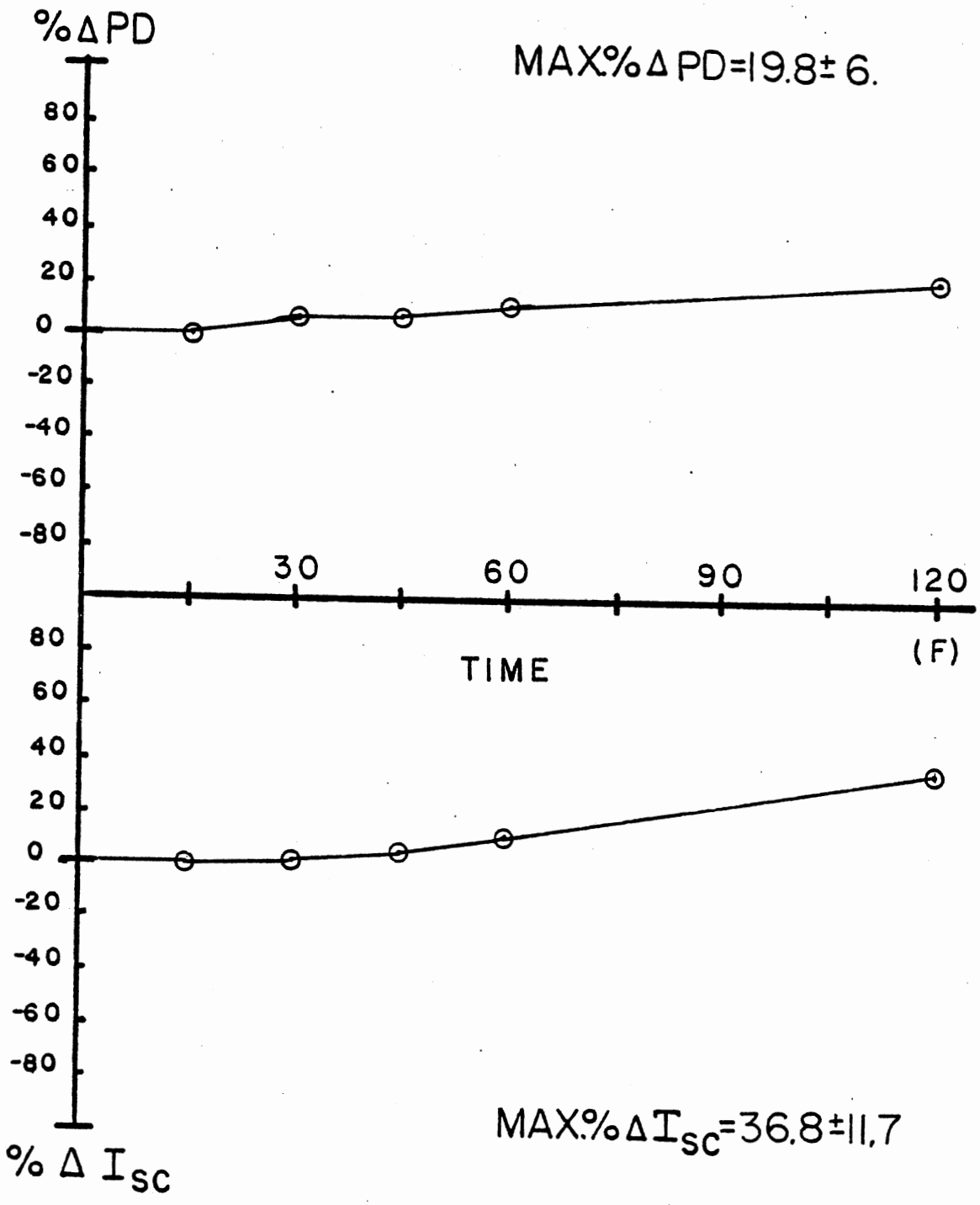

Figure 16. Electrical Response to 1.0 Microgram/Liter (PPB) Cadmium

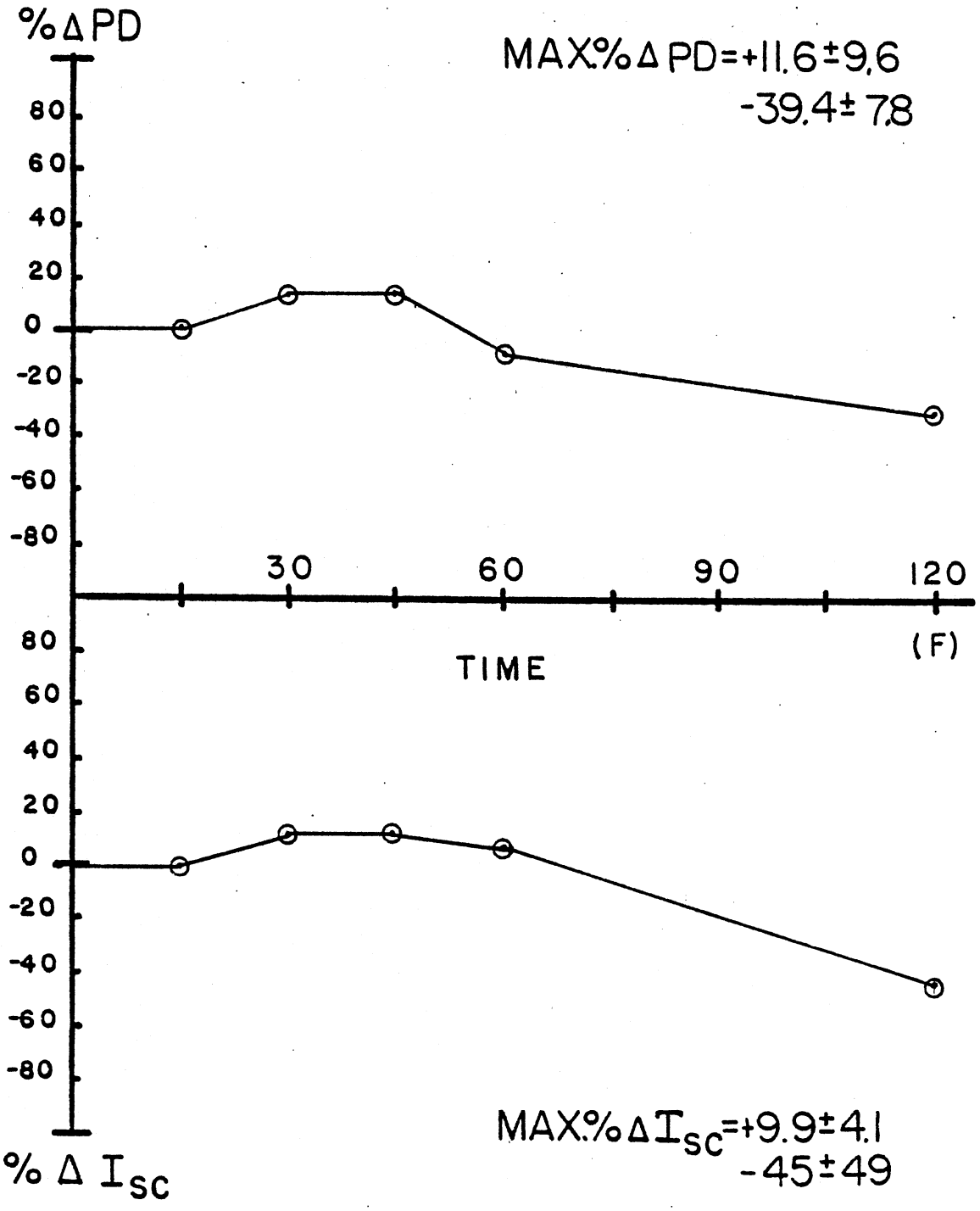

Figure 17. Electrical Response to 10.0 Microgram/Liter (PPB) Cadmium

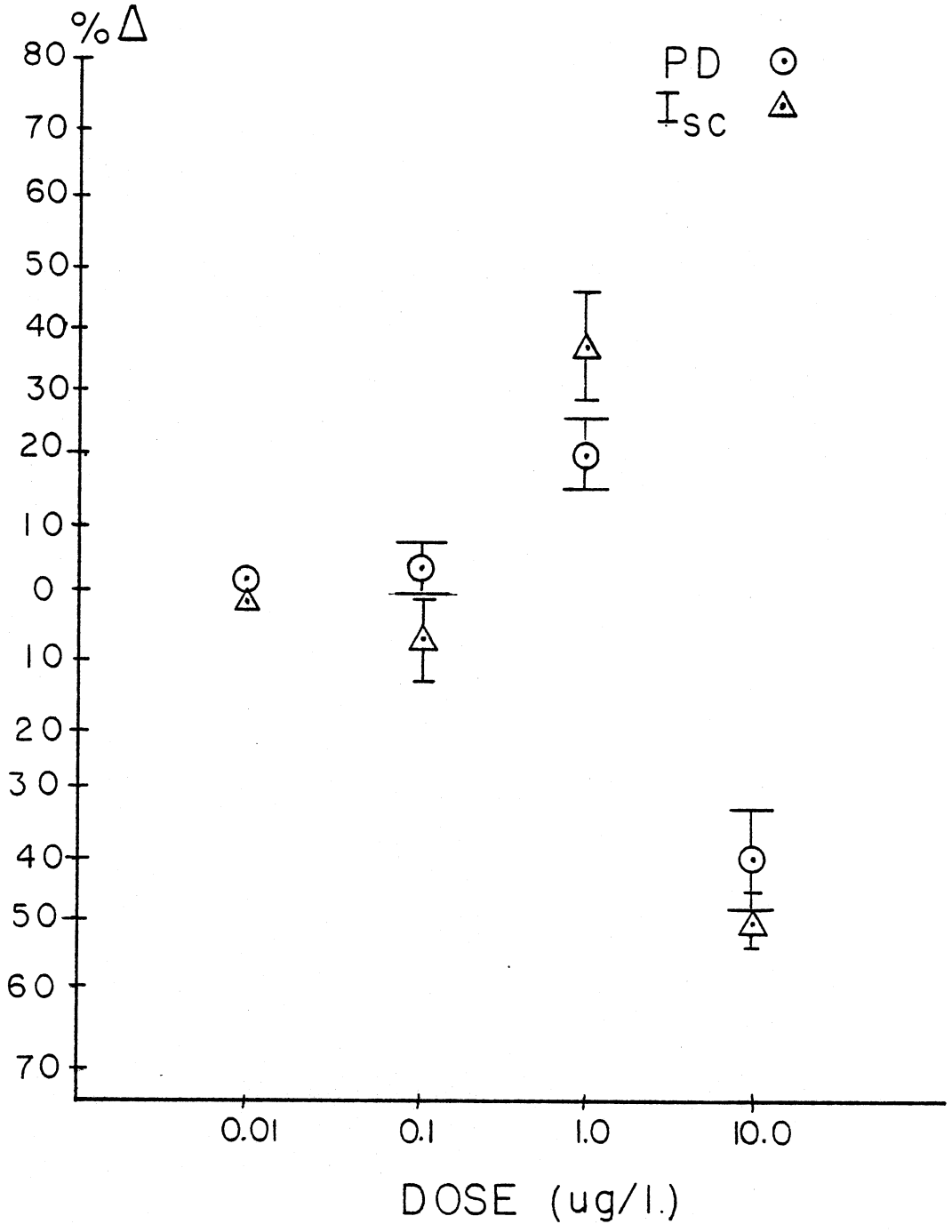

Figure 18. Cumulative Electrical Effects of Cadmium

TABLE IV

STATISTICAL EVALUATION OF POTENTIAL DIFFERENCE DATA FROM CADMIUM TREATED TISSUES

Sample	%Δ Potential Difference $\bar{X}$	S.E.	t	P	df
Cadmium (GRI 0.01)					
Control (0-15 min)	0	±0.496	--	--	--
30	-0.1	±0.75	-0.133	0.901	4
45	+0.68	±0.678	0.990	0.378	4
60	+0.32	±1.55	0.206	0.847	4
F*	+0.82	±0.405	2.02	0.113	4
Cadmium (GRII 0.1 ppb)					
Control (0-15 min)	0	±0.0	--	--	--
30	-1.16	±1.5	-0.773	0.483	4
45	-0.71	±1.78	-0.399	0.710	4
60	+2.18	±1.84	1.18	0.303	4
F	2.35	±1.93	1.22	0.289	4
Cadmium (GRIII 1.0 ppb)					
Control (0-15 min)	0	±0.323	--	--	--
30	+4.42	±2.08	2.12	0.101	4
45	+4.96	±2.92	1.70	0.164	4
60	+7.24	±1.30	5.57	0.00509	4
F	+19.78	±6.0	3.30	0.0299	4
Cadmium (GRIV 10 ppb)					
Control (0-15 min)	0	±0.707	--	--	--
30	11.6	±9.6	1.21	0.293	4
45	10.7	±8.0	1.34	0.251	4
60	-4.39	±3.97	-1.11	0.329	4
F	-39.4	±7.97	-4.94	0.00782	4
Inhibitors					
Ouabain, Mucosal					
Control(0-15)	0	±1.59	--	--	--
30	5.17	±2.63	1.97	0.12	4
45	4.27	±3.14	1.36	0.245	4
60	1.35	±2.33	0.580	0.593	4
F	2.25	±1.95	1.15	0.314	4
Ouabain, Serosal					
Control (0-15)	0	±0.477	--	--	--
30	-9.52	±7.06	-1.35	0.248	4
45	-23.05	±6.67	-3.46	0.0258	4
60	-58.05	±9.91	-5.86	0.00423	4
F	-78.2	±8.99	-8.70	0.000961	4
Furosemide, Mucosal					
Control (0-15)	0	±0.125	--	--	--
30	-1.32	±0.759	-1.74	0.157	4
45	-4.53	±2.24	-2.02	0.113	4
60	-1.9	±1.41	-1.35	0.248	4
F	-1.9	±1.41	-1.35	0.248	4
Furosemide, Serosal					
Control (0-15)	0	±0.41	--	--	--
30	0	±0	--	> .05	4
45	+0.75	±1.33	0.564	0.603	4
60	-.375	±0.324	-1.16	0.311	4
F	-1.69	±1.07	-1.58	0.189	4

F = Final Time (+ 120 minutes, relative to time zero)

TABLE V

STATISTICAL EVALUATION OF SHORT CIRCUIT CURRENT DATA FROM CADMIUM TREATED TISSUES

Sample	%Δ Short Circuit Current $\bar{X}$	S.E.	t	P	df
Cadmium (GRI 0.01 ppb)					
Control 0-15 min	0	±0.494	--	--	--
30	0.240	±.214	1.12	0.320	4
45	-0.640	±0.620	-1.03	0.361	4
60	-2.40	±1.867	-1.28	0.270	4
F*	-2.94	±1.945	-1.51	0.206	4
Cadmium (GRII 0.1 ppb)					
Control 0-15 min	0	±1.63	--	--	--
30	-0.800	±4.80	-0.167	0.875	4
45	-5.80	±3.18	-1.82	0.143	4
60	-8.00	±5.63	-1.42	0.229	4
F	+1.00	±4.16	0.240	0.822	4
Cadmium (GRIII 1.0 ppb)					
Control 0-15 min	0	±1.38	--	--	--
30	+0.820	±1.71	0.479	0.651	4
45	5.08	±3.21	1.58	0.189	4
60	11.71	±3.65	3.20	0.0329	4
F	36.8	±1.7	21.6	0.0000272	4
Cadmium (GRIV 10 ppb)					
Control 0-15 min	0	±0.834	--	--	--
30	+9.60	±3.80	2.53	0.0647	4
45	9.85	±4.07	2.42	0.0728	4
60	7.60	±5.02	1.51	0.206	4
F	-45.0	±4.87	-9.24	0.000763	4
Inhibitors					
Ouabain, Mucosal					
Control 0-15	0	±1.24	--	--	--
30	1.75	±1.52	1.15	0.314	4
45	-0.875	±2.65	-0.330	0.758	4
60	-0.150	±2.58	-0.0581	0.956	4
F	-0.150	±2.58	-0.0581	0.956	4
Ouabain, Serosal					
Control 0-15	0	±2.65	--	--	--
30	-5.25	±5.42	-0.969	0.387	4
45	-18.2	±8.23	-2.215	0.0916	4
60	-40.7	±1.63	-25.0	0.0000152	4
F	-64.5	±4.39	-14.7	0.0000125	4
Furosemide, Mucosal					
Control 0-15	0	±0.492	--	--	--
30	+1.23	±0.67	1.83	0.141	4
45	10.6	±6.80	1.56	0.194	4
60	9.79	±6.86	1.42	0.229	4
F	9.79	±6.86	1.42	0.229	4
Furosemide, Serosal					
Control 0-15	0	±0.37	--	--	--
30	-0.400	±0.346	-1.16	0.311	4
45	-0.925	±0.471	-1.96	0.121	4
60	+0.475	±1.10	0.432	0.688	4
F	+0.890	±1.12	0.795	0.471	4

F = Final Time (+ 120 minutes, relative to time zero

potential begin at 0.01 ppb cadmium. Short circuit current is significantly elevated at the 1.0 ppb dose level. At a level of 10 ppb, the heavy metal effect irreversibly lowers both PD and Isc by 39% and 45%, respectively.

Under open circuit conditions, PD increased by approximately 50% while transepithelial resistance increased 322Ω to 1250Ω over the same period of time when exposed to 1.0 ppb cadmium.

Naphthalene

Naphthalene doses were applied based upon solubility as well as toxicity. Since there would be little practical value to using concentrations of naphthalene higher than normal water solubility, dosages were diluted from 30 ug/l which is the solubility of naphthalene in water. Figure 19 shows the effects of naphthalene upon electrical parameters at four dose levels. Although a significant change appears to have taken place in short circuit current, the % change is caused largely by one tissue in a group of five. Table VI shows the change is not significant at high levels of exposure.

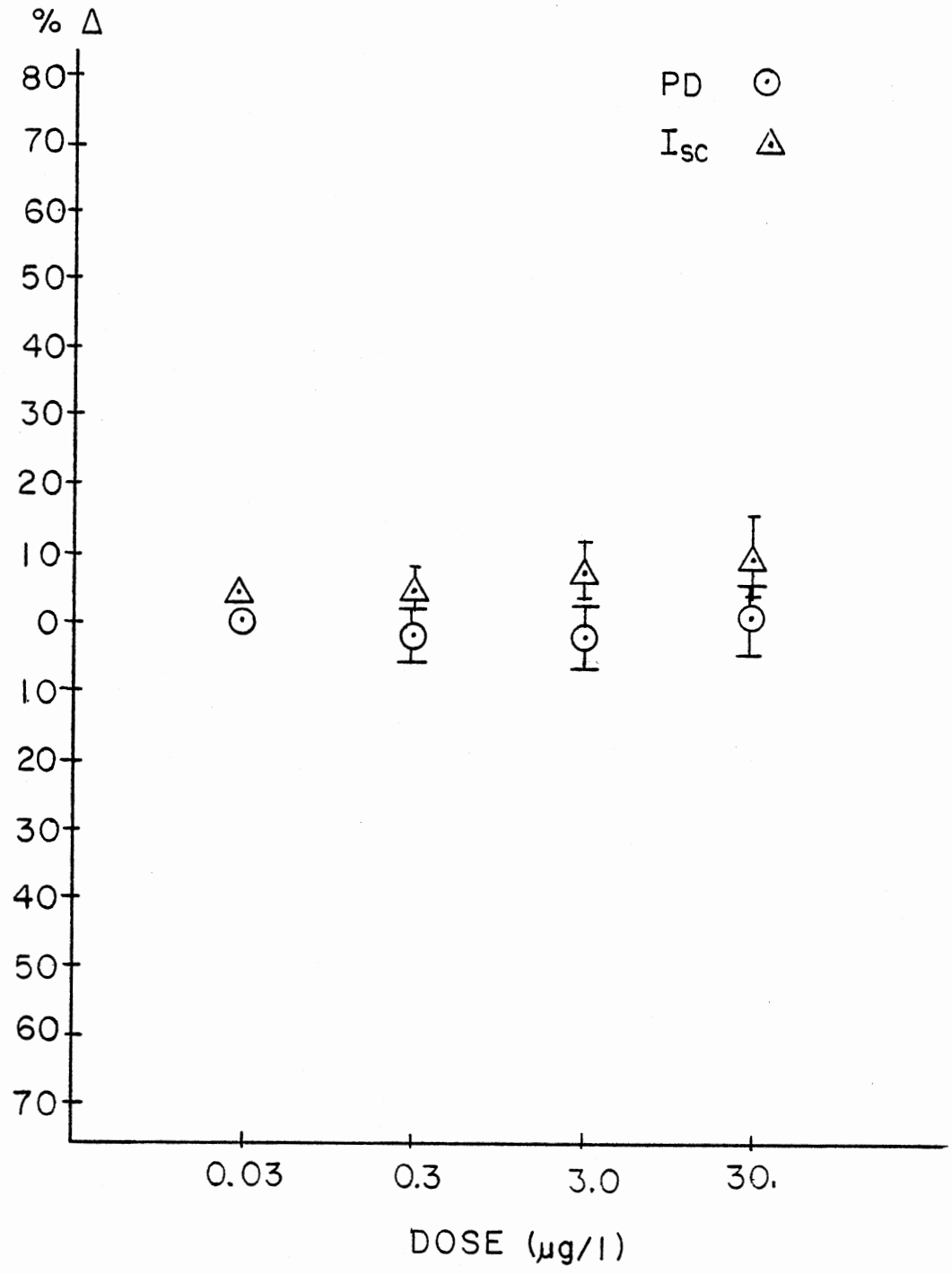

Figure 19. Cumulative Electrical Effects of Naphthalene

TABLE VI

STATISTICAL EVALUATION OF DATA FROM NAPHTHALENE TREATED TISSUES

Napthalene						
Test Group		%Δ Mean	S.E.	t	P	n
Control	(PD)	0	0.55	--	--	10
	(Isc)	0	0.33	--	--	10
0.03 ug/l	(PD)	0.35	2.06	-0.164	>0.05	10
	(Isc)	4.73	1.44	-3.2	<0.05	10
0.3 ug/l	(PD)	-2.58	3.65	0.699	>0.05	10
	(Isc)	4.58	1.95	-2.317	<0.05	10
3.0 ug/l	(PD)	-2.43	4.14	0.582	>0.05	10
	(Isc)	7.68	4.33	-1.766	>0.05	10
30. ug/l	(PD)	0.26	4.9	0.058	>0.05	8
	(Isc)	9.97	5.75	-1.732	>0.05	8

CHAPTER V

DICSUSSION AND CONCLUSIONS

It was determined that the catfish opercular epithelium is similar in structure and function to the same tissue in euryhaline teleosts described in the literature. Karnaky, Ernst, and Philpott (30) described the opercular epithelium of the fundulus to be composed largely of chloride cells. It was found that the catfish has a low density of chloride cells concentrated over vascular beds. Conversely the catfish seems to have a larger population of transport cells on the gill filaments themselves. Since euryhaline fish continuously adjust to changes in external salinity, the presence of numerous transporting cells would be advan-tageous. The catfish also lives in a wide range of salinities but cannot adapt to salinities above 135 milli-equivalents of sodium per liter.

When channel catfish were slowly adapted to increased salinity, little effect was noted in stress behavior until the level of salinity exceeded 135-145 milliequivalents/liter as sodium ion. At this level, 100% mortality was observed. This was the same level of sodium ion as measured in the catfish blood. As long as the osmolarity of the external medium was below that of blood, net ion movement across

exposed epithelial tissues was outward. No adaptation has taken place at the epithelia. When the point in external salinity was reached where passive net ion movement was inward, death occurred. The catfish appear to lack adaptive mechanisms to actively transport monovalent ions outward across the epithelia.

The models of ion movement in osmoregulation were discussed earlier. In reviewing the literature, it was found that proponents of each model made few references to the other. Maetz (42) suggested that the freshwater fish exchange mechanisms probably occurred in both fresh and saltwater fish. With this as a reference point, it was possible to evaluate both models for compatibility.

Figure 20 represents a composite of both inward and outward pumping models for monovalent ion transport. The level of activity of the outward pumping system is much higher than that of the inward pumping waste exchange system. It is accepted for the purpose of this investigation that this is a representation of the chloride cell of the euryhaline fish. The freshwater fish seems to lack some portion of the outward pumping mechanisms, probably the component necessary to transport the neutral NaCl complex across the basal cell membrane. This overview model complies fully with inhibitor data from Table I.

A study of the inhibitors, furosemide and ouabain, was used to show the compatibility of the catfish opercular epithelial chloride cells with the composite model. Furosemide

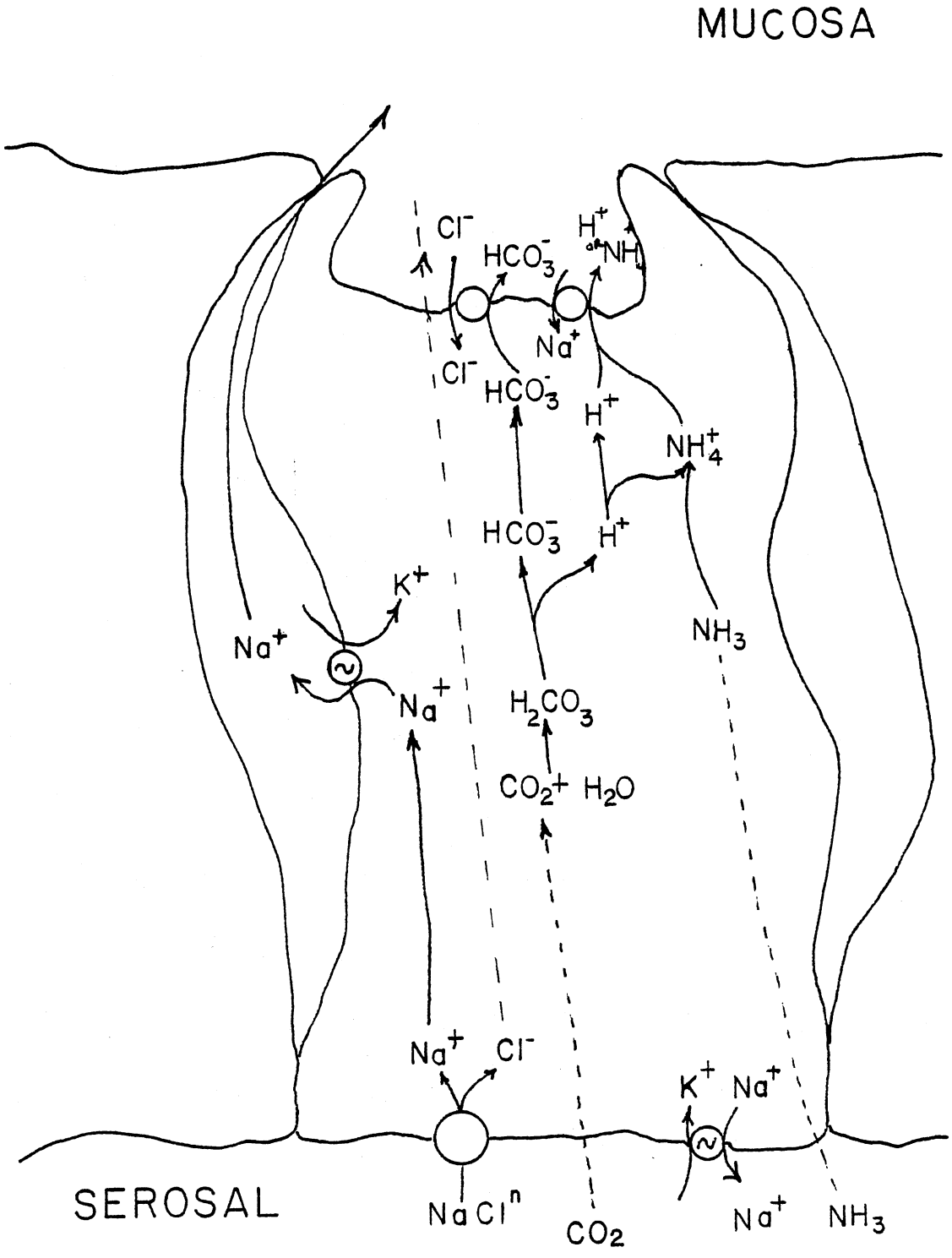

Figure 20. Complete Osmoregulatory Cell of Euryhaline Fish

is a chloride ion transport inhibitor. In the catfish opercular epithelium, furosemide has no significant effect on potential diffence or short circuit current when introduced into the bathing solutions of either the mucosal or serosal side of the tissue. In the model for active osmoregulation in freshwater fish, there is no active transport of Cl^- and the results obtained with furosemide treatment in the catfish are consistent with the model.

Ouabain is a universally accepted inhibitor of active sodium transport. It binds to and disrupts the function of the Na^+, K-ATPase enzyme protein, effectively halting the exchange of sodium for potassium across the basolateral cell membrane (in the fish gill epithelia). When introduced to the serosal facing medium of the catfish, ouabain irreversibly decreased both PD and Isc to near zero. It is concluded from this that the driving force of ion exchange in the inward pumping portion of the osmoregulatory system is a serosally located Na^+, K^+-ATPase pump. As expected, ouabain at concentrations as high as .01 M, had no effect at the mucosal surface of the opercular epithelium. Ouabain does not readily penetrate the mucosal barrier established by the cell membrane and occluding junctions.

The effects of these two inhibitors support the desription of the channel catfish opercular epithelium as a typical inward pumping freshwater osmoregulatory tissue. The following discussion of toxic effects is based upon the portions of the composite osmoregulatory model identified previously with typical freshwater fish.

Cadmium

Cadmium appears to be a unique heavy metal, having two effects on the osmoregulatory system. At cadmium concentrations as low as 0.1 micrograms per liter, statistically significant increases in PD reflect what appears to be a stimulatory effect. Since there is very little time delay in the onset of these changes, this effect probably takes place at the mucosal surface of the epithelium. A similar effect in the frog skin was explained by Takada and Hayashi (60) as an inhibition of sodium movement through occluding junctions. The orientation of the transepithelial potential difference in catfish epithelium is mucosa negative relative to serosa. Cadmium may increase the permeability of the mucosal facing cell membrane to sodium, stimulating the activity of the sodium "pump". This would result in an increase in both potential difference and short circuit current.

As the concentrations of cadmium in the external medium increased to that of the LC_{50} for cadmium (10 ug/l) the effect dramatically changed. Both short circuit current and potential difference decreased markedly. As the concentration increases, it is proposed that cadmium penetrates the epithelium to the ATPase pump on the serosal cell membrane. At this concentration, cadmium acts as a typical heavy metal. The effect is that of a sulfhydryl agent, disrupting the tertiary structure of the ATPase protein and markedly decreasing the short circuit current and potential across the epithelium. This duality of effect explains the dose

response relationship seen in Figure 18.

Naphthalene

Surprisingly, at water concentrations up to 30 ug/l, which is the saturation point of naphthalene in water, naphthalene showed no significant effect on the electrical parameters in the channel catfish opercular epithelium. Naphthalene itself is not a sulfhydryl agent. An explanation for the lack of effect on osmoregulatory active transport is that sulfhydryl action is only reported in the secondary metabolite naphthalene epoxide, which is produced in the liver. Therefore, the protein molecules responsible for osmoregulatory transport are not exposed to sulfhydryl naphthalene compounds, in an in vitro study.

The fact that napthalene is a very lipid soluble may result in the cell membrane functioning as a sink for the toxicant at these low levels. It is possible that a chronic build-up in naphthalene in the lipid matrix of the cell membrane may eventually disrupt transport function by altering membrane permeabilities. The duration of tissue life in this in vitro technique limits the evaluation of toxicants to relatively rapidly occurring effects of primary, nonmetabolite substances, although known metabolites could be added to the tissue preparation as a primary toxicant.

Recommendations For Further Study

The electrophysiological technique used for this study

shows great promise for development as a toxicologic test. It was initially believed that the osmoregulatory active transport system in freshwater fish was simply a reversal of the system present in saltwater and euryhaline fish. It was believed that by acclimating freshwater fish to saltwater conditions the transport system could be reversed and stimulated to a constant level, reflected in high and consistent values of PD and Isc. This adaptation was believed necessary because of the low values measured in the freshwater fish.

It was soon discovered that the osmoregulatory transport system was much more complicated than thought, and that the low levels of PD and Isc represent a low activity, inward pumping transport system. It was not possible to stimulate a high activity outward pumping system. The values for electrical parameters were relatively small and reflected large variation from fish to fish. This makes it very difficult to establish normal values of PD and Isc for the channel catfish and limits the experimental techniques to experiments using internal controls.

A euryhaline fish such as the stripped bass would probably have higher and more consistent electrical activity. It would be advantageous to compare toxic effects from the freshwater catfish and the euryhaline fish, to determine possibilities of modeling freshwater toxicity tests around the more electrophysiologically acceptable euryhaline teleost. Standardization of techniques and

equipment by continued evaluation would be advantageous to the development of a repeatable rapid toxicity test procedure.

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VITA[2]

Larry Lloyd Zinn

Candidate for the Degree of

Doctor of Philosophy

Thesis: THE EFFECTS OF CADMIUM AND NAPHTHALENE ON OSMOREGULATORY TRANSPORT IN THE OPERCULAR EPITHELIUM OF THE CHANNEL CATFISH, ICTALURUS PUNCTATUS

Major Field: Physiological Sciences

Biographical:

Personal Data: Born in Hobart, Oklahoma, June 18, 1948, the son of Mr. and Mrs. Domby L. Zinn.

Education: Graduated from Northwest Classen High School, Oklahoma City, Oklahoma, in May 1966; received Bachelor of Science degree in Zoology from Oklahoma State University in 1974; received Master of Science degree in Natural Sciences from Oklahoma State University in 1979; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in July, 1982.

Professional Experience: Analytic Toxicology Section, Oklahoma Animal Disease Diagnostic Laboratory 1977-79.