QUANTITATIVE ANALYSIS OF THE LOCATION AND DISTRIBUTION OF INTERCELLULAR JUNCTIONS IN FROG SKIN

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER V

CHAPTER I

General Introduction

Epithelia are sheets of organized layers of cells that cover surfaces and line cavities of living organisms. Epithelia separate the internal from the external environment and regulate the exchange of specific substances between the organism and its surroundings.

The epidermis of frog skin is a classic stratified squamous epithelium which has been used extensively as an experimental tool for studying many aspects of the salt and water transport across asymetric biological membranes (eg. Reid, 1892; Huf, 1935; Krogh, 1938; Ussing, 1949; Koefoed-Johnsen and Ussing, 1958; Herrera, 1971; Erlij and Ussing, 1978; Macknight,et al., 1980; Civan, 1983). Frog skin epithelium actively transports Na ions from the mucosal (pond) side to the serosal (blood) side of the skin (Krogh, 1938; Ussing, 1949). It is generally accepted that this transepithelial sodium transport is a two step process that occurs across two barriers: a passive entry of Na ions across the sodium-selective amiloride-sensitive apical membrane, and an active extrusion of sodium ions across the potassium-selective, ouabain-sensitive basolateral membranes of the epithelium

(Koefoed-Johnsen and Ussing,1958). The cellular compartment that is located between the two barriers is usually termed the sodium transport compartment.

One of the most studied, yet unresolved problems of the active sodium transport in frog skin is the determination of the sizes and locations of the sodium-containing compartment that participates in the transepithelial sodium transport.

Historically, the cellular transport compartment was ascribed to: the cells of stratum germinativum (Koefoed-Johnsen and Ussing, 1958), to a small fraction of the total population of the epithelial cells (see Erlij and Ussing, 1978), to a specific single layer of cells (the first reacting cell layer) provided with an active transport mechanism (Voute and Ussing, 1968 and 1970), or to all cells in the epithelium (Ussing and Windhager, 1964; Biber,et al., 1966; Farquhar and Palade, 1966). In recnt years, many lines of evidence have been accumulated to suggest that the cells of all epithelial layers are engaged in the Na-transport process forming a syncytial sodium-transport compartment (Helman, 1979; Nagel, et al., 1981; Rick,et al., 1984). However, the morphological basis for such cell-to cell coupling in this epithelium has not been established.

Epithelial cells are joined to each other by junctional complexes that serve different functions (Loewenstein,1966, 1981). Thus,the morphological investigation of intercellular junctions in a sodium-transporting epithelium like that of frog skin is exeedingly important in relation to: (1) the

determination of the magnitude of cell-to cell coupling by means of gap junctions which are widely known to be the main route through which small water-soluable molecules can be exchanged between the cells (2) the identification and localization of the physiological permeability barrier confined to the presence of tight junctions, and (3) the understanding of the functional role of the different cell layers of the epithelium.

The general organization and fine structure of the frog skin epithelium have been previously described (Voute, 1963; Parakkal and Matoltsy, 1964; Farquhar and Palade, 1964 and 1965; Dewey and Barr, 1964 ; Carrasso,et al., 1971). Two types of intercellular junctions (tight junctions and desmosomes) have been identified and fully described in this epithelium (Farquhar and Palade, 1965), and a third type ("nexus") was introduced (Dewey and Barr, 1964). However, the results of these studies do not seem to be conclusive because: (1) the morphological basis for cell-to cell coupling has not been seriously investigated, (2) other techniques beside the classical thin section methods like freeze-fracture technique for example, have not been employed for the morphological studies on this tissue, (3) a comprehensive evaluation of the location and distribution of the intercellular junctions have not been quantitavely established. and (4) the precise localization of the physiological permeability barrier and the relative contribution of the lamellar bodies to this barrier have not been

determined with certainity.

The failure to detect classical gap junctions among the cells of frog skin epithelium (Farquhar and Palade, 1965; Martinez-Palomo, et al., 1971) is difficult to reconcile with the syncytial nature of this tissue. One possible reason for the lack of gap junctions in this epithelium might be due to the fact that this tissue has junctional complexes that are functionally, but not morphologically similar to gap junctions. Another possibility is that the fixation procedures used so far is not adequate to reveal gap junctions in this epithelium.

In this study I have used different electron microscopic and immunofluorescence techniques in an attempt to study the location and frequency of the intercellular junctions in frog skin epithelium. The thin section and freeze-fracture data revealed the presence of some junctional complexes with criteria that are similar to those of gap junctions recognized in other tissues. Furthermore, the immunofluorescence technique shows that antibodies raised against rat liver gap junctions cross-react with special areas of the frog skin epithelial membranes, confirming the presence of classical gap junctions in this epithelium. I have also reported the presence of some lamellar structures in the intercellular space of the epithelium and within the epithelial cells. Finally, I have provided a comparative quantitative analysis of the location and frequency of all the intercellular junctions in the different layers of the frog skin epithelium.

CHAPTER II

REVIEW OF LITERATURE

Frog Skin As An Experimental Object

Among other visionary statements Krogh's lecture (Krogh, 1929) which was delivered on the occasion of the thirteenth International Physiological Congress at Boston included the following:

> " .. For a large number of problems there will be some animal of choice, or a few such animals, on which it can be most conveniently studied."

Based on this prescient remark of Krogh, Krebs (1965) introduced the basis for " the August Krogh Principle",by giving a few examples of plant and animal species which have served as favorable model systems for studying different aspects of certain problems. In this sense, amphibian skin and to the same extent bladders have been conveniently utilized as a useful in-vitro preparation for studying the general properties and the different mechanisms of salt and water

transport across asymetric biological membranes, especially polar epithelia.

A. Why Frog Skin ?

Maintaining a constant ionic environment is an urgent necessity not only for mammals, but also for all other forms of life. Kidneys in vertebrates are the major controlling site of electrolyte and water "traffic" between the internal and the external environment (reabsorption and excretion of salt and water). However most amphibians, due to some deficiency in the function of their kidneys (Adolf, 1931), have to rely on other sources to conserve salt and water. Therefore, they are able to reabsorb sodium and water through their skins. Indeed it has been long appreciated that frog skin is able to regulate water intake (Townson,1799). Since then a great deal of experimental work has been carried out on this epithelial tissue to study not only salt and water transport, but also to gain some insights into the molecular basis of the bioelectric phenomena recognized in all living cells.

B. Advantages of Using Frog Skin

Amphibian skin is characterized by a number of unique features that render it as one of the most privileged biological models for the study of transport physiology across asymetric epithelial cells: First and above all, frog skin is a salt-transporting organ (Krogh, 1938) which possesses

the capability of actively transporting sodium ions from the external environment towards the serosal side of the epithelium. Second, this tissue responds to the common hormonal regulators of the transport process, by showing an increase or decrease in the rate of ionic movement across the epithelium. This response is similar to that of the distal nephron and collecting tubules of mammalian kidneys (Herriera,1971). Third,the skin is loosely attached to the frog body and it can be easily removed and manipulated with minimum damage to the tissue (Kidder, 1973). Fourth, this tissue and the associated experimental preparations are readily available and relatively inexpensive. Fifth, the skin can be easily and rapidly cut into pieces, some of which can be mounted as a flat unstirred sheet of tissue between two bulks of solutions, while other pieces of the same skin can be treated as living controls. Sixth, such experimental preparation permits the investigator to define precisely the composition of the media bathing the two surfaces of the epithelium (mucosal and serosal side). Seventh, the isolated frog skin epithelium is able to retain "normal" transporting activities for many hours under in-vitro conditions. For example, it exhibits spontaneous electrical potential difference which is usually associated with the ionic movement and gradient established by the active reabsorption of sodium from the mucosal to the serosal side of the epithelium (Ussing,1949). Eighth, the electric parameters such as the electrical potential difference, resistance, and the ionic current can

be easily measured and uniformly manipulated throughout the entire epithelial sheet.

C. Some Anatomical Problems

Frog skin, however is still far from being a simple idealized model for studying Na-active transport (see Kidder, 1973). This tissue is comprised of an epidermis made of a stratified squamous epithelium which rests on a continuous basement membrane that separates the epithelial cells from the underlying corium. The corium represents about 80% of the total volume of the skin (Erlij and Ussing, 1978), and contains mucous glands,blood vessels, melanocytes,and other types of cells all dispersed in a thick mat of loose connective tissues. Furthermore, the epidermis which is believed to be the sole site of sodium active transport is comprised of four strata organized in 6-9 cell layers. These four strata are: stratum corneum (St.Co), stratum granulosum (St. Gr), stratum spinosum (St.Sp), and stratum germinativum (St.Ge) (see chapter III). The cells of different strata apparently have a slightly different morphology and are interlocked with each other by specific intercellular junctions that differ in their structure and intensity from one layer to the other (Shahin and Blankemeyer, 1986). Different cell types which have different transport properties such as mitochondria-rich cells (MRC) are frequently encountered among frog skin epithelium (Farquhar and Palade, 1965; Lavker, 1971; Whitear, 1975; Ehrenfeld, 1976).

This anatomical complexity of frog skin has retarded a clear understanding of the ionic transport mechanisms across such epithelia, and complicated our interpretation of the experimental results especially those associated with the determination of the route of ionic movement and the localization of the transport "pool" (Erlij and Ussing, 1978). To overcome this complexity, many investigators (Skjelvate, et al., 1960; Fishman and Macey, 1968; Aceves and Erlij, 1971; Caraso,et al., 1971; Fisher,et al., 1980) have successfully managed to separate the epithelium from the underlaying corium. The isolated epithelium seems to have the same electrophysiological properties that the whole skin has (Aceves and Erlij, 1971). Another approach to avoid using the whole skin was achieved by making the determinations (e.g. ionic concentration of the epithelial cells) on slices of skin cut parallel to the surface of the skin with a freezing microtome (Hansen and Zerahn, 1964).

Helman and Fisher (1977a), while attempting to assess the mechanisms of sodium entry into the Na-transporting pool, were able to isolate sheets of intact stratum corneum up to 14 cm. in area by incubating the skin overnight in Ringer solution containing aldosterone.

The contribution of specific cell types to the process of Na-transport or to the osmoregulatory function of this tissue can be studied separately by investigating the morphological and physiological characteristics that differentiate one type of cell from another. For example, carbonic

anhydrase activity was demonstrated only in one specific type of cells called mitochondria rich cells (MRC) (Rosen and Friedly, 1973). This raises the possibility that these cells may have different transport properties from the rest of the epithelial cells. It's also well established that aldosterone which stimulates active epithelial Na-transport in frog skin has the ability to promote morphobioelectric changes in the above mentioned type of cells (Voute et al., 1969, Voute et al., 1975b).

It should be emphasized that these attempts were not trivial simplifications of the original highly complex model; they have served instead a reasonable purpose for improving the accuracy of our interpretations regarding the determination of the size of the transport compartment and the estimation of intracellular Na and K activities and concentration. Nonetheless, the morphological complexity of the isolated epithelium is still a chronic problem wich has complicated our interpretation of the experimental results and retarded us from unequivocally understanding whether only a single layer of cells is responsible for the transport properties of the epithelium, or whether the whole epithelium functions as a syncytium (See Civan, 1983).

Historical Background

A great deal of knowledge concerning both salt-water metabolism and Na-active transport across frog skin epithelium has been accumulated over a period of several decades of this century. It's very difficult to account for every single event in the history of this field; however the following review is merely an attempt to summarize some of the landmarks in the history of this study as found in the available literature.

A. The Early Literature

More than one hundred and eighty-five years ago, Townson recognized that the skin of certain amphibia played a significant role in regulating water intake for these animals (Townson, 1799). The electical properties of frog skin were studied as far back as 1848 by DuBois-Reymond, who first observed that the isolated frog skin was able to generate and maintain a potential difference between its mucosal and serosal sides, the serosal side being around 100 mv positive, relative to the mucosal side. Galeoti (1904,1907, quoted by Erlij and Ussing,1978) reported that the maintenance of this potential difference required sodium or lithium in the media. Later in 1937 Dean and Gatty provided a detailed study on the electrical properties of frog skin with many references to the early literature (see Dean and Gatty, 1937).

In 1892, Reid devised the first double flux chamber to study fluid transport across certain epithelia, especially frog skin. Bathing the skin between two identical saline solutions, Reid reported a net fluid transport " a few μl./ cm^2/h " in the inward direction from mucosal to serosal side of the frog skin (Reid, 1892, 1901); however, not knowing that the primary event here is the Na active transport and that water movement follows the osmotic gradient generated by ion transport, he failed to offer a convincing explanation for this "fluid tansport phenomenon" (see Huf, 1979). In a series of articles on the movement of water across epithelium membranes, Adolph was able to demonstrate that freshly isolated frog skins gain or lose water in proportion to the square root of time elapsed after immersion, and this "gain" or "loss" was linearly related to the sodium concentration in the media (Adolph, 1931, 1933).

During the 1930's, Huf studied Reid's experiments carefully (for a review, see Huf,1979) and confirmed many of his observations and conclusions. He (Huf, 1935, 1936; quoted by Huf, 1979) was the first to show that the isolated frog skin in contact with Ringer solution on both sides was able to transport hypertonic salt solution from the mucosal to the serosal side. Because analyzing for Na at his time was a "tedious procedure", Huf only measured changes in chloride ion concentration in the solutions of the two sides of the skin. Nonetheless, he accurately assumed that the tissue actively transported sodium chloride from the outer side to the

inner side of the skin. He also stated that this active salt transport ceased when cyanide was added to the bathing solution. Shortly afterward, Krogh (1937) demonstrated that salt-depleted frogs were capable of taking up appreciable quantities of chloride ions through the skin, even from a very diluted solution of KCl and CaCl2 solution in exchange for bicarbonates (Krogh, 1937b). He concluded that Cl ions were the ions which were actively transported across frog skin. Although this conclusion has turned out to be correct only for one or two species of frogs (Zadunaisky,et al., 1963; Martin and Curran, 1966), it prompted further investigation which proved to be very fruitful.

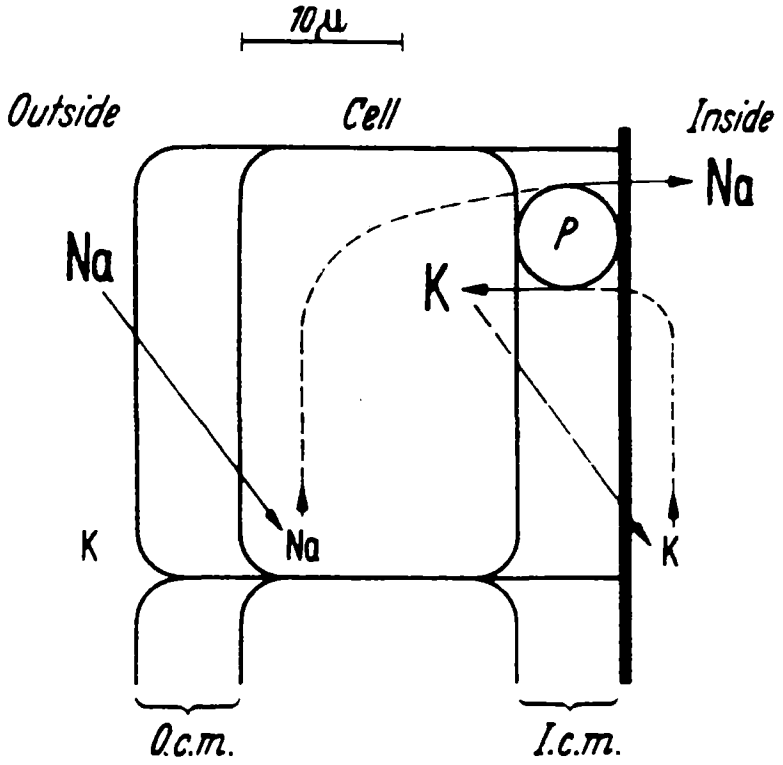
The use of radioisotopically labeled ions to measure the ionic permeability of isolated frog skin was introduced by Katzin (1939, 1940) who reported that the rate of Na influx exceeded that of Na back flux across skin bathed in normal saline solution. Ussing (1949, a and b), utilizing the same radioactive isotope technique found that isolated frog skin could actively transport sodium against a steep electrochemical concentration gradient, but passively move chloride in the same direction. The Cl is driven by the transepithelial potential difference across this tissue (Koefed-Johnsen, et al., 1952). By combining isotope flux measurements with the electrical current measurement on the short circuited skins, Ussing and Zerahn (1951) established for the first time the equality between the short circuit current and the net Na-active transport across the frog skin. Because of its

simplicity and practicality, this technique has become a standard method for measuring active transepithelial Na current. It should be mentioned however, that in some species of frogs, (ie. Leptodacrylus Ocellatus) other ions like Cl contribute to the total current flow across the skin (see Zadunaisky,et al., 1963; Martin and Curran, 1966).

B. Koefoed-Johnsen and Ussing model (KJU Model)

In 1958 Koefoed-Johnsen and Ussing introduced the "three compartment" model for explaining the transepithelial sodium transport across frog skin. This model has marked the beginning of a new era in the field of epithelial transport. According to this model (Fig.1), The transport of sodium ions across the epithelium involves a two-step process across two different barriers placed in a series with each other. Briefly, sodium ions passively enter the epithelial cells through the highly Na-selective amiloride- sensitive apical membranes and actively leave the cells through the K-selective ouabain-sensitive basolateral membranes which posses Na-K ATPase that facilitates the forced exchange of Na ions against K ions (Na-K pump). Because of its simplicity and practicality, this model has received a great deal of attention and soon became the conceptual framework that has guided investigators working in the field of epithelial transport for many years (Macknight, et al., 1980; Schultz,1983).

Figure 1. The Koefoed-Johnsen and Ussing three compartment model for explaining the transepithelial Na-transport across frog skin. O.C.M. and I.C.M. are the outer (Mucosal or apical) and the inner (basolateral or serosal) cell membranes respectively. P is the Na-K exchange pump which was assumed to be electrically neutral. This model was proposed to illustrate the origin of frog skin potential. It depicts that the outer membrane is selectively but passively permeable to Na and the inner membrane is selectively but passively permeable to K. Thus, the total potential across the skin is the result of the summation of the two diffusion potential Na and across the outer and the inner cellular membranes respectively. (After Koefoed-Johnsen and Ussing, 1958).


10μ
Outside
Cell
Inside
Na
P
Na
K
K
Na
K
O.c.m.
I.c.m.

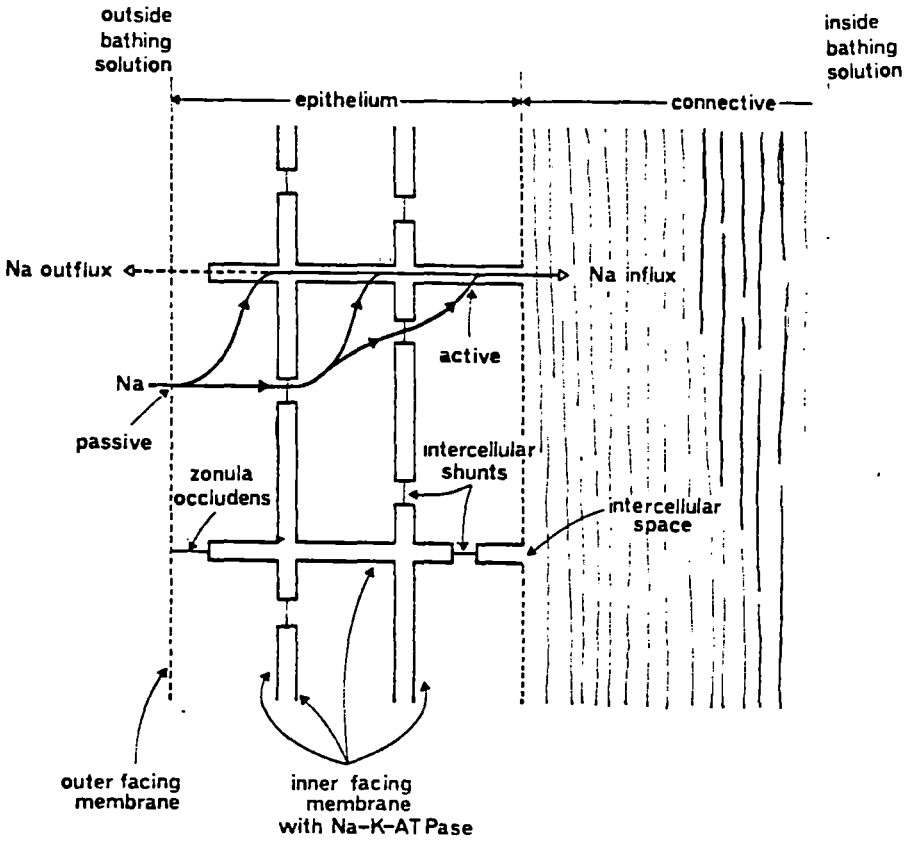
C. Revision of (KJU) Model

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

One of the first revisions of (KJU) model came from Ussing and Windhager (1964) who discovered in addition to the usual transcellular pathway, a paracellular shunt pathway in which specific ions proceeded between the cells instead of passing through the cell membranes. According to Ussing and Windhager (Fig.2), sodium ions which diffuse across the apical membranes of the skin, are conducted from cell to cell through some low-resistance intercellular junctions towards the basal layer of the epithelium, where an active mechanism (Na-K pump) transports them into the inside bathing solution. However, there might be some (pumps) in the cellular membranes of the stratum spinosum (St.Sp) which can pump sodium ions into the intercellular space; these sodium ions then become available for free communication with the inside bathing solution (Ussing and Windhager, 1964).

Thus, this new model (fig.2) introduced three elements to the original (KJU) model: 1) A possible paracellular pathway

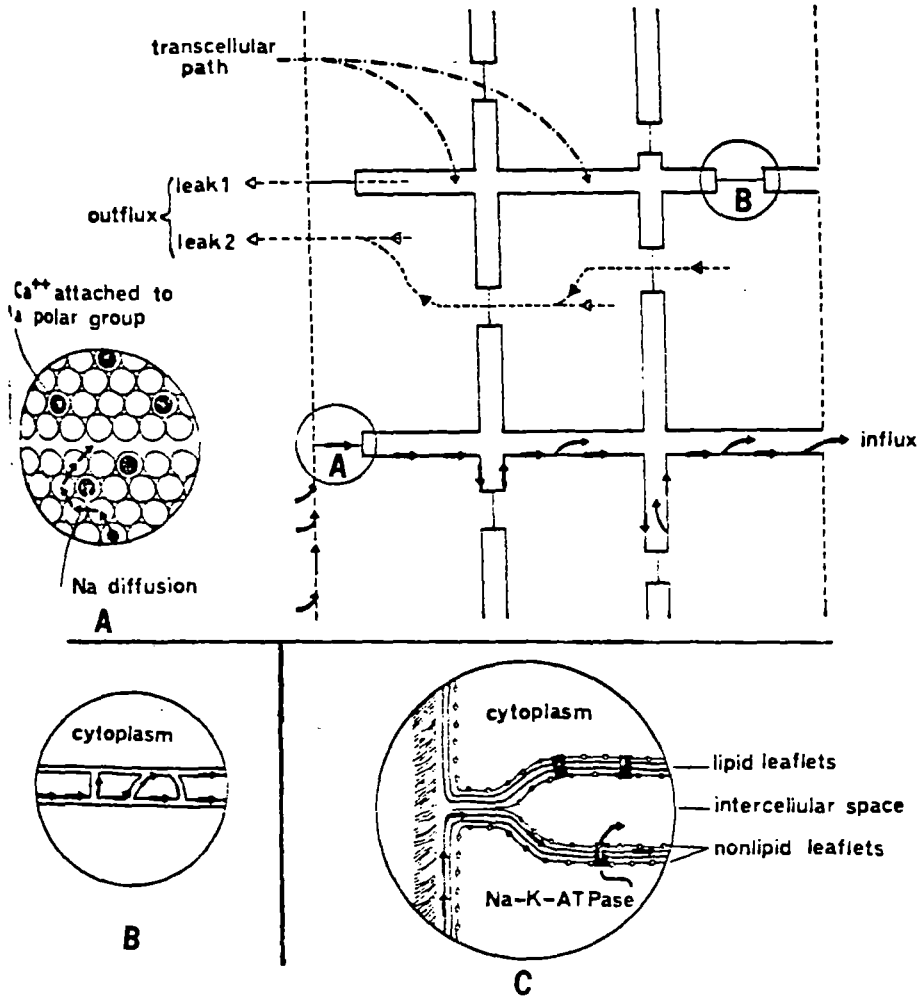
Figure 2. A schematic diagram of the Ussing and Windhager model for describing the movement of Na ions across frog skin. According to this model, Na ions passively enter the outer facing membrane into the cells of the outermost layer of the epithelium. The sodium ions then diffuse from cell to cell through specific intercellular junctions which have much higher permeability to Na than cell membrane proper. The basolateral membranes of the stratum germinativum (basal layer) and stratum spinosum (intermediate layer) represent potential sites for the active step of the Na-transport across the skin. Na ions which are actively transported from the cells of stratum spinosum into the intercellular space become freely available to the inside bathing solution. (After Ussing and Windhager, 1964).


outside
bathing
solution
inside
bathing
solution
epithelium
connective
Na outflux
Na influx
active
Na
passive
intercellular
shunts
zonula
occludens
intercellular
space
outer facing
membrane
inner facing
membrane
with Na-K-AT Pase

for Na and other ions. 2) Probable extensive cell-to cell coupling between the cells of all epithelial layers. 3) The possibility that the distribution of Na-K pumps is not restricted only to the cells of stratum germinativum (St.Ge) as was originally proposed by the (KJU) model. In fact many investigators have recently supported the notion that the ouabain-inhibitable Na-K ATP-ase are localized to the basolateral membranes of all living cells of frog skin epithelium (Farquhar and Palade, 1966; Mills, et al., 1977; DiBona and Mills, 1979).

Cereijido and Rotunno (1968) proposed a new model for the Na-transepithelial transport across frog skin epithelium in which the transepithelial movement of Na occurs as a result of sodium movement around but not through the cells. This model (fig.3) suggested that sodium ions, after being attached to the lipid leaflet of the outer facing membranes, had the tendency to travel around the cells by jumping from one fixed polar site to another in a triplet saltatory mechanism rather than penetrating the cell membranes. This route for sodium ion movement seemed to represent a lower energy barrier (see Cereijido and Rotunno, 1968; Cereijido, et al., 1974). However, sodium ions had to pass the diffusion barrier for saltatory movement formed by tight junctions and desmosomes at the outermost layer of stratum granulosum (St.Gr) in order to continue their saltatory movement towards the serosal side of the basal cells where an active transport mechanism usually pumps

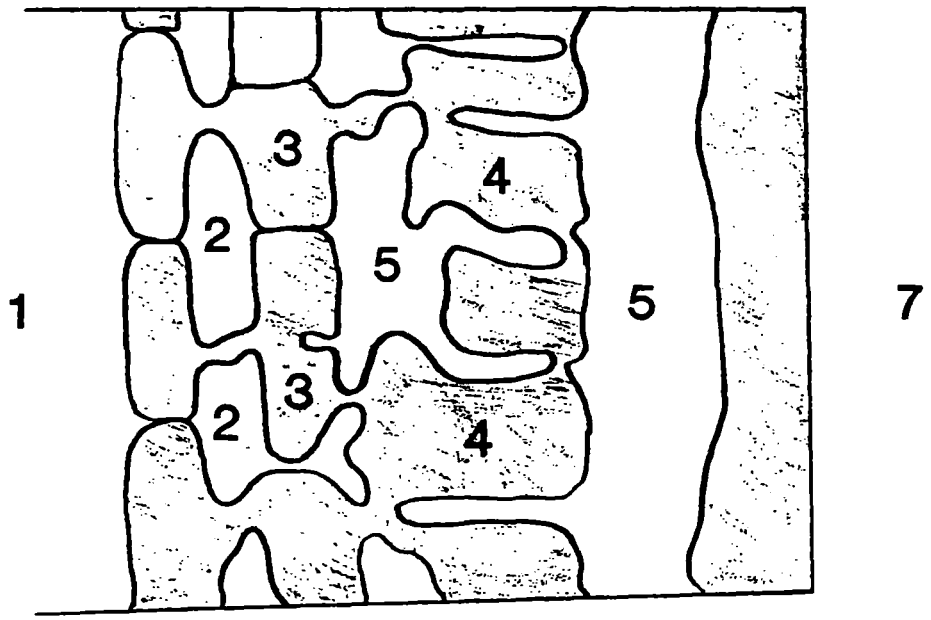
Figure 3. The Cereijido and Rotunno model for explaning the distribution and movement of Na ions across frog skin epithelium. This model favors an intramembraneous route for the Na ion movement rather than the usual trans-cellular route (see text). Circle A illustrates the jumping movement of Na ions from a fixed charge to another of the epithelial membranes; this path can be blocked by another ions such as calcium. Circle B shows that Na ions can cross from one cell membrane to another by diffusing on the extracellular side of the intercellular bridges. Circle C depicts the locations of the Na pumps. These pumps activly translocate Na ions into the intercellular space. (After Cereijido and Rutnno,1978).


transcellular path
leak1
outflux
leak2
B
Ca++ attached to polar group
A
influx
Na diffusion
A
cytoplasm
B
cytoplasm
lipid leaflets
intercellular space
nonlipid leaflets
Na-K-ATPase
C

sodium ions into the inside bathing solution.

The model proposed by Huf and Howell (1974) treated frog skin as a multi-compartmental tissue which consisted of 7 compartments (Fig.4). Huf and Howel tested their model using computer simulation compared with previously published data. All the simulations found that the fraction of the Na influx pool size was much lower (7-20%) than that of the efflux pool size (93-80%). Thus, they concluded that this compartmentalized tissue had at least two types of Na-K transporting functions: the transepithelial transport compartment which correspond to compartment 3 and the maintenance compartment which correspond to compartment 4 (see Fig.4). Furthermore, amiloride which decreases the net flux of Na ions across the tissue and the size of sodium pool exchangable with the mucosal side, has little effect on the size of sodium pool exchangable with the serosal side (Nagel and Dorge, 1970). This observation suggested to Huf and Howel that there must be a pretransport pool in the epithelium. They stated that compartment 2 was likely to be the pretransport compartment. Compartment 5 is the sodium found in the extracellular space of all cells below the permeability barrier of the epithelium, and compartment 6 is the bound sodium in the entire epithelium. In all simulations, Huf and Howel found that the sodium concentration was the highest in compartment 4 (the maintenance compartment) and the lowest in compartment 3 (the transepithelial transport compartment). This finding suggests that the sodium entry across the apical membrane

Figure 4. The Huf and Howell multicompartmental model for explaning the Na-transepithelial transport across frog skin. This model depects that the transepithelial movement of Na across frog skin involves seven compartments. Compartment 1 and 7 correspond to the mucosal and serosal bathing solution respectivly. Compartment 2 is the extracellular volume between thecells of (St.Co) and the outermost layer of (St.Gr) cells. Compartment 3 represents the intracellular volume of the first layer of (St.Gr), while compartment 4 represents the intracellular volume of the remaining epithelial cells. Compartment 5 is the volume of the intracellular space serosal to the permeability barrier created by the tight junctions of (St.Gr). Compartment 6 (not shown) is the non-exchangable Na in the epithelium. This model was tested by using computer simulation. (see the text for details about the mechanisms which this model operates). (After Huf and Howell, 1974).


1
2
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2
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4
7

occurs by a simple diffusion which agrees with the original (KJU) model.

Finn (1976) using kinetic and electrophysiological data, seriously questioned the (KJU) model stating that this model "was no longer adequate to explain the transepithelial potential difference in this type of epithelia " Finn (1976, P. 463). However, Finn did not propose any alternative model; he offered instead, some criteria for which any new model for studying Na-active transport in tight epithelia must have (Finn, 1976). Shortly afterwards, Nagel (1977), using new microelectrode techniques studied the dependency of electrical potential differences across the membranes of frog skin epithelium upon the concentration of sodium ions in the mucosal solution, and he obtained data which was not in accordance with the (KJU) model. He stated;

> "Neither the polarity of the postulated Na electrode at the outer border could be obtained, nor the expected slope of the dependency between potential gradient and [Na] of the mucosal bathing solution" Nagel (1977, P.777).

D. The Sodium Transport Compartment

The localization of the Na-transport compartment in this multilayered type of epithelium and the determination whether this transport compartment constitutes the whole population of the epithelial cells or just a small fraction of them, had been under intensive investigation during the 1970's. The experimental data concerning these issues during recent years

has established at least two trends for explaining the mode of Na-active transport and the site of its occurence.

The first trend implicates that the first reacting cellular layer (the stratum granulosm) represents the main body of the Na transport compartment. This trend is supported by Voute and Ussing (1968), Voute and Hanni (1973), and Voute et. al. (1975) who tried to correlate between different states of Na transport and the morphological changes in the epithelium at light and electron microscopic levels. Their data showed that the morpholgical changes in response to different physiological treatments of the skin were restricted to the first reacting cell layer. Thus, they assumed that this layer of cells was the main site of Na active transport. Some radiochemical analysis of the whole skin (Cereijido and Rotunno, 1967; Dorge and Nagel, 1970; Nagel and Dorge,1970 and 1971) and isolated epithelia (Aceves and Erlij, 1971) favored this view by showing that only a fraction of cellular Na was exchangeable with the outer bathing medium, however they did not attribute this fractional Na-compartment to a specific layer. It should be mentioned however,that recent data (Schultz,1983 indicated that most previously reported chemical analysis markedly overestimated the true value of cellular Na. Thus, the observations and conclusions based on chemical analysis and extracellular markers could have been misleading. Finally, the presence of tight junctions between the cells of stratum granulosm excludes the possibility of paracellular movement of sodium through this stratum. Therefore, the movement of Na

through the cellular membranes of (St.Gr) must be assumed. However, whether the cells of stratum granulosum are able to carry out the active transport of all the coming sodium , or deliver some of it to the cells of underlying layers is still an open question.

The second trend depicts that the cells of all epithelial layers share equally in the transport of sodium. In support of this notion, Farquhar and Palade (1966), using histochemical methods to localize ATP-ase activity in frog skin and Mills, et al. (1977) and DiBona and Mills (1979) using a variety of autoradiographic techniques on a wide spectrum of epithelia, including frog skin, obtained data suggesting a homogeneous distribution of the Ouabain-sensitive ATPase on the basolateral membranes of all living cells of frog skin epithelium, which indicated to these investigators that all cell membranes facing the intercellular space might be potential sites of active transepithelial Na transport.

In recent years the applications of some advanced methods and techniques have yielded many observations that lent support to this trend. For example, Dorge, et.al.,(1974); Rick, et al., (1978); Rick et al., (1984) based on electron microprobe analysis data, and Nagel, (1976); Helman and Fisher, (1977); Fisher et al., (1980); Nagel et al., (1981) based on electrophysiological data, showed that the cells of all layers of frog skin epithelia responded uniformally and homogeneously to many physiological conditions that affect the transepithelial movement of sodium ions, which indicated to them that all

cells of this epithelium were linked together as a functional syncytium. One serious drawback to this theory is the fact that the morphological studies based on electron microscopic techniques (Farquahr and Palade, 1965; Martinez-Palomo et al., 1971;) have not revealed a homogeneous distribution of gap junctions between the cells of all the layers of the epithelium.

Experimental Preparations and Methodology

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

The chambered preparations originally introduced by Du-Bois Remond (1848) and recently established as a routine technique by Ussing (1949) have facilitated various kinds of in-vitro studies on epithelial transport. A number of studies have used frog skin as a simple, but useful "black box" device which has served as an example of epithelium that performs both passive and active transport (Elij and

Ussing, 1978).

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

A. The Morphological approaches

Morphologic information concerning the general organization and fine structure of the frog skin epithelium has been previously reported (see section III). Some aspects of the correlation between the structure of this epithelia and its transporting function has been investigated by Voute and his collaborators (Voute,and Ussing, 1968,1970; Voute and Hanni, 1973; Voute et al, 1975a) who found that changing the rate of Na-active transport was accompanied by a number of morphological features (ie. changes in cell volume, presence of vacuoles, and "scalloped sacs") that were restricted only to the first reactive cell layer. Detailed electron microscopic analysis of this tissue has revealed various types of intercellular junctions (Farquhar and Palade, 1965), some of which (tight junctions) play a significant role in the determination of the permeability barrier of the

epithelium (Martinez-Palomo,et al., 1971a), while some others (desmosomes) function as cohesive elements that mechanically connect the cells to each other to protect the physical integrity of the whole tissue. The morpholocial basis for the electrical coupling between neighboring cells by way of gap junctions (see Loewenstein, 1966) has not been fully investigated in frog skin epithelium. The existence of such junctions in this tissue may provide an important transport pathway which serves to integrate the whole epithelium into one transporting unit. A qualitative and quantitative study of the intercelluar junctions in frog skin epithelia and the frequency of their distribution is provided in chapter IV of this text.

Generally, it has been clear that the morphologial approaches to studying epithelial transport have provided us with fundamental information about the characteristics of the transport cells. Some of these characteristics are:

1. The epithelial transporting cells have a highly folded plasma membrane, fuzzy coat on the apical side, numerous mitochondria and small particles on the inside surface of the membrane (Oschman, 1978).
2. The epithelial cells in most cases are not a homogeneous population of identical cells. Instead, they exhibit cellular specificity in response to various physiological conditions. For example, aldosterone stimulates some morphological changes in only one type of cell in frog skin epithelium, namely mitochondria-rich cells (Voute,et al.,

1969; Voute, et al., 1972; Voute, et al., 1975b).

3. By examining the cell interior, it is possible to indicate different subcellular organelles which may accumulate solutes at different rates (Lindemann and Voute, 1976; Oschman, 1978). This compartmentalization of the cell interior may stimulate the notion that one or more subcellular compartments may contribute to the conductive pathway of transcellular Na-transport (see for example, Voute, et al., 1975a).
4. The structural basis for the transepithelial movement of solutes and water may assume two routes: through the cell (transcellular pathway), and between the cells (paracellular pathway).
5. The rate limiting barrier for the transcellular pathway is the plasma membrane and that of the paracellular pathway is the apical tight junctions (Martinez-Palomo, et al., 1971a).
6. There seems to be an inverse relationship between the number of sealing strands of tight junctions as revealed by freeze fracture technique and the permeability of the paracelluar pathway (Claude and Goodenough, 1973). However, this might not always be the case (Martinez-Palomo and Erlij, 1975) emphasizing the fact that other factors may play significant roles in the determination of epithelial permeability.

It should be mentioned however, that one serious drawback of the morphological approach is the fact that its

methodology involves many chemical processes (ie. fixation, dehydration, etc...) which may alter the natural state of the tissue or interfere with the delicate interrelationships so crucial to the normal function. Nonetheless, the recent advances in the field of immunocytology, histochemistry, electron-probe x-ray analysis and intracellular-dye injection and mass spectrometry which heavily rely on morphological information have enhanced the applications of these approaches to the sudies of epithelial transport.

B. Black Box Approaches

When mounted between two identical Ringer solutions, The epithelium of frog skin is able to:

1. Generate high spontaneous potential difference (up to 120 mv.) between the mucosal and serosal side of the epithlium; the serosal side being positive in relation to the mucosal side.
2. Exhibit a transepithelial resistance of several thousands Ohms per cm (Erlij, 1976).
3. Transport a net current of sodium from the pond side towards the serosal side of the skin against a steep electrochemical gradient (Ussing, 1949).

In order to characterize the transport mechanism(s) operating on such a system, it was useful or even necessary to treat this tissue in a pragmatic manner as a "black box membrane" with certain transepithelial electrical parameters (Erlij and Ussing,1978). For example, the introduction of the short

circuit technique by Ussing and Zerahn (1951) has been of great technical and practical importance to determine the manner by which Na ions are transported from the pond side into the blood stream of the frog. The concept of this technique is based on the fact that in the absence of all thermodynamic driving forces across the isolated frog skin bathed between two identical Ringer solutions, only ions which are actively transported can cross the epithelium in the net transport sense. This condition can be easily achieved by equalizing all factors that affect the thermodynamic movement of ions between the two external aqueous phases bathing the skin such as chemical concentration, pressure, temperature.. .etc, and then bring the potential difference generated by the tissue to O. Under such conditions, Ussing and Zerahn (1951) reported that the net unidirectional (mucosal to serosal) flux of Na ions, when expressed in the same units, is equal to the short circuit current across the skin. In frog skin, this short circuit current is highly sensitive to the lack of oxygen in the media, and to the introduction of metabolic inhibitors into the bathing solution (Huf,et al., 1957). This knowledge provided the basis to define the process of Na-transepithelial movement across frog skin as an active step which is highly dependent on metabolic energy.

In consort with the electrical parameter measurements, many other measurements can be obtained from the chambered preparations of frog sin. A very well known example of such a "black box" approach is the study which attempted to

correlate between the changes in electrical potential across frog skin epithelium and different ionic compositions of the bathing solutions. This study has yielded the famous three-compartment model for Na-transepithelial transport formalized by Koefoed-Jhonsen and Ussing (1958). This model (fig.1) visualizes the movement of Na ions across the epithelium as a two step process;the first involves the passive entry of sodium into the epithelial cells across a highly Na-selective apical membrane and down its electrochemical gradient, the second step involves the active extrusion of sodium ions across the K-selective basolateral membranes via an ATP-utilizing Na-K pump. Thus, the final results of this model is two fold; a net transepithelial Na-transport from the pond side of the skin into the blood stream, and a tight regulation of the intracellular composition of electrolytes, mainly Na and K ions. Since it was proposed, this model has been successfully applied to many other epithelia especially those classified as tight epithelia such as urinary bladders, distal convoluted tubules, ducts of rabbit salivary gland (Ussing et al., 1974).

The black box approaches however, can't assess the precise role of each membrane in the transport process; the matter which necessitated the introduction and development of many new methods that brought about some new insights into the process of understanding the molecular mechanisms of the sodium-active transport across each barrier of the asymetric epithelial membranes.

C. Some Advances in the Methodology

Both morphological and "black box" approaches have yielded useful information which has led to: (1) Outline the polar properties of the epithelial tissue, (2) detect two possible pathways for the transepithelial movement of salt and water across the tissue; paracellular and transcellular, and (3) characterize the mechanisms by which some chemicals and hormones affect the process of transport (Civan, 1983a). However, the intrinsic limitation of these approaches can't be ignored, especially when the ultimate aim of studying the epithelial transport is to understand its mechanisms at the molecular level. In recent years, new advanced approaches and techniques have been employed to gain some insight into the microscopic mechanisms of epithelial transport. An exhaustive list of these modern techniques and their applications to the epithelial transport studies is beyond the scope of this review; the following are merely some examples.

The recent advances in electrical methodology such as the use of conventional and ion-selective microelectrodes (see Nagel, 1976,1978; Helman and Fisher, 1977; Fisher et al., 1981; Nagel et al., 1981; Civan, 1983) have enabled us to focus on specific defined areas of the transporting epithelial tissue, and characterize its individual properties. Furthermore, some other "electrical" approaches such as the studies of current-voltage relations (Fuchs et al., 1977;

Delong and Civan, 1984), impedence analysis (Clausen and Dixon, 1984), and fluctuation analysis (Hoshiko, 1984) have provided very useful data to address questions such as:"Does sodium entry across the apical membrane occur by a channel or carrier mechanism?", " How do hormones affect the transport rate?", and "Does the increase in Na transport in response to (ADH) occur by increasing the number or density of active channels or by simply increasing the conductivity of single channels?". (see Li et al., 1982, Li,and Lindeman, 1983, DeLong and Civan, 1984).

Electron probe x-ray analysis techniques (Civan, 1983a) have been useful in localizing and quantifying the intracellular and extracellular elements by analyzing the x-rays emitted by a piece of tissue irradiated with a beam of electrons. Applications of this technique to a highly suitable tissue like frog skin has yielded valuable information in regard to the determination of the intracelluar concentration of Na+, k+ and Cl- and the changes in these concentrations in response to different conditions that affect epithelial transport (Dorge, et al., 1974, Rick et al., 1978; Rick et al., 1984).

The studies of the intracelluar fluorescently labeled dye injections and mass spectometry have enlightened us about the relation between structure and function in epithelia (Blankemeyer et al., 1984; Duncan, 1982) and provided a colorful and meaningful picture of the concept of cell-to cell coupling by means of gap junctions (see Loewenstein,

1981; Cereijido et al., 1984; Warner and Lawrence, 1982).

The introduction of nuclear magnetic resonance spectroscopy (NMR) as a non-invasive technique to the study of epithelial transport has provided additional valuable information about the close interrelationships between the molecular conformation of the intracellular microenvironment and the transport of certain ions. This technique can also be used to monitor noninvasively the concentration, turnover, and transport of many biologically important metabolites and ions, and to measure the degree of mobility and immobility (bound vs. free) of different intracellular elements such as Na+, k+, etc... (for reviews see Civan, 1983a and b; Balaban, 1983).

Finally, the combination of immunological methods with biochemical and structural analysis offers an advanced and promising approach to the understanding of the physiological regulation of epithelial transport. For example, considerable efforts have been devoted recently to develop some immunological probes against Na+,k+ ATPase (an enzyme that is preferentially located on the basolateral surface of the transporting epithelial cells), and many attempts to biosynthesize the subunits of this enzyme have been reported (see Rossier, 1983). These attempts have already resulted in valuable information for the process of studying the possible sites of the action of some hormones or chemicals (aldosterone, corticosteroids, ADH, etc.) that affect transepithelial transport. The development of specific antibodies against gap

junctions (Hertzberg and Skibbens, 1984) has been very useful for the determination of the site and distribution of such important junctions in various epithelial tissues (see chapter V).

Electrical Properties and Microelectrode Studies on Frog Skin Epithelium

A. General Considerations

It has long been known that the epithelium of frog skin is characterized by certain electrophysiological parameters (ie. transepithelial potential difference and resistance.) which can be used to study the behavior of the epithelial membranes in relation to the ionic mobilities across such asymetric systems (DuBois-Remond, 1848; Dean and Gatty, 1937). Electrophysiological studies on frog skin epithelium have been largely concerned with the DC properties of this tissue (Koefoed-Johnsen and Ussing, 1958). This approach involves the determination of currents, electrical potential differences, and electromotive forces which are related by the electrical resistances or conductances of the diffrent barriers of the epithelium (Schultz, 1979). These electrical measurements along with the applications of the equivalent circuit analysis have proved useful for the characterization of the sites and the mechanisms through which certain ionic species are translocated across the epithelial membranes (see Helman, 1979).

The isolated "undamaged" frog skin bathed symmetrically between two identical solutions of ordinary Ringer's is able to generate and maintain a spontaneous transepithelial potential in the vicinity of 100 mv. This potential difference (PD) is oriented serosal side positive with respect to the mucosal side. The source of this potential difference may be attributed to the active transport of sodium (Ussing and Zerahn, 1951) or to the summation of two simple diffusion potentials across the outer (Na diffusion potential) and the inner (K diffusion potential) barriers of frog skin epithelium (Koefoed-Johnsen and Ussing, 1958). The open circuit transepithelial resistance (RT) of the frog skin epithelium ranges between 3K and 25K Ohms/cm^2 (Erlij, 1976; Helman and Miller, 1971). The source of this high (RT) is most likely to be the very developed tight junctions that are confined to the apical layers of the epithelium (Erlij and Martinez-Palomo, 1978).

The magnitude of these electrical parameters is highly dependent on the composition of the Ringer slution bathing the skin. For example, when the chloride ions of the bathing solution are replaced by non-penetrating sulphate ions, the transepithelial potential difference increases to a maximum of 150 mv. (Engbaek and Hoshiko, 1957). Under similar conditions, the skin exhibits an elevated (RT) of 34.4K Ohms/cm^2. (Erlij,1976). However, the reported values of the electrical parameters of amphibian skin vary considerably among different laboratories. These variations have been ascribed to

the edge damage effects resulted from the crushing of the skin edges between hard surfaces (Dobson and Kidder, 1968). It has been suggested also, that the low open-circuit potentials (PD) and resistance (RT) reported by some investigators could have resulted from a generalized damage to the tissue during the process of dissecting and/or mounting the skin on the chambered preparations (Finn and Hutton, 1974).

Regardless of the source of damage to the epithelial tissue, there is a strong possibility that such damage introduces a sizable noncellular low-resistance pathway for the movement of ions across the amphibian epithelium (Macknight, et al., 1980). To minimize the effect of such problems,techniques such as the use of inert tissue adhesive (Helman and Miller, 1971), the use of silicon grease (Erlij, 1976), or even specific modification in the size and design of the chambers being used (Dobson and Kidder, 1968; Erlij, 1976) have been advised.

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

B. Equivalent Electrical Circuit of the Epithelium

The simplest equivalent circuit used to describe the

electrophysiologic events associated with the transepithelial Na transport across frog skin was proposed by Ussing and Zerahn (1951). This circuit (fig.5) consists of a voltage-independent resistor (R Na), connected in series with the electromotive force (ENa) which is responsible for transporting a net current of Na ions (I Na) across the tissue. In parallel with these elements lies the resistor for the shunt pathway (Rp) which represents the imbeddance of the tissue to the passive movement of ions. When both the mucosal and serosal surfaces of the skin are bathed in the same Ringer solution, different Ohm's law-derived relationships can be easily obtained from this model (see Macknight et al., 1980):

The open circuit potential difference is

$$PD = ENa * Rp\ (RNa + Rp)$$

The total tissue resistance is

$$TR = (RNa*Rp) / (RNa + Rp)$$

When we reduce the current flowing through the shunt pathwhay to zero by short-circuiting the skin, the total short circuited current of Na can be easily obtained

$$Isc = ENa / RNa$$

This model is appropriate to provide adequate "black box" information about the electrical events which accompany the transepithelial sodium transport across the whole tissue. However,This model does not provide specific knowledge about the properties of the individual cell membranes, the paracellular shunt, or about the cell interior. Furthermore,

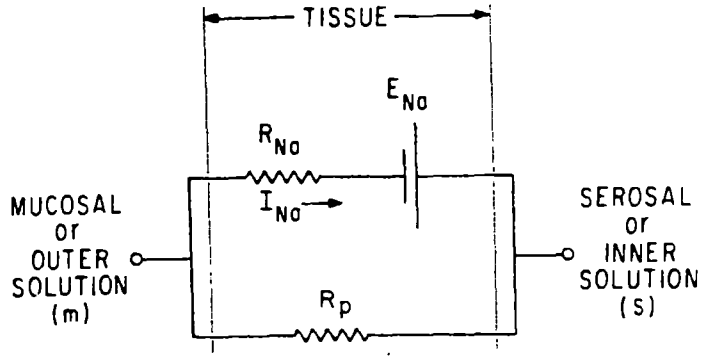
this model cannot accomodate microelectrode studies which have been proved very useful in characterizing the electrical properties of individual membranes or compartments (see Macknight et al., 1980). Therefore, more elaborate electrical circuits have been introduced to describe the electrophysiological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

An example of an electrical equivalent circuit that takes into consideration the electrophysiological events that may occur at the different barriers of the epithelium is shown on (figs.6,7). Fig.6 depects an equivelent circuit model for the Na-transepithelial transport across frog skin. This model describes the electrical events that occur across the apical and the basolateral membranes of the transporting (A) and non-transporting (B) cells. For the transporting cell (Circuit A), the apical or the outer membrane is highly permeable to Na. The Na current (INa) thus, crosses this barrier passively through its conductance (G^{o}_{Na}), and then actively traverses the basolateral mbrane via the Na-K pump. The electrogenic pump (See Helman, et al., 1979) is modeled with an electromotive fource (E pump), in series with its equivalent conductance (g pump). In parallel with the pump, there is the conductive channels for K (I_k) consisting of the conductance (g_K) and the potassium Nernst potential (E_K). Under these conditions, the short circuit current (I_{sc}) represents the net sodium-transport across the epithelium. For

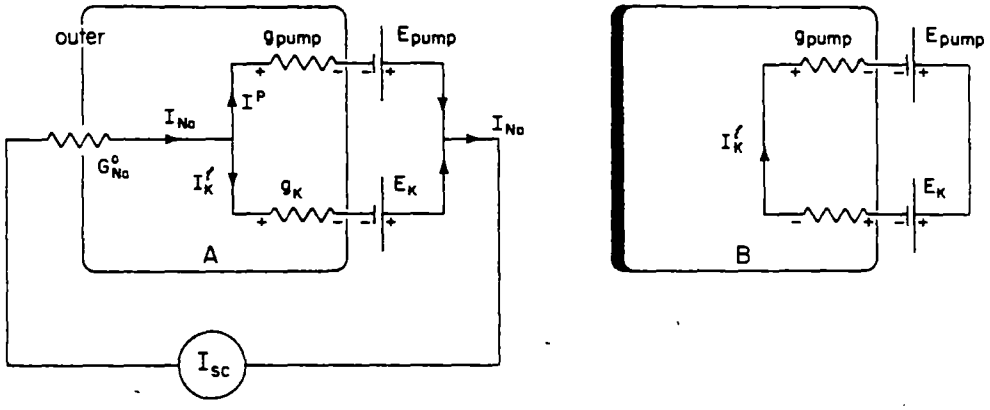
Figure 5. Simple electrical equivalent circuit for the transepithelial sodium transport across frog skin. The circuit consists of the active transcellular pathway, in parallel with the passive extracellular or shunt pathway. The active pathway is comprized of a resistance (R Na), in series with a battery that represents an operational electromotive force for sodium transport (E Na). The passive pathway include a resistance (R p) that represents the total resistance of the para cellular elements of the tissue to the passive movement of ions (After Ussing and Zerahn, 1951).

Figure 6. An electrical equivalent circuit model for the Na-transepithelial transport across frog skin, reproduced from Helman, 1979). This model describes the electrical events that occur across the apical and the basolateral membranes of the transporting (A) and non-transporting (B) cells. For more details seetext.

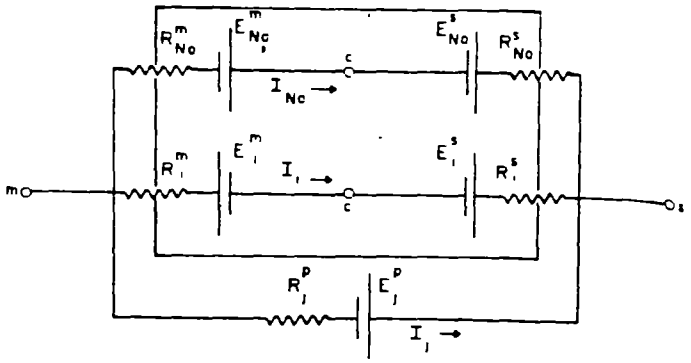
Figure 7. An electrical equivalent circuit model for the Na-transporting epithelial tissue, proposed by Schultz (1977). This model involves the movement of sodium ions and other ions across three functionally different barriers: the mucosal (m) and serosal (s) membranes which are arranged in series, and a parallel paracellular (p) shunt pathway which is parallel to the mucosal and serosal barrier. See text for more details.


TISSUE
E_{Na}
R_{Na}
I_{Na}
MUCOSAL or OUTER SOLUTION (m)
SEROSAL or INNER SOLUTION (s)
R_p

5


outer
g_{pump}
E_{pump}
I^P
I_{Na}
G^o_{Na}
I^f_K
g_K
E_K
A
I_{sc}
B

6


R^m_{Na}
E^m_{Na}
E^s_{Na}
R^s_{Na}
I_{Na}
R^m_l
E^m_l
I_l
E^s_l
R^s_l
m
s
R^p_l
E^p_l

7

the non-transporting cell (circuit B), the apical membrane (shown by a dark line) is rendered impermeable to Na by adding amiloride to the mucosal bathing solution or by making the outer bathing solution Na-free. Under these conditions, the conductance of the apical membrane (G^{o}_{Na}) is reduced to zero, the short-circuit current (I_{sc}) across the tissue is zero, and the voltage of the inner barrier is dependent on the conductances (g pump) and (g_K) and the electromotive forces (E pump) and (E_K). (For more details refer to Helman, 1979; Helman,et al., 1979).

Fig. 7 shows an equivalent circuit model for the Na-transporting epithelia, proposed by Schultz (1977). This model involves the movement of ions across three functionally different barriers: The mucosal (m) and serosal (s) membranes arranged in series, and the parallel paracellular (p) shunt pathway. The apical entry of the transcellular current of Na (I_{Na}) is driven by the electromotive force (E^{m}_{Na}) across the voltage independent resistor (R^{m}_{Na}) of the outer barrier. The basolateral extrusion of (I_{Na}) from the cell (c) into the inner bathing solution, is similarilly driven by the electromotive force (E^{s}_{Na}) across the voltage-independent resistor (R^{s}_{Na}) of the inner barrier. (I_i) represents the transcellular currents of all ions other than sodium that traverse the mucosal and basolateral membranes of the epithelium in a similar manner to that of Na ions. (I_J) represents the currents of all ions including Na which passively proceed through the extracellular pathway

of the epithelium. (For more details, see Schultz, 1979).

C. Microelectrode Studies

Despite the fact that microelectrode artifacts are a well-documented phenomenon, particularly in the epithelial tissue (Lindemann, 1975; Nelson et al., 1978), many investigators have utilized microelectrode data to gain some insight into the process of Na active transport across tight epithelia. The advantage of using a microelectrode lies in the fact that this technique enables us to impale a single cell and record its intracellular potential, or more accurately the potential of the membrane between the microelectrode tip and a point of reference. The potential profile is the recording of the voltage changes across the various barriers (membranes) encountered by the microelectrode as it is advanced through the epithelium. The resistance of the membrane located between the microelectrode tip and the mucosal bathing solution (Ro) can be easily calculated according to the Ohm's law:

$$Ro = (PDsc - PDoc)/ Isc$$

where:

PDsc is the intraepithelial potential under short-circuit condition PDoc is the potential difference between the epithelial bathing solution and the tip of the microelectrode under open-circuit conditions. Isc is the transepithelial short-circuit current.

The transepithelial resistance (TR) can be made known from

the transepithelial potential difference (PD) across the tissue and the total electrical current under short-circuit conditions (Isc). Thus, the ratio Ro/TR indicates the fraction of the resistance penetrated by the microelectrode and is usually termed as the fractional resistance ratio (Nagel, 1976). This ratio, which is usually expressed as a percentage of the total (TR) can be determined for every microelectrode position across the epithelium. For example, a fractional resistance of zero would mean that the microelectrode is in the mucosal bathing solution, while a fractional resistance of 100% would mean that the microelectrode had penetrated the entire thickness of the epithelium.

Historically, several microelectrode investigations have been carried out to measure the electrical potential profiles of frog skin epithelium and study their implications on the transepithelial Na transport in such tight epithelium (Ottoson,et al., 1953; Engbaek and Hoshiko, 1957; Ussing and Windhager, 1964; Whittenburg, 1964; Cereijido and Curran, 1965; Biber et al.,1966; Biber and Curran, 1970; Rawlins, et al., 1970; Hvid-Larsen, 1973; Nagel, 1975, 1976; Helmann and Fisher, 1977; Fisher et al; 1980; Duncan, 1982). The pattern of the electrical profile resulted from these studies is generally formed by steps; however the number, magnitude and location of these steps vary considerably from one study to another.

Ottoson, et al. (1953), using an ordinary Cl-containing Ringer solution as a bathing solution,recorded one electrical

potential jump when the microelectrode was advanced gradually from the mucosal side towards the serosal side of the skin. By using distance measurements and injection of carmine dye, they concluded that the location of the electrical jump was at the basement membrane of the epithelial tissue.

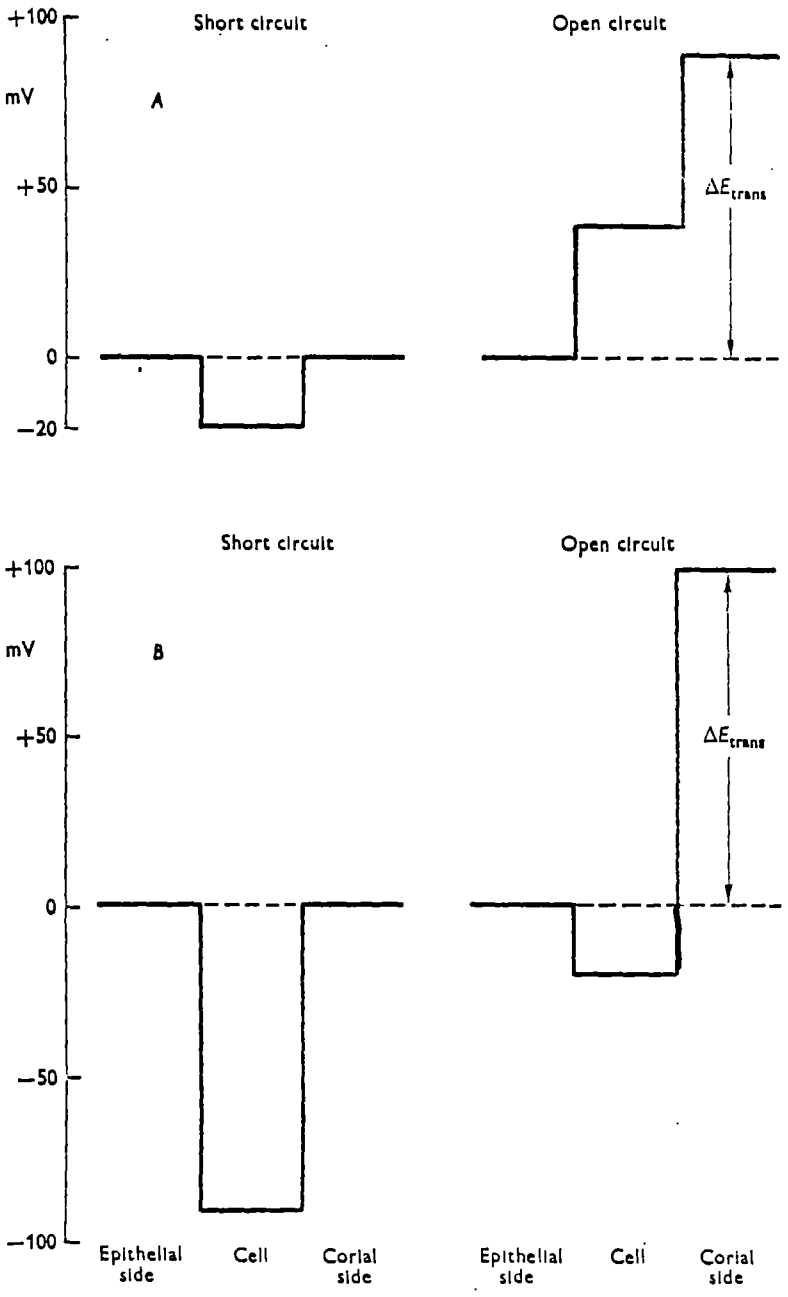
Engbaek and Hoshiko, (1957) reported that the electrical potential profile of the amphibian skin epithelium was comprised of at least two steps that were of equal magnitude and positive signs with respect to the outer bathing solution. These two steps proceeded in the same direction and summated to give the total transmural potential difference of the frog skin (see Fig.8). This pattern of potential profile is in perfect agreement with the (KJU) model for the two-membrane theory (Koefed-Johnson and Ussing, 1958) which predicts that the electrical potential difference across the frog skin epithelium is a result of the summation of the two diffusion potentials; the Na diffusion potential across the apical membrane and the K-diffusion potential across the basolateral membrane. However, it should be mentioned that Engbaek and Hoshiko discarded a negative potential difference of 4-60 mv, which was encountered every time the microelectrode tip hit the surface of the skin and penetrated into the stratum corneum. They attributed this negative potential to the membranes of "non-living cells" of the stratum corneum. They also failed to determine precisely the location of the first and second postulated jumps on the epithelium. Their indirect method of distance measurements on histological sections of

the skin was only sufficient to loosely suggest that the first step was localized at "some depth" within the epidermis, and the second was localized at the boundary between the epithlelium and the corium.

Since 1957, the electrical potential profile of amphibian skin has been recorded repeatedly (Ussing and Windhager,1964; Whittenburg, 1964; Cereijido and Curran, 1965; Biber et al., 1966; Biber and Curran, 1970; Rawlins et al., 1970; Hvid-Larsen, 1973). The result of these investigations generally supported and confirmed Engbaek-Hoshiko data and yielded an open-circuit potential profile pattern that was comprised of two positive potential steps or more that sum together the total electrical potential difference across the frog skin (fig.8). However, they disagreed on the concept of the localization of these steps inside the epithelia.

Recently, the potential profile of frog skin epithelium was re-investigated using improved techniques (see Nagel, 1975, 1976; Helmann and Fisher, 1977; Fisher et al., 1980) and a completely different pattern for the potential profile was reported. According to Nagel's experimental results (Nagel, 1976) the potential profile under open-circuit conditions was demonstrated to be trough-like instead of the two step potential pattern reported by previous investigations (see Fig.8). Thus, Nagel concluded that the earlier intracellular (PD) measurements propably resulted from more or less injured cells. Under short-circuit conditions, Nagel found that the intracellular (PD) of all epithelial cells

Figure 8. A Schematic presentation of two different patterns of the potential profile of amphibian skin epithelium. Pattern A summarizes data obtained before 1975 (see text), and pattern B illustrate recent observations by Nagel,(1976); Helman and Fisher, (1977). Under open-cicuit conditions, pattern A potential profile is comprized of two steps of equal magnitude and positive sign which summate together to give the total transepithelial potential difference (E trans). Pattern B however, shows a potential profile of one negative step that averages around -20 mv. Under short-circuit conditions, both patterens give negative intracellular potential; around -20 mv. for patteren A, and near -90 mv. for pattern B. (After Nagel, 1979).


+100
mV
+50
0
−20
Short circuit
A
Open circuit
ΔE_{trans}
+100
mV
+50
0
−50
−100
Short circuit
B
Open circuit
ΔE_{trans}
Epithelial side
Cell
Corial side
Epithelial side
Cell
Corial side

which were located beyond the stratum corneum were negative, with values that ranged between -60 and -100 mv. However, he mentioned that the deeper layers of the epithelium exhibited more negative potentials than the upper layers and the fractional resistance also increased progressively as the microelectrode was advanced towards the serosal side of the epithelia. Nagel also observed that either amiloride or the reduction of Na concentration in the mucosal bathing solution increased the intracellular negative potentials and thus the resistance by 20-40% .

Helmann and Fisher (1977) and Fisher et al., (1980) obtained data that was in perfect agreement with Nagel's results regarding the potential profile of the frog skin. However, Fisher et al., 1980 used a different approach to obtain their measurements. They impaled the isolated epithelia from either side of the skin; the stratum corneum or the stratum germanativum. They found that regardless of the routes of cell punctures, the intracellular voltage of the short-circuited isolated epithelia was markedly negative, averaging -70 mv. for the apical punctures and - 91 mv. for the basolateral punctures. Thus, they suggested that the cells of frog skin epithelium were electrically coupled as a functional syncytium.

The unstable negative potential resulting from the penetration of stratum corneum was a common feature in most microelectrode studies on frog skin (Engbaek and Hoshiko, 1975; Ussing and Windhager,1964; Cereijido and Curran, 1965;

Nagel, 1976). Nonetheless all microelectrode studies discarded and ignored this potential step, assuming that it may correspond to the intracellular (PD) of the non-living cells of stratum corneum.

More recently, Duncan (1982) using lucifer-yellow dye to correlate the various cell types in frog skin with their intracellular potentials, found that the electrical potential profile was comprised of three types of potentials that corresponded to different layers of the skin. The first potential was obtained from the cells that were directly located adjacent to the stratum corneum, and amounted to -4 to -18 mv, the second was detected from stratum granulosm cells and amounted to -48 to -80 mv., and the third cellular potential was that of the basal and intermediate layers of the skin, which amounted to 5-25 mv. Furthermore, Duncan (1982) showed that the dye injection in the first or the second cell type did not spread out to the neighboring cells, while the dye injections in the third type of cells, (stratum spinosm and stratum germinativum cells) resulted in widespread diffusion of the dye in the basal and intermediate regions of the epithelium. He concluded that the epithelial cells were compartmentalized rather than coupled together as a functional syncytium.

In summary, most microelectrode studies suggest that the the cells of all epithelial layers of the frog skin are electrically coupled. However, one drawback of this conclusion is the fact that both the intracellular dye injection data

(Duncan, 1982) and the morphological studies of the intercellular junctions in this tissue (Farquahr and Palade, 1965; Martinez-Palomo, et al., 1971; Shahin and Blankemeyer, 1986) did not show many gap junctions between the cells of stratum granulosm to couple them to each other or to the cells of the underlaying strata.

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

A. General Properties

It has been apparent, since Koefoed-Johnsen and Ussing introduced their "double membrane" model for the transepithelial sodium transport across frog skin in 1958, that the apical membrane of this epithelium is highly selective to Na. Complete replacement of the mucosal Na by other cations suggests that only lithium ions (Li) can be transported across frog skin (Benos,et al., 1980). However, when mucosal Li was substituted for Na an irreversible decrease in the transepithelial potential of the skin was observed (Lindley and Hoshiko, 1964). This effect of Li might be due to the fact that cellular Li promotes an unspecific permeability increase of the paracellular pathway of the epithelium to Na and K (Aboulafia, 1983).

The anionic composition of the mucosal medium is also known to modify the magnitude of the open-circuit potential

across the frog skin which in turn affects the apical membrane permeability to Na. For example, the addition of non-penetrating anions such as SO4, I, and NO3 to the mucosal bathing solution produes a higher potential difference across the skin than the addition of the CL or Br which are easily "shunted" across the epithelium (Koefoed-Johnsen and Ussing, 1958). The sequential effects of some of these anions on the (PD) as reported by Lindley and Hoshiko(1964) are as follows; SO4 > I > Cl > Br, with the mucosal Br producing the lowest voltage. However,the significance of this anionic substitution on the selectivity of the apical membrane to Na, and the mechanisms by which each of these anion affect this selectivity are not well established (see Civan, 1983a).

B. The Apical Entry of Sodium Ions

Recent determinations of the electrical potential profile across the apical membrane of the frog skin (Nagel, 1976; Helman and Fisher, 1977) imply that the Na-entry to the cell is a passive consequence of the driving force provided by the electrochemical potential gradient for this ion. The apical membranes of the tight epithelia such as frog skin could be studied separately by depolarizing the basolateral membranes with high K serosal activities (Fuchs et al., 1977). Under this condition, the electrical measurments of the transepithelial voltage and currents reflect the electrophysiological events that occur across the apical barrier. More direct measurements of the electrical parameters that characterize

the behavior of the apical membrane are also feasible by impaling split skin with microelectrodes and examining the current-voltage (I_V) relationships at extremely early points in the time after initiating pulses of constant transepithelial voltage (DeLong and Civan, 1984). In both depolarized (Fuchs et al.,1977) and undepolarized (DeLong and Civan,1984) preparations of the frog skin, the studies of the (I-V) relationships of the amiloride-inhibitable apical entry of Na ions across frog skin and other Na-transporting epithelia (Schultz, 1983) provide a resonably good fit with the predictions of the Goldman equation for the flux of a single cation. This indicates that the Na-entry into the epithelial cells occurs by an electrodiffusion mechanism through homogeneous channels. Consistent with this notion, are the results of current fluctuation analysis studies on frog skin epithelium (Lindemann and Driessche, 1977) which recover a high single site turnover number of Na channels, suggesting that the Na specific membrane channels are pores, and the Na-entry across the apical membrane is due to a simple diffusion process rather than to a carrier-mediated mechanism. Further support to this conclusion have been reported by other investigators who used various approaches (Van Driessche and Lindemann, 1979; Li, et al., 1982; Palmer, et al., 1982; Benos, et al., 1983).

However, the short circuit current or net Na transport across frog skin epithelium is not a simple linear function of the mucosal sodium concentration (Kirschner,1955; Lindley

and Hoshiko, 1964). This relationship exhibits a curvilinear pattern instead. This means that the short circuit current increases as mucosal Na concentration increases at first, but when the concentration reaches a certain value, the transepithelial Na current levels off (Macknight et al., 1980). Several neurotropic compounds such as curare, local anaesetics, atropine, pilocarpine, and others (for a review see Herrera, 1971) reversibly increase Na transport across frog skin by increasing the Na permeability of the apical membrane. The application of such agents on the mucosal side of the frog skin causes also an increase in the Na-K pump activities, which suggests that the curvilinear relationship between the net Na current and the mucosal Na concentration is not a result of the saturation of the Na pumping activities at the basolateral side of the epithelium. Thus, it's most likely that the process responsible for such saturation operates only at the level of the apical membrane (Macknight et al., 1980; Civan, 1983a). Nontheless, the mechanism(s) that underlies this apparent saturation of the entry step of Na is still not Known. One possibility is a diffusional process modified by self-inhibition of the apical membrane permeability as Na concentration increases either in the mucosal medium (Erlij and Smith, 1973) or in the cells (Cuthbert and Shum, 1977; Shultz, 1981, 1983).

The direction of the net Na movement across frog skin is basically from the pond side to the blood side of the skin. Flux measurements on frog skin (Moreno et al.,1973; Rick et

al.,1975) reveal a virtual identity between Na influx across the apical membrane, net transepithelial Na influx, and the short-circuit current. However, some evidence for a bidirectional movement of Na across the apical membrane in tight epithelia is available (see Macknight et al., 1980; Benos et al.,1983). For example, when ouabain is added to the serosal bathing solution of frog skin, an amiloride-sensitive back flux of Na into the mucosal bathing solution occurs (Rick et al., 1978; O'Neil and Helman, 1976). However, such back flux of sodium across the apical membrane does not take place under normal experimental conditions with normal Ringer solution on both surfaces of the epithelium (Macknight et al., 1980).

C. The Regulation of the Na-Entry Across The Apical Membrane

In addition to the amiloride which completely blocks the sodium entry across the apical membrane of frog skin epithelium (Dorge and Nagle, 1970), there are many other factors that regulate the sodium movement across this barrier. These regulatory agents include hormones (ADH and aldosterone), chemical agents (local anesthetics, novobiocin, diphenyldantion, polyene antibiotics and others), mucosal and serosal sodium and other alkali metals, mucosal and intracellular pH, and some physical factors (electrochemical potential difference across the outer barrier, cell volume, etc.). It is beyond the scope of this review to provide a detailed account of the mechanisms by which each one of these agents affects

the sodium entry across the apical membrane of frog skin. Among the excellent reviews in this field are Herrera (1971), Erlij and Ussing (1978), Macknight, et al., (1980), Civan (1983a).

Properties of the Basolateral Membranes and Mechanisms of Active Transport

A. Properties of the Basolateral Membrane

The basolateral membranes in frog skin and other Na-transporting epithelia include all the epithelial membranes that are not passively permeable to Na (Koefoed-Johnsem and Ussing,1958; Macknight and Leaf,1978). Two distinct features are usually ascribed to this barrier. First, it's passively and selectively permeable to K. Second, it's the site of the energy-dependent active transport step that drives transepithelial sodium transport.

Ion-substitution experiments (Lindly and Hoshiko, 1964; Hoshiko, 1973) reveal that the relative permeability of the basolateral membrane to the following series of cations K: Rb: Cs: Li: Na, is 1: .74: .22: .12: .09 respectively. The swelling of the epithelial cells as a responce of K substitution for Na in the serosal bathing solution (MacRobie and Ussing 1961) is another evidence for the high K-conductivity of the basolateral membrane. Chloride ions are also known to cross the inner barrier of the epithelium (Koefoed-Johnsen,et al., 1952). Complete substitution of the serosal medium Cl by

other anions (I,No,Br) causes different degrees of stimulatory effect on the transepithelial Na-transport (Ferriera, 1968; Huf, 1972). On the other hand, the substitution of sulfate ions for chloride ions in the serosal bathing solution depresses Na-transport (Huf, 1972). This kind of anion substitution is believed to affect the transepithelial Na-transport through some "unknown" mechanisms that operate at the basolateral membrane (see Macknight et al, 1980).

When the serosal bathing solution is made Na-free, Ca ions from the extracellular medium passively cross the basolateral membrane of the frog skin and accumulate in the epithelial cells (Grinstein and Erlij, 1978). Under these conditions, a significant reduction in the transepithelial Na-transport is evident. If Ca is eliminated from the serosal bathing solution, the reduction in the transepithelial Na-transport caused by Na-free serosal solution becomes unnoticable (Grinstein and Erlij, 1978). These results suggest that Ca ions play a significant role in the regulation of the permeability of the basolateral membrane, and thus in the determination of the rate of the Na-transport across the epithelium. Other multivalent cations such as Zinc, barium, cadmium, and manganese are also known to modify both the permeability of the basolateral membrane, and the net Na-transport in tight epithelia (for a review see Macknight, et al., 1980).

B. Localization of the Active Site

Due to the morpholigical complexity of the frog skin, the localization of the active site of Na-transport has been and still is a chronic problem. Historically, the active site of Na-transport was ascribed to many different locations; the basement membrane of the epithelial tissue along the boundary between the basal layer and the corium (Ottosen, et al., 1953), the serosal border of the stratum germinativum cells (Engbaek and Hoshiko, 1957; Koefoed-Johnsen and Ussing, 1958), the basolateral membranes of all epithelial cells facing the inside solution (Ussing and Windhager, 1964; Farquhar and Palade, 1966) or the innermost membranes of the stratum granulosum cells (Voute and Ussing, 1968 and 1970). However, none of the above stated results is by any means conclusive (Erlij and Ussing, 1978).

The proposal that the active site of sodium-transport is mainly localized at the basolateral membranes of the (St.Gr) cells is particularily attractive since there is a group of morphological findings that lend support to it (Erlij and Ussing, 1978). It should be mentioned however, that most recent electrophysiological studies on frog skin suggest an extensive electrical coupling between the cells (Nagel,1976; Hellman and Fisher, 1977; Fisher et al., 1980), indicating that all the epithelial cells participate in the process of active Na-transport rather than a specific layer or specific type of cells. Some drawbacks of this conclusion are: the microelectrode technique which has been usually used to draw

such conclusion is a technique with very well documented artifacts (Nelson, et al., 1978), the electron microscopy studies on such epithelia revealed very few gap junctions among the cells (Farquhar and Palade, 1965; Shahin and Blankemeyer, 1986), and the in vitro experimental manipulation of skin (ie. incubation in Ringer solution for many hours) may introduce some serious modification of the properties of the membranes (unpublished observation).

C. The Active Transport Mechanisms

In the classical formulation of Koefod-Johnson and Ussing (1958), Na which passively enters the apical membrane of frog skin epithelium is actively extruded through the basolateral membranes via Na-K ATPase (pump). The extrusion of sodium was considered to be stoichiometrically linked to the K accumulation into the epithelial cells in a tight one-to-one relationship. This simply means that such Na-K pumps are neutrogenic and do not contribute to the electrical potential of the skin. However, if the K of the serosal bathing solution is increased so that the electochemical potential gradient for this ion is reduced or reversed, the short circuit current of the skin is not abolished (Herriera,1971) nor is the potential difference across the basolateral membrane (DeLong and Civan, 1984). This indicates that the active transport mechanism is electrogenic (or rheogenic) and a strict one-to-one Na for K exchange is unlikely to exist under such experimental conditions. Many experimental results using different

approaches (for a review see Nagel,et al.,1980; Nagel, 1981) have recently provided strong evidence for a rheogenic function of the pump; that is the stoichiometry of the pump fluxes of Na and K is different from unity and the pump generates a significant fraction of the driving force for the apical border Na uptake. In general, the Na-K exchange ratio is accepted to be somewhere in the vicinity of 3 Na for 2 K (Nielson, 1982), however there appears to be considerable scatter in the data obtained for such kind of investigations. For example, the Na-K exchange ratio obtained by Helman and Cox (1984) on frog skin varies with the short circuit current from 1:1 at lower short circuit current to 6:1 at higher short circuit current. It should be mentioned however, that none of the concerned studies exclude some partial coupling between the K uptake and the Na extrusion. Nonetheless, the coupling ratio is unlikely to be a 1:1 ratio or fixed at a constant value.

The active transport mechanism is driven by metabolic energy, since metabolic inhibitors such as fluoracetate, azide, and diethyl malonate (see Herrera, 1971) are known to be potent inhibitors of Na active transport. The cardiac glycoside, ouabain, is another potent inhibitor of the active Na transport in tight epithelia. Because of its ability to bind to the Na-K ATPase at the basolateral membrane, ouabain is a very useful inhibitor for studying different aspects of Na active transport (Farquhar and Palade, 1966; Mills, et al., 1977). When added to the serosal bathing solution, ouabain

causes inhibition of the transepithelial Na transport as measured either isotopically or electrically as the short circuit current (Koefoed-Johnsen, 1957; Nagel and Dorge, 1971). The ouabain inhibition of the transepithelial Na transport is a biphasic process (Helman et al., 1979). The first phase is characterized by rapid (5-10 min.) decrease in the short circuit current. The second phase is characterized by slow but continuous marked increase in the resistance of the apical and basolateral membranes which leads ultimately to a complete inhibition of the short circuit current. The mechanism for such inhibitory pattern is not fully understood (see Macknight, et al., 1980).

Most traditional "black box" studies on Na-transporting epithelia have regarded the basolateral membranes as a barrier with static properties and the apical membrane as the rate-limiting barrier with the dynamic properties (Schultz, 1981, 1983). However recent microelectrode studies (Helman, et al., 1979; Nagel, et al., 1980; Nagel, 1981) have revealed that this barrier responds to changes in Na transport rate across frog skin, and maintains mechanisms that contribute to the regulation of the intracellular composition of electrolytes. For example, Nagel (1981) found that the rheogenic Na transport across the basolateral border of frog skin accounts for some 20-40 mv. of the intracellular potential. He concluded that the pump generates a significant fraction of the driving force for apical border Na uptake. Helman et al. (1979) using microelectrode data to study the

effects of ouabain on the conductance of the basolateral membrane found that when ouabain is added to the serosal bathing solution, there is an abrupt decrease in the conductance of the basolateral membrane followed by further decrease but at a slower rate. The rapid phase of the decrease in conductance of the basolateral membrane is ascribed to direct inhibition of the pump activity while the slower phase is due to a decrease in cellular concentration of K. Helman and Fisher (1977) obtained data that suggest a linear relationship between the basolateral membrane conductance and the pump activity.

The physiological significance of these findings is twofold. First, the parallelism between the pump activity and K conductance enables the epithelial cells to maintain a reasonably constant level of K at different rates of pump activity (see Schultz, 1983). Second, the K conductance of the basolateral membranes along with the activities of the rheogenic pump (Nagel, 1981) provide a significant fraction of the driving force for the apical entry of Na across frog skin.

CHAPTER III

SOME MORPHOLOGICAL FEATURES OF FROG SKIN EPITHELIUM: THE PRESENCE OF LAMELLAR STRUCTURES IN THE INTERCELLULAR SPACE AND WITHIN THE CELLS

Introduction

Because of its high Na-transport capacity, the epithelium of frog skin is widely used as a model in ion and water transport studies. Amphibian skin is also known to play a major role in the adaptation to the environment, osmoregulation, and the homeostasis of these animals (Lindemann, and Voute, 1976). In order to understand the physiological significance of these important functions, some familiarity with the morphology of the skin is necessary. The correlation between the structure and functional properties of the frog skin epithelium is also very helpful in the process of characterizing the mechanisms by which this tissue executes its sodium transport.

Some aspects of the general organization and fine structure of the frog skin epithelium have been previously described (Voute, 1963; Parakkal and Matoltsy, 1964; Dewey and

Barr,1964; Farquhar and Palade,1964, 1965: Voute and Ussing, 1968; Carraso et al., 1971; Lindeman and Voute, 1976). However, a systematic effort to correlate the morphological characteristics of the skin with its functional properties is still lacking.

In this chapter,I have reinvestigated the fine structure of frog skin epithelium and provided an overview of the general organization of the tissue. I have also reported the presence of some lamellar structures which were found in the intercellular space and with in the cells. The morphology of these structures is similar to the morphology of the lamellar bodies found in other stratified epithelia however, their composition and function are still not known.

Material and Methods

Experimental Tissues

Unsexed medium frogs (Rana pipiens) from (Carolina Biological Supply Company) were used in this study. The animals were kept unfed in tanks at room temperature with access to tap water. Small pieces of abdominal skin were excised from doubly pithed frogs and rapidly immersed in the fixative solution. In certain experiments, the underlying corium was removed by the enzymatic methods of Fisher,et al., (1980).

Methods For Thin Sections

Most skins were fixed by immersion of thin slices of the

tissue in fixatives, however in some cases in situ fixation and perfusion through the subcutaneous vein were performed. Several different fixation procedures were used .

Method 1. The inital fixation was done by using a solution of 2.5% glutaraldehyde in 0.1 M cacodylate buffer for two hours, followed by three washes in cacodylate buffer for 20 minutes each. The post-fixation was performed using 1-2% osmium tetroxide for 2 hours. Some tissues were "en block" stained over night with ethanolic uranyl acetate (5%).

Method 2. Tissues were directly fixed in osmium tetraxide vapor for 3 hours at room temperature without prior aldehyde fixation, and "en block" stained over night with uranyl acetate (5 %).

Method 3. A mixture of tannic acid and glutaraldehyde was used according to the following protocol (Deurs, 1975); a solution of 16 % tannic acid in Na-cacodylate buffer (PH 7.4) was prepared at 50 C°. Solutions of 8 %, 4 % , and 2 % were made by diluting the original solution with cacodylate buffer at room temperature. Two and one half ml of each of these solutons was mixed with equal volume of (5%) glutar-aldehyde in 0.2 M sodium-cacodylate buffer obtaining a final concentration of 8 %, 4 %, 2 %, and 1 % tannic acid with 2.5 % glutaraldehyde. These fixatives were applied to the tissue by immersing small pieces of the tissue in the solution for 2 hrs. The post fixation was performed by using 1 % osmium tetraxide in 0.1 M cacodylate for 2 hrs. Post fixed tissues were either processed for dehydration

or "en block "stained with uranyl acetate.

Method 4. Tissues were fixed in Karnovsky's fixatives (Karnovsky, 1965) containing 1 % formaldehyde, 3 % glutaraldehyde, and approximately 0.5 mM $CaCl_2$ buffered to pH 7.4 with 0.1 M sodium-cacodylate for 3-4 hours at room temperature, then washed in the same buffer for 2 hours. The post fixation was performed by using 1-2 % osmium tetraxide in 0.1 M cacodylate buffer, the tissues were then incubated in 5% uranyl acetate with 5% sucrose for 2 hours.

All fixed tissues were quickly dehydrated in graded ethanol and embedded in polybed resin. Silver to gray sections were cut with diamond knives on a Porter-Blum Sorval MT-2 ultramicrotome. They were collected on carbon-coated grids, stained with lead citrate and aqueous uranyl acetate, and examined either by using a Philips EM 200 or JEOL 100 CX electron microscope.

Estimating the Epithelial Thickness

Sections of 1 μm. thick were used to estimate the thickness of the epidermis. The tissue was oriented to permit subscquent cutting of sections that were perpendicular to the surface of the epidermis. The thickness of the epidermis was estimated from 75 measurements made on 15 photos (5 measurements on each photograph). The measurements were made at 100 μm. intervals of the length of the epidermis on each photograph.

Estimating the Number of the Mitochondria-Rich Cells (MRC)

The number of (MRC) was expressed as the total number of (MRC) per unit length of the epidermis. Perpendicular sections were used. Ten micrographs of each epidermis were selected randomely to be used in the count. In few cases the number of (MRC) was expressed as a percentage of the total number of the epithelial cells of each micrograph. Six micrograph of each epidermis were used for such counts.

Results

Epithelial Thickness

The total thickness of the epithelium varied slightly in the same section from one region to another (Fig.1). The mean value of the epithelial thickness in different frog skin samples ranged from 52+3 to 83+3.9 μm. with a total average of 69+2.8 μm.

The General Organization of the Epithelium

The epidermis of frog skin is a stratified squamous epithelium which is constituted by 5-9 cell layers organized in four strata (Fig.1).

The outermost stratum, stratum corneum (St.Co), is comprised of one to two cell layers of highly elongated cornified cells (Fig.2) which forms a thin protective sheath that shields and buffers against the chemical and physical impacts

Figure 1. A light micrograph of frog skin epithelium. The epithelium is constituted by 5-8 cell layers organized in four strata: stratum corneum (S.Co), stratum granulosum (S.Gr), stratum spinosum (S.Sp), and stratum germinativum (S.Ge). The (S.Ge) is comprized of one layer of columnar cells that resides on a basement membrane (BM). A morphologically distinct population of cells (round or pear-shaped cells), usually termed mitochondria-rich cells is associated with the upper portion of the epithelium (arrows). A number of mucous gland (MG) is distributed in the connective tissue (CT) of the dermis. X450.

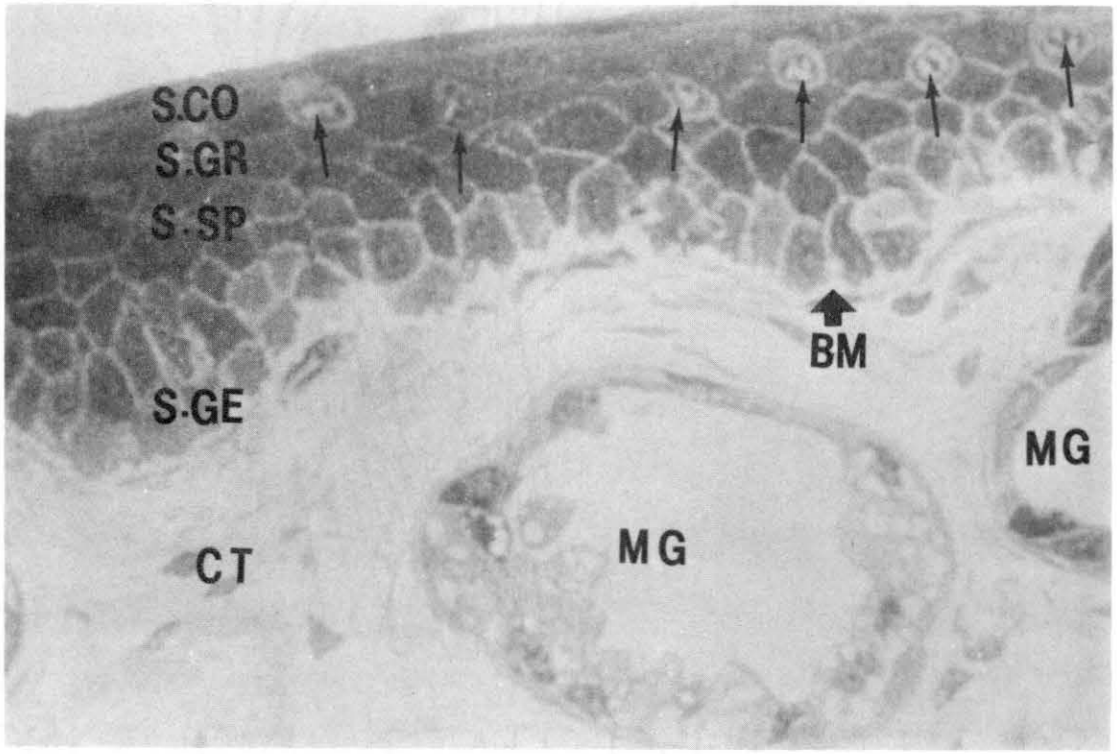
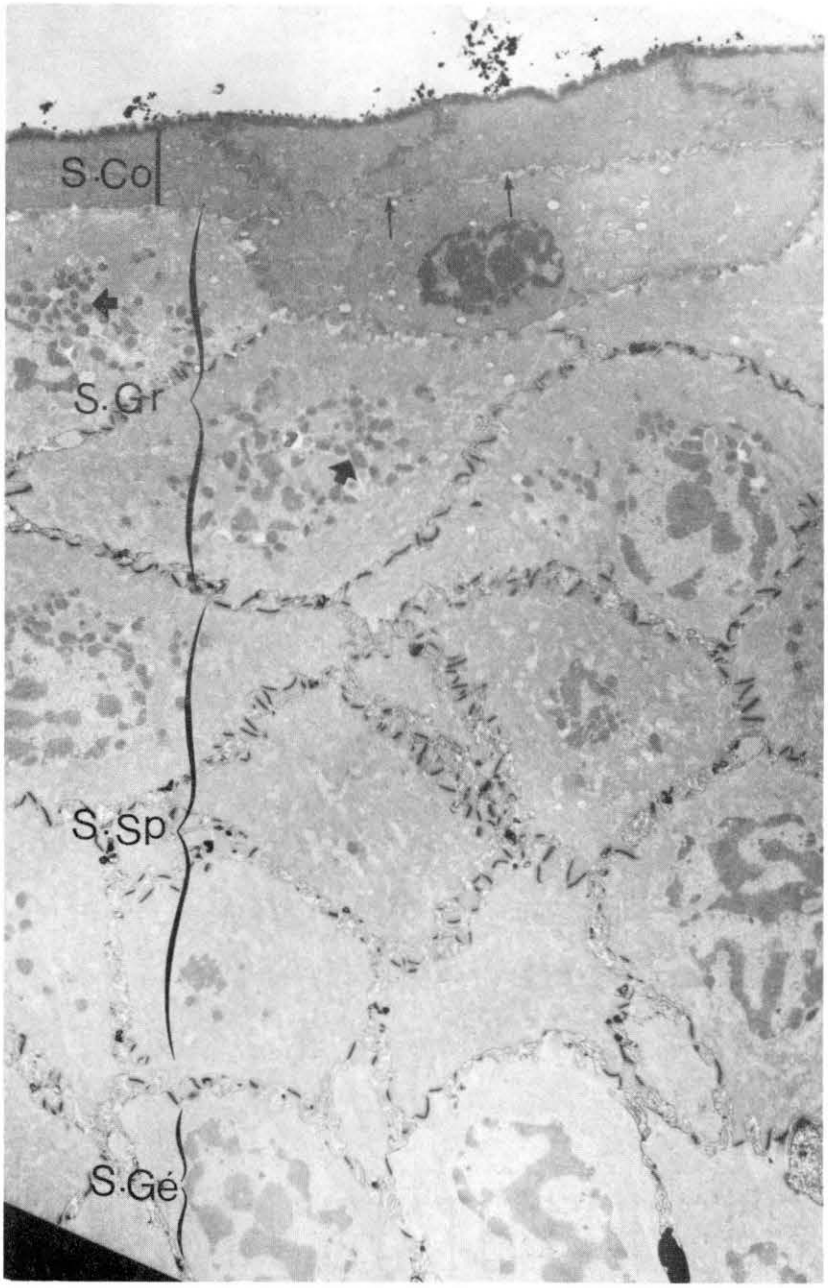

S.CO
S.GR
S.SP
BM
S.GE
MG
CT
MG

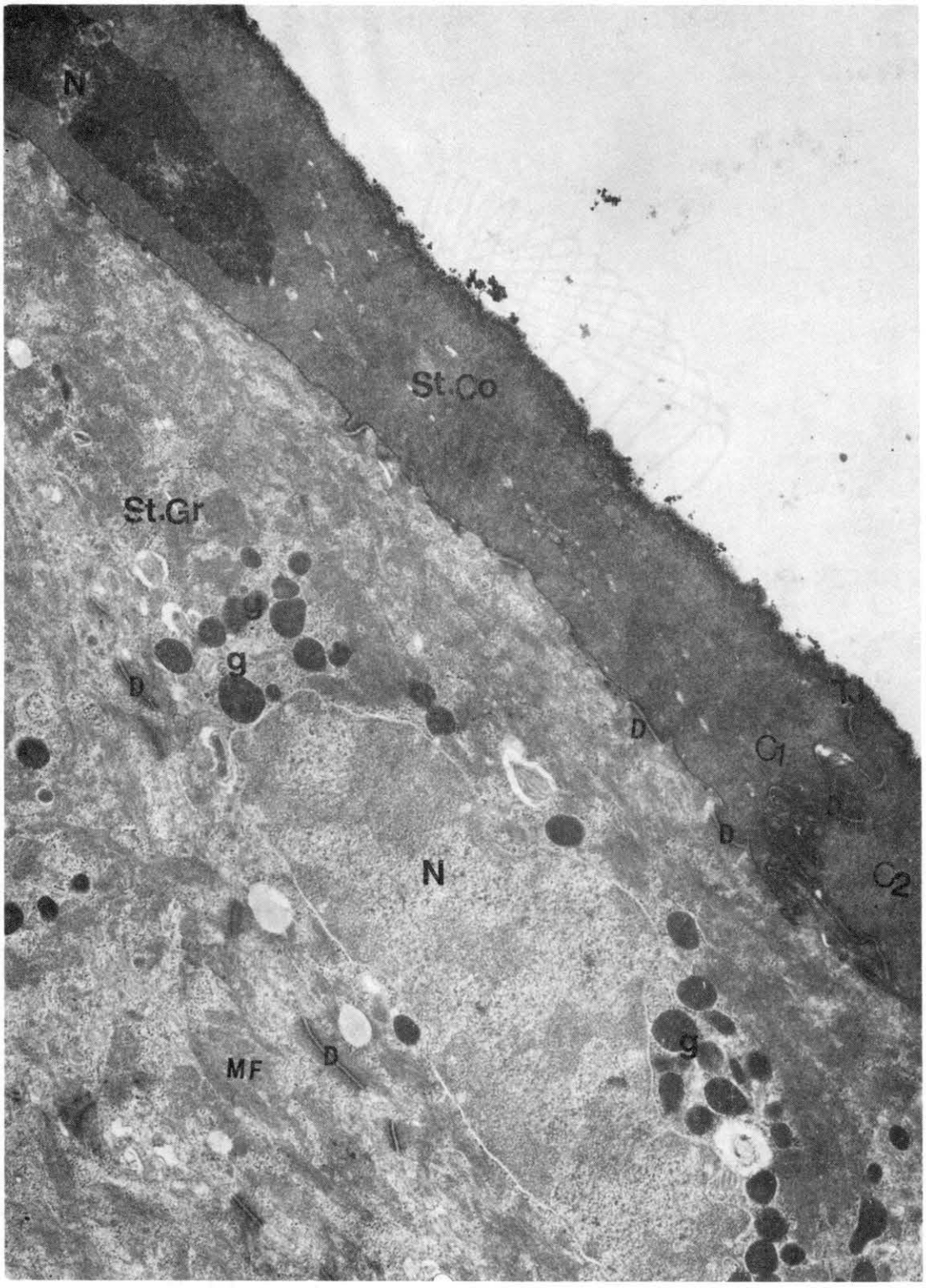
Figure 2. A low power micrograph of frog skin epithelium showing the general organization of the tissue at the level of electron microscope. The epidermis is a stratified squamous epithelium composed of: one cell layer thick of stratum corneum (S.Co), two cell layer thick of stratum granulosum (S.Gr) which contain distinct mucous granules (thick arrows), three cell layer thick of stratum spinosum (S.Sp), and a single cell layer of stratum germinativum (S.Ge). The cells of (S.Ge) resides on an extracellular structure called basal lamina (not shown here) which separates the epithelium from the corium. The thin arrows point to the intercellular space between the cells of (S.Co) and those of the replacement layer. X3500.


S.Co
S.Gr
S.Sp
S.Gé

of the external environment. The cells of (St.Co) are easily distinguished from the underlying cells by homogeneous electron-dense microfilaments which are tightly packed and uniformly distributed in the cytoplasm giving the (St.Co) a darker appearance than underlying strata (Figs.2,3), and obscuring the appearance of the subcellular organelles. However, careful examination of these cells may show some nuclear fragments, degenerating mitochondria and other hard-to define subcellular elements (Fig.3).

The stratum granulosum (St.Gr) is a one to two layers of cells which directly underlies the cells of (St.Co) with a distinct intercellular space between them (Figs.1,2). The intercellular space between the two strata is frequently filled with dense material that is probably deposited as cellular excretion (Farquhar and Palade, 1965). The attachment between the upper layer of (St.Gr), and the lower layer of (St.Co) occurs through well defined desmsomes (Fig.3). The cells of (St.Gr) tend to be horizontally elongated with centrally located nuclei that are oriented longitudinally in a parallel plane to the basement membrane (Figs.2,3). They are characterized by a low nucleus to cytoplasm ratio when compared to the cells of lower strata. A complete set of subcellular organelles: rough and smooth endoplasmic reticulum, Golgi compex, mitochondria, lysosomes, pinocytotic vesicles and specific granules of various shapes and sizes are enmeshed in dense bundles of 70-80 angstrom diameter microfilaments (Fig.4). The cells of stratum granulosum are

Figure 3. Cross section of frog skin epithelium in the region of stratum corneum (St.Co) and stratum granulosum (St.Gr). The membranes of the two cornified cells; C1 and C2 show high bending characteristics with a tight junction at the apicolateral surfaces of these cells. Many desmosomes (D) are present on the lateral aspects of the boundary between the two cornified cells. The cells of (St.Gr) which are located beneath the (St.Co) cells, have a well-defined nucleous (N), bundles of microfilaments (MF), and numerous granules (g) of different sizes and shapes. X 14000.


N
St.Co
St.Gr
g
g
D
D
C1
D
D
C2
N
MF
D
g

interlocked with each other by many desomsomes which provide the mechanical attachment between the cells (Fig.4).

The stratum spinosum, (St.Sp) is two to three layers of cuboidal or polyhedral cells that extends between the upper boundary of the basal layer and the lower boundary of (St.Gr) (Figs.1,2). These cells tend to flatten horizontally as they move towards the outer layers to incorporate with those of (St.Gr). The cytoplasm of spinosal cells contain the usual subcellular organelles found in the cells of other strata. Bundles of tonofilaments (70-80 Angstrom diameter each) are randomly distributed within the cytoplasm; they only become organized at the site of their attachment to the cytoplasmic plaque of desmosomes where they follow a predictable pattern (Fig.5). A system of "small finger-like projections" forms many labyrinthian interdigitations on the lateral aspects of spinosal cells (Figs.5,6). This system, in addition to the highly encountered desmosomes in this area seem to be responsible not only for maintaining the physical integrity of the tissue, but also providing wide surface area for cell-to-cell communication.

The basal layer of frog skin epithelium, stratum germinativum (St.Ge) is a closely packed single layer of columnar cells that rests on a well-defined basal lamina which is the only continuous structure separating the epithilial cells from the underlying connective tissue (Figs.1,7). The cell bodies of (St.Ge) appear to be laterally compressed with centrally located nuclei that tend to be oriented perpendicularly

Figure 4. Cross section of frog skin epithelium in the region of stratum granulosum. This micrograph shows a number of granulosal cells connected to each other throgh well-developed desmosomes (arrow heads). Some other junctional complexes (probably gap junctions) can also be detected (arrows). The cytoplasm contains bundles of microfilaments (mf) that are randomly distributed within the cells. The presence of some subcellular structures such as nucleus (N), Golgi complex (G), mitochondria (m), lysosomes, mucous granules (g) are also evident. The micofilaments that are attached to the desmosomes are termed here tonofilaments (TF). X21000.

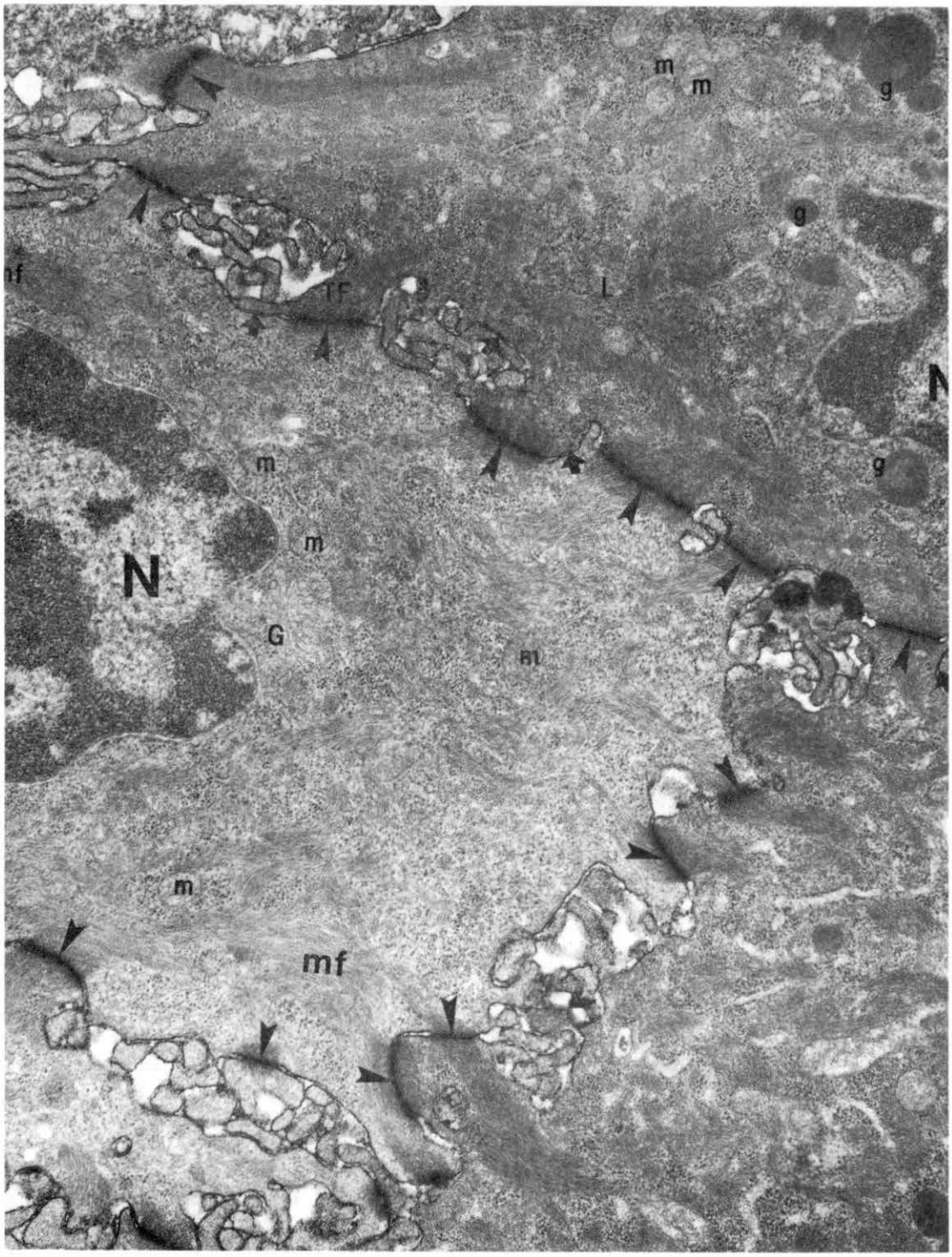

m
m
g
g
nf
TF
L
N
m
m
N
G
m
g
m
mf

Figure 5. A cross section in the mid portion of the frog skin epithelium (St.Sp). This micrograph shows that the cell-to-cell attachments occurs through complex cellular processes that interlocked themselves together to provide an increased surface area for communication among the cells. Few desmosomes (D) and other junctional complexes (arrows) are also present at the boundary between the cells. Bundles of randomly distributed microfilaments (mf) fill most of the cellular cytoplasm. The spinosal cells have fewer granules (g) than those of the upper strata. (N) is the nucleus, (TF) points to the tonofilaments, and (m) indicates the mitochondria. X40000.

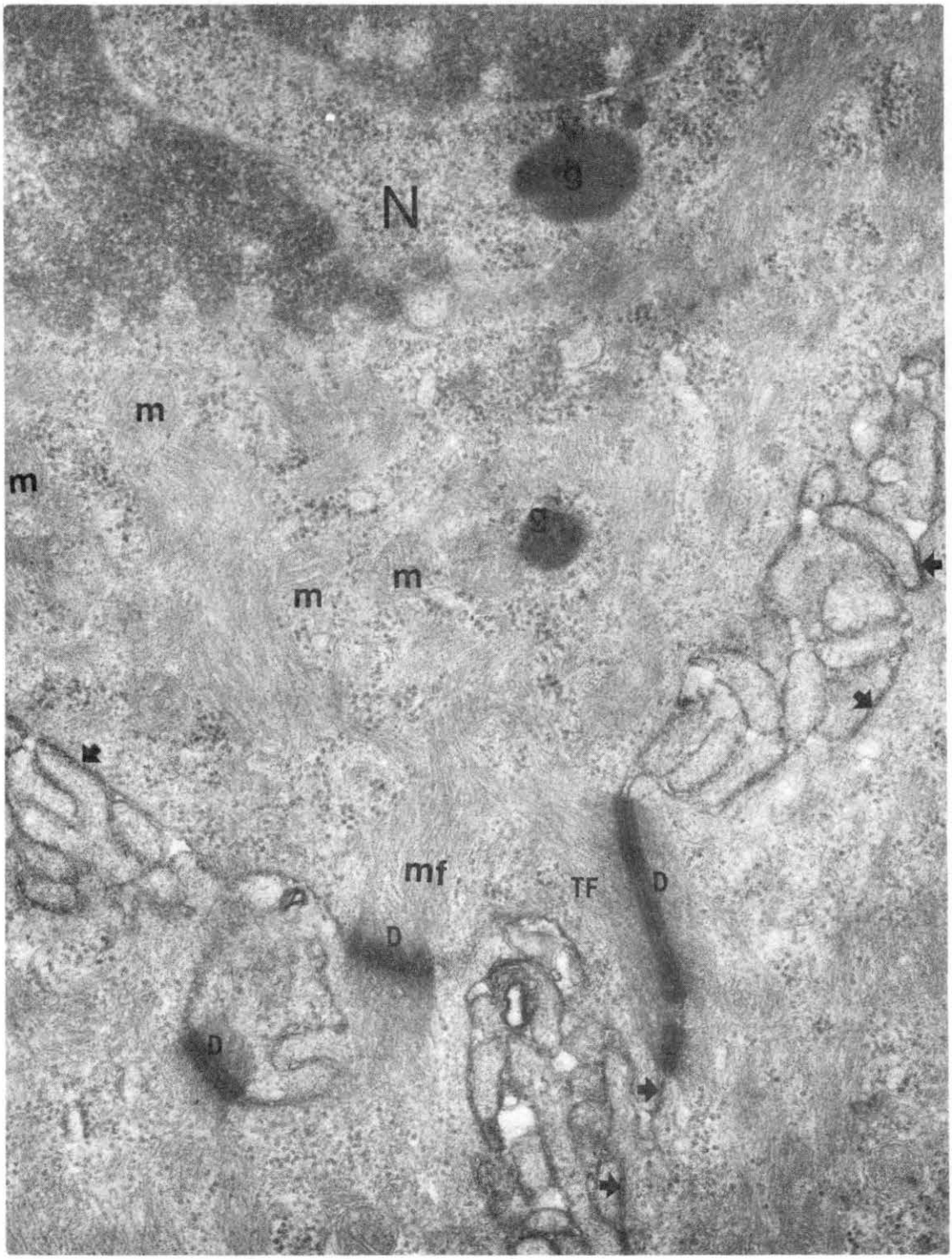
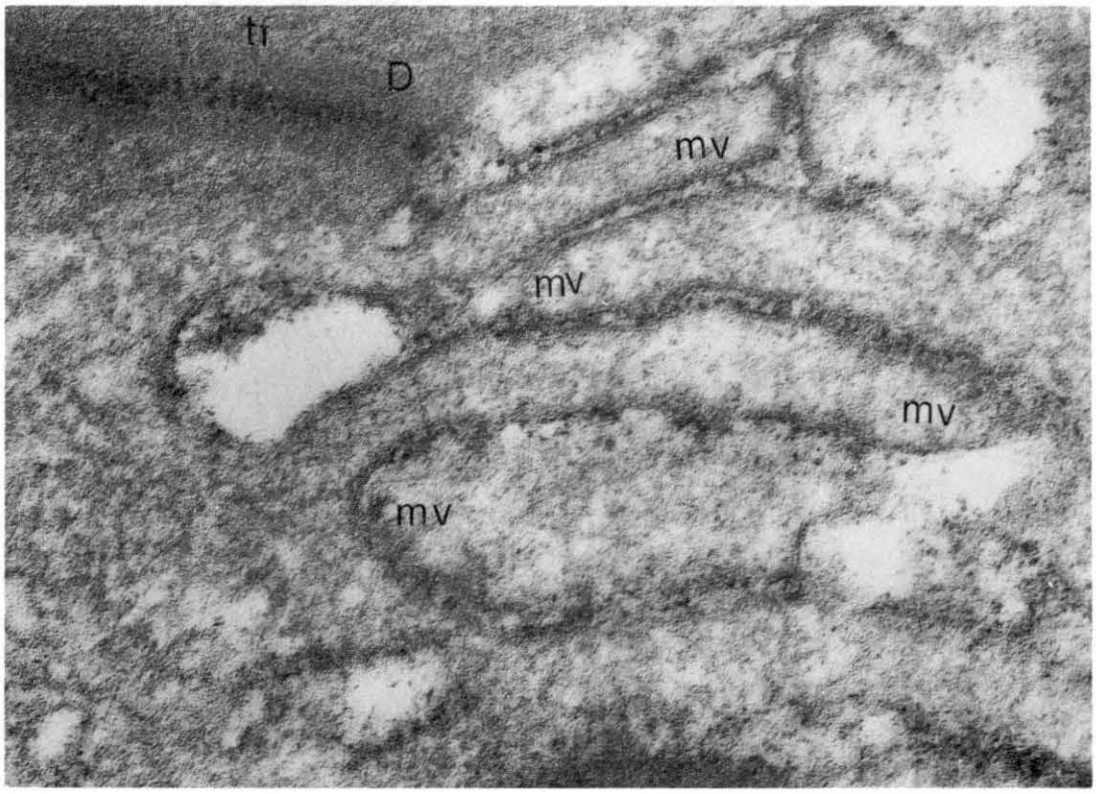

N
m
m
m
m
mf
TF
D
D
D

Figure 6. A high magnification electron micrograph of a portion of the stratum spinosum showing the finger-like projections between the cells. This type of connection has a microvilli-like (mv) appearence. (D) indicates a desomosome, (tf) indicates the site of tonofilaments. X150000.

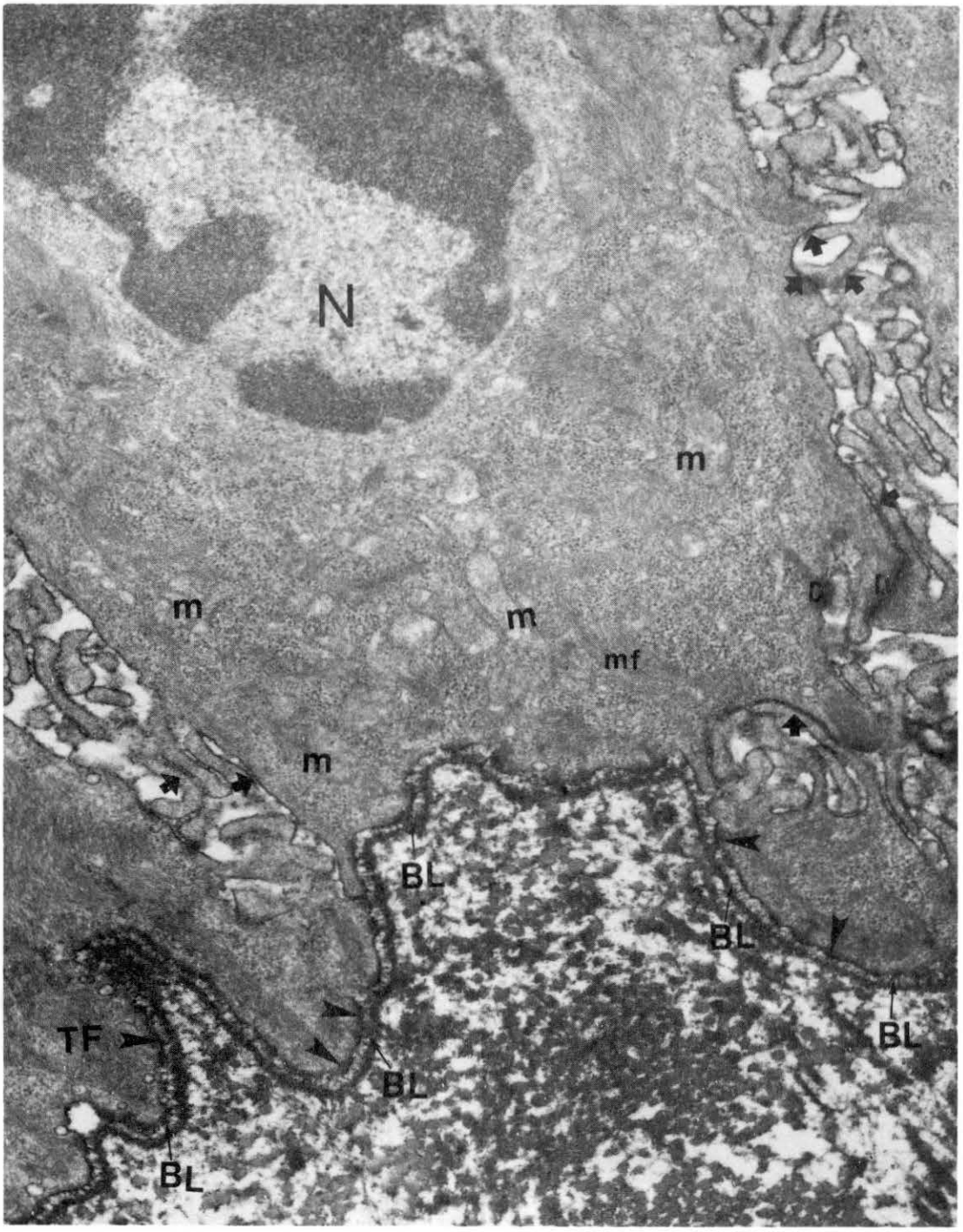

tf
D
mv
mv
mv
mv

to the basal lamina (Figs.2,7). The cytoplasm contains the usual set of subcellular organelles distributed in less developed and randomly oriented bundles of microfilments (Fig.7). Some tufts of these microfilaments stream themselves in an organized pattern to be attached to the hemidesmosomal structure which represent the basal attachment devices between the basal layer and underlying corium (Fig.7). The lateral aspects of attachment and communication between the cells of (St.Ge) seem to occur through highly complicated interdigitating cellular processes that interlock to provide an increased surface area of connection between the cells (Fig.7). Desmosomes are also present here to provide another source of mechanical adhesion among the cells, although their frequency in the basal layer is lower than that encountered in the upper strata. Due to the presence of an extensive finger-like projection system in the lateral membrane, the intercellular space in this area has a maze-like orientation with some expanded areas, which could be resulted from some shrinkage of these cells during the processing of the tissue (Fig.7).

Mitochondria-Rich cells (MRC)

Associated with the mid-portion of the epithelium (St.Gr, and St.Sp),there is a morphologically distinct population of cells which are referred to as beaker cells (Muhse, 1909), clear cells (Lavker,1971), mitochondria-rich cells (Farquhar and Palade, 1965), or flask cells (Whitear, 1975) (Fig.1).

Figure 7. The epithelial cells of stratum germinativum are separated from the underlying connective tissue by a well defined extracellular structure called the basal lamina (BL). The whole length of the (BL) is heavily populated by areas of distinct densities that represent the sites of hemidesmosomes (arrow heads). The lateral aspects of attachment and communication between the cells of stratum germinativum occurs through highly complicated cellular processes which intermingle with each other. Few desmosomes (D), and a number of gap junctions (arrows) are present along the border between the cells. The nucleus (N) is centrally located and in perpendicular oreintation to the (BL). The microfilaments (mf) are less developed in these cells than those of the cells of the upper strata. Numerous mitochondria (m) are evident. X28000.

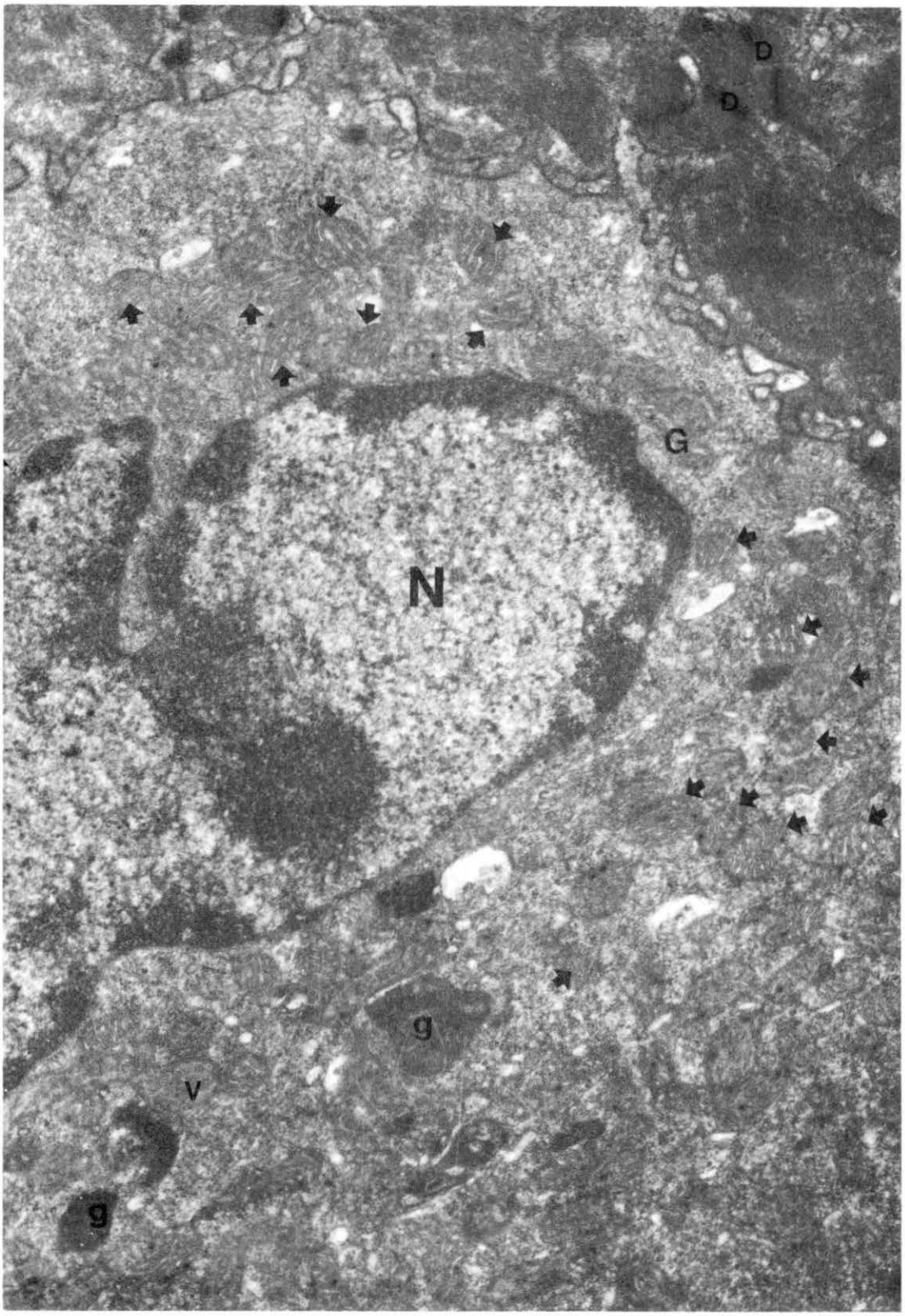

N
m
m
m
mf
m
BL
BL
BL
TF
BL
BL

Similar types of cells are usually found in the epidermis of newts (Pillai, 1962), and amphibian urinary bladders (Choi, 1963). These cells are flask-shaped or pear-like cells with large centrally located nuclei. Our calculation regarding their distribution in the epithelium showed that their highest frequency occurred among the cells of (St.Gr). About one third of the the total population of (St.Gr) cells is flask-shaped cells. We were able also, to detect very few of these cells in the (St.Sp) cells, but none in the (St.Ge) cells. Our observations concerning the fine structure of these cells, confirming previous studies (Farquhar and Palade, 1965; Lavker,1971; Whitear, 1975) showed that they contain sparse filaments, high number of mitochondria, rudimental Golgi complex, various multivesicular bodies, lysosomes, and other less well defined electron-dense granules all dispersed in a clear appearing cytoplasm (fig.8). The apical aspect of flask cells is characterized by a system of convoluted membranes in the form of micro-villar ridges that bulges out to interdigitate with the adjacent cells (Fig.8). These ridges are of different magnitude and they seem to be less pronounced in some frogs than others.

Lamellar Bodies

A system of parallel lamellar sheets similar to those described in other stratified epithelia (see Matoltsy and Bednarz, 1975; Elias, et al., 1977; Shimono and Clementi,

Figure 8. A Mitochondria-rich cell in the upper portion of the epithelium. This kind of cell is characterized by the large number of mitochondria (arrows) which are present in the cytoplasm. The cytoplasm of this cell has a clear appearence when compared with neighboring epithelial cells. Some subcellular organelles such as Golgi complex (G), vesicles (V), and some granules (g) can be identified with some dificulties. The apical aspects of the membrane of the mitochondria rich cell is characterized by a convoluted configuration. (N) is a portion of the nucleus. X30000.


D
D
G
N
g
V
g

1976) was observed in many sections of the epithelium treated with tannic acid (Figs.9,10,11). These lamellar bodies (LB) were frequently encountered in the intercellular space of stratum granulosum cells, and rarely among the cells of other strata. In certain cases the (LB) spread over a considerable area of the intercellular space, obscuring the appearance of the plasma membrane in that area (Fig.10). In some other cases these structres were found within the cells (Fig.11). The morphological structure of these bodies consisted of parallel electron opaque layers, 65-75 angstroms thick,embedded in an electron-lucent homogeneous background. The precise structure of these bodies and their functions in this kind of epithelium is not known. It seemed to us that the differentiation of the spinosal cells into granulosal cells was accompanied by discharging such structures inside the intercellular space where they were most frequently observed.

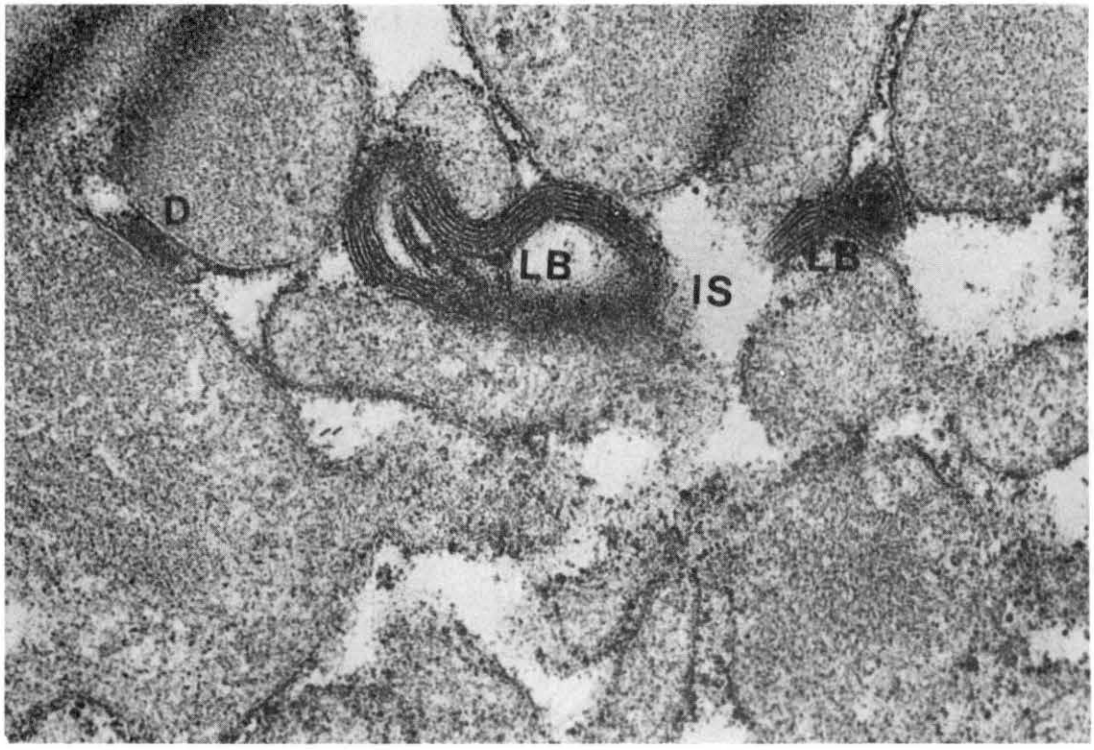
Discussion

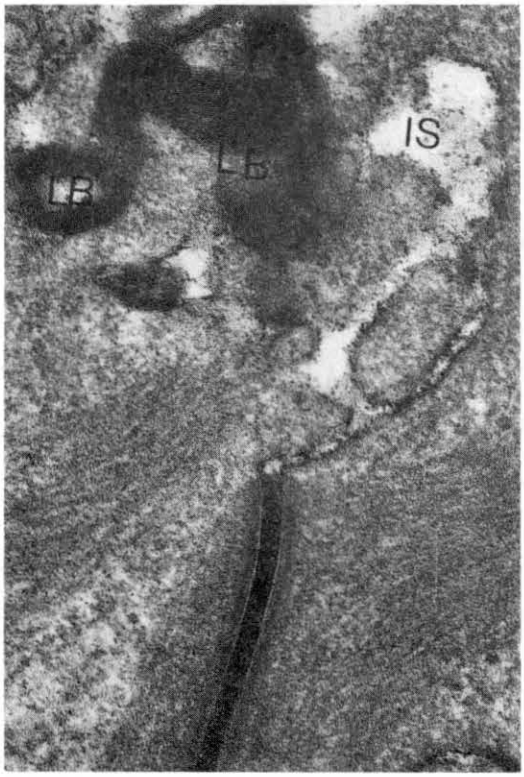
The general organization and fine structure of frog skin epithelium have been previously described and well documented (Voute,1963; Parakkal and Matoltsy,1964; Farquahr and Palade, 1964 and 1965; Dewey and Barr, 1964; Carrasso et al., 1971). It suffices here to report that a mixture of tannic acid and glutaraldehyde (see material and methods) can be used successfully to preserve the tissue and increase the density of the

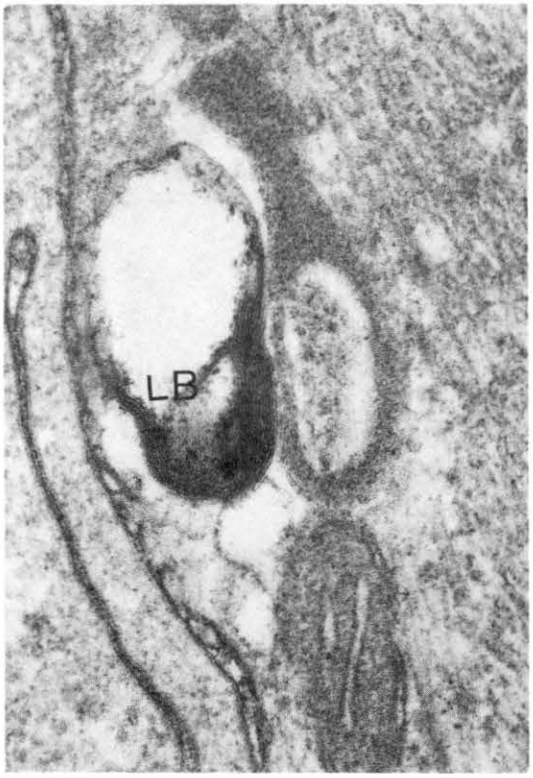
Figure 9. A cross section of frog skin epithelium in the region of stratum granulosum, showing two lamellar bodies (LB) in the intercellular space (IS) of this region. (D) is a desmosome. (glutaraldehyde-tannic acid fixation) .X 99000.

Figure 10. This micrograph shows a portion of two cells the stratum granulosum sharing a desmosome. Considerable volume of the intercellular space (IS) is occupied by lamellar bodies (LB). See text for more details. (Glutaraldehyde-tannic acid fixation). X 85000.

Figure 11. A lamellar body (LB) inside a cell of frog skin epithelium. The (LB) is at the periphery of the cells in a close position to the membrane. (Glutaraldehyde-tannic acid fixation. X99000.


D
LB
IS
LB


IS
LB
LB


LB

cellular periphery, thus enhancing our ability to visualize the plasma membranes of the epithelial cells and their junctional complexes. In fact, in all tannic acid-fixed skins a reasonable intercellular space of 250-350 angstroms wide was maintained throughout the epithelium. However, the intercellular space compartment, in many cases, was usually bridged by many stubby processes or interdigitations that extend between the cells. Such processes were highly encountered along the lateral aspects of (St.Ge) and (St.Sp) cells,they were less pronounced or most frequently absent between the cells of (St.Gr) and (St.Co).

The morphology and functions of the subcellular structures of frog skin epithelium have been previously dealt with (for reviews see Carrasso,et al., 1971; Farquahr and Palade, 1965; Parakkal and Matoltsy,1964; Lindimann and Voute,1976), however, some observations are useful to be discused here. The rough and smooth endoplasmic reticulum appeared to lose its structure during the process of differentiation to become less developed or distributed in the upper strata of the epithelium(especially in St.Co and the uppermost lyer of St.Gr) than in the lower strata (St.Ge, and St.Sp). This also indicates that the upper layer of (St.Gr) and all cells of (St.Co) tend to lose their synthetic capability during the process of diffirentiation. Various membrane-limited granules of different shapes and sizes were encountered only in the cells of (St.Sp and St.Gr). These granules are most likely to be involved in mucous production (Parakkal and Matoltsy, 1964).

Farquahr and Palade (1965) reported the presence of two distinct population of granules and classified them based on their size into "large" and "small" granules. They furthermore suggested that these two types of granules may be involved in different functions; for example, they might be responsible for the production of dense material that fills the intercellular space between the (St.Gr) and (St.Co). Our results showed that the sizes of these granules vary randomely with no sharp cutting point between the large and small ones. It seems that some functions of these granules may be associated with the extrusion of lamellar sheets in the intercellular space of (St.Sp) and (St.Gr) cells (see below).

Our results demonstrate the presence of numerous lamellar bodies in the intercellular space of the upper portion of the epithelium espicially in the region of the intercellular space between (St.Co) and (St.Gr). Some of these structures are also present within the cells of (St.Sp) and in their intercellular space. However, the number and size of these bodies seem to be much higher in the upper portion of the epithelium than their size and number in the lower portion of the tissue. Such lipid-containing structure have been known to be typical in keratinizing epithelia (Matoltsy and Parakkal, 1965; Ohashi et al., 1973; Weinstock and wilgram, 1970; Matoltsy and Bednarz, 1975; Shimono and Clementi, 1976). Their functions in other keratinized stratified squamous epithelia have been frequently associated with the permeability barrier of these tissues (see Elias and Friend, 1975; Elias

et al., 1977; Shimono and Cleminti, 1976). However, these structures are being seen for the first time in frog skin.

The Lamellar bodies seen in this study are segregated pockets of lamellar sheets that are confined to descrete areas of specific regions of the intercellular space. Thus, they are unlikely to play a siginficant role as a permeability barrier in this kind of epithelium. Furthermore, the frog skin epithelium is characterized by very well developed tight junctions (Farquhar and Palade, 1965) which establishes the main structure for the permeability barrier in such Na-transporting epithelium (Martinez-Palomoet al., 1971). It is our interpretation that these lamellar bodies are originally intracellular granules (probably mucous granules) which aggregate and incorporate within the plasma membranes to be finally extruded in the intercellular space of the upper layers of the epithelium where they are most frequently encountered.

CHAPTER IV

LOCATION AND DISTRIBUTION OF INTERCELLULAR JUNCTIONS IN FROG SKIN EPITHELIUM

Introduction

Frog skin, a "tight" epithelium with a high transepithelial resistance, actively transports sodium ions from the mucosal (pond) side to the serosal side. It is generally accepted that this transport process involves two steps: a passive entry of sodium across the Na-selective amiloride-sensitive apical membrane and an active extrusion of sodium ions across the K-selective ouabain-sensitive basolateral membranes (Koefoed-Johnsen and Ussing, 1958; Ussing and Windhager, 1964).

The determinations of the size and location of the Na-containing compartment(s) that participates in the Na-active transport is very essential for the understanding of the mechanism(s) by which the active transport takes place. Historically, the size of the cellular transport compartment has been ascribed to: a small fraction of the total population of the epithelial cells (see Erlij and Ussing, 1978),

to a specific single layer of cells (St.Gr) provided with a very active transport mechanism (Voute and Ussing, 1968, 1970; Voute and Hanni, 1973; Voute et al., 1975), or to all cells in the epithelium (Ussing and Windhager,1964 ; Biber, et al., 1966; Farquhar and Palade, 1966). Recently, many lines of evidence have converged to suggest that the cells of all epithelial layers are engaged in the Na-transport process forming a syncytial Na-transport compartment (see Helman, 1979; Nagel, et al., 1981; Rick,et al., 1984). However, the morphological basis for such cell to cell coupling in this epithelium has not been established .

The general organization and fine structure of the frog skin epithelium have been previously described (Voute,1963; Parakkal and Matoltsy, 1964; Dewey and Barr,1964 ; Farquhar and Palade, 1964 and 1965; Carrasso,et al.,1971). Two types of intercellular junctions (tight junctions and desmosomes) have been identified and fully described in this epithelium (Farquhar and Palade, 1965) and a third type ("nexus") was introduced (Dewey and Barr, 1964).

The failure to detect classical gap junctions among the cells of frog skin epithelium (Farquhar and Palade, 1965; Martinez-Palomo et al.,1971) is difficult to reconcile with the syncytial nature of this tissue. Possible reasons for the difficulties in locating gap junctions in this epithelium might be due to the fact that this tissue has junctional complexes that are functionally, but not morphologically similar to gap junctions. Another possibility is that

the fixation procedures used so far were not adequate to reveal gap junctions in this epithelium.

In this study I have used different electron microscopic techniques to study the location and frequency of the intercellular junctions in frog skin epithelium. The thin section and freeze-fracture data demonstrrated the presence of some junctional complexes having structures similar to that of gap junctions. A quantitative analysis of the location and distribution of these junctions and the other types of the intercellular junctions in frog skin epithelium was performed.

Material and Methods

Experimental Tissues

Unsexed medium frogs (Rana pipiens) from (Carolina Biological Supply Company) were used in this study. The animals were kept unfed in tanks at room temperature with access to tap water. Small pieces of abdominal skin were excised from doubly pithed frogs and rapidly immersed in fixative solution. In certain experiments, the underlying corium was removed by the enzymatic methods of Fisher,et al., (1980).

Methods For Thin Sections

Most skins were fixed by immersion of thin slices of the tissue in fixatives. However in some cases "in situ" fixation and perfusion through the subcutaneous vein were performed.

Several different fixation procedures were used.

Method 1. Inital fixation in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for two hours, followed by three washes in cacodylate buffer for 20 minutes each. The post-fixation was performed using 1-2% osmium tetroxide for 2 hours. Some tissues were "en bloc" stained over night with ethanolic uranyl acetate (5%).

Method 2. Tissues were directly fixed in osmium tetroxide vapor for 3 hours at room temperature without prior aldehyde fixation, and "en bloc" stained over night with uranyl acetate (5%).

Method 3. A mixture of tannic acid and glutaraldehyde was used according to the following protocol (Deurs,1975): a solution of 16% tannic acid in Na-cacodylate buffer (pH 7.4) was prepared at 50 C°. Solutions of 8%, 4% , and 2% were made by diluting the original solution with cacodylate buffer at room temperature. Two and one half ml of each of these solutions was mixed with equal volume of (5%) glutaraldehyde in 0.2 M Na-cacodylate buffer obtaining a final concentration of 8%, 4%, 2%, and 1% tannic acid with 2.5 % glutaraldehyde. These fixatives were applied to the tissue by immersing small pieces of the tissue in the solution for 2 hrs. The post fixation was performed by using 2% osmium tetraxide in 0.1 M cacodylate for 2 hrs., Post fixed tissues were either processed for dehydration or "en block "stained with uranyl acetate.

Method 4. Tissues were fixed in Karnovsky's fixatives

(Karnovsky, 1965) containing 1% formaldehyde, 3% glutaraldehyde, and approximately 0.5 mM CaCl2 buffered to pH 7.4 with 0.1 M Na-cacodylate for 3-4 hours at room temperature, then washed in the same buffer for 2 hours. The post fixation was performed by using 1-2% osmium tetroxide in 0.1 M cacodylate buffer; the tissue was then incubated in 5% uranyl acetate with 5% sucrose for 2 hours.

All fixed tissues were quickly dehydrated in graded ethanol and embedded in polybed resin. Silver to gray sections were cut with diamond knives on a Sorvall MT-2 ultramicrotome. They were collected on carbon-coated grids, stained with lead citrate and aqueous uranyl acetate, and examined either by using a Philips EM 200 or JEOL 100 cx electron microscope.

Freeze-Fracture

Small pieces of whole skin or isolated epithelium were used. The tissue was fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate (pH 7.4) for 1 hour, rinsed in cacodylate buffer for 15 minutes and then transported into 30 % glycerol in cacodylate buffer for 90 minutes. The specimens then were frozen by placing them in liquid freon 22 cooled by liquid nitrogen and then quickly transferred into liquid nitrogen. Cubes of tissue were mounted on specific holders, fractured and etched at - 105 C°, and replicated in a Balzer freeze-etch apparatus. The platinum-carbon replicas were cleaned by bleach or by sequential treatment with absolute methanol

and bleach and then were picked up on collodion-carbon coated 200 mesh copper grids. The replicas were examined in a Philips EM 200 or JEOL 100 cx electron microscope.

Quantitative measurments

The frequencies of intercellular junctions per unit length of the membrane were determined according to a procedure adapted from Garfield, et al., (1980). THe length of membrane surveyed for each portion of the epithelium was determined from a series of photographs (15-20 photos for each tissue) taken and printed at 20,000-35,000 magnification on 20 by 25 cm. paper. The cellular membrane length of each stratum of the epithelium was estimated separately by superimposing a lined transparent grid over the appropriate area of the photograph. The total length of membrane in the examined area of each photograph was calculated by counting the intersections between the cellular membranes and the probe lines of the transparent grid using the following formula:

$$B = \pi/2 * I/L * A * CF$$

where:

B is the length of the cell membrane in the area examined.

I is the average number of intersection points determined from two estimates taken at right angles to one another.

L is total length of probe lines on the transparent grid.

A is the area of the photograph examined in the grid system.

CF is the correction factor for the magnification.

π is 3.146

Desmosomes were easy to locate and calculate. However, for distinguishing tight junctions and gap junctions, we had to enlarge each suspected gap or tight junction from its original magnification of 20,000-25,000 to around 120,000. The distribution and frequency of junctions were calculated as the number of junctions in each strata per each um of plasma membrane length (No./micron).

Statistical Comparison

The Student t test was used to make pair-wise and among group comparisons of the frequency of intercellular between the cells of different layers of the epithelium.

Results

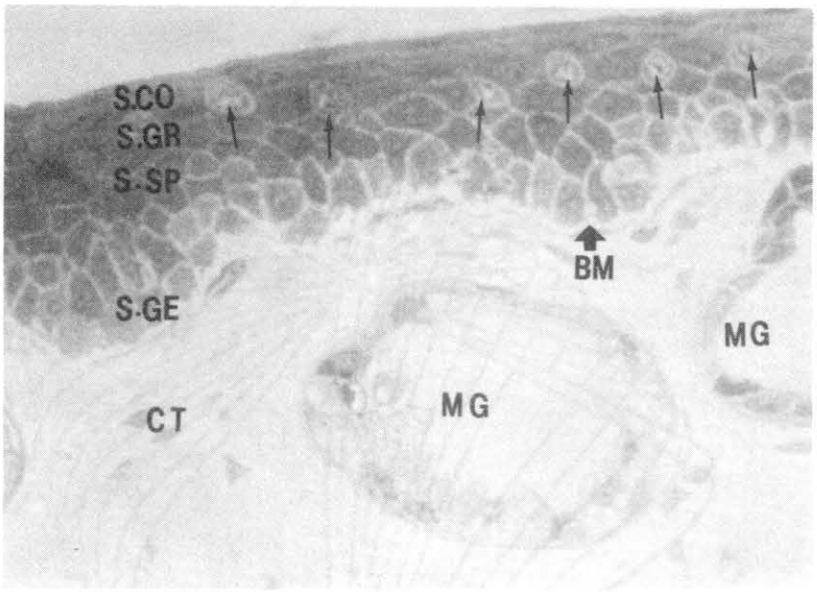
General Organization

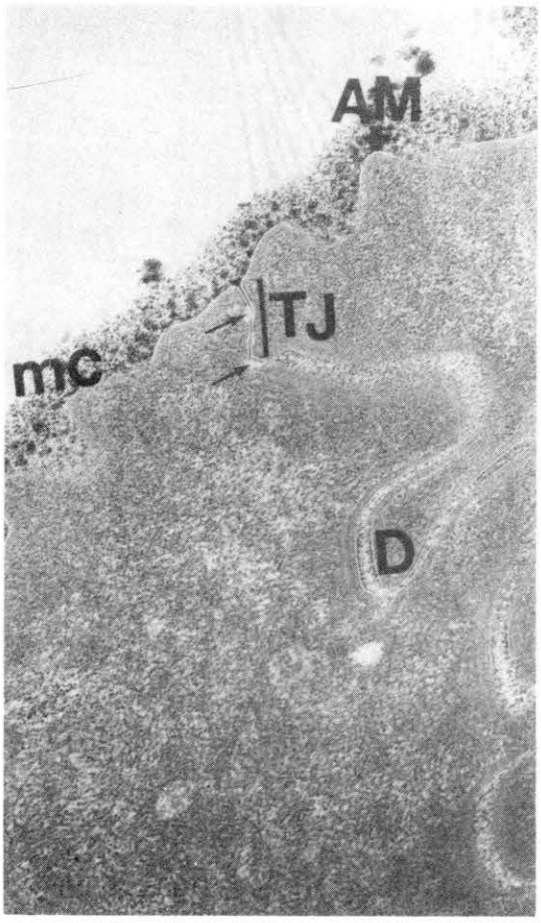
Histologically, the frog skin epithelium consists of a stratified squamous epithelium composed of 5-9 cell layers organized in four strata: stratum corneum (St.Co), stratum granulosum (St.Gr), stratum spinosum (St.Sp), and stratum germinativum (St.Ge) see Fig.1.

Figure 1. A light micrograph of frog skin epithelium. The epithelium is constituted by 5-8 cell layers organized in four strata; stratum corneum (S.Co), stratum granulosum (S.GR), stratum spinosum (S.SP), and stratum germinativum (S.GE) which is comprised of one layer of columnar cells that resides on the basement membrane (BM). A morphologically distinct population of cells (round or pear shaped cells) which are usually called mitochondria rich cells is associated with the upper portion of the epithelium (arrows). A number of mucous glands (MG) is distributed in the corium which is constituted by lose connective tissue (CT). X 400.

Figure 2. A tight junction (TJ) between two cells of (St.Co) The apical membrane (AM) is covered by a layer of mucous called mucous coat (mc). The small arrows indicate the sites of focal splitting of the fusion line of the tight junction. A number of desmosomes (D) can be easily detected on the border line between the two cornified cells. X57000.

Figure 3. Shows a tight junction (TJ) and a number of desmosomes (D) between two cells of stratum granulosum which is located directly below stratum corneum (St.Co). (Tannic acid-glutar aldehyde fixation). X40000.


S.CO
S.GR
S.SP
BM
S.GE
MG
CT
MG


AM
TJ
mc
D

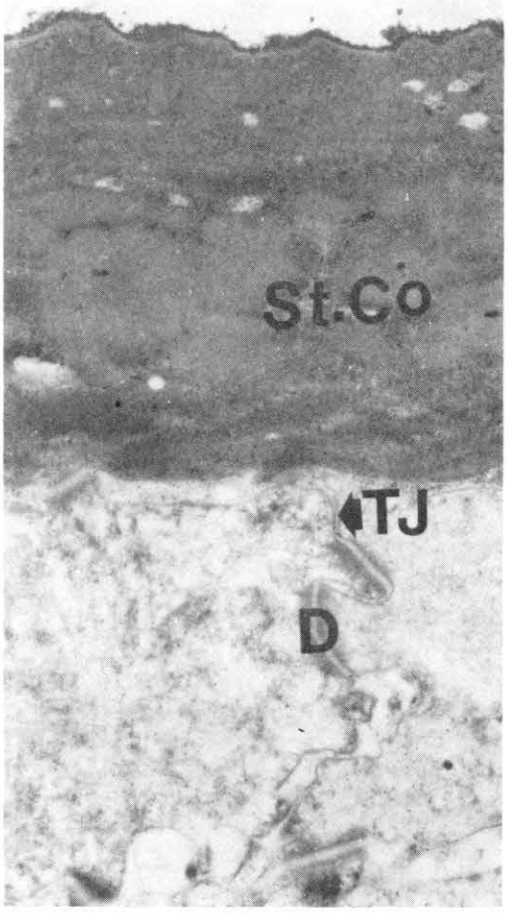
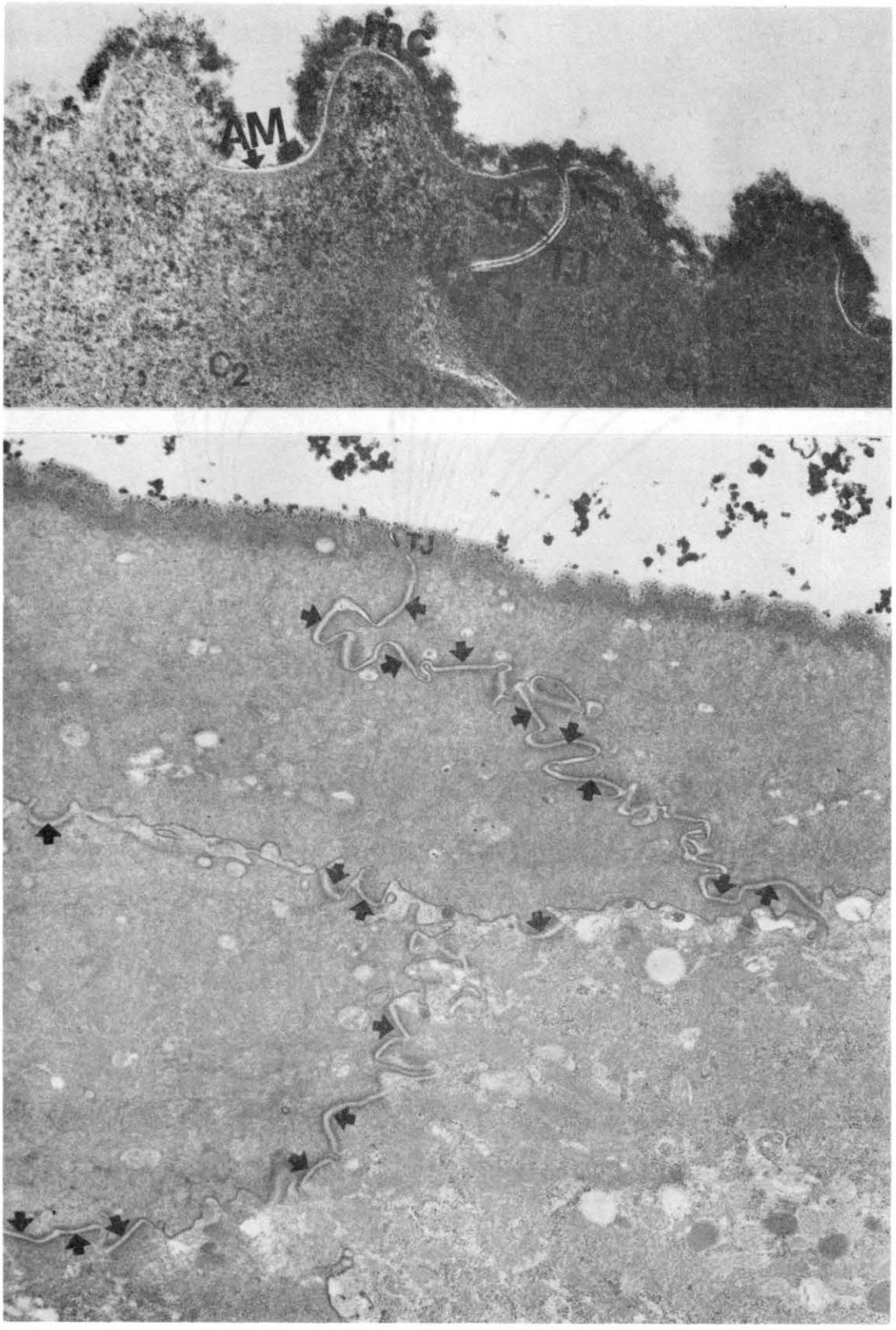

St.Co
TJ
D

Figure 4. A higher magnification of a tight junction (TJ) between two cornified cells of frog skin epithelium (C1 and C2). The upper arrow indicates the convergence of the two membranes and the lower arrow indicates the end point of the junction. AM; apical membrane, mc; mucous coat, and (dl) is the fusion line of the two membranes forming the junction. X140000.

Figure 5. A cross section of frog skin epithelium in the region of stratum corneum . The membrane of the cornified cells in the upper region show highly bending configuration with the tight junction (TJ) at the apicolateral surfaces of the cornified cells. Desmosomes (indicated by arrows) are numerous. Gap junctions are absent in the stratum corneum. (Tannic acid and glutaraldehyde fixation). X18000.


mc
AM
TJ
C_2
TJ

Intercellular Junctions

Tight Junctions

Tight junctions (zonula occludens) were observed at the apico-lateral surfaces of (St.Co and St.Gr) cells (Figs.2,3). They were exclusively localized between the superficial cells of these two strata, and never found in other layers of the epithelium. By using a glutaraldehyde-tannic acid mixture,the visulization of the thin section images of tight junction was greatly enhanced. In the region of the tight junctions, the plasma membranes of the two adjacent cells appeared to fuse together intimately forming a single band of 30-40 Angstroms in diameter, and leaving no apparent intercellular distance between the two membranes at the level of the junction (Fig. 4). These junctions seemed to encircle each cell providing a continuous physical barrier at the apical surface which separates the internal media of the epithelia from the external environment.

About 10-15 % of the total surface area of the cornified cell membranes was covered by tight junctions (see Table 1). This percentage was much lower (5%-10%) in granular cells.

Frequently, a series (1-3) of focal disattachment between the two membranes forming the tight junction were encountered (Fig.1). This phenomenon gave tight junctions the appearance of two membranes that were not cemented together, but rather fused together at a series of points leaving some areas of focal splittings along the length of the junction.

Figure 6. A cross section of frog skin epithelium in the region of stratum granulosum. This section shows the high frequency of desmosomes in this area (thick arrows) and the very low frequency of gap junctions (thin arrows). A number of granules of different sizes and shapes (Gr) are evident in the granulosal cells. (S.CO) is a portion of the stratum corneum. (Tannic acid glutaraldehyde fixation). X21600.

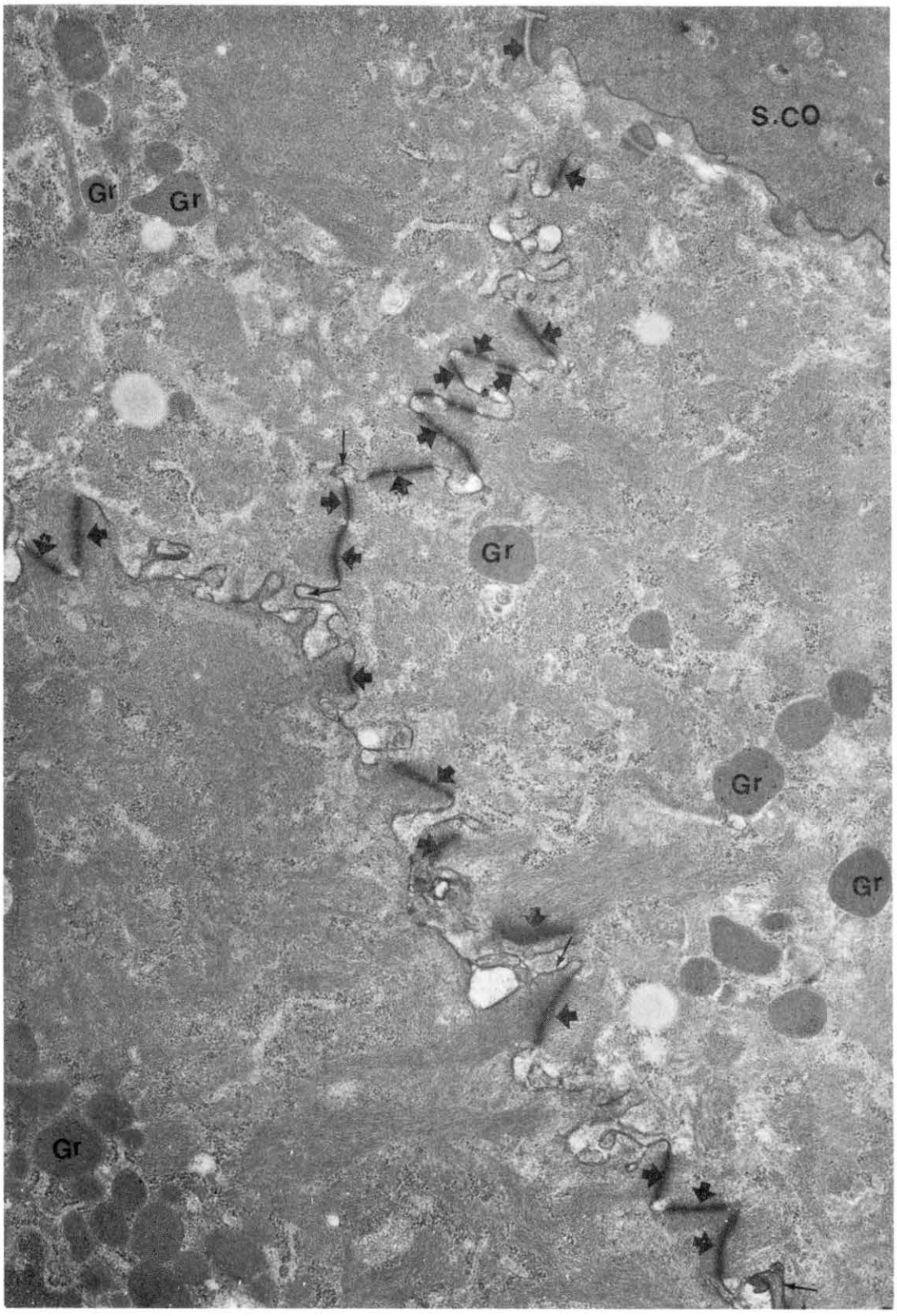

S.CO
Gr
Gr
Gr
Gr
Gr
Gr

Figure 7. A low power micrograph of a cross section in the mid portion of frog skin epithelium (St.Sp) showing the distribution of desmosomes (thick arrows) and gap junctions (thin arrows) among the cells of this area. (Tannic acid and glutaraldehyde fixation). X21000.

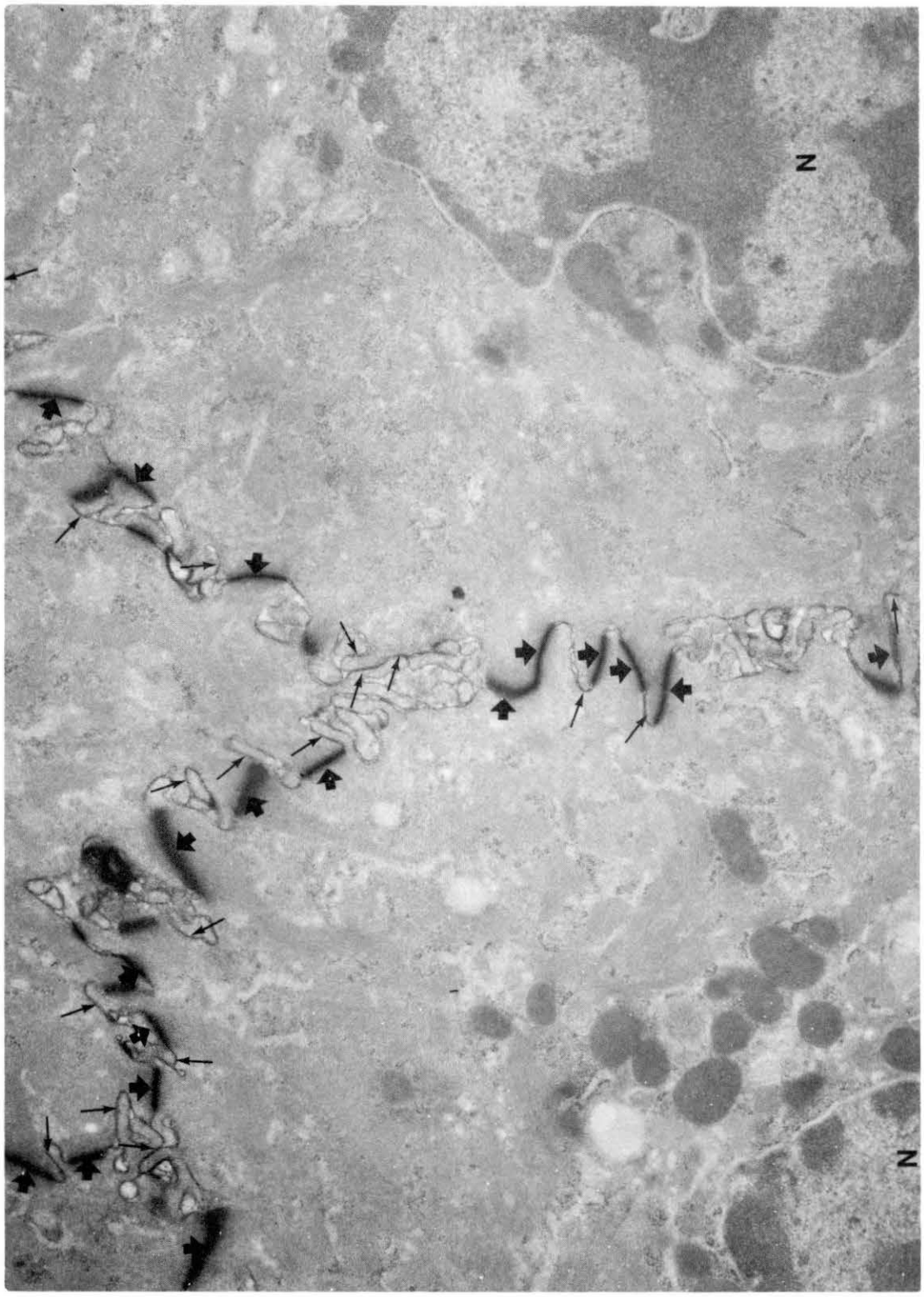
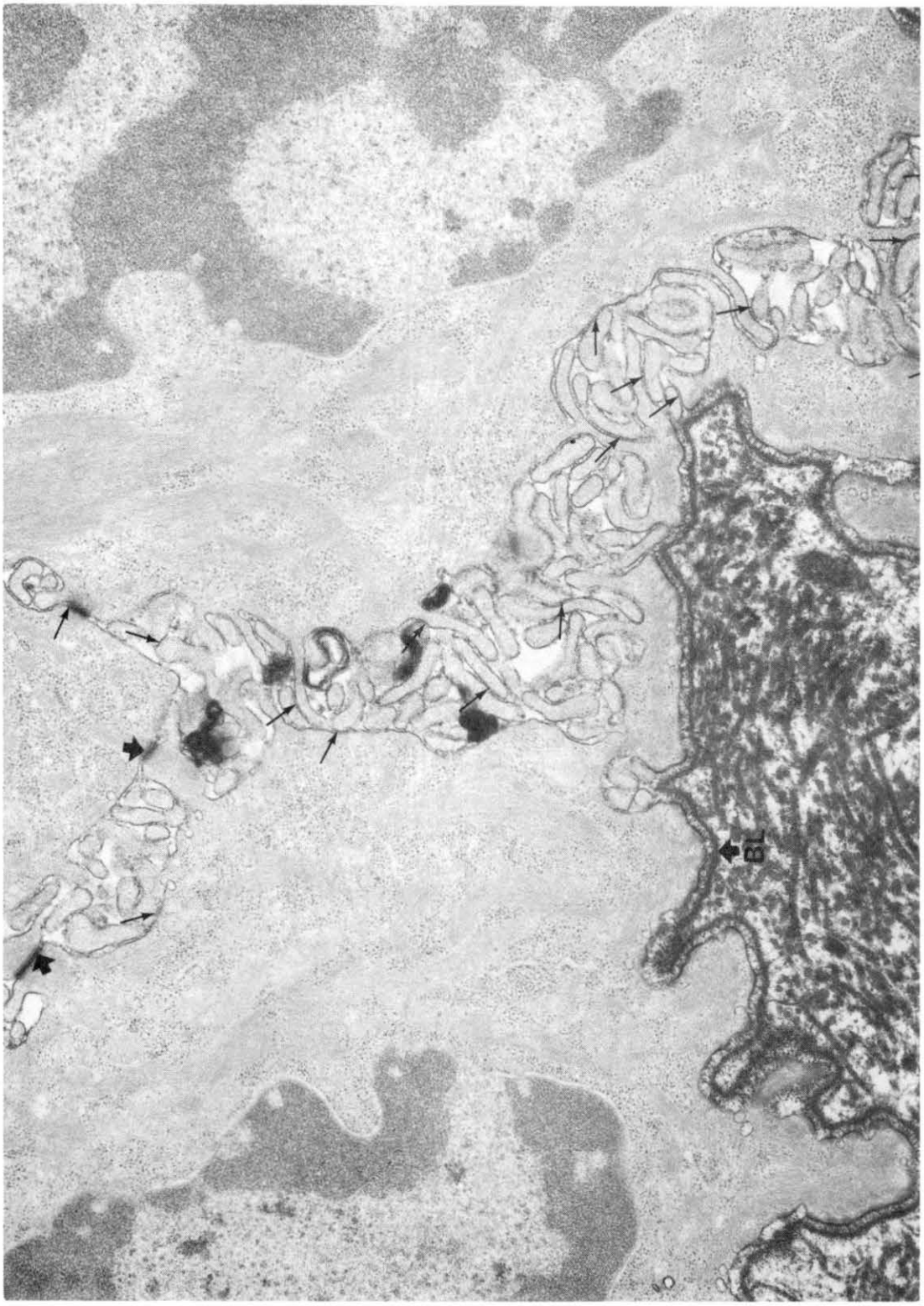

N
N

Figure 8. A cross section of a portion in the lower region of frog skin epithelium showing some cells of stratum germinativum that reside on the basal lamina (BL). Desmosomes are indicated by thick arrows and gap junctions by thin arrows. Notice that the boundary between the cells exhibit highly complex configuration. (Tannic acid-glutaraldehyde fixation). X21000.


BL

Desmosomes

In thin-section images of frog skin epithelium, desmosomes were easily found in all epithelial strata (Figs. 5-8). Their structure was essentially the same in (St.Ge),(St.Sp), and the basolateral aspects of (St.Gr); however, desmosomes that existed between the cells of (St.Co) and those connecting the cells of this strata to the underlying cells of (St.Gr) had somewhat modified structure (see Farquhar and Palade, 1965). In general, a desmosome was formed by two adjacent cell membranes that strictly lie parallel to each other separated by an intercellular gap of 250-350 Angstrom wide. This intercellular gap was usually filled with electron opaque fillamentous material that was frequently bisected by a central dense line termed central strata or central lamella (Fig.9). The inner leaflet of the membrane that shares in forming a desmosome was lined by electron dense plaque called cytoplasmic plaque (Staehlin and Hull, 1978) which represents the base for the attachment of cytoskeletal network of tonofilaments (Figs.9, 10). These tonofilaments, upon approaching the cytoplasmic plaque, had the tendency to loop in a wide arc and course back into the main stream of the tonofilament bundles of the cell. In many cases, other sets of very fine filaments adhered to the bending tonofilaments to strengthen their attachment to the cytoplasmic plaque.

Figure 9. A typical desmosome between two cells of stratum spinosum. The cytoplasmic plaque (CP) represents the base for the attachment of the tonofilaments. The central lamella (CL) bisects the intercellular space between the two membranes which form the desmosome. Bundles of tonofilaments extend randomly in cytoplasm, but become highly organized at the site of their attachment to the cytoplasmic plaque of the desmosome. (IS) is the intercellular space. X65000.

Figure 10. This micrograph shows a portion of two cells of the stratum granulosum.sharing a number of desmosomes (D) and a gap junction (GJ). Considerable volume of the intercellular space (IS) is occupied by lamellar bodies (LB). Arrows indicate some area of close apposition between two cellular membranes. (TF) indicates the tonofilamenet network of the desmosomal structure. X85000.

Figure 11. A cross section in the region of stratum spinosum showing the classical structure of an epithelial desmosome with a clear cytoplasmic plaque (CP) and distinct central lamella (CL). Some points of very close apposition between the adjacent membranes of this area can be frequently seen (arrows). The trilaminar unit membrane (UM) is evident and clear. (IS) indicates the intercellular space and (TF) indicates the site of the tonofilament attachment to the desmosome. (Glutar aldehyde-tannic acid fixation). X98000.

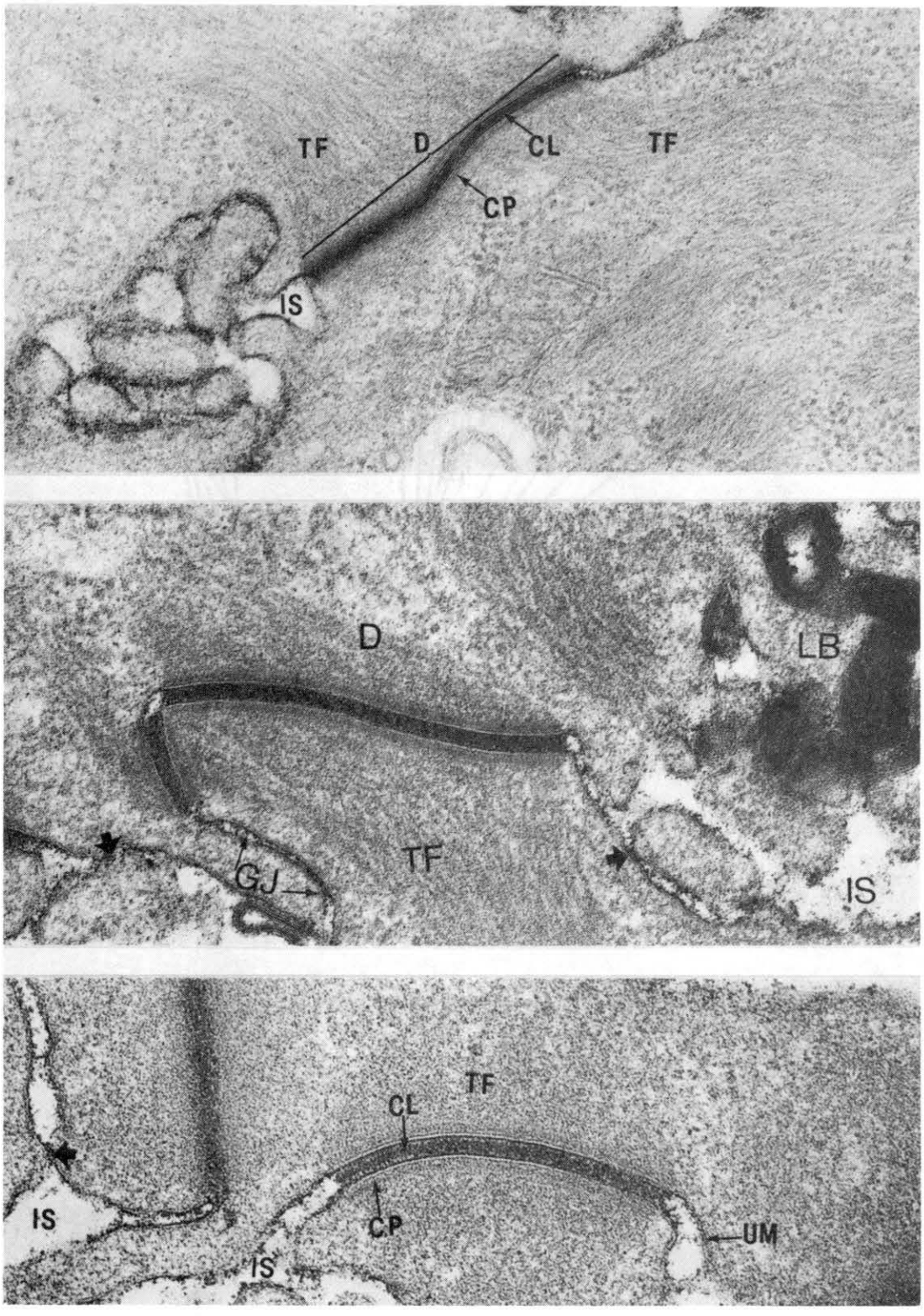
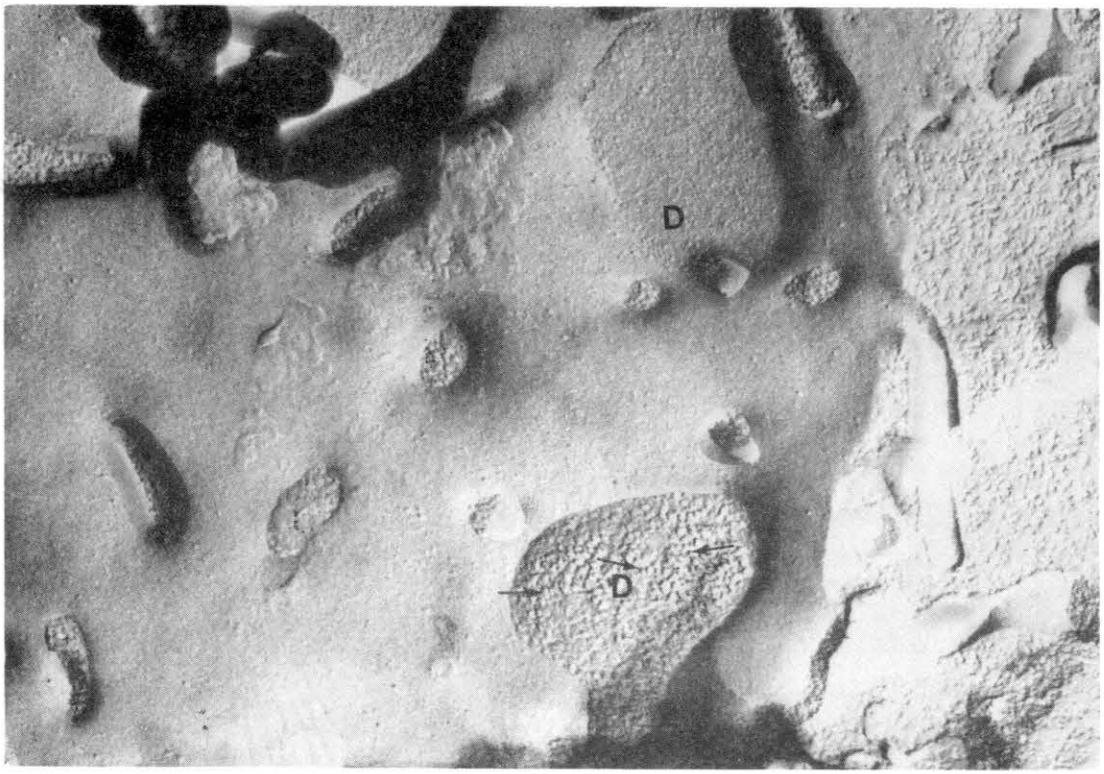

TF
D
CL
TF
CP
IS
D
LB
GJ
TF
IS
CL
TF
IS
CP
UM
IS

Figure 12. A freeze-fracture replica from the mid portion of a frog skin epithelium showing some round or elongated aggregations of closely packed particles that corrspond to the desmosomal structure in this area. Small intramembraneous particles are indicated by arrows. X35000.

Figure 13. Afreeze-fracture replica of the desmosomal structure (D) in the upper portion of the epithelium (St.Co). The desmosomes in this area seem to have fewer particles (arrows) and much smoother surface than other desmosomes belonging to other strata of the epithelium; compare with fig. 13. X35000.


D
D


D
D
D

The tannic acid-glutaraldehyde mixture fixation resulted in clearly outlined desmosomes (Figs.9-11). Tannic acid seemed to penetrate the intercellular space as an electron opaque material revealing an increased density of cell periphery and distinct intercellular space. Consistent results were obtained with a final concentration of tannic acid in the fixative mixture of 2-4 %. The trilaminar image of the desmosomal membrane was best demonstrated by using the tannic acid-glutaraldehyde mixture (Figs.9-11). The intercellular space appeared to be occupied by a moderately opaque material that is delimited by a more dense layer of the outer leaflet of the membranes sharing in the formation of the desmosome (Figs.9,11).

The "en face" views of the freeze-fracture replicas of frog skin epithelium revealed desmosomes as round or elongated aggregations of closely packed particles that measures 7-12 nm. in diameter each (Fig.12). These particles were interpreted as intramembraneous particles and/or the cleaved ends of the tonofilaments as well as the other finer microfilaments in the region of cytoplasmic plaques of the desmosomal structure. Frequently small particles (60-70 Angstroms each) could be found along the two fracture faces at the level of intercellular gap suggesting the presence of a mechanical coupling structure that cross-bridge the desmosome to act as transmembrane linkers (Fig.13). However, in contrast to what thin section images of desmosomes can easily reveal, the freeze-fracture image did not show

structures that correspond to the cytoplasmic plaque or to the central lamella. We were also not able to detect any significant difference between desmosomes that belong to different layers of the epithelium. However, desmosomes from (St.Co) seemed to have fewer particles, and much smoother surface than other desmosomes belonging to other strata (Fig.13).

Gap Junctions

Gap junctions were most frequently seen in the basal and intermediate layers of the epithelium (Figs.7,8), rarely observed between the cells of (St.Gr) (see Fig.6), and never encountered among the cornified cells (St.Co) (Fig.5).

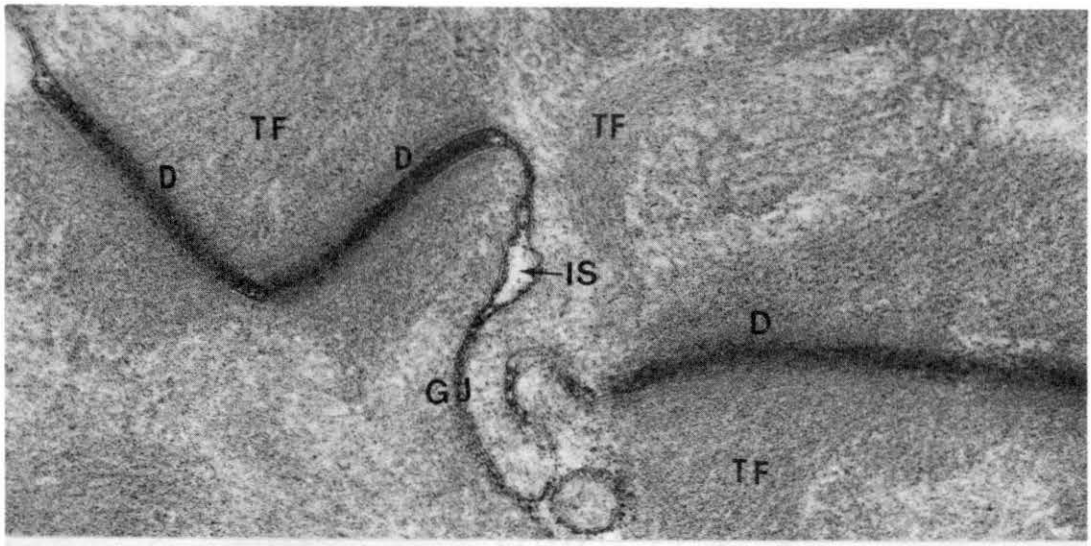
In thin sections fixed with glutaraldehyde and osmium tetraxide, each junction consisted of a modification of the two outer laminae of the adjacent plasma membranes which laid parallel to each other leaving an intercellular gap of more than 2-4 angstroms (Fig.14). In some favorable sections, the intercellular gap was seen to be interrupted by transverse small lines that extended between the two membranes forming the gap (Fig.15). These lines may correspond to the hydrophilic channeles which characterize gap junctions. The intercellular space was often obscured when the sections were cut too thick or if the membranes were inadequately stained. The en bloc staining with uranyl acetate enabeled us to see gap junctions as formed by two unit membranes that brought closely together, but still separated by more

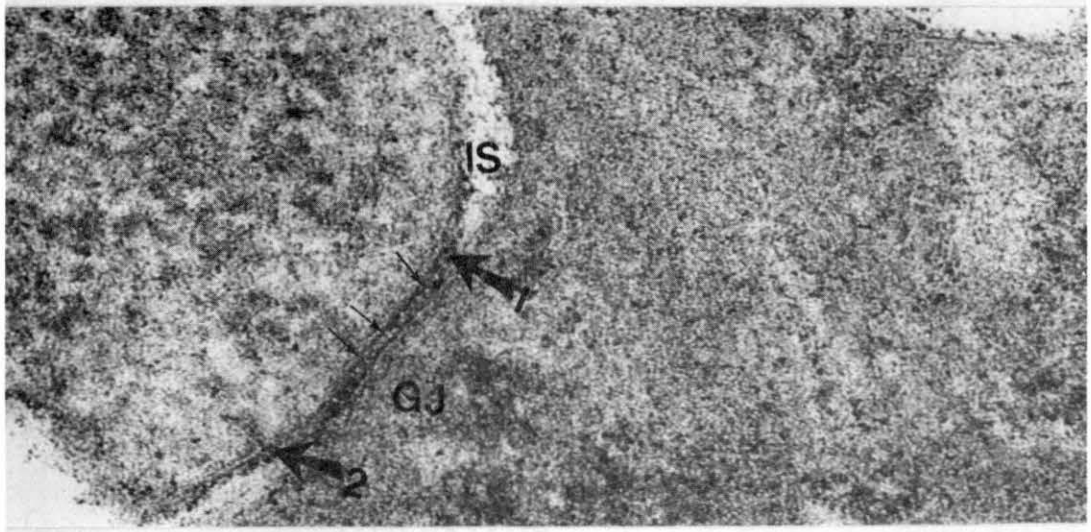
Figure 14. A gap junction (GJ) and a number of desmosomes (D) from a section in the mid portion of the epithelium. The intercellular gap in the area of gap junction seem be occupied by dense material of tannic acid. (Is) indicates the intercellular space and (TF) the tonofilaments. (Tannic acid and glutaraldehyde fixation). X85000.

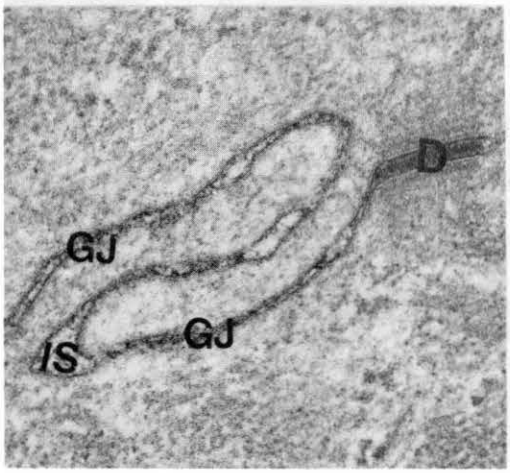
Figure 15. This micrograph shows a high magnification of a gap junction (GJ) between two cells of stratum germinativum. Some small channels (thin arrows) can be vagely seen as thin lines that cross the width of the gap. (IS) indicates the intercellular space. (Tannic acid and glutaraldehyde fixation). X160000.

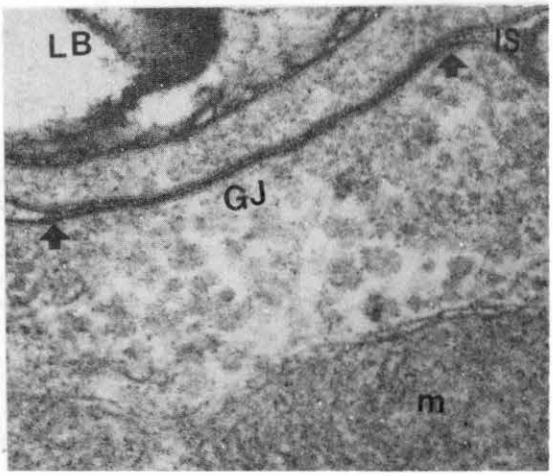
Figure 16. A cross section from the basal layer of frog skin epithelium showing some gap junctions (GJ). The intercellular gap in this section is very distinct with a diameter ranging frm 4-10 nm. This variation is probably due to the variation in the sectioning plane. (D) idicates a portion of a desmosome and (IS) indicates the intercellular space. X100000.

Figure 17. A cross section in the region of (St.Ge) of frog skin epithelium shwing a typical gap junction (GJ) between two cells of a mucous gland that is attached to the lower portion of the epithelium. (LB) is a form of lamellar body in this tissue (see text), (IS) indicates the intercellular space,and m indicates a portion of a mitochondrion. X99000.


TF
D
D
TF
IS
D
GJ
TF


IS
1
GJ
2


D
GJ
GJ
IS


LB
IS
GJ
m

than 30-40 angstroms wide electron lucent gap (Fig.16). The tannic acid-glutaraldehyde mixture fixation yielded clearly outlined gap junctions that had a width of approximately 180-190 angstroms. The gap between the two adjacent membranes in this case (40-60 angstroms) was filled with a homogeneous electron dense material (in contrast to the fluffy material found in the non-junctional space) (see Figs.14,15,17). typical gap junctions were also found frequently between the cells of the mucous glands that are attached to the lower portion of the frog skin epithelium (Fig.17).

The freeze-fracture replicas of various portions of frog skin epithelium revealed fracture faces that contained intramembranous particles (IMPs) (Fig.18). These (IMPs) were found in specific region of the cell surface in high density to form semiregular or polygonal arrays (Figs.19,20). The size of each particle on P face ranged between 60-120 angstroms with a mean value of 85+6 angstroms. The sizes of the (IMPs) reported here compared very well to the sizes of gap junction particles reported for other tissues. However, there was a slight variation in the diameter of gap junctional particles among the various cell layers. This variation may have resulted from some differences in the conditions under which the replica was prepared.

Figure 18. A low magnification micrograph of a freeze-fracture replica from the frog skin epithelium. The fracture faces of the epithelial membranes contain a number of closely packed clusters of membrane particles (arrows) which are interpreted as gap junctions. The Pt shadow is coming from the lower right. X35000.

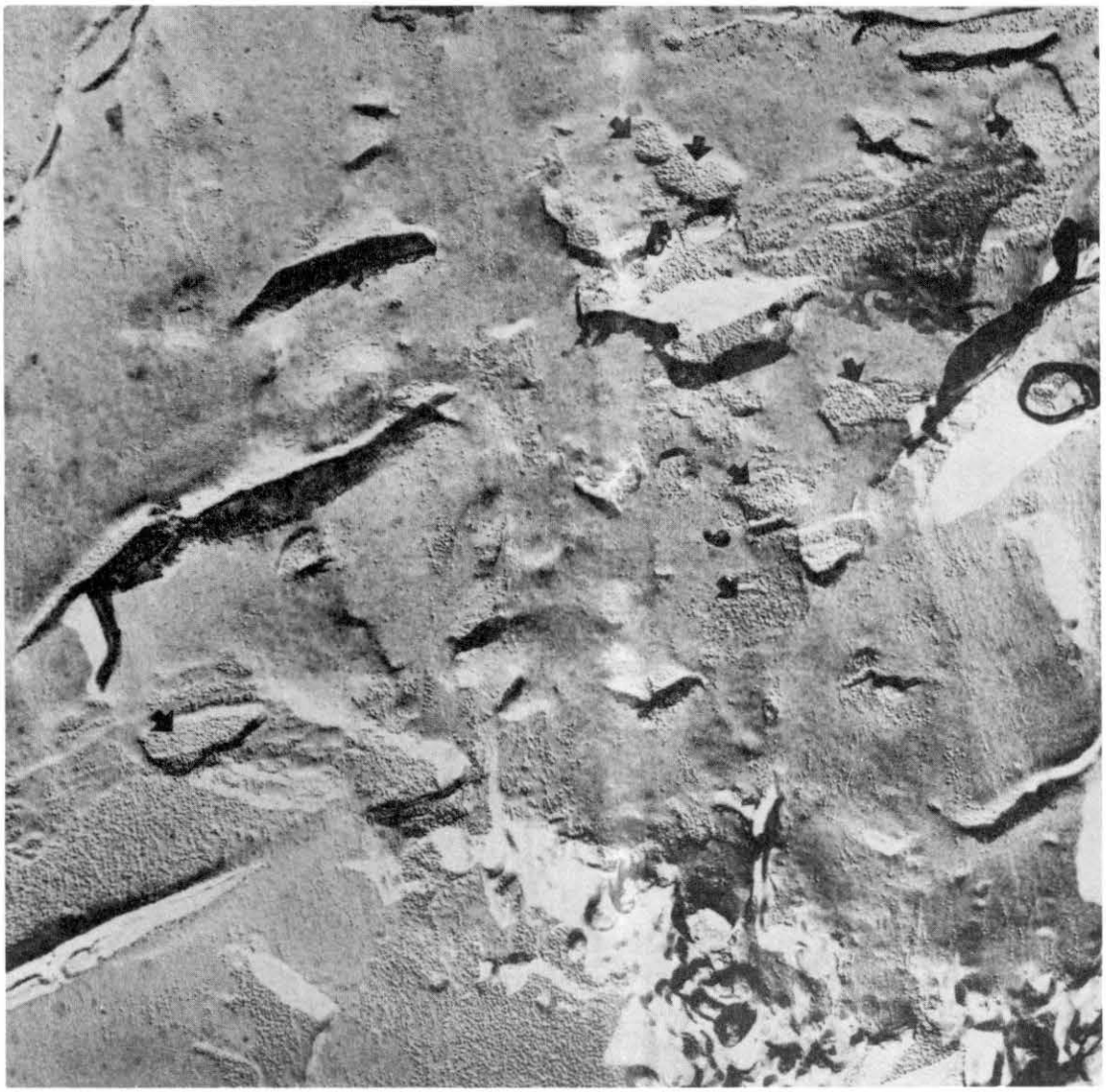
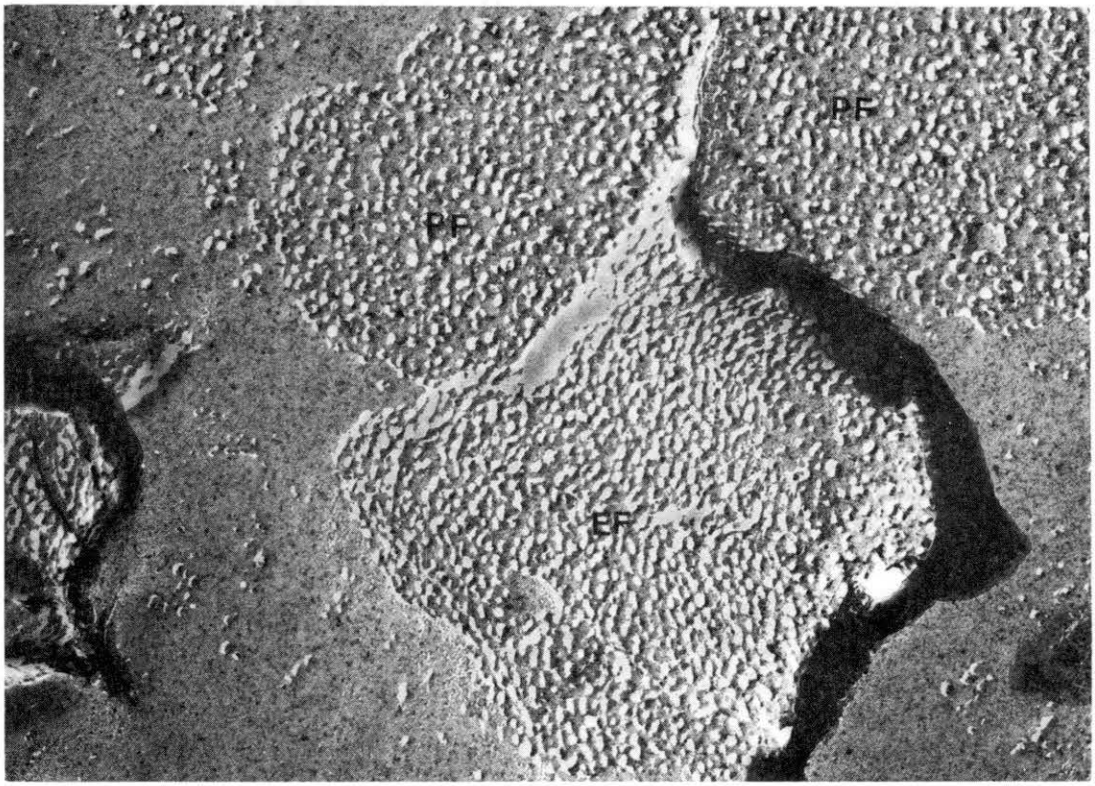


Figure 19. A freeze-fracture replica from the mid portion of frog skin epithelium showing a number of gap junctions (GJ) which appear as a disk-shaped array of closely packed particles on the P face with an ordered array of hexagonally arranged pits on the E face. The diameter of each particle on the P face is around 9 nm. The pt shadow is coming fromm the lower right. X100000.

Figure 20. A higher magnification of a freeze-fracture replica of an aggregation of some gap junctional structure of frog skin epithelium showing the individual intramembraneous particles of the junctions on P face and their complementary pits on E face. The pt shadow is coming from the lower right. X130000.


GJ
GJ
PF
GJ
EF


PF
PF
EF

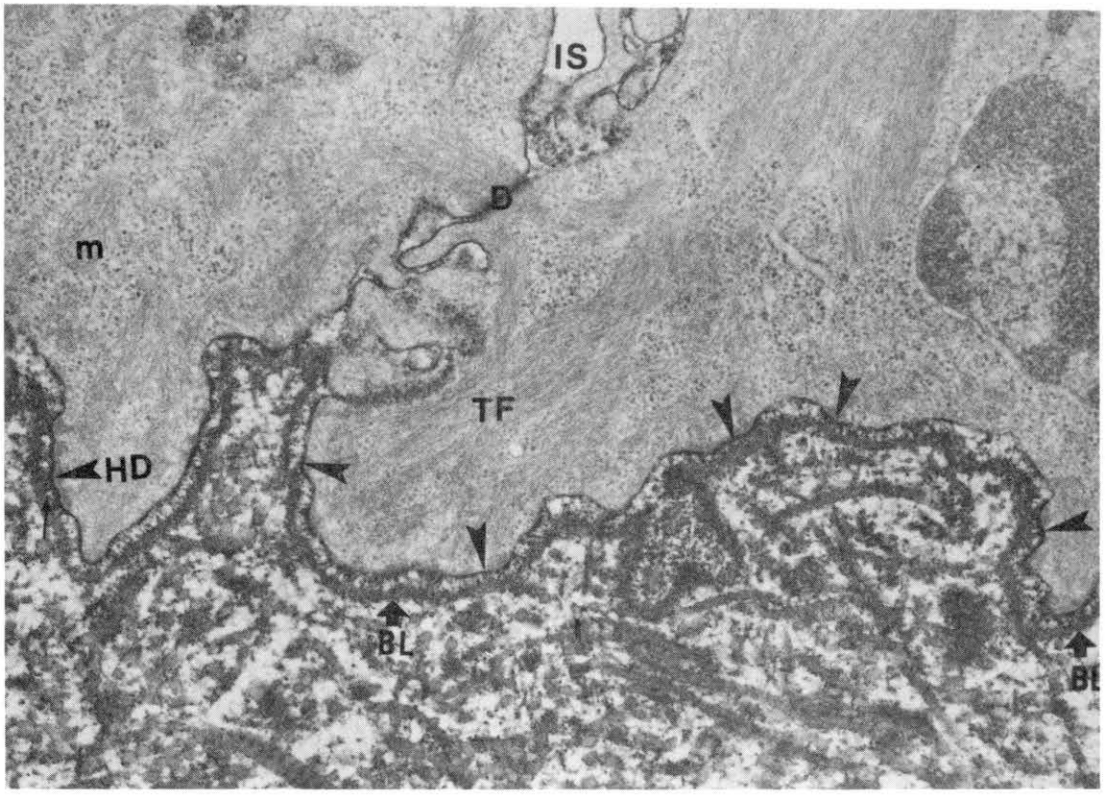
Basal Lamina and Hemidesmosomes

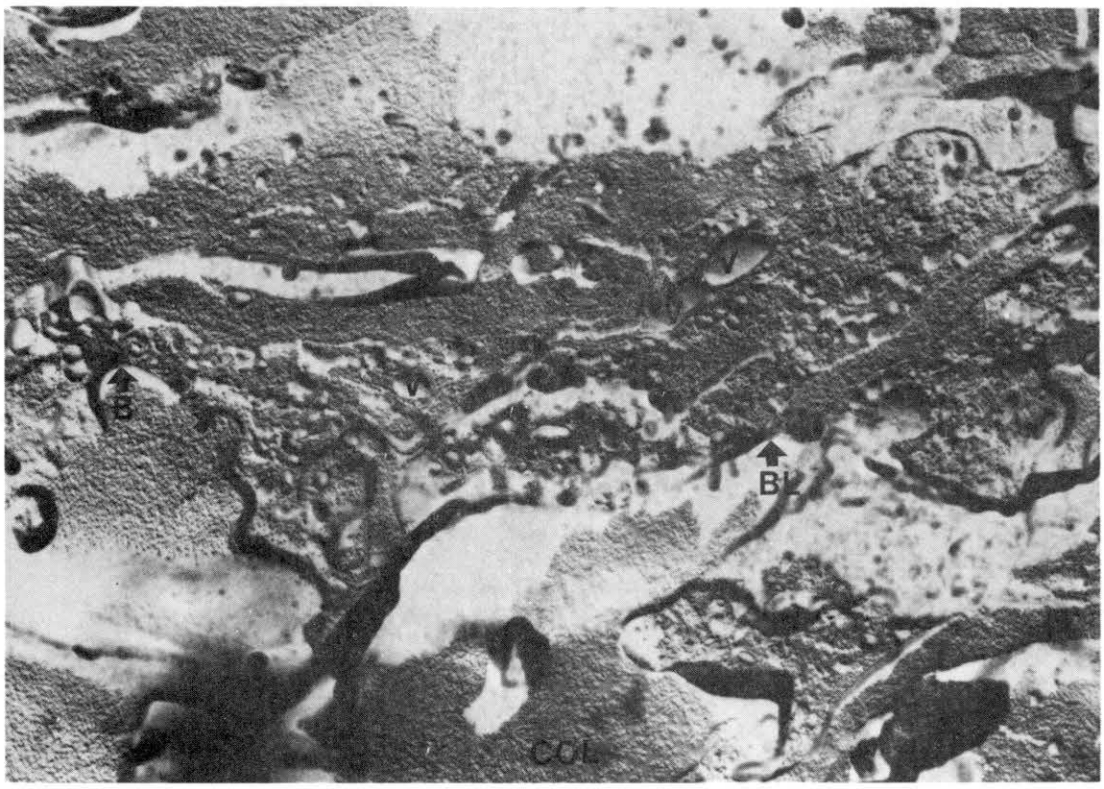
The border between the basal layer of frog skin epithelium (St.Ge) and the underlying connective tissue was characterized by irregularly plicated surface which was lined by an extracellular structure called the basal lamina (BL) (Fig.21). The whole length of the basal plasma membranes was heavily populated by areas of distinct densities which represent the site of hemidesmosomes that mechanically couple the epithelium to the underlying connective tissue. Hemidesmosomes were very well demonstrated by using tannic acid-glutaraldehyde fixation. They appeared to have distinct dense cytoplasmic plaque at which bundles of tonofilaments were attached, while the other side of this structure is shared by the (BL) (Fig.21). A set of strand-like fibrillar structures seemed to extend from the (BL) to the cytoplasmic plaque forming the mechanical aspects of epidermal-dermal junction.

The freeze-fracture image of the epidermal-dermal border did not give us much insight about this area (Fig.22). However, pinocytotic vesicles were evident all along the length of the membranes running parallel to the (BL). Hemidesmosomes appeared as clusters of smooth particles randomly distributed on the epithelial side of epidermal-dermal junction. Other features of hemidesmosomes, like cytoplasmic plaques and the tonofilament attachment to them were not distinct, however a vague trace of particles can be detected inside

Figure 21. A cross section in the region of the basal layer of frog skin epithelium showing the basal lamina (BL) which represents the only contineous structure that separates the epithelium from the underlaying connective tissue. The basal membrane of the stratum germinativum cells is separated from the (BL) by lamina lucida which is occupied by transverse fibers (thin arrows) that connect the (BL) to the basal membranes of the (St.Ge) cells. The basal plasma membrane is heavily populated by electron dense structures which is termed hemidesmosomes (HD) which act as mechanical couplers between the epithelium nd the underlayng connective tissue. (D) is a desmosome, (m) is a mitochondrion,(IS) designates the intercellular space, and (TF) indicates the tonofilamenets. (Tannic acid-glutar aldehyde fixation). X28000

Figure 22. A freeze-fracture replica of a portion of the epithelium in the dermal-epidermal region. some pinocytotic vesicles (V) are distributed all along the length of the border line between the epithelium and the underlayng connective tissue (arrows). Some collagene fibers (COL) can be seen in the dermal region of the skin. Other structures characterizing this area are absent or less distict (see text). X45000.


IS
D
m
TF
HD
BL
BL


B
V
BL
COL

lamina lucida (LL) which probably correspond to the fine fibrillar structures that link the plasma membranes with the (BL).

Quantitative Analysis of The Frequency of The Intercellular Junctions in Frog Skin Epithelium

Table 1 shows the frequency of intercellular junctions (tight junctions, gap junctions, and desmosomes) found in different strata of the epithelial tissue of adult frog skin fixed immediately after removal from the animals. Tight junctions were present only between the cells of (St.Gr) and (St.Co). Their frequencies (No.of junctions per unit of membrane length) were 0.07±.01 and 0.14±.06 respectively. This type of junctions was not observed in any stratum below the first layer of (St.Gr). The significant difference in tight junction frequency between (St.Co) and (St.Gr) can be ascribed to the fact that (St.Gr) in most cases is comprised of two cell layers with the tight junctions present only in the upper most layer of this strata, while (St.Co) is mainly constituted by one single layer of very thin cells that have tight junctions on all their apico-lateral surfaces.

Desmosomes were present in all epithelial layers. Their frequency tended to increase from the basal layer (St.Ge) where they have their lowest frequency (.09±.02) to the upper layers of the epithelium reaching their highest frequency at

Table I. The Frequency of (IJ) in the different Strata of Frog Skin Epithelium

Region	N	L μm.	Frequency of D	Frequency of G	Frequency of T
St. Ge	35	1199.63	.09±.02	.23±.02	00
St. Sp	55	1343.10	.34±.03	.20±.018	.001
St. Gr	35	1411.92	.37±.032	.04±.009	.07±.01
St. Co	31	948.543	.30±.024	000	.14±.06

Table I. The frequency of intercellular junctions (IJ) in different strata of frog skin epithelium. N is the number of photos examined; L is the total length of the membranes examined for each strata; D is desmosomes; G is gap junction; and T is tight junctions. The freqency is calculated as the number of junctions per unit length of the membrane.

Table II. Among group comparisons of the Frequency of Desmosomes in Different Strata of Frog Skin Epithelium.

Group vs Group	N	Frequency Mean±SE	T Calculated	P	Status
St. Ge	35	.09±.026			
St. Sp	55	.34±.023	7.47	<.05	Significant
St. Ge	35	.09±.026			
St. Gr	36	.37±.032	7.28	<.05	Significant
St. Ge	35	.09±.026			
St. Co	31	.30±.024	6.52	<.05	Significant
St. Sp	55	.34±.026			Not
St. Gr	36	.37±.032	0.78	>.05	Significant
St. Sp	55	.34±.026			Not
St. Co	31	.30±.024	1.11	>.05	Significant
St. Gr	36	.37±032			Not
St. Co	31	.30±.24	1.60	>.05	Significant

Table II. The result of among group t test comparison of the frequency of desmosomes (number of desmosomes per unit length of the membrane; means ± standard error) between each two strata of the epithelium. N; is the number of photos examined of each strata, and p represents the statistical probability.

the stratum granulosum (.37±.032), however their frequency declined in the stratum corneum, but not significantly (.30± .024) see (Table 1). The statistical comparisons of the desmosomal frequency among different strata of the epithelium indicates that the desmosomal frequency in (St.Ge) is significantly less than their frequency in each of the other strata when considered separately (Table 2). Comparisons of desmosome frequency among cells of (St.Sp), (St.Gr), and (St.Co) showed no significant differences among these three strata (Table 2)

Gap junctions were observed in the (St.Ge), (St.Sp), rarely in (St.Gr), but never in the (St.Co). Their frequency decreased from the basal layers of the epithelium where they exhibited their highest frequency (.23±.02) see Table 3 to the upper layers where they rarely encountered in stratum granulosum with frequency less than .04±.009 or never found in stratum corneum. Table 3 shows among strata comparisons of the frequency of gap junctions. The frequency of gap junctions in (St.Gr) is significantly less than that of either (St.Sp) or (St.Ge) however, there was no significant difference in gap junction frequency between (St.Ge) and (St.Sp). On the other hand, gap junction frequency between the cells of (St.Gr) was significantly higher than that encountered between the cells of (St.Co).

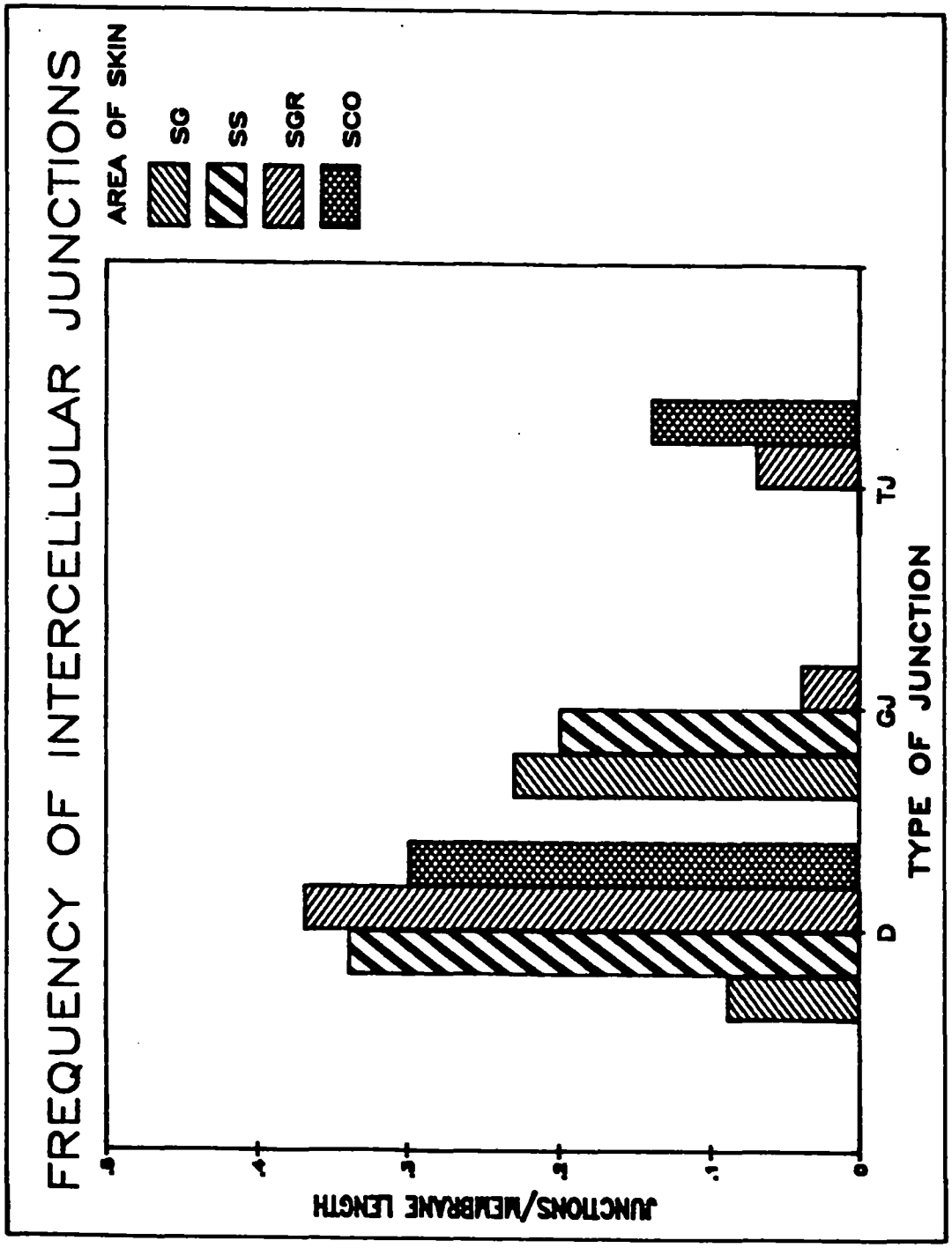
The histogram on (Fig.23) summarizes our data regarding the frequency of intercellular junctions in the different strata of frog skin. The frequency was calculated as the

Table III. Among Group Comparisons of the Frequency of Gap Junctions in Different Strata of Frog Skin Epithelium.

Group vs Group	N	Frequency Mean±SE	T Calculated	P	Status
St. Ge	35	.23±.026			Not
St. Sp	55	.20±.023	.96	>.05	Significant
St. Ge	35	.23±.026			
St. Gr	36	.04±.009	6.98	<.05	Significant
St. Ge	35	.23±.026			
St. Co	31	000	8.39	<.005	Significant
St. Sp	55	.20±.023			
St. Gr	36	.04±.009	6.59	<.05	Significant
St. Sp	55	.20±.023			
St. Co	31	000	8.05	<.005	Significant
St. Gr	36	.04±009			
St. Co	31	0000	3.73	<.05	Significant

Table III. The result of among group t test comparison of the frequency of gap junctions in different strata of frog skin epithelium. The frequency was calculated as means ± standard errors of the number of gap junctions per unit length of the membrane examined. N is the total number of photo examined for each strata, and P represents the usual statistical probability.

Figure 23. A histogram showing the frequency of the intercellular junctions in different strata of frog skin epithelium. The examined areas are: stratum germinativum (SG), stratum spinosum (SS), stratum granulosum (SGR), and stratum corneum (SCO). Tight junctions are present only in the upper two strata of the epithelium (SCO and SGR). Desmosomes (D) are found in all epithelial layers. Desmosome frequency tend to increase from the basal to the upper layers of the epithelium. Gap junctions (GJ) are are found in the basal (SG) and the intermediate layers (SS) of the epithelium. However, there are few gap junctions among the cells of (SGR), and none between the cells of (SCO). The frequency is expressed as the number of junctions per unit length of the membrane.


FREQUENCY OF INTERCELLULAR JUNCTIONS
AREA OF SKIN
SG
SS
SGR
SCO
JUNCTIONS/MEMBRANE LENGTH
0
D
GJ
TJ
TYPE OF JUNCTION

number of junctions per unit length of the membrane. The region examined are: stratum germinativum (SG), stratum spinosum (SS), stratum granulosum (SGR), and stratum corneum (SCO). This histogram shows that tight junctions are present only between the cells of (SCO) and (SGR). The frequency of desmosomes icreases as we move from the basal layers of the epithelium towards the upper layers. On the other hand, the frequency of gap junctions tend to decrease in the same direction. For pairwise and among group comparison between different strata of the epithelium refer to (Tables 2 and 3).

Discussion

Tannic acid has been used successfully as a tracer for localizing intercellular junctions in other biological tissues (Deurs, 1975). By applying this technique on frog skin, tight junctions appeared as a fusion of two plasma membranes which seals the intercellular space completely preventing the entrance of tannic acid in the intercellular spase. Tannic acid however,precipatated in the non junctional intercellular space.

The presence of tight junctions in a sodium-transporting epithelium like that of frog skin is very essential for the maintenance of the electrochemical gradient across the epithelium. Thus, they are usually associated with the structural aspects of the permeability barrier in this tissue. Martinez-Palomo et al.,(1971), using tracer techniques,have

determined that the tight junctions at the apicolateral surfaces of both (St.Co and St.Gr) of the epithelium are true occluding zonules (impermeable to lanthanium and ruthanium red). However, since the cells of (St.Co) are usually considered dead or dying cells [for example; they do not restrict the movement of solutes and water across their membranes and do not regulate their ionic intracellular contents of electrolytes (Rick,et al., 1978)], the actual site of the physiological barrier in frog skin epithelium is confined to the set of tight junctions localized between the cells of the outer most layer of (St.Gr).

The fine structure of desmosomes and their modifications have been previously investigated in frog skin epithelium (Farquhar and Palade, 1965), and other various epidermis (Odland, 1958; Hibbs and Clark, 1959; Listgartin, 1964; Matoltsy and Parakkal, 1965; Kelly, 1966; Frithiof, 1970; Orwin,et al., 1973; Allen and Potten, 1975; Raknerud, 1975; Shimono and Clementi, 1976). In frog skin epithelium, the morphological characteristics of desmosomes as revealed by thin section technique are essintially the same in (St.Ge), (St.Sp),and (St.Gr) and they are in general similar to those described in other epidermis (see above). However,desmosomes that exist between the cells of (St.CO) and those present along the boundary between (St.Co) and (St.Gr) exhibit some slight modifications in their characteristics from the rest of desmosomes that are encountered in the lower strata of the epithelium (Farquhar and Palade,1965). It is mostlikely

that these modifications are introduced to the desmosomes during the process of epithelial cell differentiation (see Frithiof, 1970) and it is conceivable also that the lamellar bodies which are extruded from the cells into the intercellular space may play an important role in the occurrence of these modifications through some kind of incorporation or exchange between the lipids of these lamellar bodies and that of the plasma membranes.

The quantitative analysis of the frequency of desmosomes in different regions of the epithelium (Table 1) showed that their frequency (number of desmosomes per unit length of the membranes) was minimal between the cells of (St. Ge) and increased towards the outward layers of the epithelium until they reached their highest frequency among the cells of (St.Gr), then their frequency began to decrease slightly. This indicates that the formation of new desmosomes takes place during the process of differentiation until this process reaches its peak at (St.Gr) where the structure of desmosomes begins to decay, and their number decreases. The high frequency of desmosomes in (St.Gr) can also be explained on the basis that the cells of this strata are more subject to physical stress and environmental impact than the lower strata. This explanation is conceivable because it is widely accepted that the main function of desmosomes in the epithelial tissue is to provide adhesive forces that mechanically connect the cells together to protect their physical integrity and unify them to act as a complete uninterrupted unit.

The fact that the cells of (St.Ge) exhibit a significantly lower frequency of desmosomes than other strata (Table 2) can only be explained on the basis that these cells are at an early stage of differentiation,and have not yet developed desmosomes. The average depth of desmosomes that join the cells of (St.Ge) is much less than the average depths of those in the upper strata (Compare Fig.8 with 6 and 7). This observation also seems to support the notion that desmosomes grow in number and size as the process of differentiation takes place.

Compelling evidence has been accumulated throughout the last twenty years to suggest that the cells of all epithelial layers of frog skin epithelium (MRC and gland cells seem to be exceptions Rick et al.,1984) are coupled together forming a functional syncytium for sodium transepithelial transport (Ussing and Windhager, 1964; Rick et al., 1978; Helman, 1979 Nagel,et al., 1981; Rick, et al., 1984).

Gap junctions are widely accepted to provide low resistance cell to cell pathways for the movement of ions, water, and other small water-soluble molecules, thus playing an essential role in electrotonic coupling, metabolic cooperation and regulation of growth and differentiation (Sheridan, 1971 Bennett, 1973; Lowenstein, 1966,1979,1981; Finbow, 1982). The failure to detect such classical gap junctions among the cells of frog skin epithelium (Farquahr and Palade, 1965; Martinez-Palomo,et al., 1971) is difficult to reconcile with the syncytial nature of this tissue. Possible reasons for

the difficulties in locating gap junctions in this epithelium might be due to the fact that these junctions are too small to be detected by the available methodology, or that the fixation procedure is not adequate to reveal such junctions.

Using a new fixation procedure constituted of glutaraldehyde and tannic acid, we have demonstrated the occurrence of reasonable numbers of gap junctions which have similar characteristics to those reported in tissues of some non-mammalian species (Martinez-Palomo and Mendez, 1971). These junctions are characterized by intimate appositions of adjacent cell membranes leaving an intercellular gap of 4-6 nm. This gap is frequently filled by tannic acid revealing a homogeneous electron-dense material that separates the two plasma membranes sharing the formation of the junctions. In favorable sections some electron-lucent lines can be seen crossing the intercellular gap . The depth of these junctions varies from 200 nm. to 1 μm. Their frequency (number of junctions per unit of membrane length; see Table 1) shows that their highest frequency occurs between the cells of (St.Ge) and decreases gradually as we move towards the upper layers of the epithelium. The among strata comparisons of gap junctional frequency (Table 3) demonstrates that (St.Ge) and (St.Sp) exhibit almost the same number of gap junctions per unit length of the membrane (the t test shows no significant difference in the gap junctional frequncy between the two strata, $P>.05$). However, a significant difference in the gap junctional frequency is evident between the (St.Ge) and the

(St.Gr) cells (P<.005). Similar significant difference does exist also between (St.Sp) and (St.Gr) (P<.o5) (see Table 3). The cells of (St.Co) completely lack gap junctions thus, the frequency of gap junctions in each stratum of the epithelium (including St.Gr) is sig- nificantly higher than that of (St.Co).

Assuming that the cell-to cell coupling in this tissue occurs solely through gap junctions, this study demonstrates that the cells of (St.Ge) and (St.Sp) are well coupled to each other however, the cells of (St.Gr) do not have as many gap junctions as those of the lower strata (St.Sp and St.Ge) (see Fig.24). If gap junctions from different tissues have the same molecular structures as some suggest (Hertzberg and Skibbens, 1984), it would not be unreasonable to conclude that partial uncoupling may exist in frog skin epithelium. This means that sodium ions which diffuse across the apical membranes of the epithelium into the cells of (St.Gr), have very few channels to traverse in getting into the cells of the underlaying strata. In support of this conclusion, previous work from this laboratory using microelectrode back filled with lucifer yellow dye (Duncan, 1982) showed that the injection of lucifer yellow (MW.450) into some cells of (St.Gr) did not result in the transfer of the dye to the neighboring cells in (St.Gr) or to other cells in the lower strata. However, injecting the same dye from the serosal side (in the cells of St.Ge) resulted in a wide spread of the dye in the epithelial cells of stratum germinativum.

Recently, Rick et al., (1984)° demonstrated that after lowering the Na concentration in the internal bath, the frequency of the Na concentration increases in the outer most living layer of the epithelium and drops in the deeper epithelial layers. They concluded that partial uncoupling of the transport syncytium does occur under such conditions.

It is of interest to document the similarity between our observations on gap junction frequncy in frog skin epithelium and that of other epidermis such as human epidermis (Siegenbeek Van Heukelon, et al., 1972; Breathnach, 1972; Caputo and Peluchetti, 1976), and oral epithelium (Shimono and Cleminti, 1976). In these tissues gap junctions were only observed in the basal and intermediate layers of the epidermis, while the upper layers were completely devoid of such junctions.

It's not surprising to find that (St.Co) is devoid of gap junctions,since the cells of this strata are considered dead or dying cells that exchange their electrolytes freely with the outer bathing solution (Rick,et al., 1981). However, the puzzling finding is the significantly low frequency of gap junctions between the cells of (St.Gr) and between them and their neighbors of (St.Sp) cells. This finding does contrast with the general belief that the cells of all epithelial layers are actually involved in the transepithelial sodium transport forming a coherent functional syncytium for sodium transport compartment.

CHAPTER V

IMMUNOFLUORESCENCE LOCALIZATION OF GAP JUNCTIONS IN FROG SKIN EPITHELIUM

Introduction

Gap junctions are specialized areas of plasma membranes. Gap junctions are usually associated with the low-resistance pathways that allow the exchange of small water-soluble molecules between the cells (Gilula, et al., 1972; Sheridan, 1976; Bennet and Goodenough, 1978). Gap junctions from a variety of mammalian (Friend and Gilula, 1972; MucNutt and Weinstein, 1973) and non-mammalian tissues (Martinez-Palomo and Mendez, 1971; Larsen, 1977) show many ultrastructure similarities with minor variations. Isolated gap junctions contain a major polypeptide of 27K dalton (Hertzberg,1984). Antibodies raised against the 27000 dalton rat liver gap junction protein seem to cross-react with similar gap junctional protein from tissues of a variety of species including mammals, fish, and birds (Hertzberg and Skibbens, 1984). This structural and biochemical homology between gap junctions of different tissues explain the association of the cell-to cell communication with the presence of gap

junctions in most electrically coupled epithelia (Loenstein, 1966, 1981; Michalke and Lowenstein, 1971).

The epidermis of frog skin is a sodium-transporting epithelium which has served as a traditional experimental model in epithelial transport studies. Electrophysiological studies on this tissue indicate that there is an extensive electrical coupling between the cells of all the epithelial layers of the skin (Nagel, 1976; Helman and Fisher, 1977; Helman, 1979; Fisher et al.,1980). However, morphological studies (Farquhar and Palade, 1965; Martinez-Palomo et al., 1971) do not show classical gap junctions between the cells of this epithelium.

Using different electron microscopic techniques, we have demonstrated the presence of junctional complexes that have structural features similar to those of classical gap junctions (see section IV). The frequency and regional distribution of these junctions show specific patterns in which some cell layers of the epithelia have more junctions per unit membrane length than others. The purpose of this study is to confirm the presence of gap junctions in this tissue by applying other techniques. We have adapted an immunofluorescent procedure (Hertzberg and Skibbens, 1984) to localize the gap junctions and study their distribution in the epithelium of frog skin.

The antibodies raised against rat liver gap junction seem to cross-react with corresponding antigen in frog skin epithelium. This cross-reaction exhibits a distributional pattern that is similar to that demonstrated by thin

section and freeze fracture techniques.

Material and Methods

A relatively pure preparation of rat liver gap junctions was obtained according to a procedure originated by Hertzberg (Hertzberg, 1984). Antibodies to this gap junctional fraction were raised in sheep (see Hertzberg and Skibbens, 1984) and affinity purified for use in immunofluorescence lacalization of gap junctions in biological tissues.

The cross-reaction of the antibodies with the the gap junctional polypeptide of frog skin epithelium was evaluated by indirect immunofluorescence method on frozen sections of the skin. Healthy unsexed medium frogs (Rana Pipiens) were used in this study. Small pieces of abdominal skin were mechanically scraped by a razor plate and rapidly frozen in liquid nitrogen. Cryostate sections of 2-4 microns thick were prepared from the frozen blocks. Before use, the sections were air dried and then incubated in a blocker of either 10% rabbit serum or 10% fetal calf serum (FCS) for 35 min. The sections then were incubated with the affinity purified antibody (Kindly provided by DR. E.L. Hertzberg, the Medical center, Houston,Texas) at concentration of 1:50 in (FCS) for 2 hrs., washed in PBS for 1 hr. and then incubated with the second antibody (Fluorescein-conjugated rabbit anti-sheep IgG; Cappel laboratory) at a concentration of 1:60 in PBS for 45 min.

The sections were examined with a Nikon Labophat microscope equiped with a 100 watt mercury lamp and a fluorescence epiillumination system.

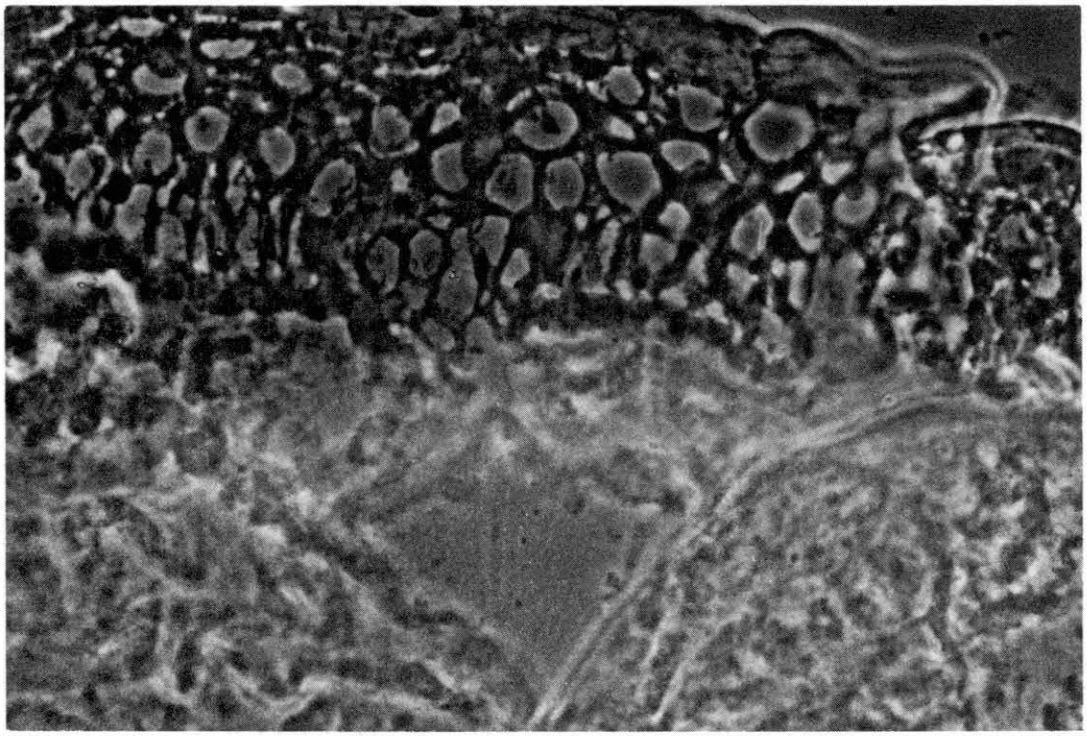
Results

The phase-contrast image of a cryostat section of frog skin showed that the epithelium is constituted by a number of organized layers of cells (Fig.1) which corresspond to the epithelial image in thin section preparation (see chapter III). The fluoresence image of the same preparation showed identical organization (Fig.2).

Consistent with the dispositions of gap junctions in frog skin epithelium as demonstrated by thin section and freeze fracture techniques (see chapter IV), the immunofluorescence staining of frozen sections of frog skin revealed a similar distributional pattern of cross-reactions between the antibody and the epithelial membrane. This pattern was characterized by isolated punctuates of fluorescence that usually follow random distribution (Figs. 3,4). In some cases, the boundary of the cells, especially those which are located in the lower strata of the epithelia seem to be heavily populated by very small punctuates which were interpreted as the cross-reaction between the gap junction antibody and its antigen in frog skin epithelum (Fig.5). When a preimmune serum was used instead of the affinity purified antibody,no fluorescence

Figure 1. Shows the phase-contrast image of a cryostat section of frog skin. The section was cut perpendicular to the surface of the skin. The upper part of the picture is the epithelium, and the lower part of the is the connective tissue. The epithelium consists 6-8 layers of cells. X450.

Figure 2. The corresponding immunofluorescence image of fig.1. Rat liver gap junction antibodies were not added to this section. X450.



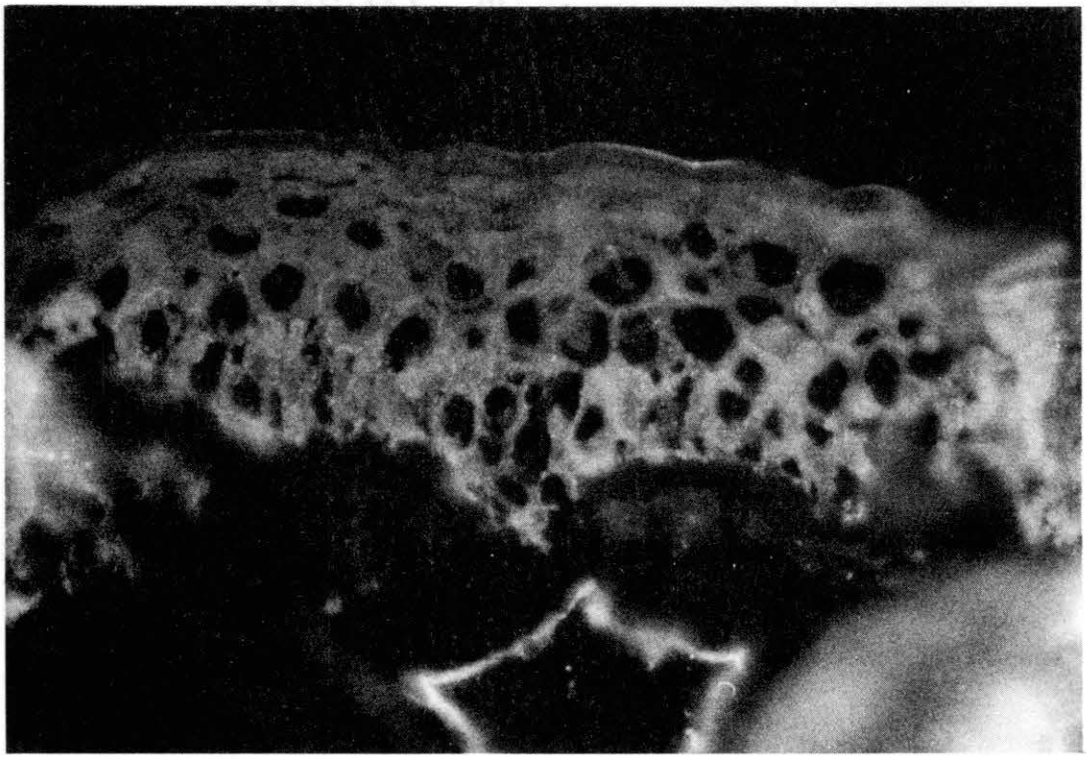
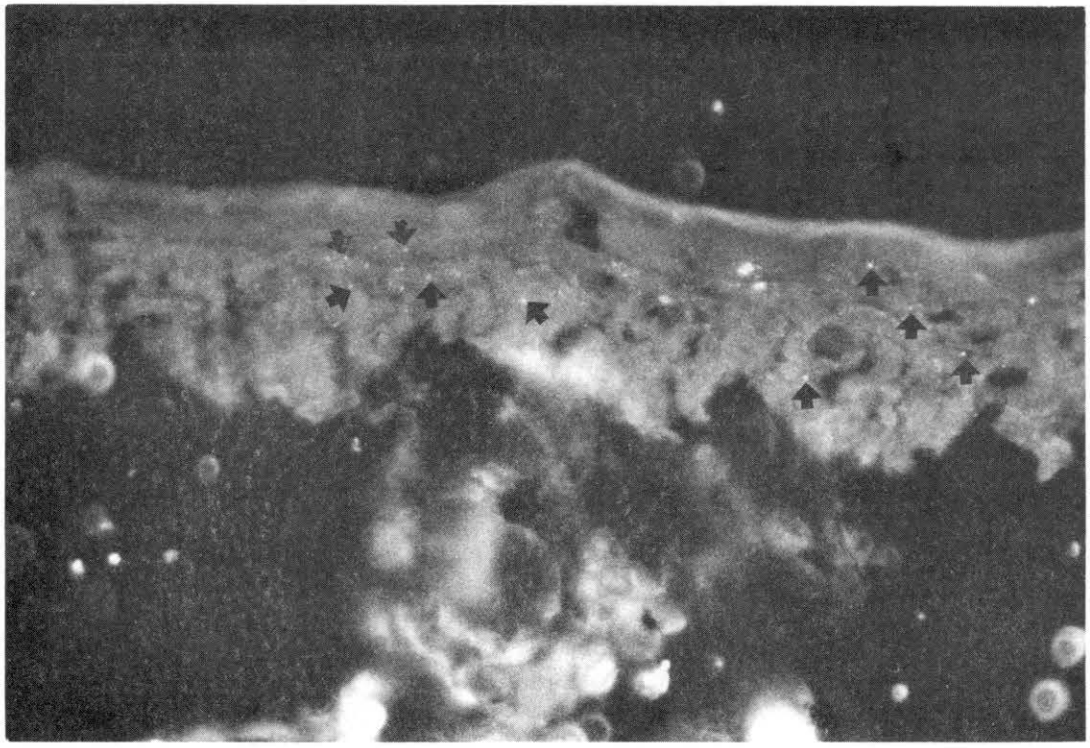


Figure 3. Indirect immunofluorescence localization of antibody-binding on frozen section of frog skin. The cryostete section was incubated with affinity purified antibodies to the 27,000 Dalton rat liver gap junction polypeptide, and then a fluorescein-conjugated rabbit anti sheep Ig G was added to the section. The cross-rection of the antibody with the epithelial membranes is demonstrated as small punctuates (arrows) which follow random distribution. X450.

Figure 4. Cryostate sections of two different frog skins were analyzed by indirect immunofluorescence staining with antibody to the rat liver gap junctions. Both the left and the right figures show cross-recactin with the gap junction antibody in the form of isolated punctuates. However, the surface area of the puctuates in the right figure is slightly higher than that of the left one. X450.



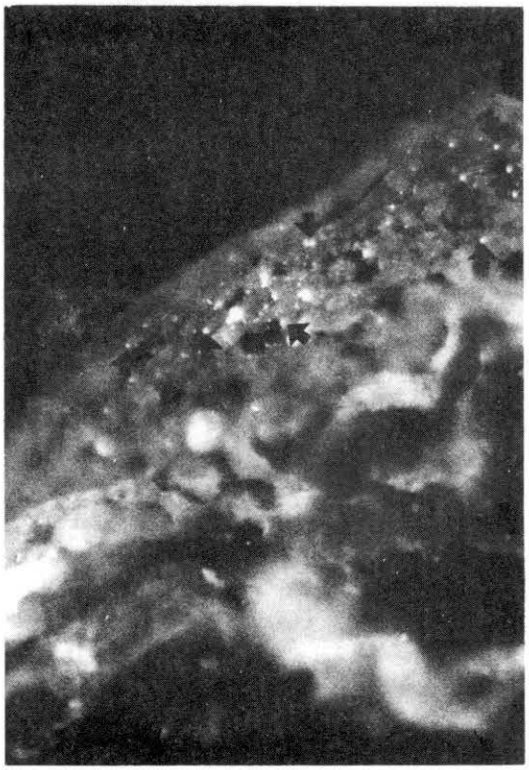
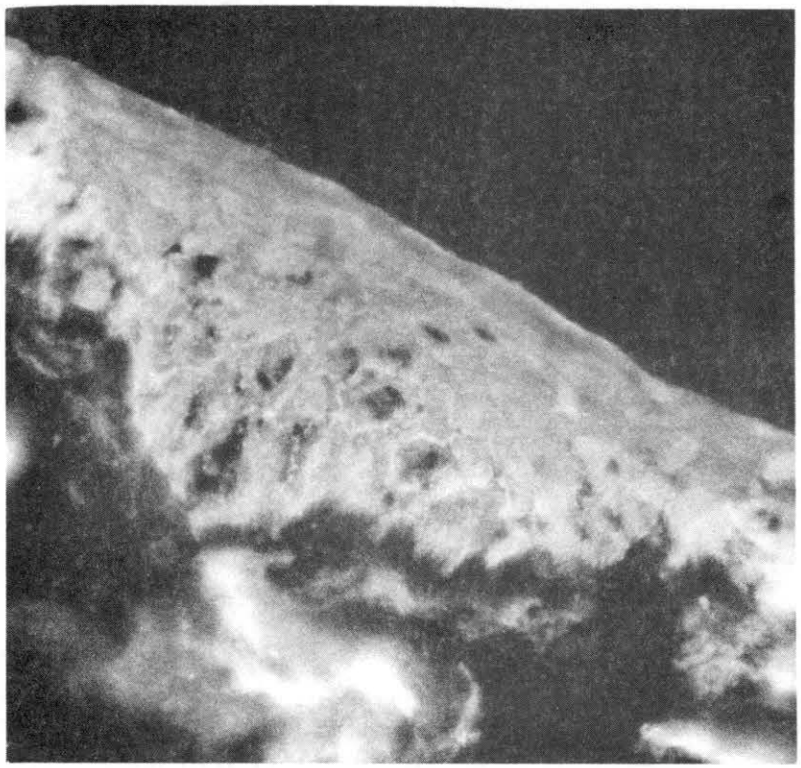
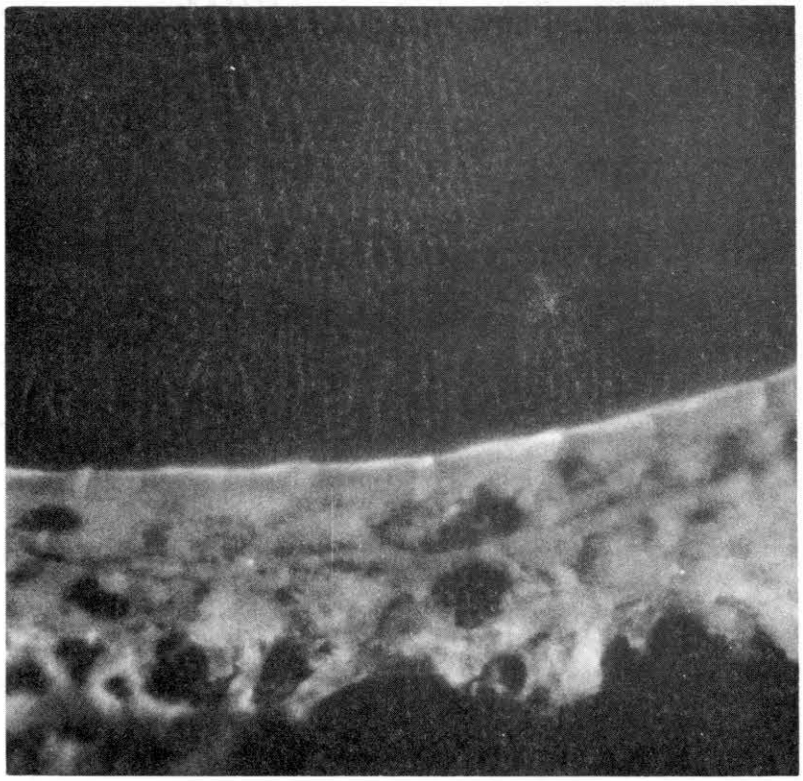


Figure 5. A cryostate section of frog skin. The section was incubated with affinity purified rat liver gap junction antibody, and then was treated with the fluorescein-conjugated rabbit anti-sheep Ig G. The periphery of the cells of the basal and intermediate layers of the epithelium contain cross-reaction punctuates between the gap junction antibody and its antigen in the frog skin epithelium. Theupper layers of the epithelium is devoid of the punctuates. X450.

Figure 6. A cryostate section of frog skin. The section was treated with a preimmune serum, and then it was incubated with a fluorescein-conjugated rabbit anti-sheep Ig G. No immunofluorescence punctuates are seen in this section. X450.





punctuates were observed on the sections of frog skin epithelium (Fig.6).

A general pattern was obtained in most of the frogs examined, however some slight variations in the total number of punctuates and in their distributional pattern were observed. The antibody-binding regions in the epithelium of frog skin were similar to the antibody-binding of other tissues such as human liver, monkey liver, sheep, goldfish and chicken, but smaller than the antibody-binding region of rat, mouse and rabbit tissue (see Hertzberg and Skibbens, 1984). Due to the low number of gap junctions in frog skin epithelium when compared to other tissues such as rat or rabbit liver, the total surface area of the punctuates in frog skin is much less than that found in rabbit, rat and mouse liver.

Discussion

Gap junctions seem to be universal features of metazoan animals (see Finbow, 1982). Their main function is to provide hydrophilic communicating channels that join the cytoplasms of neighboring cells. At the molecular level, the gap junction is a member of a whole family of membrane proteins that can be isolated and purified for use in immunological studies (Hertzberg and Gilula, 1979). Antibodies to the purified gap junctional protein can serve as a functional assay for the biochemical and cytological identification and localization

of gap junctions in biological tissues (Hertzberg, 1980; Hertzberg and Skibbens, 1984).

In this study we have adapted an immunofluorescence technique developed by Hertzberg and collaborators (Hertzberg and Skibbens, 1984; VanEldik, et al., 1985) to identify and localize gap junctions in frog skin epithelium. The data presented here demonstrate that the gap junction antibody binds to specific areas of the epithelial membrane. The antibody-binding appears as small dots which generally follow a predictable distributional pattern. This distributional pattern of gap junctions seems to correspond to their distribution as demonstrated by thin section and freeze-fracture techniques (see chapter IV). However, it should be mentioned that some variations in the distribution of the cross-reaction regions were observed. Some sections show fewer binding signals than others. The variation in the distributional pattern of frog skin gap junctions and the scarcity of their numbers might be due to the fact that this technique reveals only gap junctions which are more than 1 um in depth. Thus, most of the gap junctions that have depths of less than 1 um. escape detection by this method. Thin section measurments of frog skin gap junctions show that the depths of these junctions range from 200 nm. to 1 um. (see section IV). Therefore, many gap junctions which are usually present among the cells of frog skin epithelium do not appear on the frozen section by using the immunofluorescence technique.

The pattern of the cross-reaction of the gap junction

antibody with its specific antigenic polypeptide from frog skin epithelium compared well to that of other tissues such as rat pancrease, sheep liver, chicken liver and others (Hertzberg and Skibbens, 1984). This similarity indicates that the gap junctions of frog skin may be consisted of similar, if not identical, molecular strucures to those of other tissues. Thus, these junctional complexes represent classical gap junctions which are morphologically and functionally similar to the communicating junctions recognized in other epithelia (see Lowenstein, 1966, 1981).

These results seem to be consistent with the electrophysiological data (Nagel, 1976; Helman and Fisher, 1977; Fisher et al., 1980) which suggest extensive ionic coupling between the epithelial cells of frog skin. However, the magnitude of this electrical coupling does not seem to be homogeneous and uniform throughout the epithelial cells, since some layers of the epithelia show higher frequency of gap junctions than others (see Fig.23 of chapter IV).

CHAPTER VI

Summary and Conclusions

Amphibian skin is characterized by a number of unique features that render it as one of the most favorable experimental models to study Na-active transport across asymetric biological membranes. The intensive investigation on frog skin have yielded a great deal of valuable information regarding the cellular basis of active sodium transport by epithelia. However, our current understanding of Na-active transport across epithelial tissues has been greatly influenced by the double membrane model proposed by Koefoed-Johnsen and Ussing (1958). This model has survived a period of intense examination and skeptism, and some of its aspects have been subject to some modification; however, the basic elements of this model are still intact.

Some experimental approaches such as the morphological and "black box" approaches have been very useful in defining the general aspects of the sodium-active transport and its macroscpic mechanisms in the frog skin epithelium. Recently, the issues in epithelial transport research have gradually evolved towards molecular levels. Thus, more definitive and reliable techniques such as the conventional and ion-selective

microelectrode, noise and fluctuation analysis, X-ray microprobe analysis, fluorescently labelled dye injection and mass spectrometry, nuclear magnitic resonance spectroscopy (NMR), and immunofluorescence techniques have been developed to gain some insights into the microscopic mechanisms of sodium transepithelial transport.

One of the long-standing problems in studying Na-active transport across frog skin is the determination whether all the cells of the epithelial layers of the skin represent a homogeneous cellular transport compartment,or only a fraction of the total population of the epithelial cells (eg. one cell layer or one type of cells) constitute the sodium transport "pool" that participates in the active transport. In an attempt to contribute to the resolution of this problem, different electron microscopic techniques and immunofluorescence analysis have been used to study some morphological and biochemical aspects of the intercellular communication in frog skin epithelium.

Utilizing various methods and techniques of electron microscopy (ie. thin section, freeze-fracture, extracellular tracers), the general organization and the intercellular communication of the epithelial cells of frog skin have been investigated. Histologically, the epidermis of frog skin is a stratified squamous epithelium constituted by 6-8 layers of cells which are organized in four distinct strata: (St.Co), (St.Gr), (St.Sp), and (St.Ge). The epithelial cells represent a heterogeneous population of cells. For example,the cells of

each stratum have slightly different morphological features than those of other strata. Different cell types with different transport properties than the ordinary epithelial cells (eg. Mitochondria-rich cells) are also frequently encountered in this epithelium. The basal layer of the epithelium resides on an extracellular structure which is termed the basal lamina. The basal lamina is the only continueous structure that separates the epithelial cells from the underlaying connective tissues.

The epithelial cells of frog skin are joined to each other by different intercellular junctions which serve various functions. The application of a new fixation procedure constituted by a mixture of glutaraldehyde and tannic acid has enabled us to demonstrate the occurance of a reasonable number of new junctional complexes that have similar criteria to those of gap junctions. The quantitative analysis of the location and distribution of these junctions shows that they are present between the cells of all epithelial strata except the upper most strata (St.Co) which shows no gap junctions among its cells. The distribution of these junction however, is not homogeneous among the different layers of the epithelium. Some layers (St.Ge and St.Sp) have more junctions per each unit membrane length than others (St.Gr and St.Co). Consistent with the thin section data, the freeze-fracture technique reveals fracture faces of the epithelial cells that contain intramembraneous particles that have the size and the shape of the gap junctional particles known for other

vertebrate tissues.

Furthermore, the use of immunofluorescence technique shows clear cross-reaction between the gap junctional antibody and specific areas of the frog skin epithelial membranes. The antibody-binding to the corresponding antigen in frog skin exhibits a distributional pattern which is similar to that demonstrated by thin section and freeze-fracture techniques.

The fact that two quite different approaches (electron microscopy and immunofluorescence techniques) have obtained basically the same distributional pattern of gap junctions in frog skin offer strong support for the proposal that these junctions are classical gap junctions. The gap junctions are known to provide the main source of cell-to cell communication in all electrically coupled tissues, thus it has been proposed that their function in frog skin epithelium is associated with low-resistance pathways for electrolytes to be exchanged between the epithelial cells. The presence of classical gap junctions in a sodium-transporting epithelium like that of frog skin, is compatable with the electrophysiological and X-ray microprobe analysis data which suggest extensive ionic coupling between all the epithelial layers of frog skin.

Due to the low frequency of gap junctions in the first reactive cell layer of the epithelium, Na ions which passively diffuse across the apical membranes into the cells have very few channels to traverse. Under these conditions,

it seems that the cells of the first reactive layer (St.Gr) represent the dominating location of NA-active transport. However, under high rate of sodium ion influx (as is the case in most in vitro experiments), some sodium ions diffuse through a limited number of gap junction channels into the deeper cell layers to be transpor- ted into the inside bathing solution.

Beside gap junctions, the location and distribution of other intercellular junctions such as desmosomes and tight junctions have been quantitavely evaluated. A steriological method has been design to calculate the frequency of each type of junctions (the number of junctions per unit membrane length) in the different strata of the epithelium. The results show that tight junctions are present between the apico-lateral surfaces of the cells of both (St.Co) and (St.Gr). This type of junction is not observed between the cells of any stratum bellow the first layer of (St.Gr). The presence of tight junctions in a Na-transporting epithelium like that of frog skin is very essential for the maintenance of the electrical gradient across the frog skin. Thus, tight junctions are usually associated with the structural aspects of the permeability barrier in this tissue. Desmosomes are present in all epithelial layers however, their frequency in the basal layer is significantly less than that of any other layer of the epithelium. It's widely accepted that the functions of desmosomes in epithelial tissues is to provide adhesive forces that mechanically

connect the cells togother to protect their physical integrity and unify their functional ability. Gap junctions are most frequently seen in the basal and intermediate layers of the epithelium and less frequently between the cells of (St.Gr). Their function is usually to provide low-resistance pathways for the exchange of ions and small water-soluble molecules among the cells.

A system of lamellar structures have been frequently encountered in the intercellular space and within the cells of the epithelium. Morphologically, these structures are comprised of parallel electron opaque layers (each layer is 65-75 angstroms thick) which are embedded in an electron-lucent background. The presence of these lamellar bodies is restricted to the upper two strata of the epithelium, and they are rarely found in the intermediate or the basal layer of the epithelium. The precise composition of these lamellar bodies and their function in this kind of epithelium is not known. It has been proposed that these structures are originally intracellular granules which aggregate and incorporate within the plasma membranes to be finally discharged in the intercellular space of the upper layers of the epithelium. Due to their lipid contents, they might play a role as a permeability barrier to the water loss from the frogs through their skin.

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