IDENTIFICATION OF VARIOUS CELL POTENTIAL TYPES AND THEIR RELATIONSHIP TO PERMEABILITY BARRIERS IN THE FROG SKIN

By

RANDALL L. DUNCAN

Bachelor of Science
Southwestern College
Winfield, Kansas
1977

Master of Science
Oklahoma State University
Stillwater, Oklahoma
1980

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
December, 1982

IDENTIFICATION OF VARIOUS CELL POTENTIAL TYPES

AND THEIR RELATIONSHIP TO PERMEABILITY

BARRIERS IN THE FROG SKIN

Thesis Approved:

James T Blakeney
Thesis Adviser

Calvin A. Beams, Jr.

Anne C Rusoff

John R. Lawn

Norman N Durham
Dean of Graduate College

ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. James T. Blankemeyer, my major professor, for his suggestions, guidance and encouragement during the course of this research. I would also like to express my appreciation to Dr. Anne Rusoff, whose technical information and advice made this research possible. I wish to thank the other members of my committee, Dr. Calvin Beames and Dr. John Sauers, for their assistance and comments. I would also like to express my appreciation to Dr. Walter Stewart of the National Institute of Health in Bethesda, Maryland for the generous supply of Lucifer Yellow CH dye.

I acknowledge the financial support of this research provided by USPHS Grant AMR 21890. I also wish to thank Alan Knox for his technical assistance and critical advice.

I wish to thank my friends, Larry Zinn, Derek Mosier, Mary Bober, Howard Mass, Rhonda Gamble, Gary Bond and especially Michael M^{c}Cullough for their moral support throughout the course of this work.

I would especially like to thank my parents, Maurice and Agnes Duncan, who supported me both financially and emotionally for the past 27 years. Without their unquestioning direction and patience, this goal would never have been reached. And finally, I would like to dedicate this work to my wife, Beth, whose love, encouragement and understanding always gives me the strength to press on.

TABLE OF CONTENTS

TABLE

LIST OF FIGURES

SECTION I

GENERAL INTRODUCTION AND LITERATURE REVIEW

GENERAL INTRODUCTION

The frog skin epithelium is capable of moving sodium from the pond or mucosal side of the skin to the blood or serosal side (Huf, 1935; Krogh, 1937). This transport is an active process, (ie.) requires energy, and can produce a potential difference (PD) of 100 mV, blood side positive (Ussing, 1949). The PD can be inhibited by the cardiac glycoside, ouabain (Koefoed-Johnsen, 1957) and requires Mg^{+2} dependent, Na^{+}-K^{+} ATPase (Bonting et al., 1962). These properties of sodium transport found in the frog skin are quite similar to those of active sodium transport in mammalian tissues. For this reason, the frog skin has become the principal model of active sodium transport.

In the past, a majority of the frog skin transport studies have made the assumption that cells of the epithelium act as a functional syncytium, (ie.) all cells of the epithelium have similar functions. Unfortunately, the frog skin is a multicellular epithelium arranged in four distinct layers which makes this assumption a major source of controversy. Several experimental methods have been devised to attempt to correlate the various cell types with their functions. One method which has been extensively used is the electrical potential profile measurements using microelectrodes. These measurements are made by advancing a microelectrode through the epithelium and monitoring the resulting potential changes. Early studies using this technique found that under open circuit, two equal and positive potential steps existed (Engbaek and Hoshiko, 1957; Ussing and Windhager, 1964; Cereijido and

Curran, 1965; Biber et al., 1966). Using distance measurements, they concluded that the first potential step encountered corresponded to the penetration of the more mucosal layers of the epithelium while the second step was attributed to penetration of the epithelial-corial border on the serosal side of the epithelium. This two step profile suggested that the frog skin is a functional syncytium (Ussing and Windhager, 1964). However, electrochemical gradient estimates made using low sodium concentrations in the bathing medium (Rotunno et al., 1966) have shown that, assuming the Koefoed-Johnsen and Ussing (1958) model, passive diffusion of sodium into the epithelium would be unlikely without compartmentalization of sodium. These estimates imply that there must be at least two different cell types in the epithelium. At first glance, recent potential profile measurements by Nagel (1976) seem to support this compartmentalization concept. He found that the epithelium became increasingly negative as the microelectrode advanced toward the serosal side until reaching a potential of approximately -100 mV. As the microelectrode moved beyond this potential, a positive step was observed. From this, Nagel concluded the highly negative potentials were found throughout the epithelium and are characteristic of the transporting cell modelled by Koefoed-Johnsen and Ussing.

All previous profile studies ignored potentials which were observed upon initial contact of the microelectrode with the surface of the epithelium. These potentials ranged from -4 to -60 mV, were highly unstable and had low fractional resistances. Therefore, these potentials were interpreted as artifacts caused by the depression of the tissue by the microelectrode before an impalement was made.

Another factor which contributes to the controversy surrounding the functional state of the cell types in the epithelium is the presence of zonulae occludens or tight junctions between the cells. In adult frogs, these junctions are predominantly located between the cells of the outermost layer of the stratum granulosum (Farquhar and Palade, 1964). These complexes have been shown to be impermeable to concentrated protein solutions (Farquhar and Palade, 1963) and lanthanum (Martinez-Palomo et al., 1971) which suggests that these complexes act as a passive barrier to paracellular diffusion.

The purpose of this study is to determine the membrane potentials of each type of cell in the frog skin epithelium and to locate these types of cells in relation to the diffusion barrier. While the use of distance measurements during a profile study may give some idea as to which layer of cells is responsible for a certain potential, the accuracy of these measurements is subject to question. By using iontophoretic dye injection of cells characterized by their membrane potentials, this study will show three separate cell potential types in the frog skin epithelium. The relationship between these cell types and the permeability barrier created by the tight junctions will also be examined by combining iontophoretic dye injection with Horseradish Peroxidase diffusion.

LITERATURE REVIEW

Active transport in frog skin epithelium has been extensively studied over the past 50 years. Early work with the frog skin has produced some of the basic concepts and techniques which have become standards for the study of active ion transport. Because of the similarities between active sodium transport in both frog skin and mammalian tissues, the frog skin has become a convenient and inexpensive model of active sodium transport.

Morphology of the Frog Skin

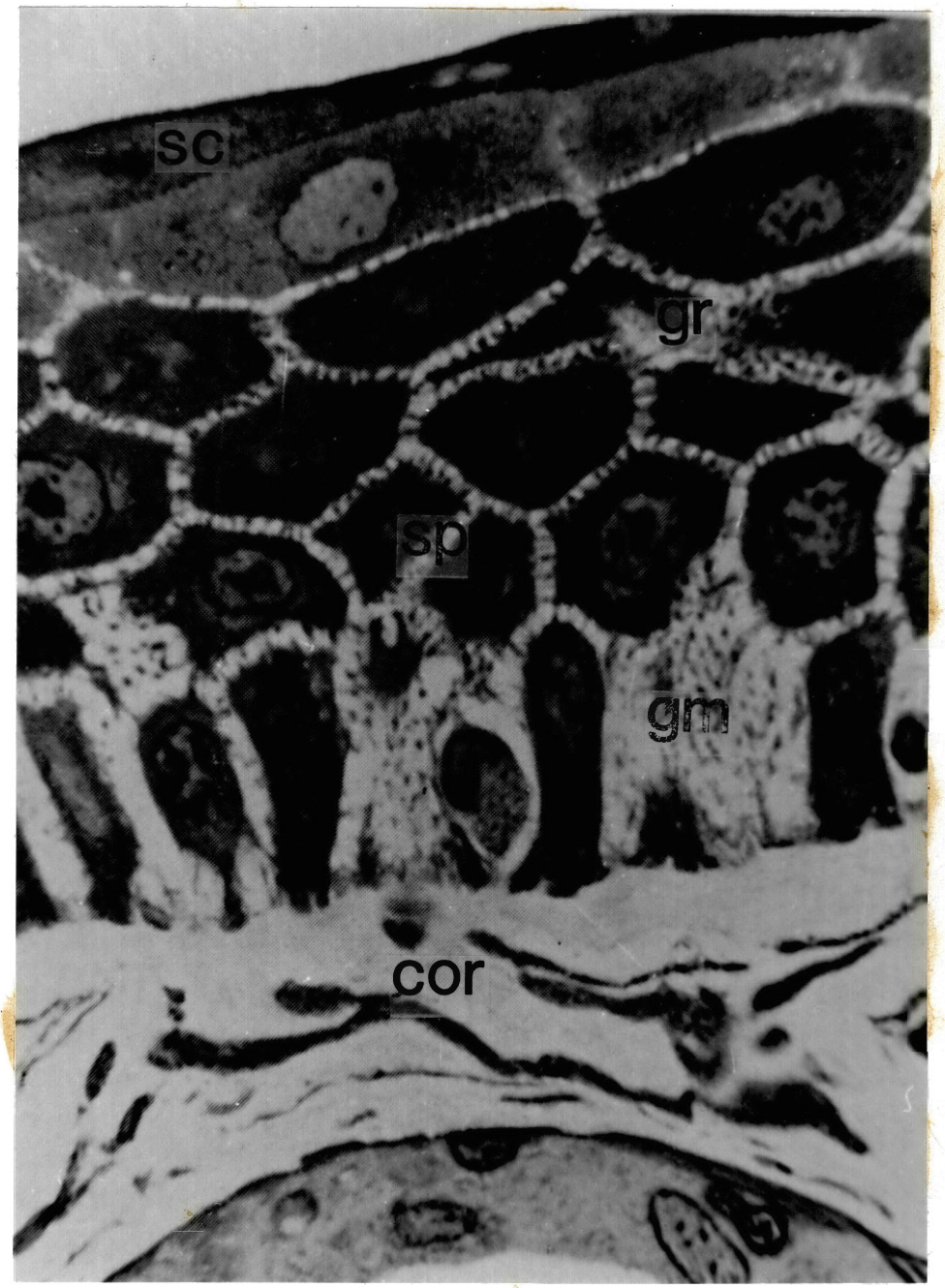
To fully understand the problems associated with studying active transport in the frog skin, one must examine the morphology of the skin (Fig. 1). The skin is composed of an epithelium, which lies on the mucosal or pond side of the skin, and the corium, which lies on the serosal or blood side. The corium is composed of connective tissue and is approximately 250 microns thick. In this region are found the blood vessels and glands of the skin. The corium can be stripped from the epidermis by bathing the skin in a Worthington's collagenase solution (Aceves and Erlij, 1971). Removal of the corium does not change the potential difference (PD) of the frog skin; therefore, the corium is not considered to be involved in the active transport mechanism.

The epithelium of the frog skin is 50 to 100 microns thick and is composed of four cell layers. The outermost layer of the epithelium is a cornified layer termed the stratum corneum. This layer is one to two

cells thick and is typical of cornified epithelium; it contains fibrillar cytoplasm, shrunken nuclei and degenerating mitochondria. The layer of cells immediately serosal to the stratum corneum, termed the stratum granulosum, is considered to be the first layer of living cells in the epithelium. This layer is two to three cells thick. The cells are elliptical, 15 to 20 microns in length and 3 to 5 microns in depth. The third cell layer of the frog skin epithelium is called the stratum spinosum. This layer is made up of one to two layers of pentagonally shaped cells which are 15 to 20 microns in diameter. The most serosal layer of the epithelium is the germinal layer, termed the stratum germinativum. The cells of this region are shaped much like epithelial columnar cells and are tightly anchored to the basement membrane of the epithelium.

Cells in both the stratum corneum and the stratum granulosum interlock with neighboring cells by multiple "interdigitations" and desmosomes (Voute, 1963). These interdigitations, termed zonula occludens or tight junctions, completely ring the cells in this region and form the only continuous barrier between the blood side and the mucosal side of the epithelium. These junctional complexes have been shown to be impermeable to concentrated protein solutions (Farquhar and Palade, 1963) and lanthanum (Martinez-Palomo et al., 1971) which suggests that one function of this region may be to act as a diffusion barrier. These tight junctions, while present in the rest of the epithelium, become less and less frequent in the more serosal layers. The principal type of junctional complex found in the more serosal layers of the epithelium is the desmosome, which primarily anchors the cells together. These are fewer and less well developed in the stratum

Fig. 1. Morphology of the Frog Skin. The frog skin is composed of two types of tissue, epithelial and connective, which are arranged in distinct layers. The epithelium consists of four cell layers and is on the mucosal or outside of the skin. The outermost layer is a non-living cornified layer termed the stratum corneum (SC). The stratum corneum is a single cell layer thick and is typical of cornified epithelia; fibrillar cytoplasm, shrunken nuclei and degenerating mitochondria. The first living cell layer of the skin is the stratum granulosum (GR) which is 2-3 cell layers thick. These cells are elliptical and interconnected with junctional complexes termed zonulae occludens. The pentagonal shaped cells serosal to the granulosum form the single cell layer of the stratum spinosum (SP). The germinal layer is the last layer of the epithelium termed the stratum germinativum (GM). This layer is made up of columnar shaped cells which adjoin the connective tissue portion of the frog skin, the corium (COR). This photomicrograph, taken from Voute and Hanni (1973), does not adequately represent the corium. The epithelium is approximately 100 microns thick while the corium is 250 microns thick.


sc
gr
sp
gm
cor

germinativum and fully developed and numerous in the stratum granulosum. The extracellular spaces of the epithelium are complex and vary greatly from layer to layer of the epithelium. The spaces form large "extracellular lakes" in the serosal layers of the epithelium, particularly at the stratum germinativum where the desmosomes are least developed (Farquhar and Palade, 1964). These extracellular spaces form a continuous network which is blocked from the external environment by the tight junctions of the outermost layer of the stratum granulosum. This network is much larger at the epithelial-corial border, opening to the basement membrane of the epithelium in 200 Å pores.

"Classical" Studies of the Frog Skin

As previously mentioned, early studies in the frog skin provided many of the techniques which have become standard methods for determination of active ion transport. The earliest studies on the frog found that one-third of the frog's body weight in water was exchanged daily through the skin (Adolf, 1933) and that a salt-depleted frog could absorb sodium chloride from very dilute bathing solutions (Krogh, 1937). Huf (1935) demonstrated that an isolated frog skin with both sides bathed in frog Ringer's transported sodium chloride from outside to inside and that this transport was related to the potential difference (PD) measured across the skin.

The advent of radio-isotopes made it possible to measure this transport in terms of flux; (ie.), "the amount of a given ion species which crosses a specific area of membrane in one direction per unit time" (Ussing, 1949b). By using labelled tracers, Ussing found that the sodium influx; that is, the movement of sodium from outside to inside, was much higher than sodium efflux, the movement of sodium from

inside to outside. This difference, which is approximately 10:1, influx to efflux, occurred even when the outside or mucosal sodium concentration was as low as 1 mM. The chloride fluxes were found to parallel those of sodium, although the rate of movement was much lower. The ratio of sodium fluxes confirmed that sodium is actively transported across the frog skin. This method is still used in determination of which ions are actively transported in a tissue.

One question which arose from the Ussing study was how much of the potential difference generated across the skin was caused by active sodium transport. Previously, Ussing (1949) and Teorell (1949) had demonstrated that the passive diffusion of an ion was in response to electrical and chmical gradients across a membrane. Therefore, Ussing and Zerahn (1951) predicted that if the electrical and chemical gradients across the skin were zero, the only movement of ions across the skin would be the result of the active transport of that ion. In order to nullify the chemical gradient, Ussing and Zerahn bathed both sides of the skin with frog Ringer's solution. The volume on each side was large so that the ions which were transported would not alter the total concentration in the bath significantly. Also, the baths on both sides of the skin were stirred with gas lift pumps to prevent a concentration build-up in unstirred layers. To abolish the electrical potential gradient, they applied current to Ag-AgCl electrodes on each side of the skin so that for every monovalent cation transported from the mucosal to the serosal side of the skin, a chloride ion was driven off the serosal electrode and another chloride ion was reabsorbed onto the mucosal electrode. Ussing and Zerahn postulated that the current required to maintain the transepithelial PD at zero, which they termed

the short circuit current (I_{sc}), would be the algebraic sum of the net fluxes of all of the actively transported ions. Using radioisotopes, Ussing and Zerahn found the influx of sodium to be 105% of the I_{sc} and the efflux of sodium to be 5%. This meant the net flux of sodium was in 100% agreement with the I_{sc} or, in other words, sodium active transport was solely responsible for the I_{sc} across the frog skin.

Inhibitors of Sodium Transport

Investigators have surmised that active transport in the frog skin can best be studied by inhibiting the active transport pump and noting the characteristics of the viable, but non-transporting skin. Fuhrman (1952) was the first to use inhibitors on the frog skin, however, the inhibitors which he used were metabolic inhibitors, e.g. DNP, which shut down not only the pump, but also every other metabolic process in the cell. Therefore, the search began for a specific sodium transport inhibitor. Two substances, ouabain and amiloride, were found. Both inhibit active sodium transport, but at different sites. These drugs have been used extensively since their discovery. Therefore, the effects of these inhibitors will be discussed at this point to clarify the remainder of the review.

Ouabain, or g-strophanthin, belongs to the family of cardiac glycosides which are used clinically to reduce the excitability of cardiac muscle. Schatzmann (1953) demonstrated that ouabain, among other cardiac glycosides, inhibited the active transport of sodium in erythrocytes. This inhibition has been shown in numerous types of transporting epithelia (Caldwell and Keynes, 1959; Bonting and Canady, 1964). Koefoed-Johnsen (1957) first demonstrated the effects of

ouabain in the frog skin and found that the decrease in short-circuit current in response to ouabain corresponded to a decrease in sodium influx. In addition to observing a decrease in short-circuit current and potential difference, Herrera (1966) found that ouabain was only effective from the serosal side of the toad bladder. The effectiveness of ouabain was shown to be the result of the specificity of ouabain binding for Na^+-K^+ ATPase (Skou, 1957), which is found at the serosal border of most transporting epithelial cells (Farquhar and Palade, 1966; Mills et al., 1977).

In order to better understand the effects of ouabain on the tissue, Nagel and Dorge (1971) measured the change in the sodium transport pool size in response to ouabain. The sodium transport pool is the amount of sodium in the frog skin that is awaiting active transport by the pump. By adding labelled sodium ($^*Na$) to the mucosal bath and observing the rate of $^*Na$ appearing on the serosal side, the pool size can be calculated. Nagel and Dorge determined the sodium pool size was unaffected by ouabain; in other words, sodium did not accumulate in the cells in response to pump inhibition. Zerahn (1969) also found the pool size did not change with ouabain. Because of the large intracellular and extracellular volume of the frog skin, Zerahn reasoned that one could not be sure whether the sodium in the skin was a pre-transport pool or a transported pool. By assuming that the sodium which diffused back into a low sodium mucosal bath was from a pre-transport pool, Zerahn concluded that the sodium in the frog skin was primarily a transported pool and that the pre-transport pool was quite small. The sodium did not change in response to ouabain because the pool was in a compartment sersal to the pump and past the point of

inhibition.

Nagel and Dorge also found that the net sodium flux was equal to the short circuit current both before and after the addition of ouabain. Huf et al. (1982) agreed that I_{sc} and net sodium flux were equal but they found this to be true only after reaching steady state and not during the transient effects of ouabain. Ferriera (1979) showed that ouabain produces a biphasic effect as the I_{sc} drops; (ie.), the I_{sc} continues to decrease at a steady rate but the sodium influx increases slightly before continuing down to the steady state level. This result has largely been ignored. Huf et al. (1982) stated that this discrepancy between I_{sc} and sodium flux is caused by two distinct pools in the epithelium in which a loop pathway, which connects these pools, causes an increase in sodium flux, but does not effect I_{sc}.

Helman et al. (1979) used microelectrodes to study the intracellular effects of ouabain. By measuring the resistances of the membranes of cells with potentials of approximately -80 mV and also measuring the E_{Na} of the pump, they found that ouabain had a biphasic effect. The first phase occurred within 5-10 min after addition of ouabain. In this phase, the serosal membrane resistance increased rapidly while the E_{Na} decreased. During the second phase, the resistance of the mucosal membrane increased. The second phase of ouabain treatment may not be an actual effect of ouabain but a result of the interdependence of the mucosal and serosal membranes of the cell.

Using labelled ouabain, Cala et al. (1978) looked at the ouabain binding versus the change in short circuit current. By separating skins into high and low currents in which a high skin was greater than

20 $\mu A/cm^2$, they found low current skins bound at least one-third of the ouabain before I_{sc} decreased while high current skins bound very little ouabain before a reduction in I_{sc} was observed. They concluded that at low currents, inhibition of the pump sites cause "recruitment" of inactive pumps to increase sodium output. Cala et al. also suggested that since the number of ouabain molecules bound was not related to I_{sc}, then variations seen in I_{sc} may not be caused by the number of pumps, but by their turnover rate.

Amiloride, or 3,5-diamino-6-chloropyrazinoylguanidine, is considered to be a potassium-sparing diuretic, (ie.) upon administration, amiloride reduces sodium reabsorption and potassium excretion by the renal distal tubules (Baer et al., 1967). Bentley (1968) found that amiloride inhibited the short circuit current and sodium influx across the toad bladder. This inhibition differed from ouabain inhibition in that amiloride was 1000 times more effective from the mucosal side than from the serosal side. Bentley found amiloride was effective down to 10^{-5} M, had no effect on the osmotic permeability of the bladder and blocked the stimulatory effects of vasopressin. From these results, he concluded that the mechanism of action of amiloride may be to block sodium entry into the tissue. In support of this assumption, Nagel and Dorge (1970) found that amiloride caused a significant drop in sodium content of the frog skin without affecting water content, extracellular volume or intracellular potassium concentration. Erlij and Smith (1973) found that amiloride inhibited sodium transport. However, the extent of this inhibition was dependent on sodium concentration of the mucosal bathing solution and the rate of sodium transport across the frog skin. They also determined that the

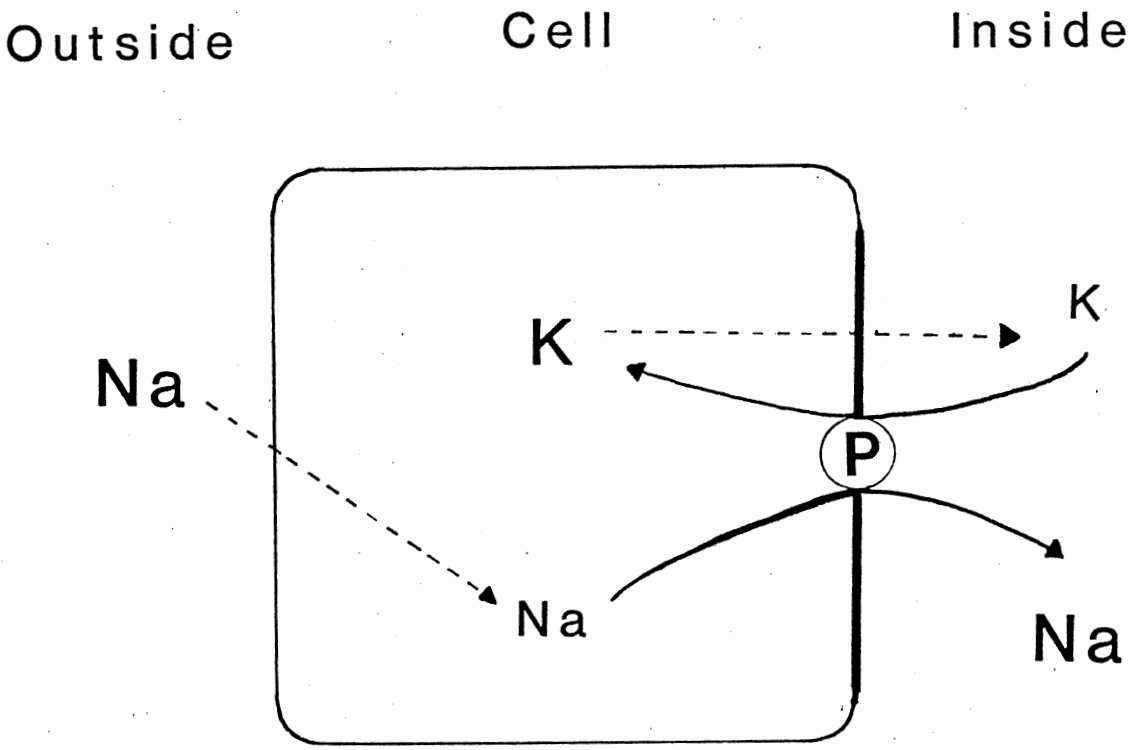
I_{sc} and PD elicited in an ouabain treated frog skin by increasing sodium concentration in the mucosal bath is abolished upon addition of amiloride.

Benos et al. (1979) used amiloride dose response curves coupled with sodium concentration changes in the mucosal bath to determine if amiloride competed with sodium for an entry site into the epithelium. They theorized that if amiloride competed with sodium for the same entry site, less amiloride would be required at low sodium levels to elicit the same inhibitory response obtained at higher sodium levels. If amiloride did not compete with sodium, amiloride would be equally effective at all sodium concentrations. Benos et al. determined that amiloride did not compete for entry sites with sodium but changed the sodium permeability by some other method.

The Koefoed-Johnsen and Ussing Model

One of major goals of investigators of epithelial transport is to compose a working model of the system they are studying. Linderholm (1954) discussed a "hypothetical" skin derived from noting changes in PD in relation of sodium concentration changes. Huf et al. (1957) proposed a model based on the effects of potassium on the frog skin. However, models of the frog skin were not considered seriously until Koefoed-Johnsen and Ussing (1958) proposed a model based on the effects of changes in cation concentration on transepithelial PD which was to become known as the "classic" model of frog skin transport (Fig. 2). By substituting various alkaline cations for sodium in the mucosal bath, they determined that the mucosal membrane of the transporting cell was essentially impermeable to all cations except sodium and lithium. When similar changes were made on the serosal side of the

Fig. 2. The Koefoed-Johnsen and Ussing (KJU) Model. This model is based on cation substitution and concentration change studies on both the mucosal and serosal side of the frog skin. From these studies, Koefoed-Johnsen and Ussing made the following observations: 1. Only Li^+ and Na^+ were permeable to the mucosal membrane of the transporting cell. 2. Na^+ would still enter the cell from the mucosal side at 1 mM concentrations. 3. Only K^+ was permeable to the serosal membrane and produced a Nernst slope. From these observations, they made the following statements: 1. The Na^+ active transport mechanism is an exchange pump with K^+. 2. Since K^+ produces a Nernst slope on the serosal side, this membrane must house the active transport mechanism. 3. The intracellular concentration of Na^+ must be low to permit passive diffusion of Na^+ into the cell and K^+ must be high due to the active transport exchange pump. 4. The transepithelial potential difference is created by the diffusion potential of Na^+ across the mucosal membrane and the diffusion potential of K^+ across the serosal membrane.


Outside
Cell
Inside
K
K
Na
P
Na
Na

skin, potassium was found to produce a Nernst response (ie.) approximately 60 mV/decade change of potassium, while all other cations, including sodium, had no effect. In addition, when the bathing solutions were aerated with 5 to 7% CO_2 rather than atmospheric air, the potassium exchange between the serosal bath and the cells decreased as did the potential difference. In this situation, the serosal membrane had little, if any, potassium selectivity. From these observations, Koefoed-Johnsen and Ussing made several statements about transport in the frog skin: 1) the mucosal membrane of the cells were impermeable to all cations except sodium and lithium, 2) the serosal membrane was selectively permeable to potassium and impermeable to sodium from the serosal side of the skin, 3) since the potential difference of the skin decreased when the potassium selectivity of the serosal membrane was abolished, the active transport mechanism was located on the serosal membrane and 4) because the potential difference was maintained at low mucosal sodium levels, the intracellular sodium concentration was low and the intracellular potassium concentration was high. The potassium concentration in the cell would diffuse across the serosal membrane in response to the concentration gradient. Therefore, Koefoed-Johnsen and Ussing predicted the transepithelial PD is the sum of two diffusion potentials, the movement of sodium across the mucosal membrane into the cell and the movement of potassium across the serosal membrane out of the cell. These passive diffusion potentials are the result of concentration gradients set up by the active transport mechanism on the serosal membrane.

Modifications to the KJU Model

As Schultz (1981) stated, "the beauty of the KJU model and certainly one of the reasons for its immediate elevation to the status of paradigm is that it did not involk more 'demons' than had been previously involked to explain ion transport." In other words, it was the simplest model which comformed to the data. However, since over the past 25 years, epithelial transport studies have revolved around this model, many modifications to the KJU model have been proposed.

One of the first modifications to the model was proposed by one of the original authors. Ussing and Windhager (1964) used microelectrode impalements to record the electrical potential profile of the epithelium (See Microelectrode studies of the frog skin). The potential profile showed that the transepithelial PD was reached in two positive steps when the microelectrode was referenced to the mucosal bathing solution. Because this two step profile comformed to the two membrane KJU model, Ussing and Windhager postulated that the epithelium acted as a functional syncytium. The functional syncytium theory received further support from a study by Rick et al. (1978). Using an electron microprobe to measure cellular electrolyte distribution, they found that the intracellular composition of all cells of the epithelium was uniform. Furthermore, changes in the electrolyte composition in response to alteration of active sodium transport by ouabain, amiloride and changes in the outer bathing solution sodium concentration, occurred consistantly throughout the epithelium. Ussing and Windhager also determined that sodium movement in the frog skin followed two pathways, the transported path and a passive shunt path. They measured the shunt path conductance assuming that the conductance of the mucosal

bath is sodium free. Therefore when sodium was replaced by potassium in the mucosal bathing solution, the measured skin resistance would be equal to the shunt resistance. They also assumed that it was unlikely for sodium to be forced through the pump backwards and therefore, the efflux of sodium was the result of the shunt conductance. In this way, the percentage of the shunt conductance for which sodium was responsible for was found. They determined that the total shunt conductance of the skin was approximately 14 mho/cm^2 and that sodium made up 46% of this conductance. To find whether the shunt path was intracellular or extracellular, urea was added to the mucosal bath and the conductance of the tissue measured. The conductance of the shunt path increased greatly while that of the transport path was relatively unaffected. Also, the influx of sodium was unchanged; therefore, Ussing and Windhager concluded the shunt pathway was paracellular.

One of the best critiques of the KJU model was made by Finn (1976). Finn, while maintaining the basic premises of the KJU model, made several alterations based on discrepancies between predicted and observed changes in PD in response to sodium and potassium concentration changes. One discrepancy Finn found in the KJU model was the assumption that the transepithelial PD was the summation of the diffusion potential of sodium across the mucosal membrane and potassium across the serosal membrane. Finn and Rockoff (1971), using short term flux studies in toad bladder preparations, found the reduction in I_{sc}, which accompanies increased serosal potassium concentration, was not caused by serosal potassium uptake but by a decrease in transepithelial sodium influx. Finn (1974) also predicted that if the

transepithelial PD is the result of diffusion potentials, then changes in sodium and potassium concentrations should have the same effect on PD whether an inhibitor is present or not. He demonstrated that when inhibitors such as ouabain, amiloride and low temperature were administered to the skin, sodium and potassium concentration changes on either side of the tissue had no effect on the transepithelial PD, which decreased to zero. This suggested that the transepithelial PD is a direct result of the active transport of sodium.

Another assumption of the KJU model which Finn contested was the independence of the mucosal and serosal membranes. Flux studies have shown that both the serosal and mucosal membranes of the cells are affected when inhibitors such as ouabain and amiloride are administered to the skin, even though these substances work on only one side of the tissue (Finn, 1971; Finn and Sutton, 1974; Finn and Rockoff, 1971). For example, ouabain, which inhibits the pump from the serosal side by blocking Na^+-K^+ ATPase, greatly decreases the serosal efflux, as expected. However, ouabain also inhibits the sodium uptake on the mucosal side under three conditions; 1) when the mucosal sodium concentration is less than 20 mM (Biber, 1971), 2) when the serosal potassium concentration is lowered to 1 mM or 3) when transepithelial PD is clamped at 100 mV (Finn, 1975). This interdependence of the membranes has been investigated further by Reuss and Finn (1975) using microelectrodes. By pulsing amiloride onto the mucosal surface of the tissue, they found that not only did the mucosal membrane potential decrease, but the serosal membrane potential as well.

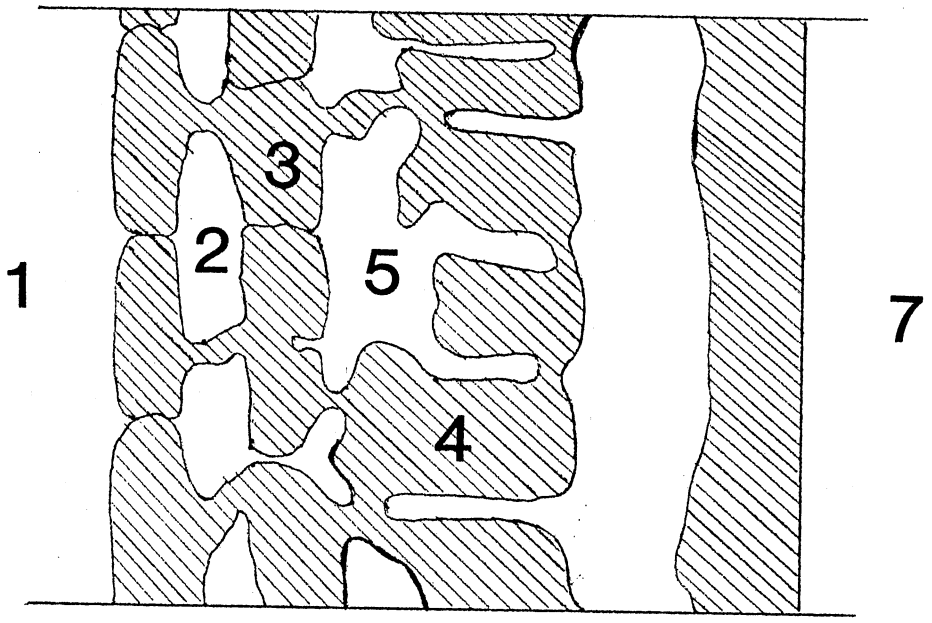
Compartmentalization of the Frog Skin

Most of the studies of the frog skin from 1958 to the present have

maintained that the frog skin epithelium acts as a functional syncytium. However, to a number of investigators, this premise seemed inconsistant with the morphology of the frog skin. This belief led to several "compartmentalization" theories of sodium transport in the frog skin.

Although the KJU model received the attention of the scientific community, it was quite similar to a model proposed by Huf et al. (1957) a year earlier. Both groups noted that sodium, and not potassium, could enter the cell compartment from the mucosal side, while potassium was required for active sodium extrusion on the serosal side. However, the primary difference between the two models pertained to the transporting compartment. While the KJU model treated the frog skin epithelium as a functional syncytium, in that all cells of the epithelium were closely associated and function in transporting sodium, the Huf model stated only that "some compartment within the epithelium" was engaged in active transport. Huf and Howell (1974) later refined this earlier model and proposed a multicompartmental model comprised of seven compartments (Fig. 3). Compartments 1 and 7 of this model correspond to the mucosal and serosal bathing solutions, respectively. Compartment 2 is the extracellular volume between the stratun corneum and the outermost cells of the stratum granulosum, termed the first Reactive Cell Layer (RCL). The third compartment is the intracellular volume of the RCL while compartment 4 is the intracellular volume of the remaining epithelial cells. Compartment 5 is the volume of the intercellular space, serosal to the permeability barrier created by the zonula occludens of the RCL. Compartment 6 is considered to be the non-exchangeable sodium in the epithelium. Huf and Howell tested their

Fig. 3. The Huf and Howell Model. Huf and Howell proposed a multicompartmental model consisting of seven compartments which they tested using computer simulation. Compartments 1 and 7 are equivalent to the mucosal and serosal baths, respectively. Compartment 2 is a pretransport pool found in the subcorneal space which is the extracellular space between the stratum corneum and the reactive cell layer (RCL). Compartment 3 is the transepithelial Na^+ transport pool which moves Na^+ from the mucosal to serosal side. This compartment is found in the RCL. Compartment 4 is termed the "maintenance" pool which also transports Na^+, but not across the epithelium. This compartment is composed of the remaining cells of the epithelium. Compartment 5 is the sodium found in the extracellular space and compartment 6 (not listed here) is the bound sodium in the entire epithelium.


1
2
3
4
5
7

model using computer simulation. By using published values to set the parameters for the computer simulation, Huf and Howell obtained values which agreed with studies conducted by Nagel and Dorge (1970) and Aceves and Erlij (1971) that found that only 10 to 12% of the exchangeable sodium in the frog skin exchanges with labelled sodium in the mucosal bathing solution. Furthermore, all simulations found the sodium influx pool size fraction was much lower (7-20%) than the efflux pool size fraction (93-80%). The difference in pool sizes suggests that the frog skin must be compartmentalized and has two types of Na^+-K^+ transporting compartments. The first compartment would be the transepithelial transporting compartment and the second would be a "maintenance" compartment. Additionally, flux studies, using amiloride to block mucosal sodium uptake (Nagel and Dorge, 1970; Salako and Smith, 1970), demonstrated that amiloride decreased the net flux of sodium across the tissue and the sodium pool exchangeable with the mucosal side, but had little effect on the sodium pool exchangeable with the serosal side. This suggested to Huf and Howell that there must be a pretransport pool in the epithelium. Therefore, based on these previous reports and the results of the computer simulation, Huf and Howell stated that compartment 2, the subcorneal space, was likely to the the pretransport pool; compartment 3, the intracellular volume of the RCL, was the transport pool; and compartment 4, the intracellular volume of the remainder of the epithelium, was the maintenance pool. In all simulations, Huf and Howell found the sodium concentrations to be $[Na^+]_4 > [Na^+]_2 > [Na^+]_3$. These simulations agree with the KJU model which states sodium enters the main active transport pool passively. Also, Huf and Howell demonstrated

that the steady state of the frog skin can be reached in 20 min., which is in accordance with the general consensus that, experimentally, steady state is reached in 30 min. Thus, the Huf and Howell model is in agreement with both previous results and computer simulation. Mikulecky et al. (1979) has since added a shunt pathway and has further tested the model using network thermodynamic analysis. While this model has received little attention among the scientific community, the results of the current study closely conform to the Huf and Howell model.

Another study which contested the functional syncytium assumption was one by Rotunno et al. (1966) which used electrochemical gradient estimates to calculate the intracellular concentration of sodium. They found that at low sodium levels in the mucosal bath, the concentration of sodium inside the cells would be at least 50 mM if they assumed the frog skin was in syncytium. If the intracellular sodium concentration was this high, sodium could not passively diffuse into the cells as required by the KJU model. Rotunno et al. and Farquhar and Palade (1966) used Na^+-K^+ ATPase analysis in an effort to localize the pump site since Na^+-K^+ ATPase is required to convert energy for the pump. These two groups found no ATPase on the mucosal border of the epithelium. Since active transport of sodium would be unlikely without ATPase and the high intracellular sodium concentrations prohibited sodium passive diffusion, Cereijido and Rotunno (1968) suggested a two compartment model. This model stated that the outer leaflet of the membranes of the stratum corneum cells contains polar groups of fixed charges which are highly selective for sodium and lithium. As the ions attach to these polar groups from the

mucosal bathing solution, the sodium starts to travel around rather than into the cell by moving from polar group to polar group by saltatory conduction. The sodium would continue to move in this fashion until it reaches the zonulae occludens or tight junctions located at the outermost layer of the s. granulosum. These junctional complexes form a diffusion barrier to the saltatory movement which has an order of selectivity of $Li > Na >> K$ (Kidder et al., 1964; Lindley and Hoshiko, 1964). This is the same selectivity the KJU model shows for the mucosal membrane of the transporting cell. Once sodium passes through the tight junctions, it remains attached to the polar groups in the outer leaflet of the cell membrane and continues to move toward the serosal side of the transporting cell. This serosal membrane is covered by a non-lipid layer which is impermeable to sodium but highly selective for potassium, the same selectivity as the KJU model shows for the serosal membrane of the transporting cell. Active transport pumps are located in the non-lipid layer which pump sodium into the intercellular space between the cells of the more serosal layers of the epithelium. As a sodium ion is transported, the following sodium ions move forward to replace it and another sodium attaches at the mucosal surface. Therefore, in this model, sodium does not enter the cells, the transport pool size is quite small and the intracellular sodium concentration does not play a role in active sodium transport.

Voute and Ussing (1968 & 1970) and Voute and Hanni (1973) also argued with the idea of a syncytial frog skin epithelium. By observing morphological changes in the frog skin in response to transepithelial current, they found current to affect only one cell layer. By either hyperpolarizing or short-circuiting the epithelium, then quickly

freezing and sectioning it, they found that only the outermost cells of the stratum granulosum were affected. This layer, which they termed the first Reactive Cell Layer (RCL), would shrink when the skin was hyperpolarized and swell when the skin was short-circuited. None of the other cells of the epithelium were affected, not even the more serosal cells of the same morphological layer. This is the same region which Farquhar and Palade (1964) found the highest concentration of tight junctions that formed the only continuous barrier to diffusion in the epithelium. Voute and Ussing interpreted that this change in volume of the cells in the RCL was caused by the majority of the transepithelial current passing through these cells. The remainder of the epithelium did not change in volume because the current was passed through the paracellular pathway. This suggested to Voute and Ussing that the RCL was the probable site of active transport. Farquhar and Palade (1966) lent support to this theory by finding the highest concentration of Na^{+}-K^{+} ATPase on the serosal border of the RCL.

Zerahn (1969) also found that the observed data did not conform to the syncytial concept of the frog skin. By placing ^{22}Na in the mucosal bathing solution and allowing the frog skin to equilibrate, he was able to measure the total amount of sodium in the skin. Comparing this value with the extracellular volume, he was able to determine the total amount of cellular sodium. By assuming that a transported sodium pool;(ie.), sodium which has already been actively transported, would be unable to diffuse back to the mucosal bath, Zerahn postulated that the sodium which did diffuse back to the mucosal side would be from a pre-transport sodium pool. He found that in comparison with the total

cellular sodium, the pre-transport pool was very small. Therefore, he stated that there was probably two cellular compartments. Aceves and Erlij (1971) agreed with this conclusion when they found that the amount of sodium which equilibrated with the mucosal solution was 0.009 eq/cm^2 in frog skins which had the corium removed. This value was approximately ten times smaller than totals in the whole epithelium.

Morel and Leblanc (1975) also found evidence for a compartmental frog skin. In control experiments in which the pump was not inhibited by ouabain, the transport pool was characterized by a rapid filling rate ($t_{\frac{1}{2}}$ varying from 0.4 to 0.7 min). When the transport pump was inhibited by ouabain, the epithelia behaved as if there were two sodium pools, a rapid one ($t_{\frac{1}{2}}$ of 0.5 min) and a slower one ($t_{\frac{1}{2}}$ of 4 to 7 min). Since the filling times of the sodium pool in the transporting epithelia and the rapid pool in the non-transporting epithelia were similar, Morel and Leblanc concluded that these were probably the same pool. From this data, they postulated that when ouabain blocks the pump, sodium accumulates in all of the cell water via intercellular junctions. In transporting epithelia, sodium is transported to the extracellular space by the active transport pumps and only a small cellular compartment is involved.

Microelectrode Studies of the Frog Skin

One of the predominant tools used in the study of epithelial active transport has been the microelectrode. The advantage of a microelectrode lies in the ability of the microelectrode to impale a single cell without damage to that cell and to record the potential of the membrane between the microelectrode tip and a point of reference. In the past, microelectrodes primarily have been used to measure the

electrical potential profile of assorted epithelia and the voltage divider ratios of the individual membranes of the epithelium. The potential profile is the recording of the voltage drops across the various membranes encountered by the microelectrode as it is driven through the epithelium. The voltage divider ratio is the ratio of the resistance caused by the cell membranes between the microelectrode tip and its reference to the total transepithelial resistance. The resistances are measured by pulsing a known current across the epithelium and recording the resultant changes in potential across the epithelium and the potential drop measured by the microelectrode. The ratio of the membrane resistance to the total transepithelial resistance is called the fractional resistance ratio and is usually expressed as a percentage of the transepithelial resistance. A fractional resistance of zero would mean the microelectrode had not penetrated a resistance barrier and a fractional resistance of 100% would mean the microelectrode had crossed the entire tissue. The current, which is pulsed across the epithelium, flows along two routes, a transcellular pathway and a paracellular or shunt pathway. The resistance which is indirectly measured by the microelectrode is a combination of the membrane resistance and the shunt resistance. The shunt resistance is 5 to 10 times greater than the transcellular resistance (O'Neil and Helman, 1976; Macchia and Helman, 1974). Since the shunt resistance and the transcellular resistance are parallel to one another, Kirkoff's Law predicts that the majority of the current will pass through the transcellular pathway and the shunt resistance will contribute little to the voltage divider ratio. However, if the transcellular resistance is increased, through the use of amiloride or

ouabain, and the shunt resistance remains constant, the contribution of the shunt resistance to the voltage divider ratio increases.

Ottosen et al. (1953) were one of the first to use microelectrodes to study the frog skin. As the microelectrode traversed the epithelium, they observed a gradual potential change, which increased until the microelectrode potential approximated the transepithelial PD. By using distance measurements and injection of carmine dye, Ottosen et al. found the majority of the potential change to take place at the submicroscopic basement membrane between the epithelium and the corium. Therefore, they postulated that the active transport site was located at this basement membrane.

Based on several assumptions made by the KJU model, Engbaek and Hoshiko (1957) disagreed with the profile observed by Ottosen et al. Since the model predicted an intermediate cell potential bounded by two membranes of different ionic permeabilities, a skin which conformed to this model would exhibit a two step profile. The model also stated that the potential difference is created by the sodium diffusion potential across the mucosal membrane into the cell and the potassium diffusion potential across the serosal membrane out of the cell. The calculation of the sodium and potassium equilibrium potentials across each of these membranes shows that each step of the two step profile would be positive with respect to the mucosal bathing solution. Therefore, Engbaek and Hoshiko looked for, and found, a two step potential profile. The first potential they encountered as the microelectrode was advanced toward the epithelium was a slightly negative potential of -4 to -60 mV. Distance measurements placed this potential in the cornified outer layer of the epithelium, the stratum

corneum. Engbaek and Hoshiko considered the stratum corneum a non-living layer and ignored these potentials. The first step they felt was a legitimate step in the profile was a positive step of approximately one-half of the total transepithelial PD. This potential step occurred 16-100 microns deep in the epithelium. As the microelectrode was advanced past this step, a second positive step, which approximated the transepithelial PD, was seen 20-75 microns more serosal to the initial positive step. From distance measurements, Engbaek and Hoshiko stated that the second step of the profile corresponded to the epithelial-corial border while the first step was in a more superficial region. Although the distance between the first and second steps of the profile, approximately 50 microns, exceeded the height of the cells of the stratum germinativum, They assumed that the potential was across these cells and explained the difference in distance as mechanical distortion of the tissue by the microelectrode.

This potential profile was reiterated in a multitude of studies on amphibian skin (Ussing and Windhager, 1964; Whittembury, 1964; Cereijido and Curran, 1965; Biber et al., 1966; Biber and Curran, 1970; Rawlins et al., 1970). In each of these studies, a slightly negative potential was observed prior to the recognized "initial step". Whittembury (1964), using iontophoretic dye injection of carmine, found these potentials to lie in the stratum corneum. Nunes and Lacaz-Vieira (1975) demonstrated that these negative potentials were in the outer layers of the toad skin and were mucosal to the major resistance barriers of the epithelia. In all of the early profile measurements, these potentials were considered to be in non-living cells and were ignored.

The two step profile suggested that the frog skin epithelium is a functional syncytium as the KJU model predicts (Ussing and Windhager, 1964). However, the electrochemical gradient estimates by Rotunno et al. (1966) implied there should be at least a three step profile to the epithelium. Recent studies by Nagel (1976) lent support to this compartmentalization concept. By using a step drive which advanced the microelectrode at one micron increments, Nagel found the profile became increasingly negative as the microelectrode advanced toward the serosal side of the epithelium until reaching a potential of -100 mV. Not surprisingly, the fractional resistance also increased as the microelectrode advanced. When the microelectrode was advanced beyond this potential, a positive step was seen which was within 10 mV of the transepithelial PD. Nagel concluded these highly negative cells were deep in the epithelia and the positive step corresponded to the epithelial-corial border.

Nagel also observed the slightly negative potentials found in all of the previous profile studies. These potentials were highly unstable and had fractional resistances of less that 25% of the total transepithelial resistance. Nagel suggested two possibilities for these potentials. One possibility was that these potentials could be the result of newly converted stratum corneum cells in freshly molted epithelium which could still be maintaining a potential. A second possibility was that these potentials could be caused by depression of the tissue by the microelectrode tip before the microelectrode penetrates the cell membrane.

The highly negative potentials found by Nagel were later confirmed by Helman and Fisher (1977) and Fisher et al. (1980). However, neither

group observed the slightly negative potentials found in all previous studies. Fisher et al. removed the corium and found that regardless of whether the epithelium was impaled from the mucosal or serosal side, highly negative potentials were observed. Impalement potentials averaged -70 mV from the mucosal side and -91 mV from the serosal side. Because they found highly negative cell types when impaling from either side of the tissue, they concluded the cells of the epithelium are in functional syncytium.

Based on the assumption that the frog skin is in syncytium, Nagel et al. (1981) measured the intracellular ion activities of these highly negative cells. Using ion-selective microelectrodes, they calculated the activities of sodium, potassium and chloride in cells with an average potential of -90 mV. They found the activity of sodium to be low, approximately 14 mM, while that of potassium was 132 mM and chloride activity was 18 mM. When amiloride was added to the mucosal bathing solution, the only changes observed was the activity of sodium which dropped to 8 mM after one hour. During this treatment, the intracellular potential became more negative, decreasing to -130 mV. An obvious drawback to this study was the use of single barrel rather than double barrel microelectrodes. The characteristics of an ion-selective microelectrode do not allow a direct measurement of the ion activity, instead the measurement is a combination of the intracellular potential and the ionic activity. To obtain ionic activity with a single barrel microelectrode, one must use an average of the intracellular potential and calculate ionic activity. To avoid this problem, one could use a double barrel microelectrode which measures the activity of each cell impaled.

Microelectrode studies of the frog skin have led to the general conclusion that the intracellular potentials of the frog skin are in accordance with the KJU model which implies that the epithelia is a functional syncytium. This conclusion has been drawn despite all previous profile studies which observed slightly negative cell types before the highly negative cell potentials.

Location of the Active Transport Site

One of the most extensively studied, but as yet unsolved, problems of frog skin research has been locating the site of the active transport pump. Almost every study of the frog skin has suggested a possible site of this mechanism. Early attempts to locate this site were hampered by the lack of a complete view of the morphology of the skin. Ussing (1948) thought the active site was on the serosal border of the stratum germinativum. However, he considered the epithelium to be composed of the stratum germinativum with "some cells which may resemble normal epithelial cells whereas others are being keratinized". With the advent of the electron microscope, Ottosen et al. (1953) gave a more comprehensive view of the morphology of the skin. However, profile measurements, which were previously discussed, found the profile potential to approximate the transepithelial PD at the basement membrane between the epithelium and the corium. The point at which the microelectrode PD was equal to the transepithelial PD was considered to be the site of active transport. This was supported by Engbaek and Hoshiko (1957) and many of the other profile studies previously mentioned.

The debate between the syncytial and the compartmental frog skin has also influenced efforts to locate the active sodium transport site.

The KJU model, which treated the frog skin as a single cell layer, placed the active transport pump on the serosal membrane of the transporting cells. This assumption was supported by studies which showed that ouabain, which blocks the potassium site of the pump, was only effective from the serosal side of the frog skin (Herrera, 1966). When Ussing and Windhager (1964) proposed that the epithelium acted as a functional syncytium, the general consensus became that the sodium transport pump was on the serosal border of all cells of the epithelium.

While proponents of the compartmentalized frog skin did not disagree with a serosally located active transport pump, the morphological characteristics of the epithelium indicated that a consistant concentration of active transport mechanisms throughout the epithelium seemed unlikely. Farquhar and Palade (1964) reported that the only continuous barrier to diffusion, a network of tight junctions, was found in the outermost cell layer of the stratum granulosum. Farquhar and Palade (1966) also found this region to have the highest concentration of Na^+-K^+ ATPase, although some Na^+-K^+ ATPase was found throughout the epithelium. Voute and Ussing (1968 & 1970) demonstrated that the only cell layer to respond to external transepithelial current was the outermost layer of the stratum granulosum or the RCL. They stated the this response was probably caused by the majority of current passing through only this cell layer, which suggested that these cells were the site of active sodium transport. The model proposed by Huf and Howell (1976) also places the site of active transport in this outermost layer. The model also predicts that a second compartment or "maintenance" compartment is

located in the deeper layers of the epithelium. The cells of this maintenance compartment also transport sodium but only to maintain intracellular sodium concentration. This premise agrees with the ATPase localization results of Farquhar and Palade. Therefore, a large portion of the research which supports the compartmental frog skin places the active transport site on the serosal border of the outermost cells of the stratum granulosum.

This review has dealt with the majority of studies of active sodium transport in the frog skin and other transporting epithelia. It has omitted research on some aspects of the frog skin such as chloride, potassium and calcium transport and research on inhibitors. However, the topics covered in this review are most pertinent to the current study.

SECTION II

IDENTIFICATION OF THE CELL TYPES CORRESPONDING TO THE ELECTRICAL POTENTIAL PROFILE OF THE FROG SKIN

IDENTIFICATION OF THE CELL TYPES CORRESPONDING TO

THE ELECTRICAL POTENTIAL PROFILE OF THE FROG SKIN

Summary. Iontophoretic dye injection with Lucifer Yellow CH was used to correlate the various morphological cell types in the frog skin epithelium with microelectrode-derived membrane potentials. The first potential encountered from the mucosal side, -4 to -18 mV, was obtained from cells in the first cell layer of the stratum granulosum, adjacent to the stratum corneum. The second potential observed, -48 to -80 mV, was obtained from cells in the remaining layers of the stratum granulosum (ie.) at least one cell layer serosal to the stratum corneum. The final potential, +5 to +25 mV, was found in cells in the deeper layers of the epithelium, in either the stratum spinosum or the stratum germinativum. Since the Lucifer Yellow CH dye did not spread, The cells of these various layers do not appear coupled indicating a compartmental rather than syncytial frog skin.

Key Words: Frog skin, microelectrodes, membrane potentials, electrical potential profile, dye injection.

Introduction

The frog skin has been used extensively as a model for active transport since it was shown to actively transport sodium from the mucosal or pond side of the tissue to the serosal side or blood side (Ussing, 1949). One source of controversy in the study of the frog skin, which has remained unresolved for 25 years, is whether the frog skin acts as a functional syncytium or a multicompartmental epithelium;

(ie.), different cell types have different functions. However, since the frog skin is a multicellular epithelium any correlation between the various cell types and their function has been difficult.

One of the principle methods used to try to correlate cell types with function has been electrical potential profile measurements made with microelectrodes. Early studies using this technique found that under open circuit, two equal and positive potential steps existed in the frog skin epithelium (Engbaek and Hoshiko, 1957; Ussing and Windhager, 1964; Cereijido and Curran, 1965; Biber, et al., 1966). Engbaek and Hoshiko, using distance measurements, determined that the first legitimate potential step corresponded to a superficial layer of the epithelium 16 to 100 microns below the mucosal surface of the epithelium. The second step was associated with penetration of the epithelial-corial border. This potential profile suggested that the epithelium is a functional syncytium (Ussing and Windhager, 1964). However, electrochemical gradient estimates at low sodium levels have shown that passive sodium diffusion into the epithelium, assuming the Koefoed-Johnsen and Ussing (1958) model, would be unlikely without compartmentalization of sodium (Cereijido and Rotunno, 1968). The compartmentalization of sodium would suggest that at least two different cell types must be present in the epithelium. Recent potential profile measurements (Nagel, 1976; Helman and Fisher, 1979) support the compartmentalization concept. They demonstrated that, as the microelectrode advanced through the epithelium, the cell membrane potentials increased in negativity to approximately -100 mV. When the microelectrode was advanced beyond this -100 mV potential, a positive step was observed. This positive step was interpreted as the

microelectrode penetration of the epithelial-corial junction.

All early studies discounted potentials which were found when the microelectrode first penetrated the tissue from the pond side. Ranging from -4 to -25 mV, these potentials were unstable and had low fractional resistances which led to the interpretation that these potentials were artifacts caused by depression of the tissue as the microelectrode first came in contact with the epithelium.

The use of distance measurements during a profile study may give some idea as to which layer of cells is responsible for a certain potential. However, due to depression of the tissue by the microelectrode as the impalement is made, the accuracy of these measurements is subject to question. To more closely associate the various cell types in the frog skin with their membrane potential, we have combined potential profile measurements with iontophoretic dye injection. With this technique we have found three cell types based on their membrane potential and have been able to associate these types with cell layers in the epithelia.

Material and Methods

The abdominal skin of the Northern grass frog, Rana pipiens was removed and mounted on a modified plexiglass flat-sheet chamber which was designed for microelectrode impalements perpendicular to the skin surface. The skin was perfused on both sides with aerated frog Ringer's (110 mM NaCl, 2.5 mM KCl, 2.5 mM TRIS, 1 mM $CaCl_2$, pH 8.2) at a rate of 5 ml/min. The skin was tied to the chamber lip to prevent edge damage. To measure transepithelial potential difference, two calomel electrodes were connected to the bathing solution on each side of the skin via Ringer-agar bridges. Ag-AgCl wires located on each

side of the chamber 0.5 cm from the skin, served as electrodes to supply the current for fractional resistance measurements.

Microelectrodes with a sharply tapered tip (diameter approx. 0.5 microns) were pulled from single barrel capillary glass (1.5 mm O.D., A-M Systems) on a horizontal puller. The microelectrode tip was partially backfilled with Lucifer Yellow CH (Aldrich) and the remainder of the barrel was backfilled with 250 mM KCl. The criteria for a usable microelectrode was a tip resistance of 10^{15}-10^{20} ohms and a tip potential below 7 mV. Ag-AgCl wires connected the microelectrode to a 3431J Burr-Brown electrometer and a constant-current pulse generator. The microelectrode potential and the transepithelial PD were recorded on a dual-pen chart recorder (Linear Instruments).

The microelectrode was mounted in a plexiglass holder in a hydraulic drive micromanipulator (Frederick Haer) and advanced toward the skin from the mucosal side until a potential change was observed. The criteria for a successful impalement was a stable impalement potential for 30 sec and less than 2 mV change from zero after withdrawal of microelectrode. Fractional resistance was measured by transepithelial current pulses (500 msec duration) with 0% of the total resistance found on the pond side and 100% on the blood side.

After the cell was impaled, the Lucifer Yellow CH dye was iontophoretically injected into it by pulsing a 5 to 7 nA current through the microelectrode 75 to 100 times. After the injection of the dye, the microelectrode was withdrawn, moved and a subsequent impalement and dye injection made. Each skin was impaled and injected 5 to 10 times, but of only one potential type per skin. The skin was removed from the chamber and fixed for 2-6 hours in 4% formaldehyde in

0.1 M Na_2HPO_4. The skin was then frozen and sectioned in 15 micron sections on a cryomicrotome (American Optical). The sections were mounted on slides and allowed to dry. The sections were observed on a Nikon Labophot microscope with an epi-illumination attachment. For fluorescent photomicrography, the speciment was illuminated with an Oscram 30W halogen lamp. A Nikon blocking filter with a cut-off wavelength of 500 nm was used to observe the sections.

Results

A typical potential profile obtained from the frog skin epithelium is shown in Fig. 1. This profile is characterized by four potential steps as the micro-electrode advances through the epithelium. The potentials and fractional resistance of each step are summarized in Table 1.

As the microelectrode entered the epithelium from the pond side the first potential step encountered ranged from -4 to -18 mV (Fig 1a). This potential step was referred to as an α potential step. The fractional resistance of this potential step was quite low, less than 5%, which suggests that this potential step could be an artifact. However, dye injected at this step was recovered in cells located just serosal to the stratum corneum, in the outermost cell layer of the stratum granulosum (Fig. 2a). Dye injected into the -4 to -18 mV impalements was always recovered in this first layer which was referred to as the first Reactive Cell Layer (RCL) (Voute and Ussing, 1968). These cells are 15 to 20 microns in length and 5-7 microns in diameter.

The second step of the potential profile of the frog skin was referred to as the β potential step and is shown in Fig. 1b and 1c. This step is characterized by potentials ranging from -48 to -80 mV and

Fig. 1. The electrical potential profile (M_{pd}) was measured as a microelectrode traversed the skin from mucosal to serosal side. Transepithelial current pulses (arrows) were used to measure the transepithelial resistance and the membrane resistance of the cells impaled. The ratio of these two resistances was termed the fractional resistance. The first cell potential type (a) encountered as the microelectrode entered the skin from the mucosal side had a membrane potential of -4 to -18 mV and a fractional resistance of approx. 5%. The second cell potential type (b & c) ranged from -48 to -80 mV and had a fractional resistance of approx. 55%. The third cell type (d) was characterized by 5 to 25 mV potentials and a fractional resistance of 72%. The final potential step (e) approximated the transepithelial PD (T_{pd}) and had a fractional resistance of approx. 95%. It was later found that this potential step was located in the corium.

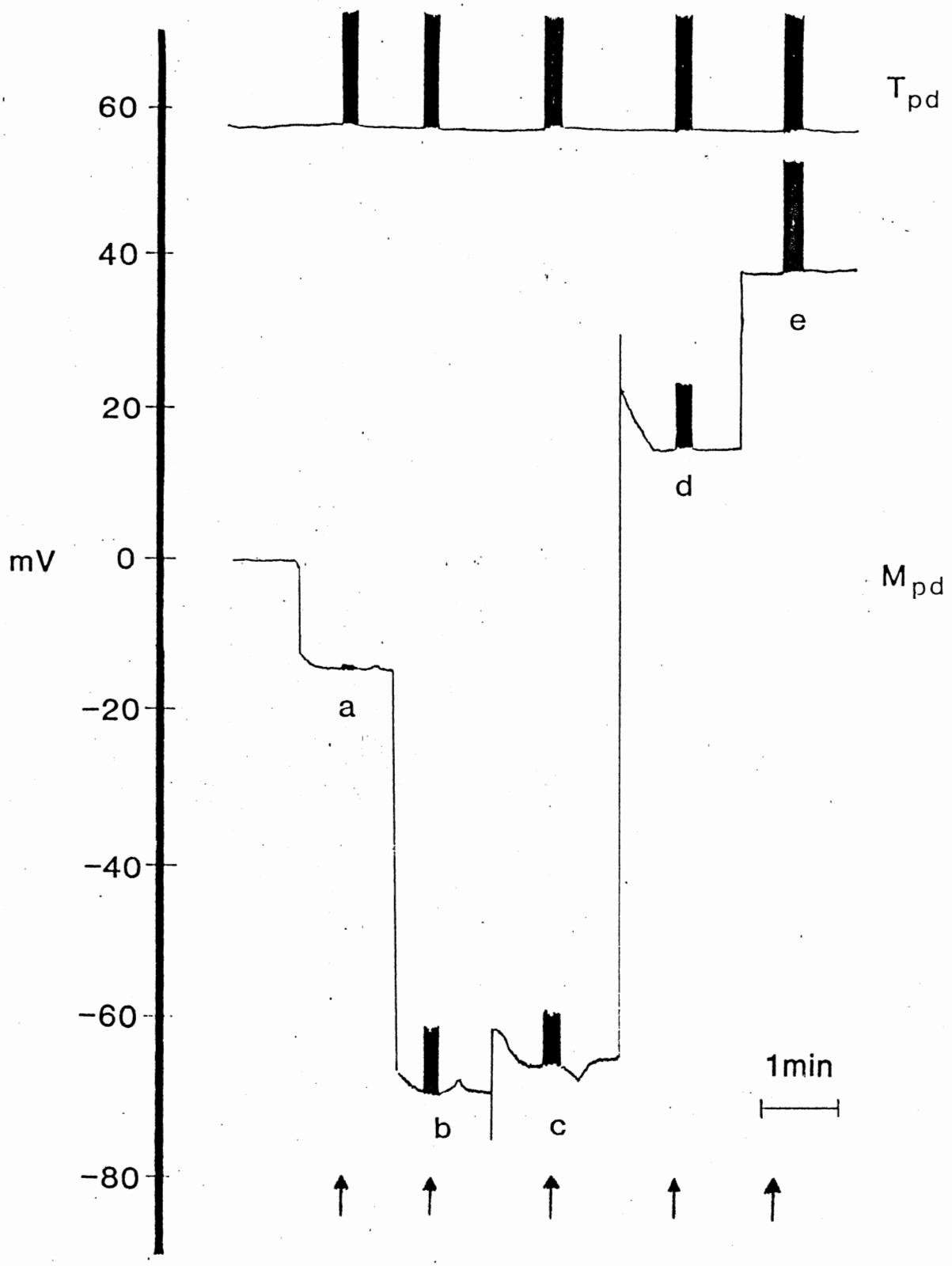
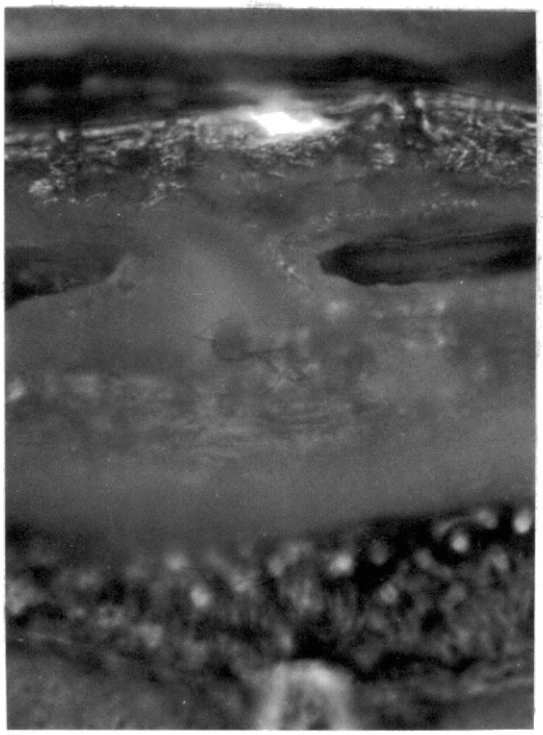
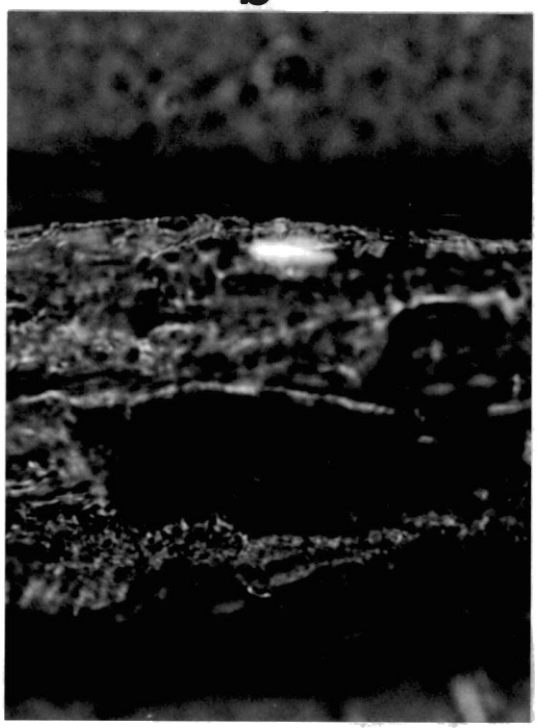

60
40
20
0
-20
-40
-60
-80
mV
T_{pd}
M_{pd}
a
b
c
d
e
1min

Fig. 2. Dye recoveries of the three cell potential types found in the frog skin epithelium. The first cell type encountered as the microelectrode entered the skin from the mucosal side had a potential of -4 to -18 mV and was termed the α cell type. Following injection of dye into this potential type, dye was recovered in the cell layer immediately serosal to the stratum corneum, in the first layer of the stratum granulosum (a). The dark area marked OCT is caused by the sectioning compound used to freeze and section the tissue. The second potential type, referred to as the β cell type and characterized by -48 to -80 mV potentials, was located in the remaining layers of the stratum granulosum (b), 12 to 25 microns below the mucosal surface of the skin. The third cell type had a potential +5 to +25 mV and was termed the γ cell type. This cell type (c) was found in the lower layers of the epithelium, corresponding to the stratum spinosum or stratum germinativum.

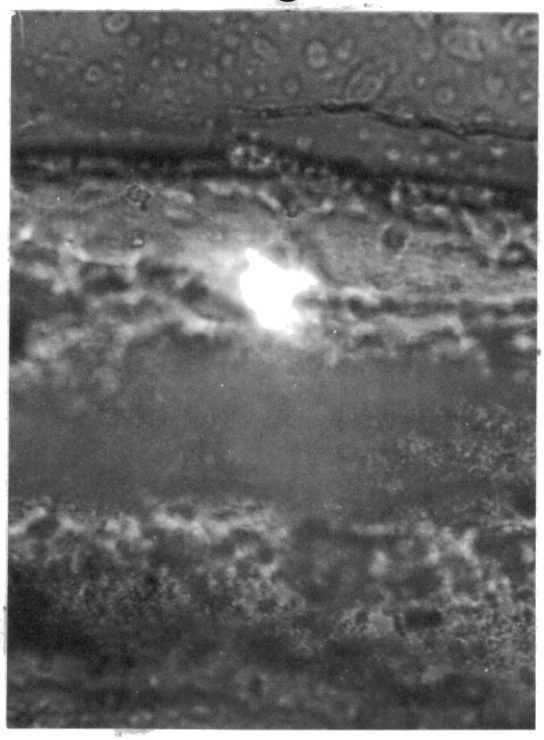
a



b



c



Cell Type	Potential (mV)	Fractional Resistance (%)	Recovery Rate (%)	Location
α	-4 to -18	4.3 ± 1.2	27	1st Reactive Cell Layer
β	-48 to -80	53.2 ± 5.9	30	Stratum Granulosum
γ	+5 to +25	72.3 ± 11.2	31	Stratum Spinosum or Stratum Germinativum

Table 1. Summary of the Membrane Potential, Fractional Resistance and Location of the three cell types found in the frog skin epithelium. The recovery rate, the number of recoveries/number of impalements, was based on 40 impalements for each cell type. Eight separate skins were used for each cell type.

a fractional resistance of approximately 50%. Dye marked cells of this potential type were found in the deeper layers of the stratum granulosum (Fig. 2b). Distance measurements from photomicrographs have shown these marked cells to be 7 to 17 microns below the mucosal surface of the epithelium or at least one cell thickness below the stratum corneum. The β cells are elliptical with dimensions similar to those of the α cell type.

The third potential step observed was positive (Fig. 1d) and was referred to as a γ potential type. This potential step ranged from +5 to +25 mV and had a fractional resistance of 65 to 75%. Marked cells were found in the region of the stratum spinosum or stratum germinativum (Fig. 2c). The line of demarcation between these two layers was indistinct and a more accurate localization of this potential step was difficult. Dye spreading or "flare" was common with this cell type although in some instances, the outline of both spinosal and germinatival cells could be seen in a flare. Often dye was observed in two or more cells. Most of the cells observed were spinosal cells and had a diameter of 15 microns.

When the microelectrode was advanced past the γ cell type, the potential was approximately that of the transepithelial PD and fractional resistance was approximately 95%. Dye injected into this potential step was found in the corium or connective tissue of the frog skin.

Only one potential type was injected in each skin preparation. All of the marked cells recovered in a given skin were found in the same layer of the epithelium. When comparing between skins, all α potential types were found in the first reactive cell layer, all the β

potential types were found in the remainder of the stratum granulosum and all the γ potential types were found in the remaining layers of the epithelium. The dye recovery rate of all cell types was 30 to 35%.

Discussion

The potential profile shown in Fig. 1 is similar to profiles reported previously (Engbaek and Hoshiko, 1957; Ussing and Windhager, 1964; Whittembury, 1964; Cereijido and Curran, 1965; Biber et al., 1966; Biber and Curran, 1970; Rawlins et al., 1970; Nagel, 1976). Impalement potentials of the α cell type were seen as the microelectrode entered the tissue by both Enbaek and Hoshiko (1957) and Nagel (1976). These potentials were discounted as artifacts due to the instability of the impalements and to the low fractional resistances. They interpreted these potentials as the result of mechanical distortion of the tissue by the microelectrode before entry. Whittembury (1964) iontophoretically injected carmine into toad skin and found these slightly negative cells in the stratum corneum. However, we have shown that this potential is the mucosal membrane potential of cells which lie in the first living cell layer below the stratum corneum (Fig. 2a).

The location of the α cell type corresponds to the Reactive Cell Layer (RCL) identified by Voute and Ussing (1968 & 1970) and Voute and Hanni (1973). They showed that under transepithelial short-circuiting or hyperpolarization, the cells in this layer would swell or shrink, respectively, while the remaining layers of the epithelium were unaffected. Using freeze etching techniques, Farquhar and Palade (1964) found tight junctions, zonulae occludens, between the cells of the RCL layer, forming a physical barrier to passive paracellular

movement. Tight junctions were not found in the more serosal cell layers of the epithelium. These results, coupled with our dye recovery experiments, suggest that the first reactive cell layer of the stratum granulosum is a separate cell layer from the remainder of the stratum granulosum.

The membrane potential and fractional resistance of the β cell type correspond to the highly negative potential step observed by previous investigators. We found this cell type in the serosal layers of the stratum granulosum, (ie.) 7 to 17 microns below the stratum corneum, which suggests this highly negative layer is two to three cells thick. Nagel (1976) reported that the potentials measured by the microelectrode became increasingly negative as the microelectrode advanced toward the serosal side of the epithelium. However, we found no such correlation.

The γ potential type was also observed in all previous studies of the potential profile of the frog skin. These studies placed this positive step at the epithelial-corial junction. However, dye injection of this step has shown marked cells located in the stratum spinosum or the stratum germinativum.

The potential profile of this study is the same basic potential profile of the frog skin that has been reported, with interpretive variations, for the past 25 years. However, by combining iontophoretic dye injectionwith intracellular potential measurements, the conclusions previously drawn as to the location of each potential step were shown to be erroneous. Our results strongly suggest that microelectrode studies of tissues which actively transport ions, particularly multicellular tissues, must be combined with dye injection techniques

in order to accurately evaluate the tissue. This study also shows three separate cell types in the frog skin which implies that the frog skin does not act as a functional syncytium, but is multicompartmental.

SECTION III

LOCATION OF THE PERMEABILITY BARRIER WITH RESPECT TO THE VARIOUS CELL TYPES IN THE FROG SKIN

LOCATION OF THE PERMEABILITY BARRIER WITH RESPECT TO THE VARIOUS CELL TYPES IN THE FROG SKIN

Summary. Based on their intracellular potentials, three types of cells have been identified in the frog skin epithelium (Duncan and Blankemeyer, in press). These three types of cells were found in the first reactive cell layer, the remainder of the stratum granulosum and the stratum spinosum. We have used extracellular diffusion of horseradish peroxidase (HRP) and iontophorectic dye injection of cells in each layer to determine the relationship between the permeability barrier in the frog skin epithelium and the various cell layers. The first reactive cell layer, characterized by -4 to -18 mV potentials, was found to be either mucosal to or at the level of the permeability barrier; the remaining two cell types were serosal to this diffusion barrier. Since only the cells of the RCL are in unrestricted contact with the mucosal bathing solution, sodium must pass through the cells of the reactive cell layer to reach the serosal side of the skin. Therefore, the reactive cell layer probably plays a major role in the active transport of sodium.

Key Words: Frog skin, permeability barrier, tight junctions, potential profile, dye injection.

Introduction

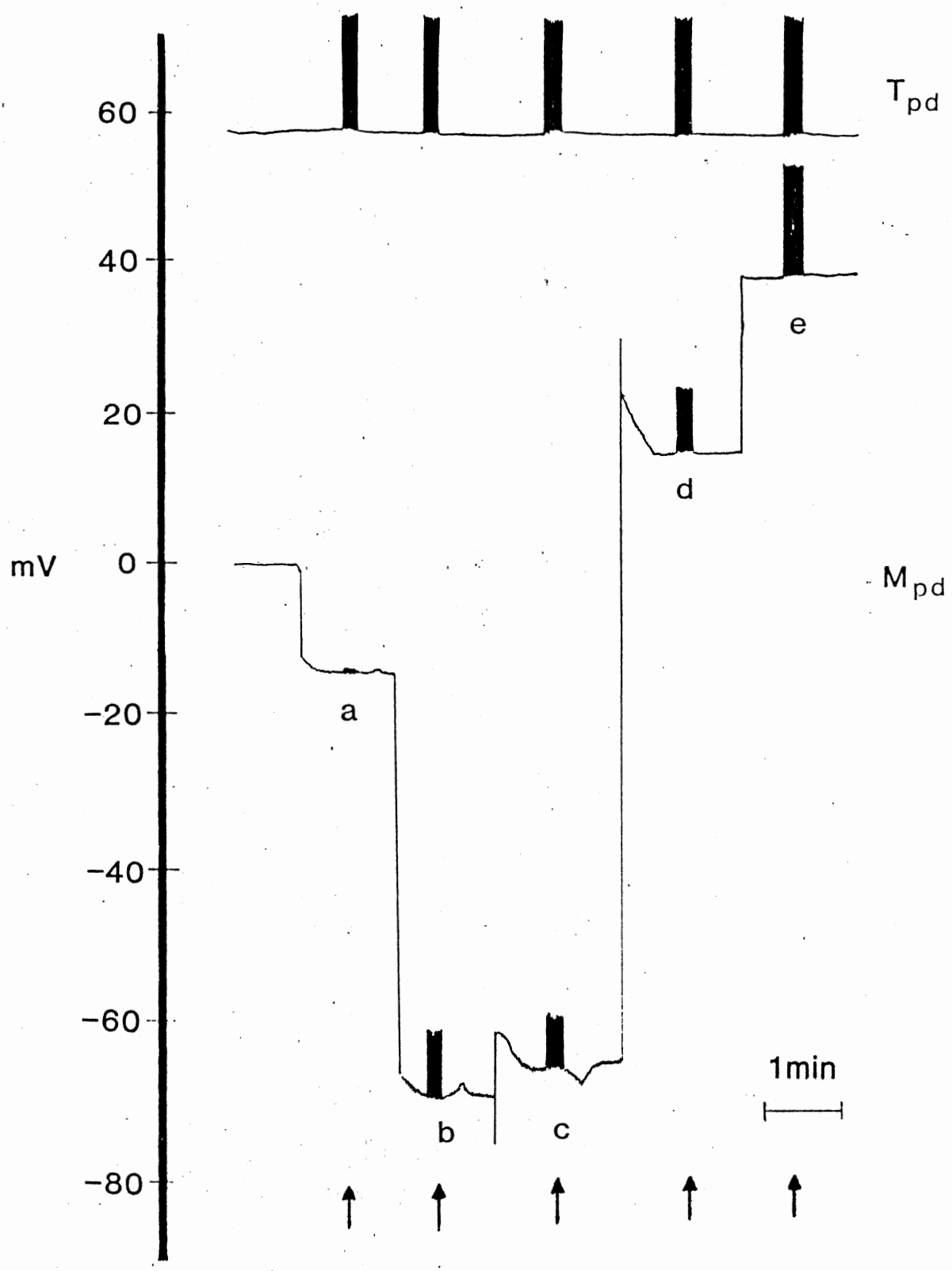
The frog skin actively transports sodium from the pond or mucosal side of the epithelium to the blood or serosal side (Ussing, 1949).

Most epithelial tissues, the frog skin included, have been shown to have junctional complexes (tight junctions) between cells which act as a permeability barrier. However, the relationship between this barrier and the active transport mechanism is still unclear.

Farquhar and Palade (1963), using light and electron microscopy, found tight junctions (zonula occludens) in amphibian epithelia. These junctional complexes were impermeable to concentrated protein solutions, suggesting that a primary function of these junctions is to act as a diffusion barrier. In the frog skin, tight junctions are predominantly found in the stratum corneum and the outermost layer of the stratum granulosum (Farquhar and Palade, 1964). Using lanthanum and ruthenium red, Martinez-Palomo et al. (1971) demonstrated that the permeability barrier is located in the outer layers of the stratum granulosum and the stratum corneum, and that this barrier is impermeable from both sides.

To better understand the relationship between tight junctions and active transport, correlation must be made between the permeability barrier and the various cell potential types of the electrical potential profile (Fig. 1). Using iontophoretic dye injection, Duncan and Blankemeyer (in press) found three types of cells in the frog skin epithelium. The first cell type encountered as the microelectrode entered the epithelium from the pond side exhibited a membrane potential of -4 to -18 mV (Fig. 1a) and was found in the outermost layer of the stratum granulosum. The second cell type which had a potential of -48 to -80 mV (Fig. 1b and 1c) was found in the remaining layers of the stratum granulosum. The third cell type was characterized by a potential of +5 to +25 mV (Fig. 1d) and was found in

Fig. 1. The electrical potential profile (M_{pd}) was measured as a microelectrode traversed the skin from mucosal to serosal side. Transepithelial current pulses were used to measure the transepithelial resistance and the membrane resistance of the cell impaled. The ratio of these two resistances was termed the fractional resistance. The first cell potential type (a) encountered as the microelectrode entered the skin from the mucosal side had a membrane potential of -4 to -18 mV and a fractional resistance of approx. 5%. The second cell potential type (b & c) ranged from -48 to -80 mV and had a fractional resistance of approx. 55%. The third cell type (d) was characterized by +5 to +25 mV potentials and a fractional resistance of 72%. The final potential step (e) approximated the transepithelial PD (T_{pd}) and had a fractional resistance of approx. 95%. It was later found that this potential step was located in the corium.


60
40
20
0
-20
-40
-60
-80
mV
T_{pd}
M_{pd}
a
b
c
d
e
1min

the region of the stratum spinosum and stratum germinativum.

The diffusion of Horseradish peroxidase (HRP) coupled with iontophoretic dye injection was used to study the relationship between the cell types of the frog epithelium and the permeability barrier. We will show that the first cell type found in the outermost layers of the stratum granulosum borders the diffusion barrier created by the tight junction.

Material and Methods

The skin from the abdominal region of Rana pipiens (Lemberger) was removed and mounted on a plexiglass, flat-sheet, horizontal chamber designed for microelectrode impalements perpendicular to the skin surface. The skin was tied to the chamber aperture to prevent edge damage and perfused on both sides with aerated frog Ringer's (110 mM NaCl, 2.5 mM KCl, 2.5 mM TRIS buffer, 1 mM $CaCl_2$, pH 8.2) at a rate of 5 ml/min.

Dye impalements were made using microelectrodes (tip diameter approx. 0.5 microns) pulled from single barrel capillary glass (1.5 mm O.D., A-M Systems) on a horizontal puller. The microelectrode tip was partially backfilled with Lucifer Yellow CH (Aldrich) and the remainder of the barrel, backfilled with 250 mM KCl. The criteria for a usable microelectrode was a tip resistance of 10^{15} to 10^{20} ohms and a tip potential less than 7 mV. The microelectrode was connected via Ag-AgCl wire to a 3431J Burr-Brown electrometer and a constant current pulse generator. The microelectrode was mounted in a plexiglass holder on a hydraulic drive micromanipulator (Frederick Haer) and advanced from the pond side of the skin until a potential change was observed. An impalement which was stable for 30 sec and gave less than 2 mV change

from zero upon withdrawal of the microelectrode constituted a successful impalement.

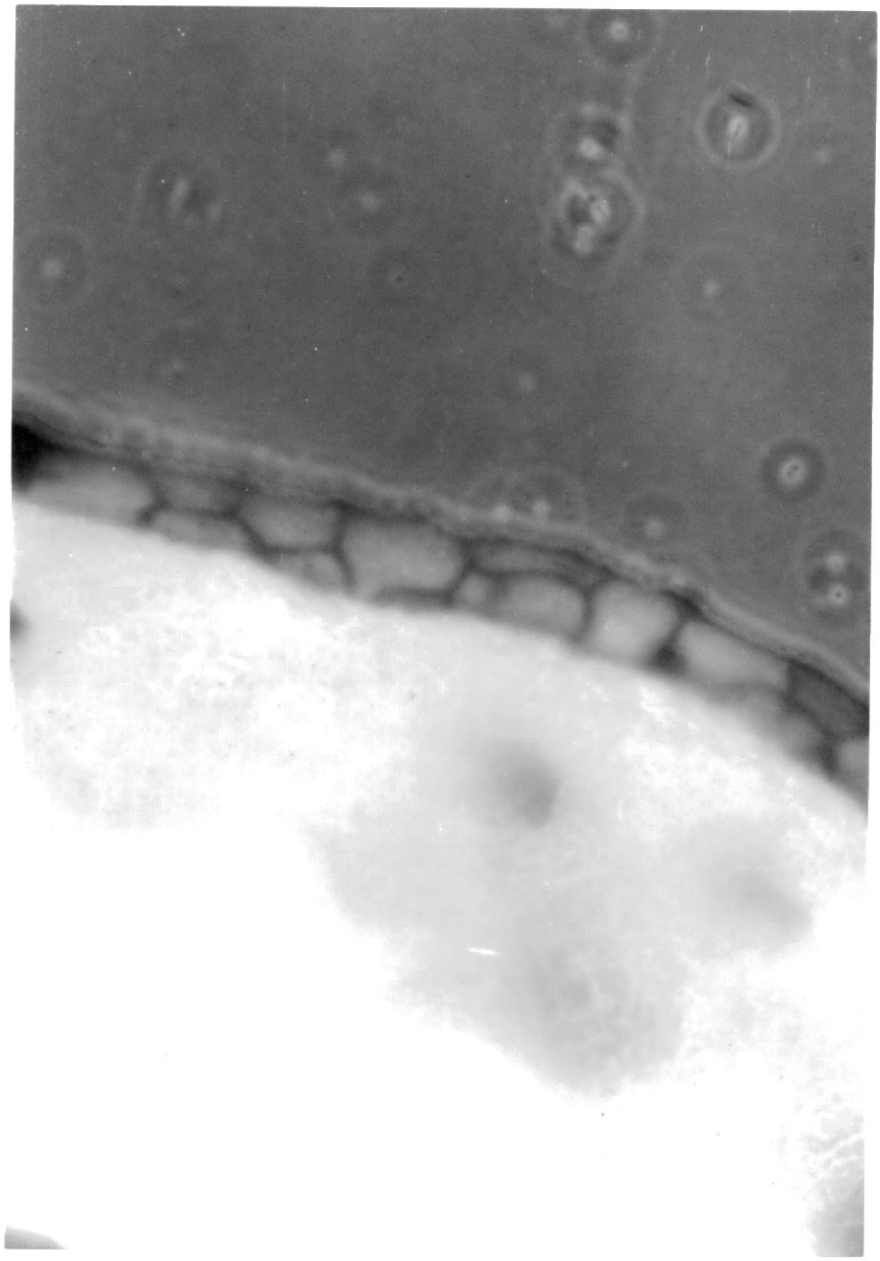
The dye was iontophoretically injected into each impalement by pulsing a 5 to 7 nA current through the microelectrode 75 to 100 times. After injection of the dye, the microelectrode was withdrawn, moved and a subsequent impalement and dye injection made. Ten injections were made in each skin but of only one of the cell potential types.

After injection of the dye, the solution bathing the pond side of the skin was removed. The blood side of the skin continued to be perfused with aerated Ringer's. Horseradish peroxidase (HRP, Boehringer) was placed on the pond side surface of the skin and allowed to diffuse for two hours. The frog skin was then removed from the chamber, fixed for two hours in 2% gluteraldehyde, frozen and sectioned in 15 micron sections on a cryomicrotome (American Optical). Sections were then reacted with o-dianisidine (Sigma) and peroxide (A. Rusoff, personal communication) to reveal the distribution of HRP in the tissue.

Results

In one series of experiments, HRP was placed on the pond side of the skin and allowed to diffuse without prior iontophoretic injection of fluorescent dye. Fig. 2 is a photomicrograph of a typical HRP permeability experiment. HRP was found to diffuse 10 to 15 microns from the mucosal side of the epithelium before reaching a permeability barrier. As can be seen from Fig. 2, the barrier is one to two cells deep into the epithelium which corresponds with the stratum corneum and the outermost layer of the stratum granulosum.

Fig. 2. Photomicrograph showing the extent of HRP diffusion in the frog skin from the mucosal side. The HRP is shown to diffuse 10 to 15 microns into the skin before reaching the permeability barrier created by the tight junctions. As can be seen in this figure, this permeability barrier is only one or two cell thicknesses from the mucosal surface.



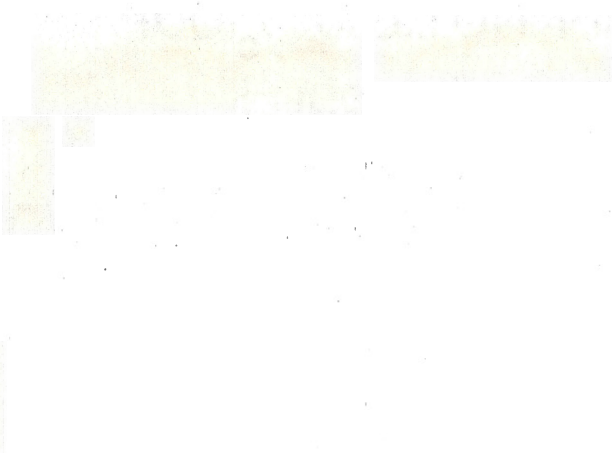
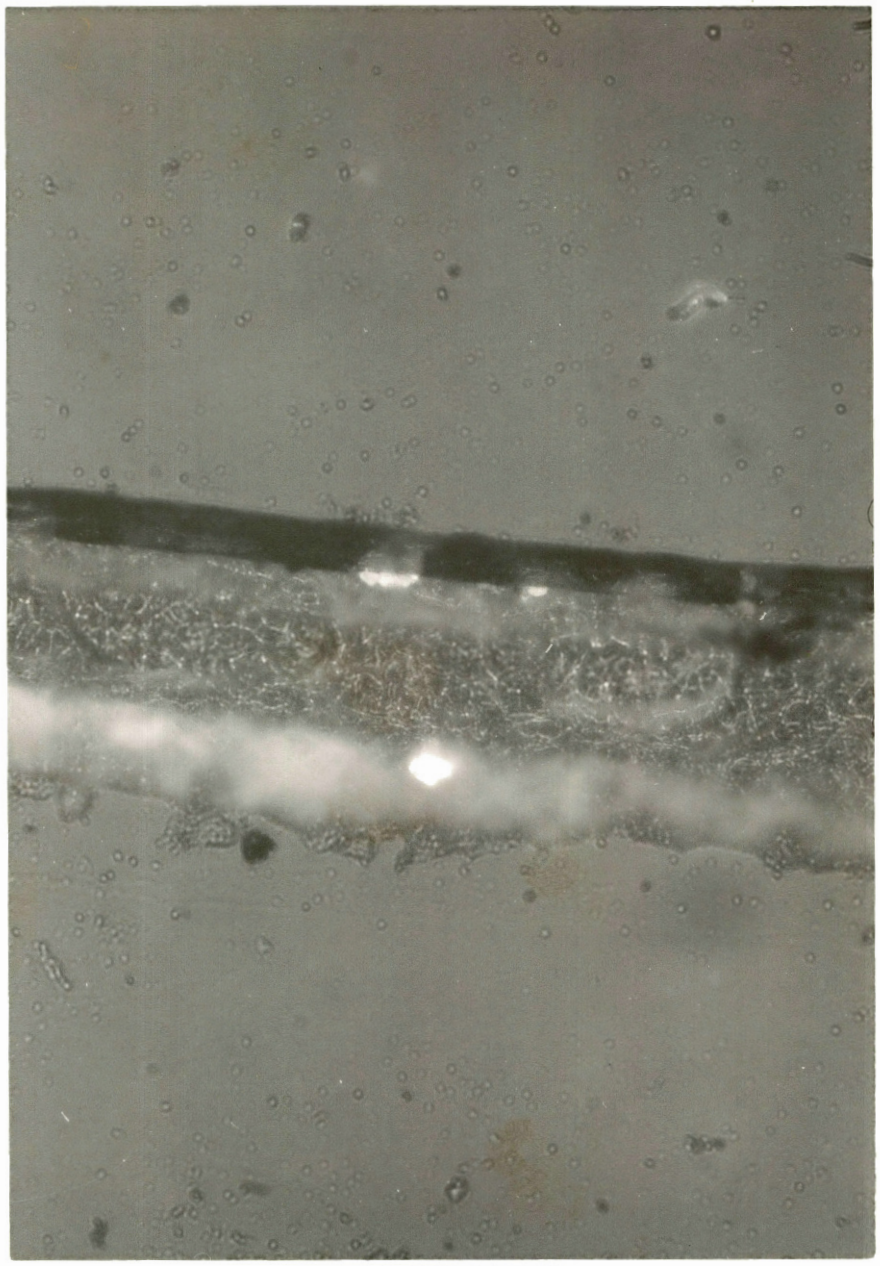


Fig 3. This figure shows the relationship between the permeability barrier of the frog skin created by tight junctions and the α cell type which corresponds to the reactive cell layer. The β and γ cell types were found serosal to the permeability barrier which suggests that sodium must pass through the reactive cell layer to reach the serosal bath. This must mean that the reactive cell layer plays a major role in sodium active transport.



In specimens which HRP diffusion was coupled with iontophoretic dye injection, HRP again diffused 10 to 15 microns below the mucosal surface of the epithelium. When cells characterized by -4 to -18 mV potentials, were injected, the cells filled with fluorescent dye were found either to the mucosal side or at the level of the permeability barrier (Fig. 3). Eighteen marked cells of this potential type from six different skins were found in this region. These cells were never found more serosal than the level of the permeability barrier. When the second cell type, characterized by -48 to -80 mV potentials, was injected, dye filled cells were found at least one cell layer serosal to the permeability barrier.

Discussion

The results of the horseradish peroxidase permeability experiments show a diffusion barrier in the same region as described by Martinez-Palomo et al. (1971). Using electron microscopy, they found lanthanum and ruthenium red could not penetrate the tight junctions of the outermost layer of the stratum granulosum from either the mucosal or serosal side. Like lanthanum, HRP did not penetrate the tight junctions found 10-15 microns from the mucosal surface of the epithelium. This is in agreement with Rawlins et al. (1970). By measuring resistance changes with respect to distance from the serosal side, they found the high resistance barrier to be 5-15 microns from the mucosal surface.

In this study, HRP was found in the cells mucosal to the permeability barrier as well as the extracellular spaces. As Martinez-Palomo et al. stated, this suggests a small compartment, separate from the remainder of the epithelium, which exhibits a very

low resistance to ionic diffusion. Whittembury (1964), using iontophoretic carmine injection, found that the stratum corneum had a very low membrane resistance indicating that the permeability of these cells is quite large. A low membrane resistance is typical of the first cell potential type encountered upon entry from the mucosal side of the skin (Duncan and Blankemeyer, in press). This cell type is characterized by a fractional resistance (membrane resistance/total transepithelial resistance) of less than 5%. Fig. 2 shows the location of this cell type in relation to the permeability barrier. As can be seen, this cell type borders the barrier forms the outermost layer of the stratum granulosum. Voute and Ussing (1968 and 1970) demonstrated that the cells in this layer swelled or shrank in response to changes in the rate of transport. This suggested to them that a majority of the transport takes place across these cells. Since the permeability barrier is between these cells, the movement of sodium from the mucosal side of the epithelium to the serosal side must cross these cells.

This study shows that the stratum corneum and the outermost cells of the stratum granulosum are separated from the remainder of the epithelium by a highly resistive permeability barrier and that the slightly negative cell types found previously are either mucosal to or at the permeability barrier. This implies that for sodium to move from one side of the barrier to the other, the slightly negative cell type must have a definite function in the active transport mechanism.

SECTION IV

GENERAL DISCUSSION AND SUMMARY AND CONCLUSIONS

GENERAL DISCUSSION

The results of this study show a three step potential profile of the frog skin epithelium, which at first glance, is quite different from previous profile studies. Early profile studies were influenced by the Koefoed-Johnsen and Ussing (1958) model of frog skin transport. This model states that the potential difference across the skin is the result of diffusion potentials created by the passive movement of sodium into the cell from the mucosal side and the passive movement of potassium out of the cell to the serosal side. The Nernst equation predicts that the potential of each membrane would be positive when referenced against the mucosal bathing solution. Since the model also treats the frog skin as a monolayered epithelium in syncytium, Engbaek and Hoshiko (1957), along with numerous other investigators, expected and found a two step potential profile in which both steps were equal and positive. A different profile was reported by Nagel (1976) who described the frog skin profile as "trough-like". This profile became increasingly negative as the microelectrode advanced through the epithelium until reaching a maximum of -100 mV. Helman and Fisher (1977) concluded that these highly negative cells are the probable site of active sodium transport.

The difference between the previous profiles and the profile shown in this study is not in the actual recorded potentials, but in the interpretation of the general profile. All of the earlier studies

reported slightly negative potentials encountered upon initial penetration of the epithelium from the mucosal side. These potentials ranged from -4 to -60 mV, had very low fractional resistances and were highly unstable. This potential type was ignored in all previous studies for various reasons. By using distance measurements, Engbaek and Hoshiko located these potentials within 16 microns of the mucosal surface which placed these potentials in the non-living layer of the epithelium, the s. corneum. Nagel also considered these potentials to be an artifact and suggested two possibilities for their cause. He thought these potentials could either be caused by newly formed s. corneum cells in freshly molted skin which still retained a fraction of their intracellular potential and resistance or by depression of the epithelium around the tip of the microelectrode prior to penetration.

The intracellular potentials, fractional resistance and location of these slightly negative cells reported in earlier studies are all similar to the α cell potential type shown in this paper. This cell type is characterized by potentials ranging from -4 to -18 mV and a fractional resistance of less than 5%. Whittembury (1964), using iontophoretic dye injection of carmine in toad skin, found these slightly negative cells in the stratum corneum. However, this study shows that the α cell type is within 7 microns of the mucosal surface which corresponds to the outermost layer of the stratum granulosum termed the first reactive cell layer (Voute and Ussing, 1968).

The second step of the profile shown in this study, the β cell potential type, also corresponds to potentials recorded in earlier studies. Enbaek and Hoshiko (1957) did not observe a highly negative step; however, the profiles they show illustrate that as the

microelectrode advanced 16 microns into the epithelium, the potentials decreased to approximately -60 mV, well within the -48 to -80 mV range of the β cell type. The fractional resistance of the β cell, about 55%, is also similar to fractional resistance values of 59% recorded by Nagel (1976) from the highly negative cells. Dye recovery places this cell type 7 to 17 microns from the mucosal surface of the epithelium. This agrees with the assumption of Helman and Fisher (1977) that the highly negative cells are located in the stratum granulosum.

The third step of the profile is characterized by 5 to 25 mV potentials and is referred to as the γ cell potential type. This cell potential type is much like the step which Engbaek and Hoshiko (1957) assumed was the first legitimate step of the profile. Although Nagel failed to discuss a positive step, the profile illustrated in his study has a positive step of approximately 30 mV with a fractional resistance of 80%. This is near the fractional resistance measurement of 72% which I measured for the γ cell type. Distance measurements of early studies place this cell potential type 16 to 100 microns from the mucosal surface, in the stratum germinativum. This is basically the same area as reported in this paper. Dye recovery shows the γ cell to be 25 to 40 microns from the mucosal surface in the s. spinosum or s. germinativum. Because of the "flare" of dye associated with injected cells of the γ cell type, an argument has arisen that perhaps the microelectrode, and thus the potentials recorded, may be in the extracellular space in this region rather than in the cells (Nagel, Helman; personal communication). If the extracellular spaces are as large in this region of the epithelium as Farquhar and Palade (1964) have observed, one would assume the diffusion of the dye throughout

these spaces would be much more extensive than my photomicrographs show.

The second study of this paper uses horseradish peroxidase (HRP) to determine the relationship between the permeability barrier and the various cell types of the frog skin. Diffusion of HRP from the mucosal side was stopped by a permeability barrier located 10 to 15 microns below the mucosal surface of the epithelium. This is the same region which Farquhar and Palade (1963) report the highest density of tight junctions between the cells of the outermost layer of the s. granulosum. This barrier was also found to be impermeable to concentrated protein solutions (Farquhar and Palade, 1963) and lanthanum (Martinez-Palomo, 1971). HRP diffusion coupled with iontophorectic dye injection shows that the α cell type borders on or is mucosal to this diffusion barrier while the β and γ cell types are serosal to the barrier. This result agrees with Nunes and Lacaz-Vieira (1975) who found the cells with slight negative potentials are mucosal to the large resistance barriers of the frog skin. A possible function of this barrier is to resist the passive paracellular diffusion of the shunt pathway. Since the only cell type in unrestricted contact with the mucosal bathing solution is the α cell type or the reactive cell layer, then the α cell must either play a major role in or is the actual site of active sodium transport in the frog skin. This would also explain the results of current-voltage studies with microelectrodes which found the breakpoint to occur on the mucosal of the highly negative cells rather than the serosal side as the KJU model would predict (Fisher et al., 1980).

Many studies of the frog skin agree with the suggestion that the cells of the reactive cell layer are the site of active transport.

Localization of Na^+-K^+ ATPase finds most of this enzyme on the serosal border of the RCL (Farquhar and Palade, 1966; Rotunno et al, 1966). Voute and Ussing (1968 & 1970) and Voute and Hanni (1973) observed that cells in this layer undergo volume changes with varying transepithelial current while the rest of the epithelial cells remain unchanged. They concluded that a majority of the current must pass through these cells while shunting around the remainder of the epithelium, implying that the RCL is the site of active transport. The morphology of the frog skin also supports this conclusion. Since there are very few tight junctions serosal to the RCL and the extracellular spaces in this region form large "lakes" which open to the basement membrane in 200 Å pores, it is unlikely that any cells in this region are active transport sites. Therefore, with this supportive evidence, the assumption that the RCL is the site of active transport is not unreasonable.

Aside from the possibility of locating the site of active transport, the implications of this study should be obvious. By separating the epithelium into three cell types based on intracellular potentials, this study determines that under open circuit conditions, the frog skin is not a functional syncytium, but is multicompartmental. Also, since no more than three cells were found to contain dye at any given injection site, and no overlap was observed between cell types, one can safely assume that, under open circuit condition, very little if any intracellular coupling occurs. This would also indicate that studies, such as that by Nagel et al. (1981), which only examined the ionic activity in the highly negative cells, are incomplete and must include other cell types, particularly the cells of the reactive cell layer.

SUMMARY AND CONCLUSIONS

The previous literature concerning the electrical potential profile of the frog skin can be divided into two camps based on their interpretations of the profile. However, upon closer examination of the actual potentials recorded, it can be seen that the profiles are essentially the same. The purpose of this study was to correlate the intracellular potentials measured across the frog skin with the various types of cells in the epithelium and to find the relationship between these cells and the permeability barrier.

By using iontophoretic injection of Lucifer Yellow dye, three types of cells were found based on their intracellular potentials. The first cell type encountered as the microelectrode penetrated the epithelium from the mucosal side was referred to as an α cell. This cell was characterized by -4 to -18 mV potentials and was found in the outermost layer of the stratum granulosum, known as the first reactive cell layer (RCL). The second cell type, termed a β cell, was denoted by -48 to -80 mV potentials. This type of cell was found in the remaining cells of the stratum granulosum, serosal to the RCL. The third type of cell, the γ cell, had potentials ranging from 5 to 25 mV. Marked cells of this type were found in the region of the stratum spinosum and stratum germinativum.

The results of the horseradish peroxidase (HRP) diffusion coupled with iontophoretic dye injection show that the α cell type borders on or is mucosal to the permeability barrier. The β and γ cell types are

shown to be serosal to this barrier. This suggests that only the cell has unrestricted contact with the mucosal bathing solution.

In conclusion, these studies demonstrate that:

1. The frog skin epithelium is not a functional syncytium as the Koefoed-Johnsen and Ussing model assumes but is multicompartmental.

2. The permeability barrier is mucosal to all cell types but the α cell. Therefore, this cell type must have a major role in active sodium transport, if it is not the site of active sodium transport.

3. Microelectrode studies which have only examined the highly negative cells of the frog skin are incomplete and must include the other cell types, particularly the α cell type.

4. The basic potential profile, which has been recorded for 25 years, is essentially correct; however, using iontophoretic dye injection, the interpretation of this profile changes. Therefore, microeletrode studies of the active transport of ions in various tissues, particularly multicellular tissues, must be combined with dye injection techniques to accurately evaluate the tissue.

SELECTED BIBLIOGRAPHY

Aceves, J. and D. Erlij. Sodium Transport Across the Isolated Frog Skin. J. Physiol. (Lond.) 212:195-210. 1971.

Adolf, E.F. Exchanges of Water in the Frog. Biol. Rev. 8:224-240. 1933.

Baer, J.E., C.B. Jones, A.S. Spitzer and H.F. Russo. The Potassium-Sparing and Natriuretic Activity of N-amino-3,5 diamino-6-chloro-pyrazinecarboxamide hydrochloride (Amiloride hydrochloride). J. Pharmac. exp. Ther. 157:472-485. 1967.

Benos, D.J., L.J. Mandel and R.S. Balaban. On the Mechanism of the Amiloride-Sodium Entry Site Interaction in Anuran Skin Potential. J. Gen. Physiol. 73:307-326. 1979.

Bentley, P.J. Amiloride: A Potent Inhibitor of Sodium Transport Across the Toad Bladder. J. Physiol. (Lond.) 195:317-330. 1968.

Biber, T.U.L., R.A. Chez and P.F. Curran. Sodium Transport Across Frog Skin at Low External Concentration. J. Gen. Physiol. 49:1161-1176. 1966.

Biber, T.U.L. and P.F. Curran. Direct Measurement of Uptake of Sodium at the Outer Surface of the Frog Skin. J. Gen. Physiol. 56:83-99. 1970.

Biber, T.U.L. The Effects of Changes in Transepithelial Transport on the Uptake of Sodium Across the Outer Surface of the Frog Skin. J. Gen. Physiol. 58:131-144. 1971.

Bonting, S.L. and M.R. Canady. Na^+-K^+ Activated Adenosine Triphosphatase and Sodium Transport in Toad Bladder. Am. J. Physiol. 207:1005-1009. 1964.

Cala, P.M., N. Cogswell and L.J. Mandel. Binding of [^{3}H]ouabain to Split Frog Skins. The Role of Na^+-K^+ ATPase in the Generation of Short Circuit Current. J. Gen. Physiol. 71:347-367. 1978.

Caldwell, P.C. and R.D. Keynes. The Effect of Ouabain on the Efflux of Sodium From a Giant Squid Axon. J. Physiol. (Lond.) 148:8P. 1959.

Cereijido, M. and P.F. Curran. Intracellular Electrical Potentials in Frog Skin. J. Gen. Physiol. 48:543-557. 1965.

Cereijido, M. and C.A. Rotunno. Fluxes and Distribution of Na^+ in the Frog Skin. A New Model. J. Gen. Physiol. 51:280s-289s. 1968.

Civan, M.M. Effects of Active Sodium Transport on Current-Voltage Relationship of Toad Bladder. Am. J. Physiol. 219(1):234-245. 1970.

Engbaek, L. and T. Hoshiko. Electrical Potential Gradients Through Frog Skin. Acta Physiol. Scand. 39:348-355. 1957.

Erlij, D. and M.W. Smith. Sodium Uptake by Frog Skin and Its Modifications by Inhibitors of Transepithelial Sodium Transport. J. Physiol. (Lond.) 228:221-239. 1973.

Farquhar, M.G. and G.E. Palade. Functional Organization of Amphibian Skin. Proc. Nat. Acad. Sci. 51:569-577. 1963.

Farquhar, M.G. and G.E. Palade. Cell Junctions in Amphibian Skin. J. Cell Biol. 26:263-291. 1964.

Farquhar, M.G. and G.E. Palade. Adenosine Triphosphatase Localization in Amphibian Epidermis. J. Cell Biol. 30:359-379. 1966.

Ferreira, K.T.G. The Relationship of K^+ Efflux at the Inner Surface of the Isolated Frog Skin Epithelium to the Short Circuit Current. Biochim. Biophys. Acta 555:13-27. 1979.

Finn, A.L. and M.L. Rockoff. The Kinetics of Sodium Transport in the Toad Bladder. I. Determination of the Transport Pool. J. Gen. Physiol. 57:326-348. 1971.

Finn, A.L. The Kinetics of Sodium Transport in the Toad Bladder. II. Dual Effects of Vasopressin. J. Gen. Physiol. 57:349-362. 1971a.

Finn, A.L. Transepithelial Potential in Toad Urinary Bladder Is Not Due to Ionic Diffusion. Nature 250:495-496. 1974.

Finn, A.L. and S.A. Sutton. The Kinetics of Sodium Transport in the Toad Bladder. III. The Role of Potassium. J. Membrane Biol. 17:253-262. 1974a.

Finn A.L. The Action of Ouabain on Sodium Transport in Toad Urinary Bladder. Evidence for Two Pathways of Sodium Entry. J. Gen. Physiol. 65:503-514. 1975.

Finn A.L. Changing Concepts of Transepithelial Sodium Transport. Physiol. Rev. 56:453-464. 1976.

Fisher, R.S., D. Erlij and S.I. Helman. Intracellular Voltage of Isolated Epithelia of Frog Skin. J. Gen. Physiol. 76:447-453. 1980.

Fuhrman, F. Inhibition of Active Sodium Transport in Isolated Frog Skin. Am. J. Physiol. 171:266-278. 1952.

Helman, S.I. and D.A. Miller. In vitro Techniques for Avoiding Edge Damage in Studies of Frog Skin. Science 173:146-148. 1971.

Helman, S.I. and D.A. Miller. Edge Damage Effect on Measurements of Urea and Sodium Flux in Frog Skin. Am. J. Physiol. 226(5):1198-1203. 1974.

Helman, S.I., R.G. O'Neil and R.S. Fisher. Determination of the E_{Na} of the Frog Skin From Studies of Its Current-Voltage Relationship. Am. J. Physiol. 229(4):947-951. 1975.

Helman, S.I. and R.S. Fisher. Microelectrode Studies of the Active Sodium Transport Pathway of Frog Skin. J. Gen. Physiol. 69:127-134. 1981.

Herrera, F.C. Action of Ouabain on Sodium Transport in the Toad Urinary Bladder. Am. J. Physiol. 210(5):980-986. 1966.

Huf, E.G. Versuche uber den Zusammenhang Zwischen Stoffwechsel, Potentialbildung und Funktion der Froschhaut. Pflugers Arch. ges. Physiol. 235:655-673. 1935.

Huf, E.G., N.S. Doss and J.P. Wills. Effects of Metabolic Inhibitors and Drugs on Ion Transport and Oxygen Consumption in Isolated Frog Skin. J. Gen. Physiol. 41:397-411. 1957.

Huf, E.G. and J.R. Howell. Computer Simulation of Sodium Fluxes in Frog Skin Epidermis. J. Membrane Biol. 15:47-66. 1974.

Huf, E.G., J.R. Howell and P.A. Boswell. Kinetic Studies on the Effects of Ouabain on Na^+ Fluxes in Frog Skin. Pflugers Arch. (in press). 1982.

Kidder, G.W., M. Cereijido and P.F. Curran. Transient Changes in the Electrical Potential Difference Across Frog Skin. Am. J. Physiol. 207:935-961. 1964.

Koefoed-Johnsen, V. The Effect of g-Strophanthin (Ouabain) on the Active Transport of Sodium Through Isolated Frog Skin. Acta Physiol. Scand. 42(Suppl. 145):87-88. 1957.

Koefoed-Johnsen, V. and H.H. Ussing. Nature of the Frog Skin Potential. Acta Physiol. Scand. 42:298-308. 1958.

Krogh, A. Osmotic Regulation in the Frog (R. esculenta) by Active Absorption of Chloride Ions. Skand. Arch. Physiol. 76:60-74. 1937.

Linderholm, H. On Behavior of the "Sodium Pump" in Frog Skin at Various Concentrations of Na Ions in the Solution on the Epithelial Side. Acta Physiol. Scand. 31:36-61. 1954.

Lindley, B.D. and T. Hoshiko. The Effects of Alkali Metal Cations and Common Anions on the Frog Skin Potential. J. Gen. Physiol. 47:749-763. 1964.

Macchia, D.D. and S.I. Helman. Determination of the Shunt Resistance (R_S) and the EMF of the Sodium Pump (E_{Na}) in Frog Skin. Fed. Proc. 33:1252-1274. 1974.

Martinez-Palomo, A., D. Erlij and H. Bracho. Localization of the Permeability Barriers in the Frog Skin Epithelium. J. Cell Biol. 50:277-287. 1971.

Mikulecky, D.C., E.G. Huf, S.R. Thomas. A Network Thermodynamic Approach to Compartmental Analysis. Na^+ Transients in Frog Skin. Biophys. J. 25:87-106. 1979.

Mills, J.W., S.A. Ernst and D.R. DiBona. Localization of Sodium Pump Sites in Frog Skin. J. Cell Biol. 73:88-110. 1977.

Morel, F. and G. Leblanc. Transient Current Changes and Na Compartmentalization in Frog Skin Epithelium. Pflugers Arch. 358:135-157. 1975.

Nagel, W. and A. Dorge. Effect of Amiloride on Sodium Transport in Frog Skin. Pflug. Arch. ges. Physiol. 321:91-103. 1970.

Nagel, W. and A. Dorge. A Study of the Different Na^+ Compartments and the Transepithelial Na^+ Fluxes of the Frog Skin with the Use of Ouabain. Pflugers Arch. 324:267-278. 1971.

Nagel, W. The Intracellular Electrical Potential Profile of the Frog Skin Epithelium. Pflugers Arch. 365:135-143. 1976.

Nagel, W., J.F. Garcia-Diaz and W. M^CD. Armstrong. Intracellular Ionic Activities in Frog Skin. J. Membrane Biol. 61:127-134. 1981.

Nunes, M.A. and F. Lacaz-Vieira. Negative Potential Levels in the Outer Layer of the Toad Skin. J. Membrane Biol. 24:161-175. 1975.

O'Neil, R.G. and S.I. Helman. Influence of Vasopressin and Amiloride on Shunt Pathways of Frog Skin. Am. J. Physiol. 231(1):164-173. 1976.

Ottosen, D., F. Sjostrand, S. Stenstrom and G. Svaetichen. Micro-electrode Studies on the EMF of the Frog Skin Related to Electron Microscopy of the Dermo-Epidermal Junction. Acta Physiol. Scand. 29(Suppl. 106):611-624. 1953.

Rawlin, F., L. Maten, F. Fragachan and G. Whittembury. Isolated Toad Skin Epithelium: Transport Characteristics. Pflugers Arch. 316:64-80. 1970.

Reuss, L. and A.L. Finn. Dependence of Serosal Membrane Potential on Mucosal Membrane Potential in Toad Urinary Bladder. Biophys. J. 15:71-75. 1975.

Rotunno, C.A., M.I. Pouchan and M. Cereijido. Location of the Mechanism of Active Transport of Sodium Across Frog Skin. Nature 210:597-599. 1966.

Salako, L.A. and A.J. Smith. Changes in Sodium Pool and Kinetics of Sodium Transport in Frog Skin Produced by Amiloride. Brit. J. Pharmacol. 39:99-112. 1970.

Schatzmann, J.J. Herzglycoside als Hommstoffe fur den Aktiven Kalium and Natrium Transport Durch die Erythrocytenmembran. Helv. Physiol. Acta 11:346-354. 1953.

Shultz, S.G. Homocellular Regulatory Mechanisms in Na Transporting Epithelia: Avoidance of Extinction by "Flush-Through". Am. J. Physiol. 241:F579-F590. 1981.

Skou, J.C. The Influence of Cations on Adenosine Triphosphatase From Peripheral Nerves. Biochim. et Biophys. Acta 23:394-403. 1957.

Ussing, H.H. The Active Ion Transport Through the Isolated Frog Skin in Light of Tracer Studies. Acta Physiol. Scand. 17:1-37. 1949.

Ussing, H.H. and K. Zerahn. Active Transport of Sodium as the Source of the Electric Current in the Short Circuited Isolated Frog Skin. Acta Physiol. Scand. 23:110-127. 1951.

Ussing, H.H. and E.E. Windhager. Nature of the Shunt Path and Active Sodium Transport Path Through Frog Skin Epithelium. Acta Physiol. Scand. 61:484-504. 1964.

Voute, C.L. An Electron Microscope Study of the Skin of the Frog (R. pipiens). J. Ultrastructure Res. 9:497-510. 1963.

Voute, C.L. and H.H. Ussing. Some Morphological Aspects of Active Sodium Transport. The Epithelium of the Frog Skin. J. Cell Biol. 36:625-642. 1968.

Voute, C.L. and H.H. Ussing. The Morphological Aspects of the Shunt Path in the Epithelium of the Frog Skin. Exp. Cell Res. 61:133-151. 1970.

Voute, C.L. and S. Hanni. Relation Between Structure and Function in Frog Skin. In: Transport Mechanisms in Epithelia. Alfred Benzon Symposium V. H.H. Ussing and N.A. Thorn (eds.) Munksgaard, Copenhagen. 1973.

Whittembury, G. Electrical Potential Profile of the Toad Skin Epithelium. J. Gen. Physiol. 47:795-808. 1964.

Zerahn, K. Nature and Localization of the Sodium Pool During Active Transport in Isolated Frog Skin. Acta Physiol. Scand. 77:272-281. 1969.

VITA 2

Randall L. Duncan

Candidate for the Degree of

Doctor of Philosophy

Thesis: IDENTIFICATION OF THE VARIOUS CELL POTENTIAL TYPES AND THEIR RELATIONSHIP TO PERMEABILITY BARRIERS IN THE FROG SKIN

Major Field: Physiological Sciences

Biographical:

Personal Data: Born August 31, 1955, at Hutchinson, Kansas, the son of Maurice and Agnes Duncan. Married, January 8, 1978, to Mary Elizabeth (Beth).

Education: Graduated from Haven High School, Haven, Kansas, in May, 1973; received the Bachelor of Science degree in Biology and Chemistry from Southwestern College in May, 1977; received Master of Science from Oklahoma State University in December, 1980; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in May, 1983.

Professional Experience: Undergraduate Teaching Assistant, Department of Biology, Southwestern College, Winfield, Kansas, from January, 1975 through May, 1977, teaching Comparative Anatomy; Graduate Teaching Assistant, Department of Physiological Sciences, Oklahoma State University, September, 1978 through May 1979 and September 1979 through December 1980, teaching General Physiology. Graduate Teaching Associate, Department of Physiological Sciences, Oklahoma State University, January 1981 through May, 1982, teaching Veterinary Physiology, Cell and Comparative Physiology and Mammalian Physiology.