SODIUM TRANSMEMBRANE TRANSPORT EFFECTS OF α-CHACONINE AND α-SOLANINE ON *RANA PIPIENS*

By

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PREFACE

Frog skin is an established model for trans-epithelial active transport of sodium from the pond-side of the skin to the serosal-side. Glycoalkaloids, produced in potatoes and tomatoes have been implicated in teratogenicity. Previous studies suggested that glycoalkaloids disrupted membrane integrity and affected membrane potential. We tested the effect of glycoalkaloids on sodium active transport by using α-chaconine and α-solanine, glycoalkaloids that occur in potatoes. Our tests were performed on the isolated epithelial tissue of the *Rana pipiens* (commonly known grass frog) with an Ussing Chamber. A voltage clamp was used to record the change in the short circuit current as a result of the addition of the glycoalkaloids. The purpose of this thesis is to report those findings as well as to discuss the mechanisms of frog epithelial membrane sodium transport. The data suggest that the mechanism of action of the two glycoalkaloids is to modify the active transport of sodium.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Plants belonging to the family *Solanaceae* contain a variety of glycoalkaloids, which have been characterized on the basis of their toxic effects. Most of the available information concerning the toxic effects on this variety of glycoalkaloids pertains to α-chaconine and α-solanine, the major toxic constituents in potatoes. These alkaloids posses mild to moderate animal toxicity; however, episodes of poisoning have been reported due to an increased alkaloid content under certain conditions.

The potato makes an important contribution to the diet in many parts of the world. In addition to their nutritional value, potatoes can be grown plentifully under moderate climate and are easily stored. Potatoes rank third to wheat and rice in world annual production and serve as an economic commodity. Potatoes are a source of high carbohydrate energy and provide twenty-five percent of our daily requirement for vitamin C, in addition to significant amounts of minerals like phosphorous, potassium and calcium. On the basis of total protein production per acre, potatoes rank second only to soybeans.

Solanaceous plants, including agriculturally important crops such as potatoes, synthesize secondary plant metabolites including glycoalkaloids. In commercial potato cultivars, the primary glycoalkaloids are α-chaconine and α-solanine. These compounds can have toxic effects in animals and humans (Jelinek *et al.*, 1976; Mun *et al.*, 1975). Thus potatoes may represent a potential source of undesirable compounds, especially if improperly stored or processed (Morris, Lee, 1984).

One proposed mechanism of action for the toxic action of the glycoalkaloids is disruption of intracellular membranes. It has been reported that potato and tomato glycoalkaloids disrupted liposome membranes (Roddick *et al.*, 1988). It has also reported that mixtures of α-chaconine and α-solanine acted synergistically in lysing rabbit erythrocytes, red beet cells, and *Penicillium notatum* protoplasts.

The glycoalkaloids evaluated in this study, α-chaconine and α-solanine, are found in approximately equal concentrations in the potato plant, *Solanum tuberosum*, and in several other *Solanum* and *Veratrum* species. The carbohydrate side chain of α-chaconine has a branched *bis*-α-*L*-rhanmopyranosyl-ß-*D*-glucopyranose trisaccharide side chain. The carbohydrate side chain of α-solanine is a branched α-*L*-rhanmopyranosyl-ß-*D*-glycopyranosyl-ß-galactopyranose (Roddick *et al.*, 1988).

Isolated abdominal skin from frogs has been used as a model system for studying transepithelial active transport of sodium. *In vitro* experiments, performed by mounting the frog skin on an Ussing chamber, show that the frog skin actively transports sodium from the pond-side of the skin to the serosal-side of the skin. Active transport of sodium causes an electrical potential difference (PD), up to 140 mV, which is proportional to the logarithm of the pond-side sodium concentration (Koefoed-Johnsen, Ussing, 1958). When the solutions bathing each side of the skin are identical, and the required external current is applied to change the PD to zero, the skin is "short-circuited." Under short-circuit conditions, no driving force for ions or water exists across the skin. Thus, any net flux of ions (or water) must be due to processes internal to the skin and equal to the short-circuit current (ISC). It has been shown that the ISC correlated well with the measured net sodium flux and thus was an excellent measure of the net sodium transport through the frog skin (Ussing, Zerahn, 1951). Agents that inhibit sodium active transport in mammals act identically on the frog skin. For example, ouabain and amiloride inhibit sodium active transport whereas arginine vasopressin (ADH) increases sodium active transport.

Recently the frog skin has been used as a test system to study the effect of organic toxicants on transepithelial active transport of sodium. Naphthalene increases sodium active transport of the frog skin with an EC50 of 4.4 mg/L

(Blankemeyer, Hefler, 1990). It was also shown that the probable site of action of naphthalene was on the pond-side membrane of the frog skin. Further investigation showed the effect of other cyclic organics on frog skin active transport and that the organics decreased sodium active transport in a dose-dependent manner (Blankemeyer, Bowerman, 1992).

Using frog embryos with the CHAWQ (Cell Health and Water Quality) assay (Blankemeyer *et al.*, 1993), the glycoalkaloids affected the membrane potential of the embryonic cells (Blankemeyer *et al.*, 1992). α-Chaconine and α-solanine depolarized the membrane showing an increase in the membrane potential. The EC50 from membrane potential concentration response curves was near that of the FETAX (Frog Embryo Teratogenic Assay *Xenopus*) assay (Blankemeyer *et al.*, 1992). Depolarization of the membrane potential suggests that the mode of action of the glycoalkaloids involves specific ion channels and/or ion "pumps." To test this hypothesis, we used frog skin sodium transport as a model system to analyze the effect of the glycoalkaloids on epithelial active transport and to determine if the active transport of sodium was affected.

CHAPTER II

LITERATURE REVIEW

Potatoes and Glycoalkaloids

A normal potato contains insignificant amounts of the glycoalkaloids, α-chaconine and α-solanine. α-Chaconine and α-solanine have the same aglycone alkaloid solanidine, but differ with respect to the composition of the carbohydrate chain. However, certain environmental conditions, such as light exposure or mechanical damage, will induce synthesis of the glycoalkaloids in the potato tuber. The level of these glycoalkaloids depends upon the state of tuber development, and the environmental conditions.

There is evidence concerning potato-related poisoning in humans and farm animals attributed to the ingestion of large amounts of glycoalkaloids in the green tubers or sprouts. It has been reported that potato tubers with more than 20 mg of α-solanine per 100 g of fresh weight exceed the upper limit for food safety (Bomer, Mattis, 1924). It is also interesting to note that the glycoalkaloids of potatoes are not destroyed during boiling, baking, frying, or drying at high temperatures (Bushway *et al.*, 1980). A controversial hypothesis concerning the relationship between

imperfect potatoes and birth defects had renewed interest in the toxicological aspects of potato glycoalkaloids (Renwick, 1972).

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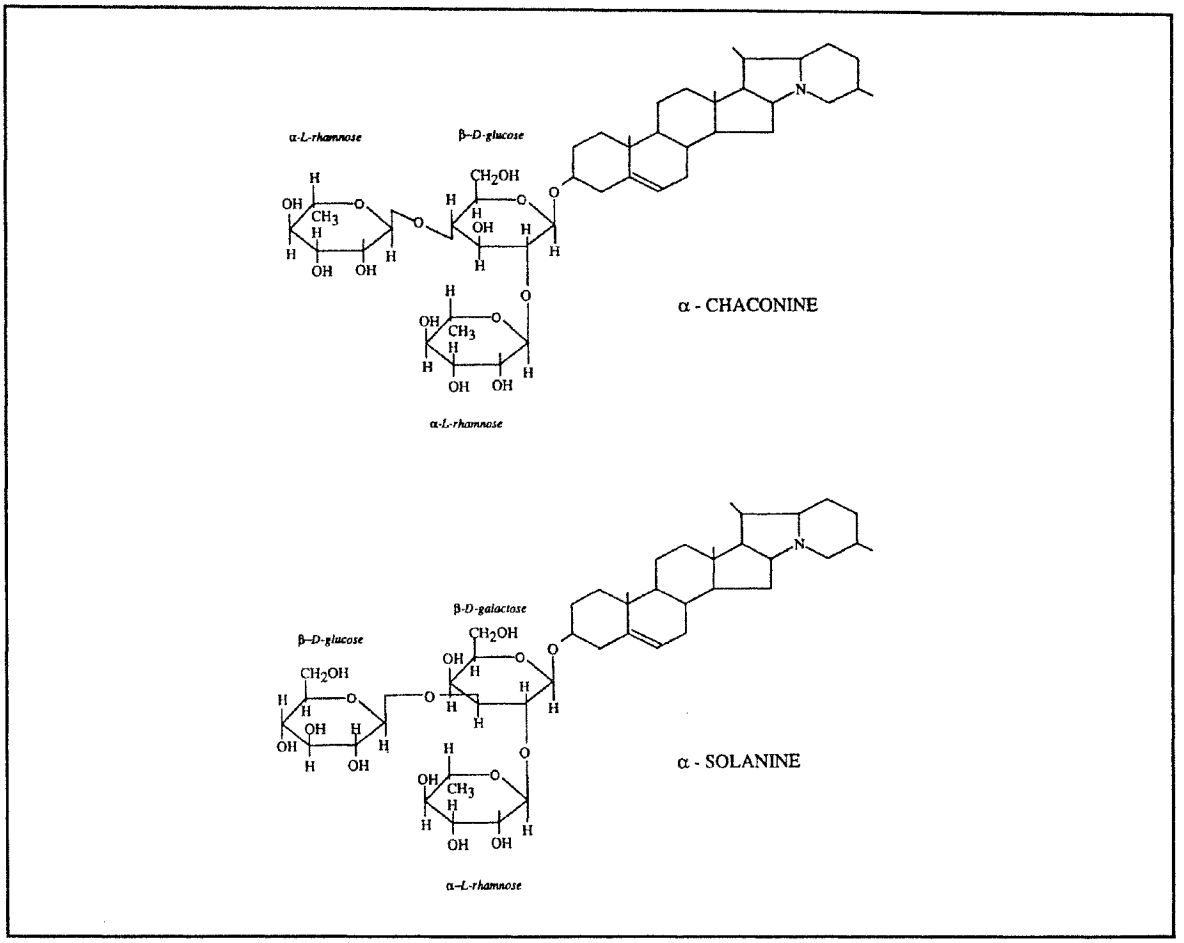

Figure 1: The chemical structure of α-Chaconine and α - Solanine.

Frog Skin As An Experimental Tool

Maintaining a constant ionic environment is an urgent necessity not only for mammals, but also for all other forms of life. Kidneys in vertebrates are the major controlling site of electrolyte and water balance maintenance between the internal and the external environment through reabsorption and excretion of salt and water. However most amphibians, due to differences in the function of their

kidneys (Adolph, 1931), have to rely on other sources to conserve salt and water. Therefore, they are able to regulate water intake. Since 1799, a great deal of experimental work has been completed using epithelial tissue to study not only salt and water transport, but also to gain insight into the molecular basis of the bioelectric phenomena of all living cells.

Advantages of Using Frog Skin

Amphibian skin is characterized by a number of unique features which make an excellent biological model for the study of transport physiology across asymmetric epithelial cells: First, frog skin is a salt-transporting organ which possesses the capability of actively transporting sodium ions from the external environment or mucosal side to the internal environment or the serosal side of the epithelium. Second, this tissue responds to common hormonal regulators of the transport processes by showing an increase or decrease in the rate of ionic movement across the epithelium. This response is similar to that of the distal convoluted tubules and collecting ducts of the mammalian kidneys (Herrera, 1971). Third, the skin is loosely attached to the frog body and it can be easily removed and manipulated with minimum damage to the tissue (Kidder III, 1973). Fourth, this tissue and the associated experimental preparations are readily available and relatively inexpensive. Fifth, the skin can be easily cut into pieces

some of which can be easily mounted as a sheet of tissue between two bathing solutions, while other pieces of the same skin can be treated as the control group. Sixth, such experimental preparations permit the investigator to define precisely the composition of the media bathing the two surfaces to the epithelium. Seventh, the isolated frog skin is able to retain its transporting capabilities for many hours under *in-vitro* conditions (for these experiments, the time-lapse did not exceed six and one-half hours). For example, the isolated skin exhibits spontaneously electrical potential difference which is usually associated with the active reabsorption of sodium from the mucosal to the serosal side of the epithelium. Eighth, the electrical parameters, such as the electrical potential difference, resistance, and the ionic current can be easily and uniformly manipulated throughout the entire epithelial sheet.

Some Anatomical Problems

The frog skin, however, is still far from being the ideal model for studying sodium active transport (Kidder III, 1973). This epithelial tissue is comprised of an epidermis made of a stratified squamous epithelium which rests on a continuous basement membrane that separates the epithelial cells from the underlying corium. The corium represents about 80% of the total volume of the skin (Erlij, Ussing, 1978), and contains mucous glands, blood vessels,

melanocytes, and other types of cells all dispersed in a thick mat of loose connective tissues. Furthermore, the epidermis, which is believed to be the sole site of sodium active transport, is comprised of four strata organized in 6-9 cell layers. These four strata are; stratum corneum, strata granulosum, stratum spinosum, and stratum germinativum. The cells of different strata apparently have a slightly different morphology and are interlocked with each other by specific intracellular junctions that differ in their structure and intensity from one layer to the next (Shahin, Blankemeyer, 1986). Different cell types which also have different transport mechanisms, such as mitochondrial-rich cells, are frequently encountered among frog skin epithelium (Farquhar, Palade, 1965). The anatomical complexity of frog skin has made it difficult to gain a clear understanding of the ionic transport mechanisms across such epithelia, and complicated the interpretation of the experimental data. Especially, those associated with the determination of the route of ionic movement and the localization of the transport "pool" (Erlij, Ussing, 1978). To overcome this complexity, some investigators (Fisher *et al.*, 1980) have successfully managed to separate the epithelium from the underlaying corium. The isolated epithelium seems to have the same electrophysiological properties as the whole skin (Aceves, Erlij, 1971). Another approach to avoid using the whole skin was achieved by making determinations (e.g. ionic concentration of the

epithelial cells) on slices of skin cut parallel to the surface of the skin with a freezing microtome (Hansen, Zerahn, 1964).

The contribution of specific cell types to the process of sodium transport or to the osmoregulatory function of epithelial tissue has been studied separately by investigating the morphological and physiological characteristics that differentiate one type of cell from another. For example, carbonic anhydrase activity was demonstrated only in one specific type of cells called mitochondria rich cells (Rosen, Friedley, 1973). This raises the possibility that these cells may have different transport properties from the rest of the epithelial cells. It's also well established that aldosterone, which stimulates active epithelial sodium transport in frog skin, has the ability to promote electrophysiological changes in epithelial tissue (Voute *et al.*, 1969).

It should be emphasized that these attempts to determine the transport properties and transport locations were not trivial simplifications of the original highly complex model. Yet the simplifications have served instead as reasonable steps for improving the accuracy of interpretations regarding the size of the transport compartment and the estimation of intracellular sodium and potassium activity and concentration. Nonetheless, the morphological complexity of the isolated epithelium is still a chronic problem which has complicated the interpretation

of the experimental results and retarded the unequivocal understanding whether only a single layer of cells is responsible for the transport properties of the epithelium, or whether the whole epithelium functions as a syncytium (Civan, 1983).

Historical Background

A great deal of knowledge concerning both salt-water metabolism and sodium active transport across the frog skin epithelium has accumulated over a period of several decades. More than one hundred and eighty-five years ago, Townsen recognized that the skin of certain amphibia played a significant role in regulating water intake. The electrical properties of the frog skin have been studied as far back as 1848 by DeBois-Reymond, who first observed that the isolated frog skin was able to generate and maintain a potential difference between its mucosal and serosal sides, the serosal side being around 100 mv positive, relative to the mucosal side. Erlij (Erlij, Ussing, 1978) reported that the maintenance of this potential difference required sodium or lithium in the bathing solutions. Later, in 1937, Dean and Gatty provided a detailed study on the electrical properties of the frog skin with many references to the earlier literature (Dean, Gatty, 1937). In 1892, Reid devised the first double flux chamber to study fluid transport across certain epithelia, but especially designed for the frog skin. Bathing the skin between two identical saline

solutions, Reid reported a net fluid transport of "a few $\mu l/cm^2/hr$" in the inward direction from mucosal to serosal side of the frog skin (Reid, 1892); however, not knowing that the primary event was sodium active transport and that water movement followed the osmotic gradient generated by the ion transport, he failed to offer a convincing explanation for his "fluid transport phenomenon" (Huf, Howell, 1935). In a series of articles on the movement of water across epithelium membranes, Adolph was able to demonstrate that freshly isolated frog skins "gain" or "lose" water in proportion to the square root of time elapsed after immersion, and this "gain" or "loss" was linearly related to the sodium concentration in the media (Adolph, 1933).

During the 1930's, Huf studied Reid's experiments carefully (Huf, Howell, 1935) and confirmed many of his observations and conclusions. He was the first to show that the isolated frog skin, in contact with Ringer solution on both sides, was able to transport hypertonic salt solution from the mucosal to the serosal side. Analyzing for sodium during his time was a "tedious procedure", therefore, Huf only measured changes in chloride ion concentration in the solutions on the two sides of the skin. Nonetheless, he accurately assumed that the tissue actively transported sodium chloride from the outer side to the inner side. He also stated that this active salt transport ceased when cyanide was added to the bathing solution. Shortly

afterward, Krogh (Krogh, 1937) demonstrated that salt-depleted frogs were capable of transporting appreciable quantities of chloride ions through the skin, even from a very diluted solution of KCl and $CaCl_2$ in exchange for bicarbonate (Krogh, 1937). He concluded that the chloride ions were the ions which were actively transported across frog skin. Although this conclusion has been proven to be correct only for one or two species of frogs (Zadunaisky *et al.*, 1963; Martin, Curran, 1966), it prompted further investigation which proved to be very fruitful.

The use of radioactively labeled ions to measure the ionic permeability of isolated frog skin was introduced by Katzin (Katzin, 1940) who reported that the rate of sodium influx exceeded that of sodium efflux across skin bathed in normal saline solution. Ussing (Ussing, 1949), utilizing the same radioactive isotope technique, found that isolated frog skin could actively transport sodium against a steep electrochemical gradient, but only passively move chloride in the same direction. The chloride is driven by the transepithelial potential difference across this tissue (Koefoed-Johnsen *et al.*, 1952). By combining isotope flux measurements with the electrical current measurement on the short circuited skins, Ussing and Zerahn (Ussing, Zerahn, 1951) established for the first time the equality between the short circuit current and the net sodium active transport across the frog skin. Because of its simplicity and practicality, this technique has become a standard

method for measuring active transepithelial sodium current. It should be mentioned however, that in some species of frogs, (ie. *Leptodacrylus ocellatus*) other ions, like chloride, contribute to the total current flow across the skin (Zadunaisky *et al.*, 1963; Martin, Curran, 1966).

Koefoed-Johnsen and Ussing Model

In 1958, Koefoed-Johnsen and Ussing introduced the "three compartment" model for explaining the transepithelial sodium transport across frog skin. This model has marked the beginning of a new era in the field of epithelial transport. According to this model (Fig. 2), the transport of sodium ions across epithelia involves a two-step process across two different barriers placed in series.

Briefly, sodium ions passively enter the epithelial cells through the highly sodium selective, amiloride sensitive, apical membranes and actively leave the cells through the potassium selective, ouabain-sensitive, basolateral membranes which contains the Na^+-K^+ ATPase that facilitates the forced exchange of sodium ions against potassium ions (Na^+-K^+ pump). Because of its simplicity and practicality, this model received a great deal of attention and soon became the conceptual framework that guided investigators working in the field of epithelial transport for many years (Macknight *et al.*, 1980; Schultz, 1983).

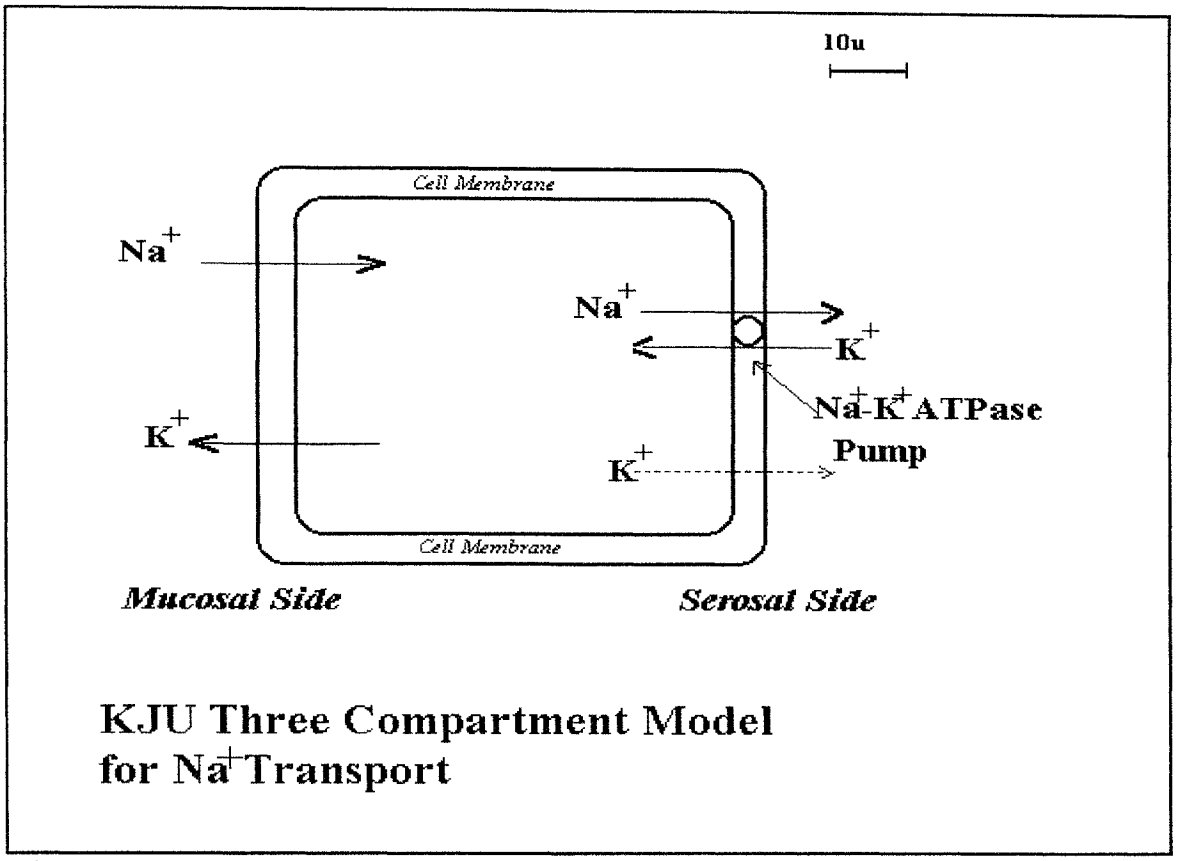

Figure 2: The KJU (Koefoed-Johnsen and Ussing) Three Compartment Model of Sodium Transport. This model proposed the origin of the frog skin potential.

Revision of (KJU) Model

Despite the fact that the KJU model was widely accepted by the community of epitheliologists, and indeed extended to many other "tight" and "leaky" epithelia (Ussing *et al.*, 1974; Macknight *et al.*, 1980), this model has been subject to many modifications in order to account for many new observations that resulted from introducing some advanced methods and techniques to the field of epithelial transport.

One of the first revisions of the KJU model came from Ussing and Windhager (Ussing, Windhager, 1964) who

discovered, in addition to the usual transcellular pathway, there was a paracellular shunt pathway in which specific ions proceeded between the cells instead of passing through the cell membranes. According to Ussing and Windhager, sodium ions which diffuse across the apical membranes are conducted from cell to cell through some low-resistance intercellular junctions towards the basal layer of the epithelium, where an active mechanism (Na^+-K^+ pump) transports them into the serosal bathing solution. However, it has been postulated that there are pumps in the cellular membranes of the stratum spinosum which can pump sodium ions into the intercellular space as well, but these such sodium ions then become available for free communication with the inside bathing solution (Ussing, Windhager, 1964)

Thus, this new model introduced three elements to the original KJU model: One, a possible paracellular pathway for sodium and other ions. Two, a probable extensive cell-to-cell coupling between the cells of all epithelial layers. And three, the possibility that the distribution of Na^+-K^+ pumps are not restricted only to the cells of stratum germinativum as was originally proposed by the KJU model. In fact many investigators have recently supported the notion that the ouabain-sensitive Na^+-K^+ ATPase enzymes are localized to the basolateral membranes of all living cells of frog skin epithelium (Farquhar, Palade, 1966; Mills *et al.*, 1977).

Cereijido and Rotunno (Cereijido, Rotunno, 1968) proposed a new model from the sodium transepithelial transport across the frog skin in which the movement of the sodium occurs as a result of sodium movement around, but not through the cells. This model suggested that sodium ions, after being attached to the lipid bilayer of the mucosal membrane, have the tendency to travel around the cells by jumping from one fixed polar site to another in a "triplet saltatory mechanism" rather than penetrating the cell membranes. This route for sodium ion movement seemed to represent a lower energy barrier (Cereijido, Rotunno, 1968). However, sodium ions had to pass the diffusion barrier for saltatory movement formed by tight junctions and desmosomes at the outermost layer of stratum granulosum in order to continue their saltatory movement towards the serosal side of the basal cells where an active transport mechanism usually pumps sodium ions into the serosal bathing solution.

The Sodium Transport Compartment

The localization of the sodium transport compartment in this multilayer type of epithelium, and the determination whether this transport compartment constitutes the whole population of the epithelial cells or just a small fraction of them, was under intensive investigation during the 1970's. The experimental data concerning these issues during recent years has established at least two trends for

explaining the mode of sodium active transport and the site of its occurrence.

The first trend implicates that the first reacting cellular layer (the stratum granulosum) represents the main body of the sodium transport compartment. This trend was supported by Voute and Ussing (Voute, Ussing, 1968), who tried to correlate between different states of sodium transport and the morphological changes in the epithelium at light and electron microscopic levels. Their data showed that the morphological changes in response to different physiological treatments of the skin were restricted to the first reacting cell layer. Thus, it was assumed that this layer of cells was the main site of sodium active transport.

Some radiochemical analysis of the whole skin (Cereijido, Rotunno, 1968) and isolated epithelia (Aceves, Erlij, 1971) supported this view by showing that only a fraction of cellular sodium was exchangeable with the mucosal bathing solution, however, they did not attribute this fractional sodium compartment to a specific layer. It should be mentioned however, that previous data (Zadunaisky *et al.*, 1963) indicated that most previously reported chemical analysis markedly overestimated the true value of cellular sodium. Thus, the observations and conclusions based on chemical analysis and extracellular markers have been misleading. Finally, the presence of tight junctions between the cells of stratum granulosum excludes the possibility of paracellular movement of sodium through this

stratum. Therefore, the movement of sodium through the cellular membranes of stratum granulosum must be assumed. However, whether the cells of stratum granulosum are able to carry out the active transport of all the sodium, or deliver some of it to the cells of underlying layers is still an unanswered question.

The second trend suggests that the cells of all epithelial layers share equally in the transport of sodium. In support of this notion, Farquhar and Palade (Farquhar, Palade, 1966), using histochemical methods to localize ATPase activity in frog skin, and Mills (Mills *et al.*, 1977) using a variety of autoradiographic techniques on a wide spectrum of epithelia, including frog skin, obtained data. The data suggested a homogeneous distribution of the ouabain-sensitive ATPase on the basolateral membranes of all living cells of frog skin epithelium, which indicated that all cell membranes facing the intercellular space might be potential sites of active transepithelial sodium transport.

Experimental Preparations and Methodology

In order to gain some insight into the macroscopic mechanisms of epithelial transport, some familiarity with the morphological aspects of the transport phenomena was absolutely necessary. Thus, the morphological approaches utilizing various methods and techniques of electron microscopy (ie. thin sections, extracellular traces, freeze fracture, x-ray diffraction etc...) have contributed a great

deal to enhance the understanding of the anatomical and physiological properties which correlate the epithelial transport process.

The chambered preparations originally introduced by DuBois and Remond, and established as a routine technique by Ussing (Ussing, 1949) have facilitated various aspects of *in vitro* studies on epithelial transport. A number of studies have used frog skin as a simple, but useful "black box" device which has served as an example of epithelium that performs both passive and active transport (Erlij, Ussing, 1978).

In recent years, the issues in epithelial transport research have gradually evolved toward the molecular level, and it is now obvious that the more classic approaches to study these issues were inadequate by themselves to address the central questions of epithelial transport. Therefore, the trend in epitholiology has shifted toward utilizing more advanced approaches and techniques that could overcome the intrinsic limitations of the earlier "black box" approaches.

Black Box Approaches

When mounted between two identical Ringer solutions, the epithelium of frog skin is able to: One, generate high spontaneous potential difference (up to 120 mv) between the mucosal and serosal side of the epithelium; the serosal side being positive in relation to the mucosal side. Two, exhibit a transepithelial resistance of several thousand

Ohms/cm^2 (Erlij, 1976). And three, transport a net current of sodium from the pond side toward the serosal side of the skin against a steep electrochemical gradient (Ussing, 1949).

In order to characterize the transport mechanism(s) operating on such a system, it was useful or even necessary to treat this tissue in a pragmatic manner as a "black box membrane" with certain transepithelial electrical parameters (Erlij, Ussing, 1978). For example, the introduction of the short circuit technique by Ussing and Zerahn (Ussing, Zerahn, 1951) has been of great technical and practical importance to determine the manner by which sodium ions are transported from the pond side to the blood side of the frog skin. The concept of this technique is based on the fact that, in the absence of all thermodynamic driving forces across the isolated frog skin bathed between two identical Ringer solutions, only ions which are actively transported can cross the epithelium in the net transport sense. This condition can be easily achieved by equalizing all factors that affect the thermodynamic movement of ions between the two external aqueous phases bathing the skin such as chemical concentration, pressure, temperature...etc., and then bring the potential difference generated by the tissue to 0. Under such conditions, Ussing and Zerahn (Ussing, Zerahn, 1951) reported that the net unidirectional (mucosal to serosal) flux of sodium ions, when expressed in the same units, is equal to the short circuit current across the

skin. In frog skin, this short circuit current is highly sensitive to the lack of oxygen, and to the introduction of metabolic inhibitors into the bathing solution (Huf *et al.*, 1957). This knowledge provided the basis to define the process of sodium transepithelial movement across frog skin as an active step which is highly dependent on metabolic energy.

In addition to the electrical parameter measurements, many other measurements can be obtained from the chambered preparations of frog skin. A well known example of such a "black box" approach was the study which attempted to correlate the changes in electrical potential across frog skin epithelium and different ionic compositions of the bathing solutions. This study yielded the famous three-compartment model for sodium transepithelial transport formalized by Koefoed-Johnsen and Ussing (Dobson, Kidder, 1968). This model (fig. 2) visualized the movement of sodium ions across the epithelium as a two step process. The first involved the passive entry of sodium into the epithelial cells across a highly sodium selective apical membrane and down the electrochemical gradient. The second step involved the active extrusion of sodium ions across the potassium selective basolateral membranes via an ATP-utilizing Na^+-K^+ pump. Thus, the final results of this model were two fold; a net transepithelial sodium transport from the pond side of the skin to the serosal side, and a

tight regulation of the intracellular composition of electrolytes, mainly sodium and potassium ions.

The black box approach however, could not assess the precise role of each membrane in the transport process; the matter which necessitated the introduction and development of new methods that provided some new insights into the process of understanding the molecular mechanisms of the sodium-active transport across each barrier of the asymmetric epithelial membranes.

Advances in Approaches of Methodology

The combination of immunological methods with biochemical and structural analysis offered an advanced and promised approach to the understanding of the physiological regulation of epithelial transport. For example, considerable efforts have been devoted recently to develop immunological probes against Na^+-K^+ ATPase (an enzyme that is preferentially located on the basolateral surface of the transporting epithelial cells), and many attempts to biosynthesize the subunits of this enzyme have been reported (Rossier, 1983). These attempts have already resulted in valuable information for the process of studying the possible sites of the action of some hormones or chemicals (aldosterone, corticosteroid, ADH, etc.) that affect transepithelial transport. The development of specific antibodies against gap junctions (Hertzberg, Skibbens, 1984) has been very useful for the determination of the site and

distribution of such important junctions in various epithelial tissues.

Electrical Properties of Frog Skin Epithelium

General Considerations

It has long been known that the epithelium of frog skin is characterized by certain electrophysiological parameters (ie. transepithelial potential difference and resistance) which can be used to study the behavior of the epithelial membranes in relation to the ionic mobilities across such asymmetric systems (Dean, Gatty, 1937). Electrophysiological studies on frog skin epithelium have been largely concerned with the DC (direct current) properties of the tissue (Koefoed-Johnsen, Ussing, 1958). This approach involved the determination of currents, electrical potential differences, and electromotive forces which are related by the electrical resistances and conductances of the different barriers of the epithelium (Schultz, 1979).

The isolated "undamaged" frog skin bathed symmetrically between two identical solutions of ordinary Ringer's is able to generate and maintain a spontaneous transepithelial potential of approximately 100 mv. This potential difference (PD) is oriented serosal side positive with respect to the mucosal side. The source of this potential

difference may be attributed to the active transport of sodium (Ussing, Zerahn, 1951) or to the summation of two simple diffusion potentials across the outer (sodium diffusion potential) and the inner (potassium diffusion potential) barriers of frog skin epithelium (Koefoed-Johnsen, Ussing, 1958). The open circuit transepithelial resistance (RT) of the frog skin epithelium ranges between 3K and 25K Ohms/cm^2 (Erlij, 1976).

The magnitude of these electrical parameters is highly dependent on the composition of the Ringer's solution bathing the skin. For example, when the chloride ions of the bathing solution are replaced by non-penetrating sulphate ions, the transepithelial potential difference increased to a maximum of 120 mv (Engbaek, Hosiko, 1957). Under similar conditions, the skin exhibits an elevated RT of 34.4K Ohms/cm^2 (Erlij, 1976). However, the reported values of the electrical parameters of amphibian skin vary considerably among different experiments. These variations have been attributed to the edge damage effects resulted from the crushing of the skin edges between hard surfaces (Dobson, Kidder, 1968). It has been suggested also, that the low open-circuit PD and resistance TR reported by some investigators could have resulted from a generalized damage to the tissue during the process of dissecting and/or mounting the skin on the chambered preparations (Finn, Hutton, 1974).

The basic electrical criteria for the *in vitro* undamaged tight epithelial tissues as listed by Macknight (Macknight *et al.*, 1980) include: high open-circuit PD, high TR, and the inverse relationship between open-circuit potential and tissue resistance, a direct proportionality between short circuit current and tissue conductances, and highly elevated resistance TR for tissues treated with amiloride.

Equivalent Electrical Circuit of the Epithelium

The simplest equivalent circuit used to describe this model cannot accommodate microelectrode studies which have proved very useful in characterizing the electrical properties of individual membranes or compartments (Macknight *et al.*, 1980). Therefore, more elaborate electrical circuits have been introduced to describe the electrophysiological behavior of the two barriers (the apical and basolateral membranes) of the epithelium (Schultz, 1979).

Properties of the Apical Membranes and the Mode by Which Sodium Enters the Cellular Compartment

General Properties

It has been apparent, since Koefoed-Johnsen and Ussing introduced their "double membrane" model for the transepithelial sodium transport across frog skin in 1958, that the apical membrane of this epithelium is highly selective to sodium. Complete replacement of the mucosal sodium by other cations suggests that only lithium ions can be transported across frog skin. However, when mucosal lithium was substituted for sodium, an irreversible decrease in the transepithelial potential of the skin was observed (Lidley, Hoshiko, 1964). This effect of lithium ions might be due to the fact that cellular lithium promotes an nonspecific permeability increase of the paracellular pathway of the epithelium to sodium and potassium.

The anionic composition of the mucosal medium is also known to modify the magnitude of the open-circuit potential across the frog skin which in turn affects the apical membrane permeability to sodium. For example, the addition of non-penetrating anions such as SO_4^-, I^-, and NO_3^- to the mucosal bathing solution produce a higher potential difference across the skin than the addition of the chloride or bromine which are easily "shunted" across the epithelium (Koefoed-Johnsen, Ussing, 1958). The sequential effects of

some of these anions on the PD as reported by Lindley and Hoshiko (Lidley, Hoshiko, 1964) are as follows; SO_4^- > I^- > Cl^- > Br^-, with the mucosal Br^- producing the lowest voltage.

The Apical Entry of Sodium Ions

Recent determinations of the electrical potential profile across the apical membrane of the frog skin imply that the sodium entry to the cell is a passive consequence of the driving force provided by the electrochemical potential gradient for sodium.

However, the short circuit current or net sodium transport across frog skin epithelium is not a simple linear function of the mucosal sodium concentration (Lidley, Hoshiko, 1964). This relationship exhibits a curvilinear pattern instead. This means that the short circuit current increases as mucosal sodium concentration increases initially, but when the concentration reaches an equilibrium, the transepithelial sodium current stabilizes (Macknight *et al.*, 1980). Several neurotropic compounds such as curare, local anesthetics, atropine, and pilocarpine (Herrera, 1971) reversibly increase sodium transport across frog skin by increasing the permeability of the apical membrane. The application of such agents on the mucosal side of the frog skin causes also an increase in the Na^+-K^+ pump activities, which suggests that the curvilinear relationship between the net current and the mucosal sodium concentration is not a result of the saturation of the

sodium pumping activities at the basolateral side of the epithelium. Thus, the process responsible for such saturation operates only at the level of the apical membrane (Macknight *et al.*, 1980). Nonetheless, the mechanism(s) that account for this apparent saturation of the entry step of sodium is still not well understood. One possibility is a diffusion process modified by self-inhibition of the apical membrane permeability as sodium concentration increases either in the mucosal bathing solution or in the cells.

The direction of the net sodium movement across frog skin is basically from the mucosal side to the serosal side of the skin. However, some evidence for a bi-directional movement of sodium across the apical membrane has been explained where the presence of tight epithelia are found (Macknight *et al.*, 1980). For example, when ouabain was added to the serosal bathing solution of frog skin, an amiloride-sensitive back flux of sodium into the mucosal bathing solution occurred. However, such flux in the opposite direction of sodium across the apical membrane does not take place under normal experimental conditions with normal Ringer solution on both surfaces of the epithelium (Macknight *et al.*, 1980).

The Regulation of the Sodium Entry Across the Apical Membrane

In addition to the amiloride which completely blocks the sodium entry across the apical membrane of frog skin epithelium (Helman, 1979), there are many other factors that can regulate the sodium movement across this barrier. These regulatory agents include hormones (ADH and aldosterone), chemical agents (local anesthetics, novobiocin, diphenylhydantoin, and polyene antibiotics), mucosal and serosal sodium and other alkali metals, mucosal and intracellular pH, and some physical factors (electrochemical potential difference across the outer barrier, cell volume, etc.).

Properties of the Basolateral Membranes and Mechanisms of Active Transport

Properties of the Basolateral Membrane

The basolateral membranes in frog skin and other sodium transporting epithelia include all of the epithelial membranes that are not passively permeable to sodium (Koefoed-Johnsen, Ussing, 1958). Two distinct features are usually ascribed to this barrier. First, it is passively and selectively permeable to potassium. Second, it is the site of the energy-dependent active transport step that drives transepithelial sodium transport.

Ion-substitution experiments (Lidley, Hoshiko, 1964) revealed the relative permeability of the basolateral membrane to the following series of cations K^+: Rb^+: Cs^+: Li^+: Na^+, is 1.00: 0.74: 0.22: 0.12: 0.09 respectively. The swelling of the epithelial cells as a response of potassium substitution for sodium in the serosal bathing solution provides evidence for the high potassium conductivity of the basolateral membrane. Chloride ions are also known to cross the inner barrier of the epithelium (Koefoed-Johnsen *et al.*, 1952). Complete substitution of the serosal medium chloride by other anions (I^-,Br^-) causes different degrees of stimulatory effect on the transepithelial sodium transport (Huf, 1972). On the other hand, the substitution of sulfate ions for chloride ions in the serosal bathing solution depresses sodium transport (Huf, 1972). This kind of anion substitution is believed to affect the transepithelial sodium transport through some mechanisms that operate at the basolateral membrane (Macknight *et al.*, 1980).

When the serosal bathing solution is made sodium free, calcium ions from the extracellular medium passively cross the basolateral membrane of the frog skin and accumulate in the epithelial cells. Under these conditions, a significant reduction in the transepithelial sodium transport caused by sodium free serosal solution became negligible. These results suggest that calcium ions play a significant role in the regulation of the permeability of the basolateral membrane, and thus in the determination of the rate of the

sodium transport across the epithelium. Other multivalent cations such as zinc, barium, cadmium, and manganese are also known to modify both the permeability of the basolateral membrane, and the net sodium transport in tight epithelia (Macknight *et al.*, 1980).

Localization of the Active Sites

Due to the morphological complexity of the frog skin, the localization of the active site of sodium transport has been a chronic problem. Historically, the active site of sodium transport was ascribed to many different locations; the basement membrane of the epithelial tissue along the boundary between the basal layer and the corium, the serosal border of the stratum germinativum cells, the basolateral membranes of all epithelial cells facing the inside solution or the innermost membranes of the stratum granulosum cell (Erlij, Ussing, 1978).

The concept that the active site of sodium-transport is mainly localized at the basolateral membranes of the cells is particularly attractive since there are groups of morphological findings that lend support to this theory (Erlij, Ussing, 1978). It should be mentioned however, that most recent electrophysiological studies on frog skin suggest an extensive electrical coupling between the cells (Nagel, 1976), indicating that all the epithelial cells participate in the process of active sodium transport rather than a specific layer or specific type of cells.

The Active Transport Mechanisms

In the classical formulation of Koefoed-Johnson and Ussing (Koefoed-Johnsen, Ussing, 1958), sodium which passively enters the apical membrane of frog skin epithelium is actively extruded through the basolateral membranes via Na^+-K^+ ATPase pumps. The extrusion of sodium was considered to be stoichiometrically linked to the potassium accumulation into the epithelial cells in a tight one-to-one relationship. This simply means that such Na^+-K^+ pumps are neurogenic and do not contribute to the electrical potential gradient for this ion. The short circuit current of the skin is not abolished, nor is the potential difference across the basolateral membrane. This indicates that the active transport mechanism is electrogenic (or rheogenic) and a strict one-to-one sodium for potassium exchange is unlikely to exist under such experimental conditions. Many experimental results using different approaches (Macknight *et al.*, 1980) have recently provided strong evidence for a rheogenic function of the pump; that is the stoichiometry of the pump fluxes of sodium and potassium is different and the pump generates a significant fraction of the driving force for the apical border sodium uptake. In general, the Na^+-K^+ exchange ratio is accepted to be 3 sodium for 2 potassium (Neilsen, 1982), however there appears to be considerable scatter in the data obtained from such investigations. For example, the Na^+-K^+ exchange ratio obtained (Helman, Cox,

1984) on frog skin varies with the short circuit current from 1:1 at lower short circuit current to 6:1 at higher short circuit current. It should be mentioned however, that none of the concerned studies exclude some partial coupling between the potassium uptake and the sodium extrusion. Nonetheless, the coupling ratio is unlikely to be a 1:1 ratio or fixed at a constant value.

The active transport mechanism is driven by metabolic energy, since metabolic inhibitors such as fluoracetate, azide, and diethyl malonate (Herrera, 1971) are known to be potent inhibitors of sodium active transport. The cardiac glycoside, ouabain, is another potent inhibitor of the active sodium transport in tight epithelia. Because of its ability to bind to the Na^+-K^+ ATPase at the basolateral membrane, ouabain is a very useful inhibitor for studying different aspects of sodium active transport (Farquhar, Palade, 1966; Mills *et al.*, 1977). When added to the serosal bathing solution, the analysis of the effects of ouabain on the conductance of sodium through the basolateral membrane indicated there is an abrupt decrease in the conductance of the basolateral membrane followed by further decrease but at a slower rate. The rapid phase of the decrease in conductance of the basolateral membrane is attributed to direct inhibition of the pump activity while the slower phase is due to a decrease in cellular concentration. Helman and Fisher (Helman, Fisher, 1977) obtained data that suggest a linear relationship between the

basolateral membrane conductance and the pump activity. The physiological significance of these findings is two-fold. First, the parallelism between the pump activity and potassium conductance enables the epithelial cells to maintain a reasonably constant level of intracellular potassium by changing the rates of the pump activity. Second, the potassium conductance of the basolateral membranes along with the activities of the rheogenic pump provide a significant contribution to the driving force for the apical entry of sodium across frog skin.

CHAPTER III

MATERIALS AND METHODS

The frogs, *Rana pipiens*, were purchased from William Lemberger (Oshkosh, WI) and shipped "second day air" when the temperature forecasted was to remain above freezing. Following arrival, the frogs were rinsed and maintained in tap water (pH 7, 20° C, 146 mg/L hardness) until use. No feeding was required because the frogs will not feed while in captivity. Experimental testing on each frog shipment was generally completed within two weeks of arrival. The frogs were randomly selected from the holding tank and then were anesthetized by injection of 2.5 ml 10% urethane into the dorsal lymph sac until they became limp. The frogs where then euthanized by decapitation posterior to the tympanic membrane, and the spinal cord pithed. Each animal procedure was performed in strict concordance with the AVMA accepted methods for euthanasia. The frog's abdominal skin was excised with forceps and iris scissors, and care given not to damage or touch the "experimental portion" of the skin with the instruments. Then the skin was placed in Nitrate Frog Ringer's Solution (110 Mm $NaNO_3$, 2.5 Mm KCl, 1.0 mM $CaCl_2$, 2.5 mM TRIS buffer, adjusted to pH 8).

The glycoalkaloids were obtained commercially (Roth, Basel) and were purified using HPLC methods to 95% purity, then stored in the dark and at zero degrees Celsius. Solutions containing glycoalkaloids were freshly prepared by lowering to pH 3 with 1 M HCl to facilitate solubilizing the alkaloids, then restoring to pH 8 with 1 M NaOH. No carrier solvents were used. A modified Ussing Chamber was used to mount the excised abdominal skin (Fig. 3) in preparation for experimental testing.

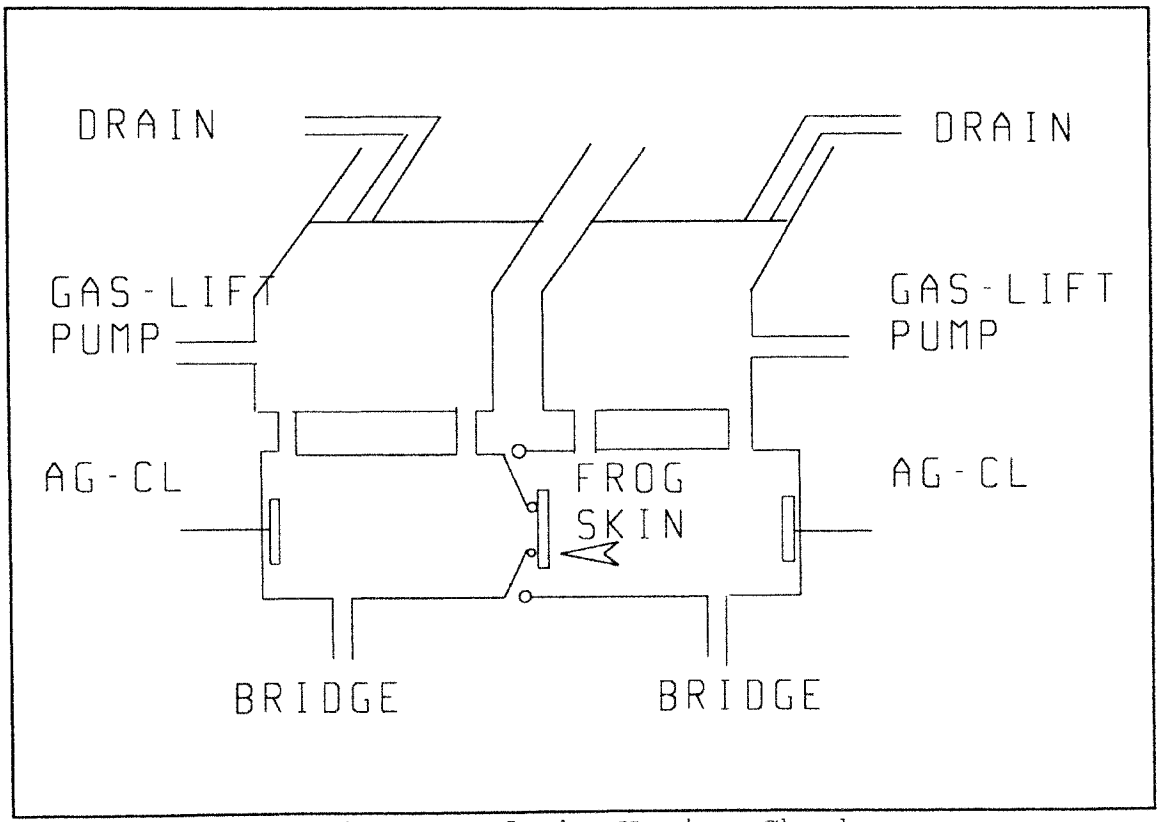

Figure 3: The Diagram of the Ussing Chamber.

The potential difference was measured by two 2.5% agar-Frog Ringer's bridges. The short circuit current (ISC) was passed by Ag-AgCl electrodes placed at the end of each chamber tank so the current density was uniform across the

skin. $NaNO_3$ Ringer's solution bathing the mucosal side and NaCl Ringer's solution (110 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 2.5 mM TRIS buffer, adjusted to pH 8) bathing the serosal side of the frog skin, were continuously stirred and aerated by gas-lift pumps facilitated by the chamber's design. Drains were connected to vacuum lines to assist in removal of changing solutions. The frog skin was secured onto the chamber lip with size 000 surgical thread. An automatic voltage clamp, generating the required ISC, maintained the PD of the frog skin at zero mV. The ISC was recorded from a digital panel meter on the voltage clamp at five minute intervals. A chart recorder (Linear Instruments, CA) set with a range of 100 mV and a chart paper speed of 0.2 cm/hour was used to trace the time course of the ISC.

The effect of glycoalkaloids on the ISC was determined by using the 30 minute period prior to glycoalkaloid administration as a control period, then calculating the change in ISC during the glycoalkaloid period as a percent of the control ISC. Data were collected from at least four frog skins for each glycoalkaloid concentration. Statistical significance was determined by calculating 't' values and evaluating at the 95% level.

CHAPTER IV

RESULTS

In an exemplary α-Chaconine experiment, the frog skin was "equilibrated" for two hours with standard $NaNO_3$ Frog Ringer's solution on the mucosal side and NaCl Frog Ringer's on the serosal side of the skin. During this period, the ISC (short circuit current)increased from near 82 μAmps to near 90 μAmps. Typically the ISC (the net sodium transport) stabilized during the two hour period. If the ISC had not stabilized, the slope for the 30 minute time period prior to the beginning of the experiment would be calculated and subtracted from the treatment readings. The treatment solution was added at 120 minutes. The mucosal bathing solution was changed to a $NaNO_3$ Ringer's solution containing the first dilution 0.75 mg/L of α-chaconine. At 125 minutes, the ISC decreased steadily for the next twenty-five minutes. At 150 minutes, the $NaNO_3$ Ringer's solution with α-chaconine was replaced with control $NaNO_3$ Ringer's. After a twenty-minute delay a slight recovery ensued. It was observed that the individual response of any particular frog skin in the presence of α-chaconine was highly variable exhibiting a minimum of a 17% change to a maximum of 44% change with dose of 7.5 mg/L. Although, the pattern of

concentration-response was clear in all of the concentration-response curves showing that increased concentration of α-chaconine produced increased response of ISC.

Figure 4 represents one experimental trial in which the effect of an α-chaconine treatment was tested.

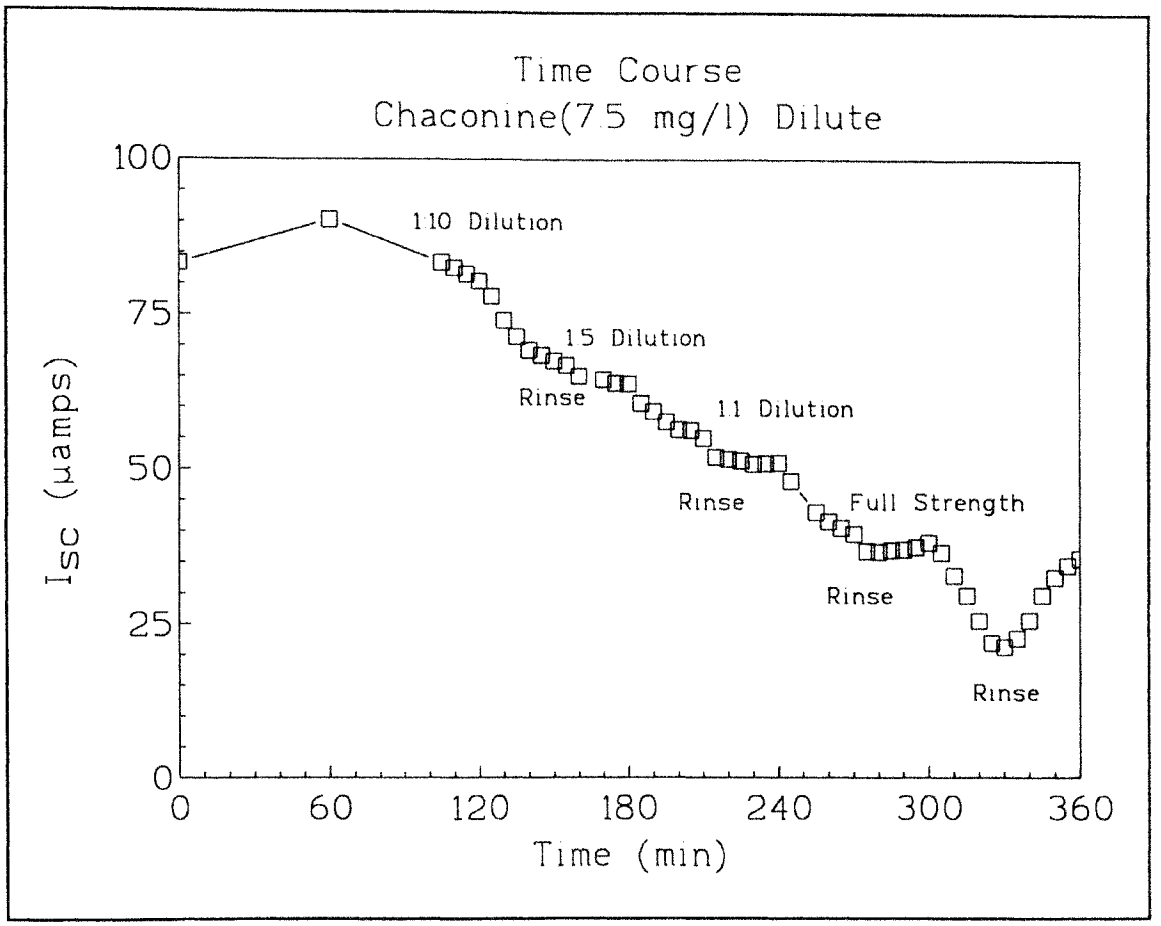

Figure 4: α-Chaconine Time Course following exposure for the *Rana pipiens* excised frog epithelia.

Figure 5 represents a Dose Response Curve for the data in figure 4. The percent response was calculated using the short circuit current before the addition of the first dilution of the 7.5 mg/L stock α-Chaconine and the lowest

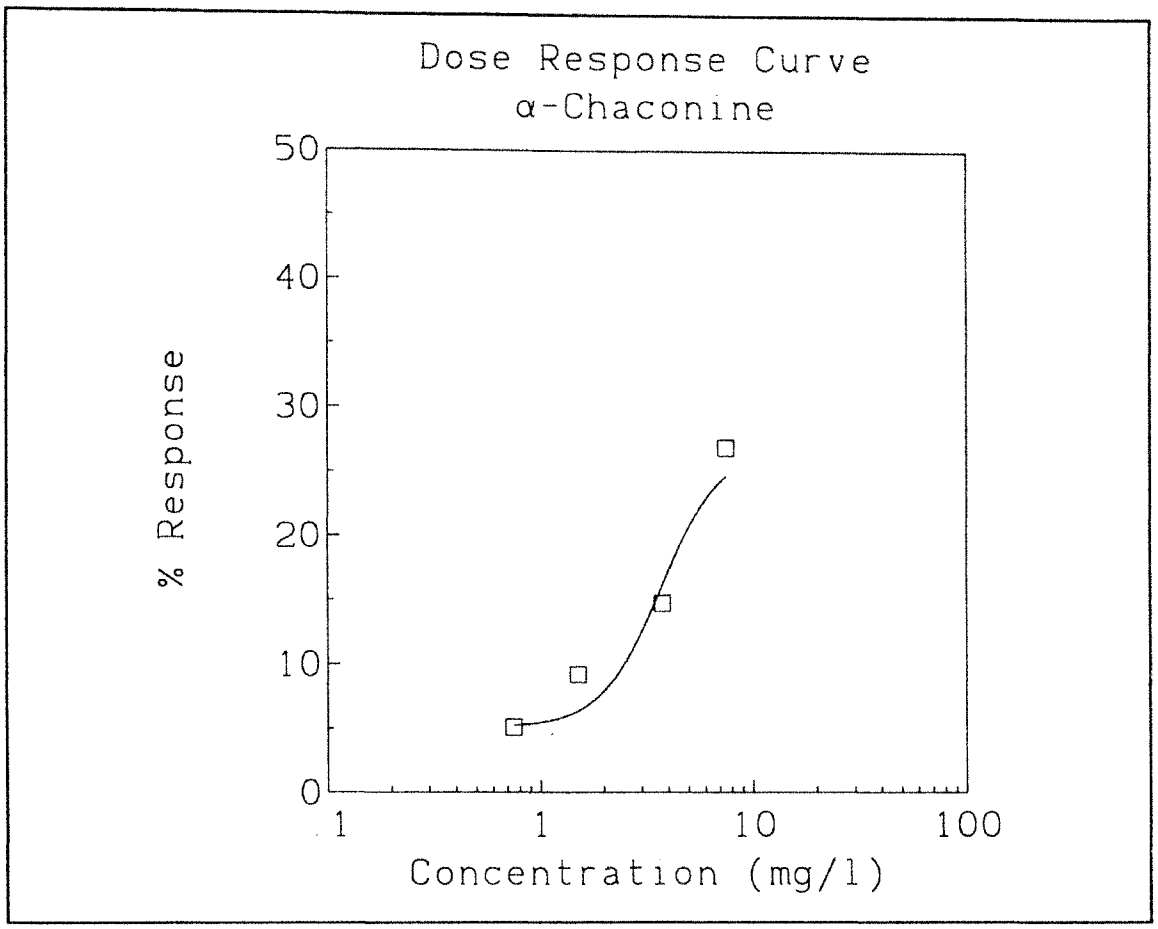

Figure 5: α-Chaconine Dose Response Curve of *Rana pipiens* excised epithelia measuring change in short circuit current.

short circuit current reading before the addition of the control solution ($NaNO_3$ Ringers Solution) 30 minutes later.

To allow direct comparison, the selected concentrations of α-chaconine reflected the selected concentration of α-solanine due the solubility maximum of α-solanine in Frog Ringer's Solution. The control experiments where only Ringer's Solution were used as the treatment showed less than a 0.5% response change between "treatments." Therefore, it appears there was a significant effect of the

lowest concentration value (0.75 mg/L) compared to the control.

The curve was fitted using the constraints of a Dose Response Model and generated using GraphPad InPlot version 4.0 sigmoidal curve fit. The curve characteristics using concentrations greater than 7.5 mg/L were not tested. The EC50 was determined to be 3.2 mg/L using GraphPad Inplot version 4.0.

Figure 6 represents one experimental trial in which the effect of α-solanine was tested.

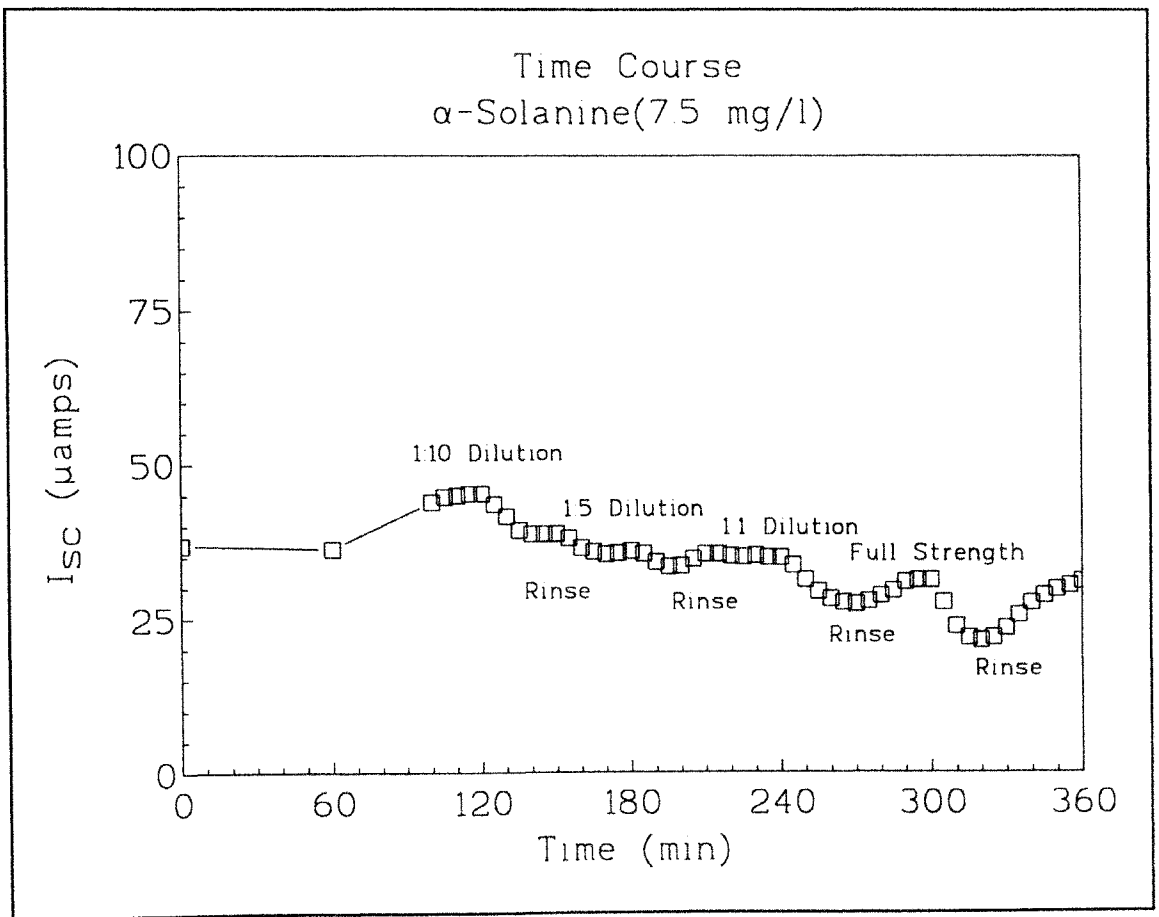

Figure 6: α-Solanine Time Course following exposure for the *Rana pipiens* excised frog epithelia.

The time course between time zero and 120 minutes looks uncharacteristic for the skin to be reaching equilibrium, but the change in the ISC 30 minutes before addition of the first dilution varied only a few μamps, denoting the equilibrium state. The same procedure for addition of dilutions was followed throughout all experiments. Again, the most significant drop in ISC was following the addition of the highest concentration of α-solanine. Figure 7 represents a Dose Response Curve for the data in figure 6.

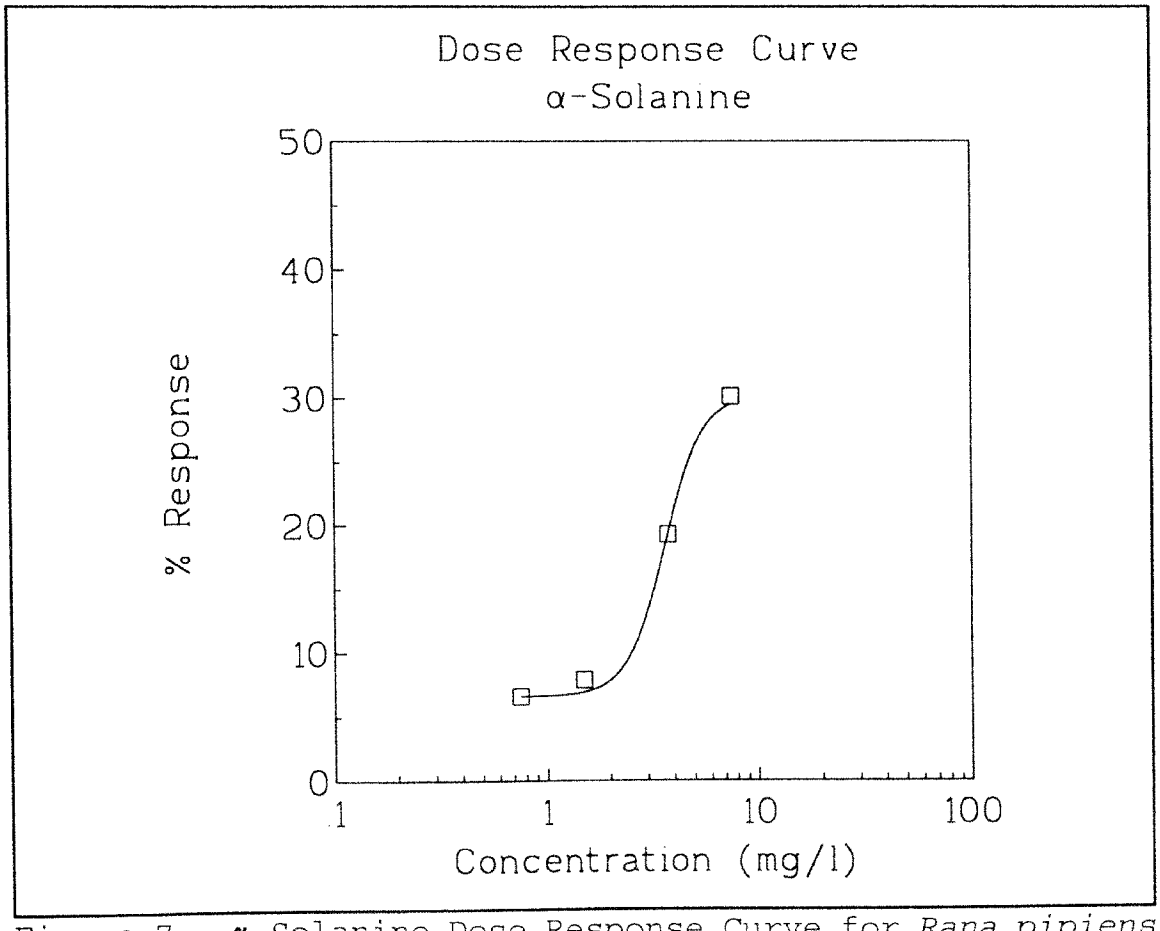

Figure 7: α-Solanine Dose Response Curve for *Rana pipiens* excised epithelia measuring change in short circuit current.

The percent response was calculated using the short circuit current before the addition of the first dilution of the 7.5 mg/L stock α-solanine and the lowest short circuit current reading before the next addition of the control solution ($NaNO_3$ Ringers Solution) to the mucosal side 30 minutes later. The same control experiments were used to determine the significant difference between the zero concentration and the 0.75 mg/L concentration Isc measurements. The curve was fitted using the same constraints as those applied to the α-chaconine experiments. The EC50 was calculated to be 2.75 mg/L using GraphPad InPlot version 4.0.

As was found for α-chaconine, α-solanine also reduced the ISC thus inhibiting sodium active transport. The slopes of the two curves appear to be similar. It was therefore suggested that perhaps the two glycoalkaloids altered the ISC by the same mechanism. To test this assumption, two more sets of experiments were completed. The first of these experiments used a stock solution of a mixture of α-chaconine at 7.5 mg/L and α-solanine at 2.5 mg/L, providing a stock solution with a ratio of 3:1. The same dilution addition method as used in the previous experiments.

Figure 8 represents the Dose Response Curve of the 3:1 (α-chaconine:α-solanine)diluted solution. Figure 9 represents the Dose Response Curve of the 3:1 (α-solanine:α-chaconine) diluted solution. The curve shape for each Dose Response Curve was constrained to the dose response model. The curves were fitted using this model and GraphPad Inplot

version 4.0. The unusual first and second point arrangements of both figures 8 and 9 are the artifacts of a few high initial readings of the ISC following the initial dilution addition. Although the % Response for the initial and final concentrations for each solution are not equal, the slopes are very similar. This similarity was believed to be the function of a similar mechanism of actions for each. The increased % Response of figure 9 could be explained as a function of the α-solanine effects on the

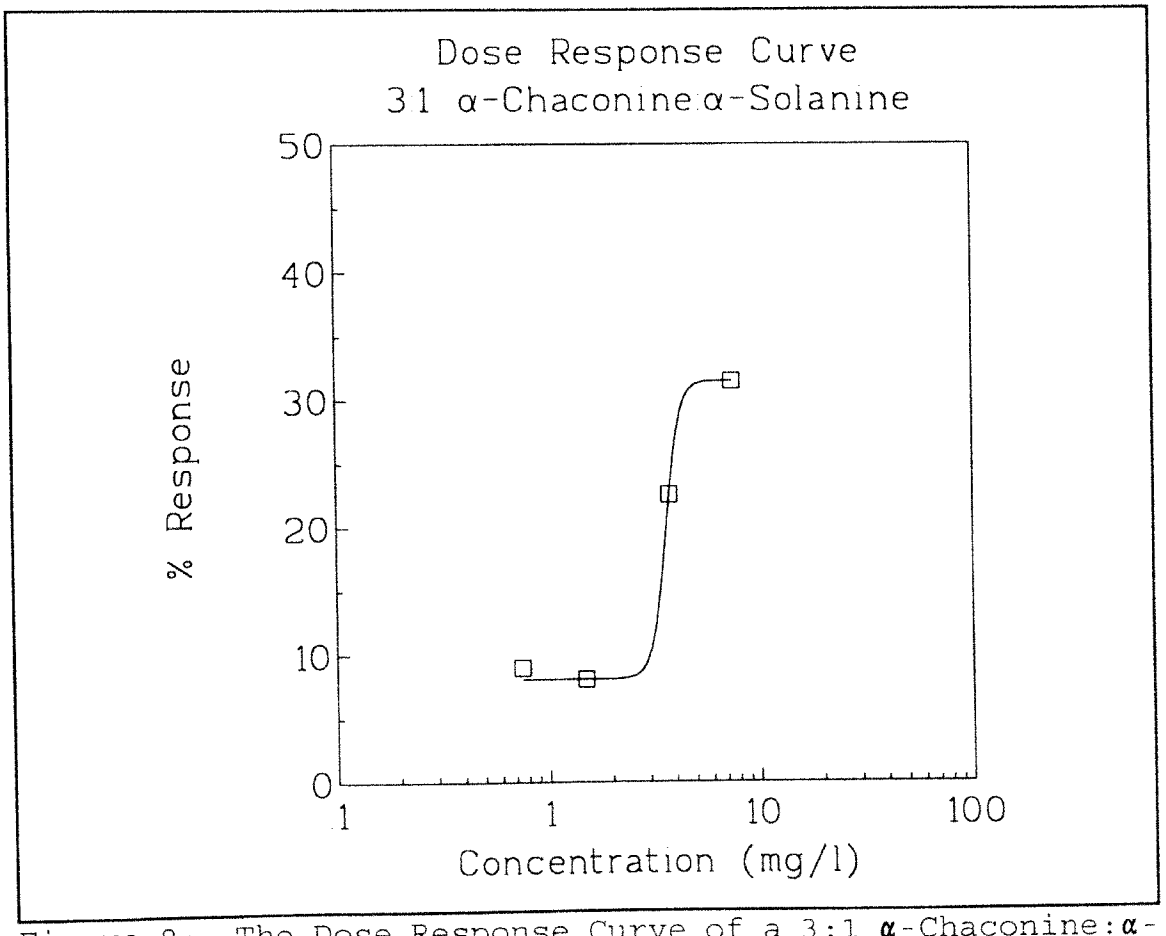

Figure 8: The Dose Response Curve of a 3:1 α-Chaconine:α-Solanine Dilution on the *Rana pipiens* excised epithelia. The concentration is of α-Chaconine.

cellular membrane that disrupt the membrane integrity more so than the α-chaconine or α-solanine alone.

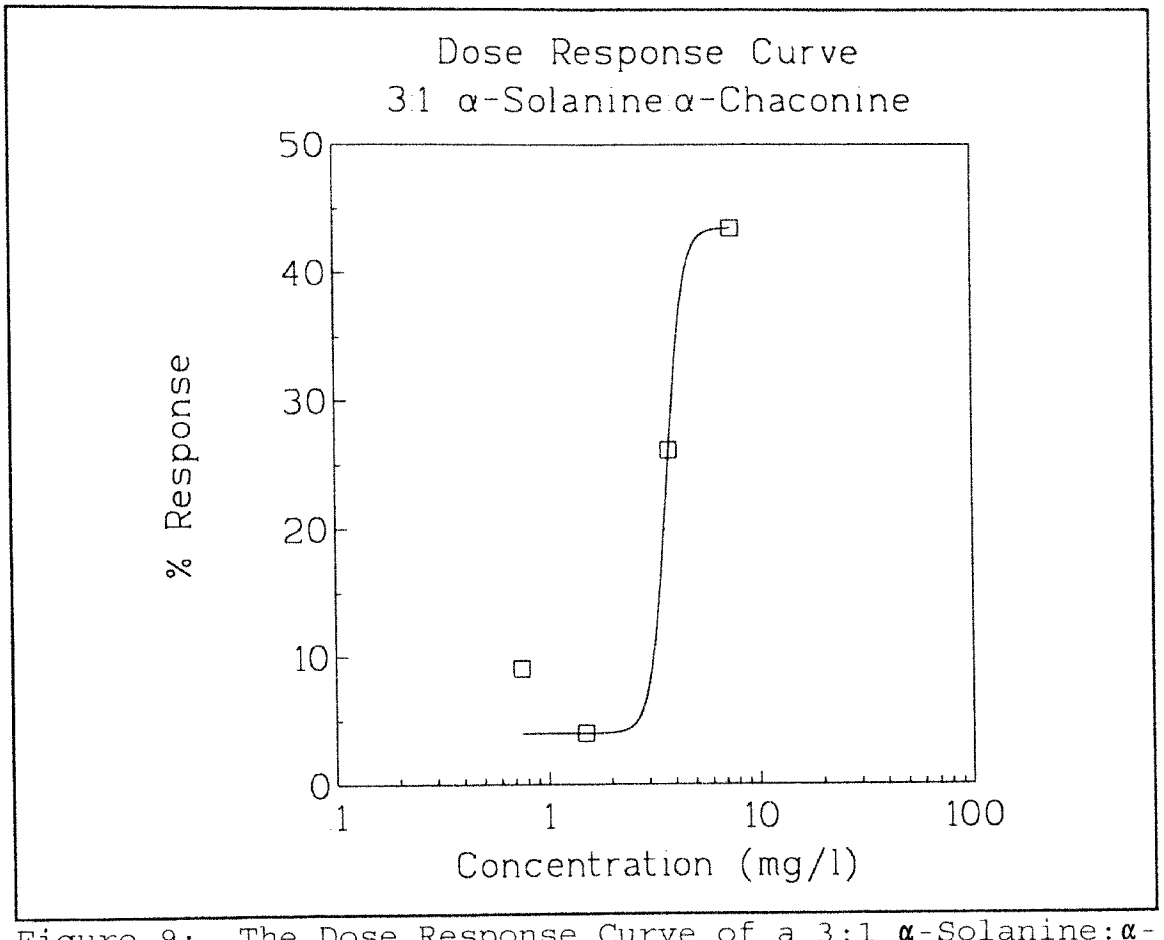

Figure 9: The Dose Response Curve of a 3:1 α-Solanine:α-Chaconine Dilution on the *Rana pipiens* excised epithelia. The concentration is of α-Solanine.

CHAPTER V

DISCUSSION

Previous studies have indicated that glycoalkaloids act on the membrane of the exposed cells or subcellular structures (Roddick *et al.*,1988). Experiments with FETAX showed that the EC50 of α-Chaconine was 2.6 mg/L (0.0036 mM) and the EC50 of α-Solanine was 8.8 mg/L (0.010 mM)(Friedman *et al.*, 1991). The EC50's were calculated using the short circuit current from frog skin were determined to be 3.2 mg/L (0.0037 mM) and 2.75 (0.0032 mM) mg/L respectively. The α-chaconine values very close, but there is no suggestion for the difference of α-solanine values.

It should be addressed that there are no error bars placed on the figures. Although the use of a % Response calculation usually allows for variation between two frog skins of a given treatment to be negated, this was not the case in these experiments. It should be noted that in each of the four experimental replicates, and among each of the four % Response determinations within a treatment, that three of the four data points for each trial were consistent with the initial and final data points for that trial.

The validity of this experimental design to use excised frog skin epithelia as a model for the determination of the

toxicity of the glycoalkaloids on the transmembrane sodium conductance, as a mechanism of action, is not questionable. However, the validity of using this model to determine the mechanism of action of the glycoalkaloids to determine the mechanism of toxicity for humans, chicken embryos or frog embryos is not known. It was the intent of this investigation to determine the effect of the glycoalkaloids on the sodium transport by measuring the short circuit current. The EC50 values determined were a function of the constraints of the Dose Response Design. The statistical significance of the Dose Response Design was not interpreted.

The concentrations selected for these experiments were determined by the solubility products of α-solanine. The highest concentrations for the glycoalkaloids were in the range of 8.5 μM, and the lowest concentration in the range of 0.85μM. The effective concentration for the alkaloids observable effects in humans as spina bifida malformations are in the μM range (Renwick, 1972). Therefore, the concentration selections are comparable.

Finally, the evidence in this study can be interpreted as a membrane effect but more likely is a specific effect on active transport of sodium since most (95-99%) of the ISC is accounted for by sodium active transport. We can extend the data in this study to speculate that the effects observed with frog embryos are due to the effects of α-chaconine and α-solanine on active transport in the embryos. The change

in membrane potential of the early embryos affected by α-chaconine and α-solanine is most easily explained by a cessation of active transport of some ion, probably sodium.

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