STUDIES ON CULTURED FRESHWATER BRANCHIAL EPITHELIA

ELECTROPHYSIOLOGICAL AND ION TRANSPORT CHARACTERISTICS OF CULTURED BRANCHIAL EPITHELIA FROM FRESHWATER RAINBOW TROUT

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree

Master of Science

McMaster University

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MASTER OF SCIENCE (1997) McMASTER UNIVERSITY

(Biology) Hamilton, Ontario

TITLE: Electrophysiological and Ion Transport Characteristics of Cultured Branchial Epithelia from Freshwater Rainbow Trout

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Number of Pages: xii, 174

Abstract:

Electrophysiological, morphological, and ion transport characteristics were examined in two cultured preparations of freshwater rainbow trout gills on permeable supports. The mitochondrial stain Rhodamine 123 (R123) revealed that single-seeded insert (SSI) preparations contained only pavement cells, and new double-seeded insert (DSI) preparations contained both pavement cells and mitochondrial-rich cells (MRCs). A series of physiological comparisons were made between the two preparations. Both preparations showed increases in transepithelial resistance (TER) with time in culture, reaching stable values (1500 - 25000 ohms.cm^2) after 5-7 days in culture. Different transepithelial potentials (TEP) were observed in the two preparations with culture media (Leibowitz L-15 supplemented with 5% foetal bovine serum (FBS)) on both surfaces of cultured preparations (symmetrical conditions). The TEP of SSI membranes prepared in Hamilton, Canada was negligible. The TEP of SSI membranes prepared in Uppsala, Sweden was -2.24 mV (serosal negative) and sensitive to vanadate and iodoacetate/KCN. DSI membranes under symmetrical conditions had mean TEP values of 1.87 mV (serosal positive). Replacing apical culture media with freshwater (asymmetrical conditions) mimicked the *in vivo* environment of gills. Under these conditions TER increased and TEP was serosal negative in all membranes. TER recovered to initial values after culture media was returned to the apical side. Replacing culture media on the basolateral side with

freshwater (opposite to *in vivo* conditions) resulted in decreased TER which was irreversible and consistent with permanent damage. Taken together, TER measurements with freshwater on the apical side or basolateral side indicate the cultured cells of SSI and DSI membranes were polarized as *in vivo*. PEG-4000 fluxes were performed to assess paracellular permeability across DSI membranes. Results show that with freshwater on the apical side, paracellular permeability increased while conductance decreased in comparison to symmetrical conditions. In contrast, apical seawater exposure produced "leaky" DSI membranes with high paracellular permeability and high conductance. Na^+K^+ -ATPase activity of cells in primary culture (flasks) and on SSI membranes was retained (approximately 3uM PO_4^{2-}/mgprotein/h) throughout culture conditions. Unidirectional Na^+ and Cl^- fluxes in DSI membranes revealed negligible net fluxes under symmetrical conditions but large effluxes and small influxes under asymmetrical conditions, similar to previous results with SSI membranes. Ion transport was mainly conductive (indicated by linear relationships between conductance and fluxes) in DSI preparations. The Ussing flux criteria indicated active Na^+ uptake and passive chloride movements in symmetrical conditions, and active Cl^- uptake and passive Na^+ movements in asymmetrical conditions. SSI membranes also demonstrated active Cl^- uptake, however DSI membranes were the first *in vitro* preparation of the gill to demonstrate active Na^+ transport. This finding suggests MRCs may be involved in Na^+ transport, and contradicts current models which suggest that pavement cells are the site of Na^+ uptake. Unidirectional calcium fluxes revealed active Ca^{2+} transport in DSI branchial epithelia under both symmetrical and

asymmetrical conditions, and no active Ca^{2+} transport in SSI epithelia. These results reinforce theories of calcium transport across chloride cells in the branchial epithelia.

Acknowledgements:

I wish to thank the following people for helping make this thesis successful. Firstly, to my supervisor Mike O'Donnell, thanks for your encouragement and guidance. Ditto for Chris Wood, but in addition I'd also like to thank Chris for the fantastic experiences abroad. As well, thanks for being such an efficient and reliable lab tech during my last couple weeks of experiments (HaHaHa)! A huge thanks to Peter Pärt for giving me the opportunity to complete a portion of this work in his lab at Uppsala University, Sweden. The whole experience was definitely the highlight of this Master's degree.

A special thanks to Katie Gilmour for our productive week together in Uppsala, and for the many times I had to rely on her resourcefulness, and thanks to my "Hood Pal" Vandy Thomas who kept both me and the hood in line. As well, I must thank my great roommate Linda Wei for providing me with wholesome fuel, especially while I was madly finishing up this project. To all my wonderful lab mates, particularly Erin (Babe) Fitzgerald, Nathan Webb, Adele Jackson, Mike Quinlan, the O'Donnell gang (Kim^3 + Mark), and of course Diarrhoeal (oops!), I mean Darryl Burgess, thanks for making my days in the lab that much more enjoyable. Finally, a thousand thanks to my favourite lab mate, my best friend Derek Alsop who helped me in countless ways over the last couple of years. I'm done!!!

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List of Abbreviations:

TER	Transepithelial Resistance
TEP	Transepithelial Potential
MRC	Mitochondrial-rich Cell
R123	Rhodamine 123
SSI	Single-seeded Insert
DSI	Double-seeded Insert
TEM	Transmission Electron Micrograph
FBS	Foetal Bovine Serum
PBS	Phosphate Buffered Saline
FW	Freshwater
SW	Seawater
CM	Culture Media
PEG	Polyethylene Glycol
RBC	Red Blood Cell

Chapter 1: Ion Transport in Freshwater Fish Gills

General Introduction:

The gills of freshwater fish are highly specialized organs that serve a primary role in gas exchange and osmoregulation. The primary epithelium which blankets the gill filaments is in close proximity to the external environment and internal blood supply making it an ideal location for ion transport. This epithelium contains a minimum of 5 cell types: pavement or respiratory cells, mitochondrial rich chloride cells, mucous cells, neural epithelial cells, and undifferentiated stem cells (Laurent, 1984). It is believed that only pavement and chloride cells are involved in the mechanisms of ion transport across the gill epithelium , and recently attempts have been made to characterize their individual functions in ion regulation (Perry, 1997). Although significant progress has been made in elucidating these mechanisms, the models are far from being resolved.

Pavement cells are either columnar or squamous shaped and comprise the largest portion of cells (up to 95%) in the branchial epithelium (Goss *et al.*, 1995). Morphologically they are characterized not only by their shape, but by the presence of apical microridges (Perry *et al.*, 1992b). Embedded among the pavement cells are chloride cells which are the second most abundant cell type (5-15%) in the gill. They are generally larger than pavement cells and exhibit variations on their apical surface which make them

distinguishable from surrounding pavement cells. For instance, chloride cells in the tilapia (*Oreochromis mossambicus*) possess a deep groove on their apical surface called an apical crypt (reviewed by Perry, 1997). Other distinguishing features of chloride cells include an abundance of mitochondria and high Na^+K^+ - ATPase activity.

There are thought to be two chloride cell subtypes in fish gills, alpha and beta. Alpha cells are thought to be precursors of seawater-adapted fish whereas the beta subtype is mostly found in the inter-lamellar regions of the freshwater gill filament (Laurent, 1984). At present, it is unclear whether the two types are actually different cells or different cell stages in development (Goss, 1995).

Transport Mechanisms

Overall, freshwater fish take up ions from their hypoosmotic environment to compensate for diffusional and urinary losses. Specifically, the branchial epithelium is responsible for the uptake of Na^+, Cl^-, and Ca^{2+}. The model for ion transport across the gill epithelium is tentative and highly based on correlational findings from many studies (Figure 1).

Chloride Uptake - Cl^-/HCO_3^- Exchanger

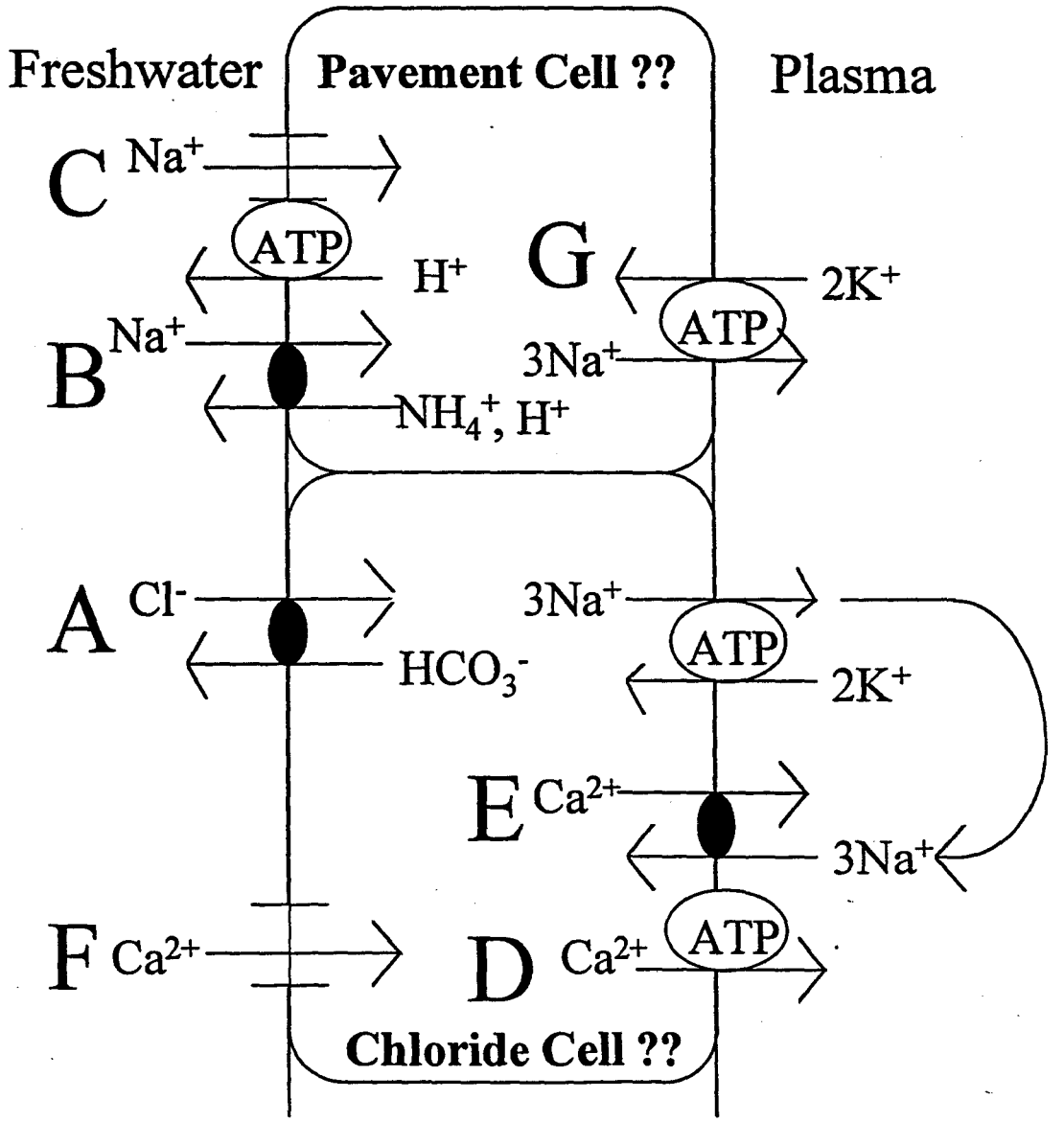
The preponderance of evidence points to Cl^- uptake occurring in exchange for HCO_3^-

across the apical membrane of chloride cells (Figure 1A) (reviewed by McDonald *et al.*, 1989). Chloride cells are well established as the site of chloride transport in seawater fish (Marshall, 1995), so it seems reasonable that these cells have a similar role in freshwater fish. Chloride transport in freshwater gills has often been investigated in response to applied acid-base disturbances. Respiratory acidosis consistently results in a reduction in chloride cell surface area (Goss *et al.* 1995). This morphological change is accompanied by reduced chloride uptake, the consequence of which is a reduction in the number of Cl^-/HCO_3^- exchangers exposed to freshwater so as to retain HCO_3^- and thereby counteract the acidosis. Similarly, respiratory alkalosis, or any condition that alkalizes the blood, causes an increase in chloride cell surface area and an increase in chloride uptake (Perry *et al.*, 1992b, Perry and Goss, 1994, Goss *et al.*, 1994). Alkalosis is corrected for more rapidly when external $[Cl^-]$ is increased, most likely because more Cl^- is available for the exchanger (McDonald *et al.*, 1989).

Molecular, biochemical, and pharmacological studies are also used to investigate Cl^- transport mechanisms. Recently, *in situ* hybridization strengthened the view that chloride uptake is connected to acid-base mechanisms by revealing that mRNA encoding the Cl^-/HCO_3^- exchanger increases during arterial $NaHCO_3$ infusion (Sullivan *et al.*, 1996). The hybridization signal is localized to cells on the gill filament and lamella, also known to be the predominant sites of chloride cells in the freshwater gill (Laurent and Perry, 1991). The addition of anionic exchange blockers or removal of Cl^- from freshwater results in decreased Cl- uptake and bicarbonate retention (reviewed by Perry, 1997). Evidence is

Figure 1: A model of ion transport mechanisms in the freshwater fish gill epithelium based on evidence from many studies. A) Cl^-/HCO_3^- electroneutral exchanger on the apical membrane, B) $Na^+/H^+,NH_4^+$ exchanger on the apical membrane, C) Na^+ channel and a H^+ -ATPase on the apical membrane, D) Ca^{2+}-ATPase on the basolateral membrane, E) Na^+/Ca^{2+} exchanger on the basolateral membrane, F) Ca^{2+} channel on the apical membrane, G) Na^+K^+ -ATPase on the basolateral membrane.

Freshwater Gill Epithelium



still lacking for the mode of chloride transport across the basolateral membrane of chloride cells.

Sodium Uptake

Mechanisms for the uptake of Na^+ and Ca^{2+} remain somewhat more controversial than for Cl^- uptake. While Na^+ is thought to cross the basolateral membrane of gill cells via Na^+K^+-ATPase (Goss *et al.*, 1995), there are two schools of thought on how Na^+ crosses the apical membrane of freshwater gills.

$Na^+/H^+,NH_4^+$ Exchanger

Originally, sodium transport across the apical branchial membrane was thought to occur in exchange for NH_4^+ (Figure 1B) (Wright and Wood, 1985). Much of the evidence for this electroneutral exchanger came from *in vivo* studies of ammonia excretion. Wright and Wood (1985) found that fish exposed to a severe acid stress experience a blockade of sodium uptake and simultaneous ammonia retention, despite the production of a gradient which favours ammonia excretion. As well, amiloride, a well known sodium transport blocker (Benos, 1982), was found to decrease sodium influx by 94% and ammonia efflux by 23%, again showing a relationship between sodium and ammonia transport. It was suggested that amiloride inhibited Na^+/NH_4^+ exchange, thereby reducing sodium influx,

and that ammonium excretion was maintained by an increase in the diffusion of NH_3.

Further evidence for a relationship between sodium and ammonia transport came from looking at changes in sodium uptake and ammonia excretion that accompany changes in external Na^+ levels (reviewed by McDonald et al., 1989). A significant 1:1 correlation between Na^+ influx and total ammonia efflux is found over a range of external Na^+ concentrations. However other studies show that Na^+ and ammonia transport were not always related (Avella and Bornancin, 1989). When external conditions are kept constant, ammonia excretion increases as a function of internal ammonia levels, whereas sodium uptake does not change in accordance with ammonia excretion. Therefore, in this case sodium and ammonia transport are uncoupled. The same study did find a correlation between sodium uptake and proton excretion favouring the hypothesis of sodium uptake in exchange for protons but ammonia excretion by simple diffusion. More recent evidence for this mechanism comes from a study on boundary layer acidification (BLA) (Wilson *et al.*, 1994). BLA facilitates the excretion of ammonia by increasing the gradient for ammonia diffusion. This is accomplished by decreasing pH on the external surface of the gill, thereby trapping NH_3 in the form of NH_4^+ in the boundary layer. By exposing the gills to amiloride and HEPES buffer (which eliminates BLA), sodium transport is inhibited as for previous studies with amiloride, but ammonia excretion remains unchanged. It appears that BLA and not the blockade of Na^+/NH_4^+ exchangers accounts for the partial decrease in ammonia excretion seen previously with amiloride, and that Na^+/H^+ exchange accounts for Na^+ uptake.

Although electroneutral $Na^+/H^+,NH_4^+$ exchange appears to contribute to the uptake of sodium (Figure 1B), analysis of the gradients suggests a role for an active mechanism across the gill. Intracellular Na^+ concentrations are in the range of 52mM (Wood and LeMoigne, 1991, Morgan and Potts, 1995) and freshwater Na^+ levels are approximately 1mM so the chemical gradient for sodium most likely did not drive the electroneutral exchangers (reviewed by Lin and Randall, 1995). Also, intracellular pH is usually in the range of 7.4 and gills are known to excrete protons into freshwater with pH 6.0, and to take up Na^+ at pH (water) as low as 4.0. To account for these recent findings it appears that an active mechanism is necessary for transporting sodium across the apical membrane.

Na^+ Uptake Via an Electrochemical Gradient Produced by a H^+- ATPase

There is increasing evidence for sodium uptake across the apical membrane occurring along an electrochemical gradient produced by the active outward pumping of protons (Figure 1C). Initially evidence for this mechanism came from studies which were able to measure P-Type H^+-ATPase activity in isolated basolateral membrane fragments from gill epithelia (reviewed by Lin and Randall, 1995). This pump, which extrudes 3 H^+ for every ATP, is thought to generate a negative potential which serves as the driving force for Na^+ uptake. More recently, immunostaining has localized a V-type H^+- ATPase on the apical surface of gill filaments (Lin *et al.*, 1994). *In situ* hybridization has further localized mRNA for this latter proton pump near the apical surface (Sullivan *et al.*, 1996). The

expression of this signal is enhanced during hypercapnic acidosis which concurrently increases Na^+ influx. The data suggests that increased gene expression is the mechanism of acid-base regulation in response to respiratory acidosis.

Calcium Uptake

The electrochemical gradient across the freshwater gill (calculated by using the electrical potential across the whole gill and water and plasma calcium activities) favours outwardly directed calcium transport (Flik *et al.* 1996). However, along with sodium and chloride, the gill takes up calcium for growth and homeostasis against its electrochemical gradient. Since the intracellular calcium concentration is in the submicromolar range (typically 100-200 $nmol.L^{-1}$), which is much lower than freshwater calcium levels (approximately 1mM), the entry step across the apical membrane is considered to occur passively. The most likely mode of apical entry is through calcium channels since La^{3+}, which blocks calcium channels, inhibits transcellular influxes of calcium (Figure 1F) (reviewed by Flik and Verbost, 1993). Paracellular calcium transport for the most part appears to be negligible since tight junctions in the gill epithelium are relatively impermeable to calcium ions (Flik *et al.*, 1995). However, transport across the basolateral membrane is an active process since ion movement is against a very steep electrochemical gradient. While Na^+K^+-ATPase appears to be the route by which Na^+ crosses the basolateral membrane (Figure 1G), evidence points to two active mechanisms which

contribute to the uptake of Ca^{2+} across the basolateral membrane: a high-affinity calcium P-type ATPase and a Na^{+}/Ca^{2+} exchanger.

Ca^{2+}- ATPase

Several studies have found calcium P-Type ATPase activity in basolateral membrane fractions of freshwater fish gills (Figure 1D) (Flik *et al.*, 1985, Verbost *et al.*, 1994, Flik *et al.*, 1996). The pump exhibits an affinity for calcium concentrations in the range of intracellular values (approximately 200 $nmol.L^{-1}$) which supports its proposed role in calcium extrusion from the cytoplasm (Flik *et al.* 1985). Calcium influx rates appear to be correlated with the activity of the pump. The relationship between Ca^{2+} influx and pump activity has been investigated in prolactin-treated fish. Flik *et al.*, (1994) found that prolactin-treated fish show an increase in Ca^{2+} influx. Purification of membranes revealed that these fish show an increase in Ca^{2+} - ATPase activity. Furthermore, the membrane vesicles also demonstrate an accumulation of calcium. Results show that influx values across membrane vesicles from 20g tilapia are 560nmol/h which compare extremely well to whole animal values (580 nmol/h) for fish of the same size. Since the isolated membranes exhibited Ca^{2+}-ATPase activity, the authors conclude that the calcium influx observed *in vivo* is attributed to the calcium ATPase pump.

Na^+/Ca^{2+} Exchanger

In tilapia, there is evidence for Na^+/Ca^{2+} exchangers as well as Ca^{2+} -ATPases in the basolateral membrane (Verbost *et al.*, 1994). This exchanger pumps out one intracellular calcium ion in exchange for 3 sodium ions (Figure 1E). The sodium gradient produced by the outward pumping of Na^+ from the Na^+/K^+- ATPase is thought to be the driving force for this mechanism. This study as well as others (reviewed by Flik *et al,* 1996) conclude that both transport mechanisms exist. The controversy lies in which mechanism is predominant in accounting for the calcium influx measured across gills. Although it seems that the Ca^{2+}-ATPase could account for all of the ion transport (Flik *et al.*, 1985), further kinetic analysis reveals that this condition is not always the case. Verbost *et al.* (1994) found that Ca^{2+}-ATPase activities greatly exceed exchanger activities in basolateral membrane fractions when intracellular Ca^{2+} concentrations were in the normal physiological range (in this case, 10^{-7} mol.L^{-1}). However, when intracellular Ca^{2+} concentrations increase, exchanger activity exceeds ATPase activity. It appears that the relative contribution of the two mechanisms depend on intracellular calcium levels, and that under "normal" circumstances the ATPase is responsible for calcium transport, but that under "dangerous" conditions of high intracellular calcium (which could lead to detrimental effects such as cell death), the exchanger mechanism is predominant.

Pavement Cells versus Chloride Cells in Ion Transport

Not only is the mechanism of ion uptake controversial in the freshwater fish gill, but the site of uptake is also the subject of debate. Most of the evidence is correlational, but overall seems to suggest that calcium and chloride uptake occurs via chloride cells which are rich in mitochondria and therefore thought to be the main active ion transporting cells in the fish gill (Perry, 1997). For instance, not only does prolactin increase calcium influx rates and Ca^{2+} -ATPase activity, but it also increases branchial chloride cell density (Flik *et al.*, 1996). In contrast, more recent evidence points to pavement cells as the site of sodium uptake.

Originally it was thought that all ions were taken up by chloride cells. Studies found correlations between chloride cell number and sodium influx rates (Avella *et al.*, 1987). More recently, measurements of intracellular ion levels and molecular techniques reveal a different story. Morgan *et al.* (1994) found that intracellular Na^+ concentrations in pavements cells but not chloride cells vary with external Na^+ levels, and since specific immunoreactivity of the H^+- pump is localized to pavement cells (Sullivan *et al.*, 1996), it appears that pavement cells could be the site of Na^+ uptake. The frog skin, which is a well characterized epithelium, possesses both principle cells and mitochondrial-rich cells that are equipped to transport sodium (Harvey and Ehrenfeld, 1988). The epithelia demonstrates Na^+ uptake across the principle cells when mucosal NaCl concentrations are high, and Na^+ uptake across mitochondrial-rich cells when mucosal solutions are dilute.

The complexity of the gill makes it difficult to determine the ion transport mechanisms and site of ion uptake. For example, in contrast to the flat opercular epithelia, isolated gills cannot be studied in Ussing chambers. Therefore much of the evidence for transport processes will remain indirect until a testable model of the gill can be established. Further investigation using *in vitro* preparations and molecular techniques in fish gills provide opportunities for detailed characterization of ion transport.

In vitro Models of Transporting Epithelia

Various Isolated Epithelia

In vitro models of other osmoregulating epithelia have contributed greatly to the understanding of ion transport in seawater fish. The opercular epithelium of teleosts is one such example, and since it contains chloride cells and demonstrates NaCl excretion from the basolateral (blood) to apical surfaces (seawater), it is currently used to model NaCl secretion in seawater gills (Marshall, 1995). Studies examining many aspects such as electrochemical gradients and hormonal control in opercular epithelia, have led to a clearer understanding of Na^+, Cl^-, and Ca^{2+} transport. The success of this model for ion transport in seawater fish has lead to the search for comparable isolated epithelia from freshwater fish.

The skin overlying the cleithrum bone of freshwater acclimated teleosts was

investigated as a surrogate model of the freshwater gill (Marshall *et al.*, 1992). Although other epithelial models were explored such as the opercular epithelium of brook trout and rainbow trout, they were considered inferior to the cleithrum skin because they lacked numerous mitochondrial rich cells which the isolated cleithrum skin possesses. Flux experiments conducted under conditions that mimicked the *in vivo* situation (freshwater on the apical side, Cortland's saline that duplicated trout plasma on the basolateral side) provides evidence for active Ca^{2+} uptake that correlate positively with the number of mitochondrial rich cells in the preparation. These findings are in accordance with the relationship established for gills of intact trout (Perry and Flik, 1988) but since the gill surface area is approximately 100-fold greater than the total surface area of the cleithrum skin, the capacity of isolated cleithrum skin to transport Ca^{2+} must be considerably smaller than the gill (Marshall *et al*, 1992). However, this was not the case, so either the cleithrum skin transports Ca^{2+} at a much higher rate than expected, or the skin is not highly representative of the intact gill. This latter suggestion is supported by the fact that this particular model does not transport Na^+ and Cl^- as *in vivo*.

In fact, a common problem with earlier attempts at freshwater gill models is the lack of net Cl^- uptake. Freshwater-adapted euryhaline species such as *Sarotherodon mossambicus* actually exhibit chloride secretion across isolated opercular membranes, like seawater-adapted animals (Foskett *et al.* 1981). Since this would be harmful to the animal *in vivo*, it was concluded that the experimental design somehow activates the chloride secretion mechanisms (reviewed by Karnaky, 1986).

Progress has been made with the development of the killifish opercular epithelium as a model system for the freshwater gill. Opercular epithelia from killifish acclimated to freshwater for 10 days demonstrate active chloride uptake (Wood and Marshall, 1994, Marshall *et al.* 1997). The epithelia was shown to take up Ca^{2+} under the same conditions, much like the cleithrum skin (Marshall *et al.*, 1992). Mitochondrial rich cells present in the epithelium were determined to be the "freshwater-type" and not the "seawater-type" since the epithelium did not develop a serosa positive potential or exhibit net Cl^- secretion when seawater replaced freshwater on the apical side. However, this model is not without problems since it cannot explain which cell is responsible for Cl^- uptake, or the relationships of ion uptake to acid/base regulation. Another major problem is that, unlike the intact gill of freshwater fish, it does not take up Na^+.

Cultured Epithelium as an *In Vitro* Model of the Gill

Cultured branchial epithelium is currently being explored as a possible model for both freshwater and seawater gills. Again, more progress has been made in the seawater version of the cultured preparation. The culturing of cells from fish actually predates mammalian cell culture and began in 1914 with the isolation of small bits of tissue from trout embryos for several hours in saline (Wolfe and Quimby, 1969). Although it took a number of years before cell culture techniques were adopted by physiologists as a research tool, it is now used for a variety of purposes ranging from the study of general cell functions (DNA synthesis, etc.) to specialized properties of specific cell types, such as ion transport

(Freshney, 1989). Many fish cell lines have been established to study fish pathology (Lannan, 1994), two of which exist for gill cells (Bols and Lee, 1991). However, fish physiologists prefer to use primary cultures which are produced directly from isolated tissues and grown *in vitro*, rather than secondary cultures (cell lines) which dedifferentiate and often lack many of their initial properties. Consequently, cell lines may not be good representations of the mother tissue.

The first successful ion transport studies across primary fish cultures were performed on cultured renal tubules from seawater flounder (Dickman and Renfro, 1986). The cultures exhibit differentiated properties of intact renal tubules, and are considered a suitable model for further investigation of renal transport properties. This culturing technique produced flat layers of epithelial cells enabling renal tubules, an epithelium of comparable complexity to the gill, to be studied using standard electrophysiological techniques, such as Ussing chambers. Such a model can now permit transport studies in regulated substrate environments with appropriate hormonal, nutrient, and acid-base conditions. Attempts have been made to mimic this type of cultured model for branchial epithelium, but progress has been slow.

Cultured Branchial Epithelium from Seawater Fishes

One of the initial studies involving cultured branchial cells employed an organ culture technique where free-floating gill filaments from seawater-adapted coho salmon remain viable for up to 4 days *in vitro* (McCormick and Bern, 1989). They were used to study

changes in Na^+K^+ -ATPase activity over time, which incidentally decreases, indicating a loss of viability in this cultured tissue. However, cortisol prevents the decline in activity and actually causes a significant dose-dependent increase in Na^+K^+ -ATPase activity over the 4 days. More success was seen with primary cultures of gill epithelial cells from sea bass (Avella *et al.*, 1994). Using an explant technique, homogenous monolayers of gill cells arose from outgrowths of isolated filaments, and subsequently grew to form a continuous epithelium that lasts for 12-14 days. Ultrastructural analysis reveals viability and similarity to intact branchial epithelium. However the preparation lacks mitochondrial-rich chloride cells and appears to consist entirely of pavement cells, and its glass support does not allow for any type of quantitative analysis or transport study. This latter disadvantage was rectified in an impressive follow-up study of the sea bass gill epithelium cultured on permeable supports.

Avella and Ehrenfeld (1997) were able to enzymatically isolate gill cells and culture them into monolayers onto permeable culture inserts which were mounted in Ussing chambers, much like the renal tubule cultures (Dickman and Renfro, 1986). This provided for analysis of electrophysiological properties and ion transport characteristics. Like the seawater gill, the cultured epithelium demonstrates chloride secretion (from the serosal to the mucosal surface) by a $Na^+K^+2Cl^-$ cotransporter and a Cl^-/HCO_3^- exchanger. Like the previous sea bass primary culture, these cultures consist of homogenous monolayers of one cell type only, pavement cells. Earlier *in vivo* studies provide evidence for both of these exchange mechanisms in the seawater gill (reviewed by Marshall, 1995), but they are

generally believed to function only in the chloride cells. The fact that an apparently pure pavement cell preparation demonstrates Cl^- secretion may necessitate a reconsideration of the current model for ion transport across the seawater gill.

Cultured Branchial Epithelium from Freshwater Rainbow Trout

Primary cultures of freshwater gill cells were first established by Pärt *et al.* (1993). Like the sea bass epithelia, rainbow trout cultures from dispersed rainbow trout gill cells yield homogenous layers of pavement cells only, as confirmed by morphological studies and mitochondrial stains. Other physiological characteristics such as intracellular pH are similar to intact gill tissue, providing hope that this cultured system could provide the basis for a fully functioning surrogate model of the intact freshwater gill (Part and Wood, 1996).

Since then, pavement cells have been successfully cultured onto permeable inserts by a two stage process (Wood and Part, 1997). Firstly, isolated cells are cultured in primary culture flasks, and secondly, they are transferred from these flasks to permeable culture inserts. These cultures develop a high transepithelial resistance (TER), characteristic of tight epithelia, and demonstrate tight junctions as seen by transmission electron micrographs (TEMs). This type of culture allows the cells to be exposed to freshwater on the apical side and plasma-like media on the basolateral side so that transport studies could be conducted under conditions close to those *in vivo*. Thus far, these cultures are able to survive up to 48 hours of apical freshwater exposure, and preliminary transport studies

indicate they actively take up chloride from freshwater, as *in vivo* (Wood *et al.*, 1997). However, in contrast to the *in vivo* situation, there is no active uptake of Na^+, and the preparation loses both Na^+ and Cl^- in large amounts on a net basis to the apical freshwater.

Despite the advances in culturing gill cells, there remain many unanswered questions concerning this freshwater model. Relationships between physiological parameters need to be addressed, ion transport mechanisms need to be explored by way of radiotracer studies, and the electrophysiological properties of the cultured epithelia need to be fully characterized. The goals of this thesis were to further characterize the cultured freshwater gill model established by Wood and Part (1997) by investigating several areas of uncertainty.

Objectives:

1. Site and Mechanism of Ca^{2+} Transport

According to current theories of the intact gill (Figure 1), a preparation of pure pavement cells should exhibit active sodium transport, but not active calcium or chloride

uptake because the latter are thought to be a function of the chloride cells. Thus far it has been shown that the cultured branchial pavement cells actively take up chloride but not sodium (Wood and Part, 1997), which is not in agreement with present models. Calcium transport across the cultured epithelia has not been studied. Therefore it is necessary to determine whether calcium is actively taken up by the cultured pavement cell preparation in order to get a fuller understanding of how the cultured branchial epithelium fits with the current theories of ion transport. This project investigated calcium movement by way of radioisotopic fluxes, similar to those performed previously for sodium and chloride transport (Wood *et al.* 1997).

2. Transepithelial Resistance (TER) and Transepithelial Potential (TEP)

Exposing the apical side of cultured gill cells to freshwater results in a large increase in TER and the production of a negative TEP, the latter condition being consistent with *in vivo* measurements (Wood and Part, 1997). However, the epithelium loses integrity during the 48 hours of freshwater exposure as indicated by a decrease in TER and the slow disappearance of the potential. Therefore more studies are needed to verify that the cultured cells are polarized properly. Tests were performed that involved substituting the basolateral plasma-like culture media with freshwater in order to examine the physiological parameters in conditions opposite to those *in vivo*.

3. Seawater Challenge

One suggestion to explain the discrepancy between the current theories of ion transport in freshwater (Figure 1) and the transport characteristics of the cultured branchial epithelium, is that since the cells are isolated and reared in isotonic conditions (with plasma-like culture media on both apical and basolateral surfaces), the cultured cells are behaving like seawater gill cells, as indicated by net sodium and chloride effluxes (Wood *et al.*, 1997). This possibility was examined in this thesis by substituting the apical media with seawater, and monitoring characteristics such as its permeability to PEG-4000 (a paracellular permeability indicator) and TER. This was done in comparison to cultured epithelium exposed to freshwater and plasma-like media on the apical surface.

4. Properties of Chloride Cells and Pavement Cells

As can be seen, there is much to be investigated before the cultured branchial epithelium can be considered suitable as a model for the functioning gill. In order to develop a proper model, the most important objective must be to incorporate chloride cells into the cultured epithelium. This has not yet been accomplished for either the freshwater or the seawater model, yet the heterogeneity of the intact organ, and the fact that chloride cells are thought to be the major site of ion transport, make it necessary to produce a model that consists of more than one cell type. This particular objective was accomplished for the first time during this project by developing a technique that involved

seeding culture inserts with gill cells from two different fish on two consecutive days. This resulted in two cell types in the cultured preparation, one rich in mitochondria and one not. The remainder of this thesis will report a physiological comparison between this new double-seeded preparation, and one resembling the pure cultured pavement cell epithelium of Wood and Part (1997). The comparison is developed by addressing the transport and electrophysiological issues presented above.

Chapter 2: Morphology, Growth Characteristics, and Electrophysiological Properties of Cultured Freshwater Branchial Epithelia: A Comparison between Two Preparations.

Introduction:

The complex shape of the teleost gill has hindered its ability to be studied with classical physiological tools such as the Ussing chamber. Thus, there remains much to be learned about its ion transporting capabilities and mechanisms. Although *in vitro* preparations such as the cleithrum skin of fresh-water adapted rainbow trout (Marshall *et al.*, 1992) and the opercular epithelium of killifish (Wood and Marshall, 1994, Marshall *et al.*, 1997) are flat structures which have a composition similar to the primary gill epithelium (ie. they contain both pavement cells and mitochondrial-rich chloride cells), they have not yet been successfully employed in elucidating the mechanisms of sodium and chloride transport in the freshwater gill. The cultured branchial epithelium of freshwater rainbow trout is currently being investigated as a possible model for studying ion transport in this epithelium.

Initial work on the cultured gill model began with Part *et al.*, (1993) who designed a protocol for producing primary cultures of branchial pavement cells. This culturing technique was later adapted by Wood and Part (1997) to include a two stage process which developed epithelial cell layers on permeable membrane supports. Typically, the cells were grown for 6 days in primary culture flasks (stage 1) and then harvested and

reseeded onto permeable filters where they developed for another 6-9 days (stage 2). These permeable supports allowed the epithelial cells to be exposed to either culture media on both apical and basolateral surfaces (symmetrical conditions) or freshwater on the apical side and culture media on the basolateral side (asymmetrical conditions). The latter situation mimics the environment of the intact gill, and therefore allows for the possibility of experimentation in realistic conditions.

Thus far, different morphological, electrophysiological, and permeability properties of cultured membranes have been investigated to determine the relationship between these various parameters, and ultimately, the cultured model's similarity to the intact gill. After initial seeding of filter inserts with harvested cells, cultured membranes with plasma-like culture media on both surfaces experienced a daily increase in transepithelial resistance (TER) which eventually plateaued around day 6-9 (Wood and Part, 1997, Wood *et al.*, 1997). No transepithelial potential (TEP) was apparent across the preparation in these symmetrical conditions, which is discouraging since a small TEP exists across the intact gill with extracellular fluid (ECF) -like media on both apical and basolateral sides (Potts, 1984). However, cross-sectional TEMs revealed several layers of healthy cells connected with tight junctions. As well, it is encouraging to note that this preparation could withstand and fully recover from several hours of exposure to freshwater on the apical side (Wood and Part, 1997). This exposure resulted in a large (approximately 5 fold) increase in transepithelial resistance (TER) and a decrease in transepithelial potential (TEP) to serosal negative values consistent with *in vivo* measurements (Wood and Part,

1997). These changes were found to be reversible after 3 hours of exposure, but not after 48 hours.

Permeability to polyethylene glycol (PEG - 4000, a paracellular permeability marker) increased in asymmetrical conditions relative to symmetrical conditions, suggesting the epithelium became "leaky" when freshwater replaced culture media on the apical surface (Wood *et al.*, 1997). Despite this finding, conductance (the inverse of TER), decreased during freshwater exposure, indicating the physiological response to this change could involve alterations in both paracellular and transcellular permeability.

This chapter further examines electrophysiological parameters, and will also demonstrate the stability of the preparation by measuring Na^+K^+-ATPase activity of the cultured branchial cells. Previous cell culture models have shown that Na^+K^+-ATPase activity decreases with time in culture (McCormick and Bern, 1989), however this is not apparent with these cultured gill cells.

During this thesis I developed a different culturing technique which omits the primary culture stage in flasks and directly seeds freshly isolated cells onto permeable inserts. This new protocol permits a major step forward in studies of freshwater branchial epithelium. Specifically, I have obtained cultures of both pavement cells and chloride cells on the same permeable support. The new double-seeded insert (DSI) preparations establish a serosal positive TEP in symmetrical solutions whereas Wood and Part (1997) found the TEP to be negligible.

Materials and Methods:

Experimental Animals:

Rainbow trout (*Oncorhynchus mykiss*) were locally obtained for experiments in both Hamilton, Canada and Uppsala, Sweden. In Hamilton, the fish were held in dechlorinated running tap water (composition: $[Na^+]$=0.55, $[Cl^-]$=0.7, $[Ca^{2+}]$=1.0, $[Mg^{2+}]$=0.15, $[K^+]$=0.05 mmol.L^{-1}, pH= 7.8-8.0). Photoperiod and temperatures (4-17°C) varied seasonally. In Uppsala, fish were also held in running temperature controlled (11-13°C) freshwater (composition: $[Na^+]$=0.6, $[Cl^-]$=0.5, $[Ca^{2+}]$=2.0, $[Mg^{2+}]$=0.04 mmol.L^{-1}, pH=7.0) and exposed to a photoperiod that matched that of Hamburg, Germany to allow for longer daylight in the winter months.

Cell Culture Preparations:

All cell culture procedures were conducted in a laminar flow hood using sterile techniques. Surgical equipment was disinfected with 70% ethanol before use, and all solutions were sterilized either by autoclaving or by passing them through 0.2um Acrodisc syringe filters (Gelman, Ann Arbor, USA). All containers that were not purchased sterile were also autoclaved.

The procedure for isolating gill cells was the same for both single-seeded insert (SSI) and double seeded-insert (DSI) preparations, and is based on methods established by Part *et al.*, 1993. The culture media and antibiotics used in Hamilton were purchased from GIBCO Laboratories, N.Y., USA. Fish weighing 50-150g (Hamilton) or 100-200g (Uppsala) were sacrificed with a blow to the head or by decapitation, and all eight gills were removed and placed in a petri dish containing phosphate buffered saline (PBS) without Ca^{2+} or Mg^{2+} (composition: KCl=0.2, KH_2PO_4=0.2, NaCl=8, Na_2HPO_4=1.15 $g.L^{-1}$, pH 7.7 with NaOH). The filaments were excised from the arches and rinsed for three consecutive 15 minute periods with 10 ml washing solution containing antibiotics and fungizone (composition: 200ul/ml PEST (#15070-063), 5000 iu/ml penicillin and 5000 iu/ml streptomycin), 400ug/ml of gentamicin (#15710-023), 2.5ug/ml of fungizone diluted in PBS). The washed filaments were transferred to a 50ml centrifuge tube, rinsed with 1 ml of trypsin solution (composition: 2.5% trypsin-EDTA stock(#15400-054) diluted to 0.05% in PBS), then incubated with 2 ml of fresh trypsin solution for 10 minutes on an orbital shaker (model 361, Fisher scientific, U.S.A). After the 10 minute period the cell suspension (containing isolated cells and trypsin) was passed through a cell strainer (80 uM nylon cloth - Falcon) into trypsin stop solution (composition: 10% Fetal Bovine Serum (FBS) in PBS). New trypsin solution was added and the procedure was repeated 3 times. The entire cell suspension from the four trypsinations was centrifuged for 10 minutes at 200g. The supernatant was aspirated off and the pellet was resuspended in a rinsing solution (PBS containing 5% FBS) and centrifuged again. After washing, the cell

pellet was suspended in 20 ml of culture media supplemented with antibiotics (composition: 20ul/ml PEST (see washing solution above for details), 200ug/ml gentamicin, 5% FBS in Leibowitz L-15 media plus 2mM.L^{-1}glutamine (Uppsala - ICN Flow Ltd., Hertfordshire, UK) which contained [Na^+]=155, [K^+]=6.0, [Cl^-]=143, [Ca^{2+}]=1.26 mM). The cells were counted and seeded onto the appropriate substrate.

Single Seeded Insert (SSI) Preparations:

The methods for establishing SSI cultured membranes was based on procedures established by Wood and Part (1997). After isolation, the cells were counted using a haemocytometer seeded at a density of 400 000 cells.cm^{-2} in 25cm^2 culture flasks (Falcon 3013 Tissue Culture Flask, Becton Dickinson Labware, N.J., USA or Nunculon Culture Dishes, Nunc, Denmark). These flasks were placed in an air filled incubator kept at 17-19°C. The day after seeding, culture medium was replaced with fresh medium of the same composition, and the flasks were returned to the incubator. Four days after seeding the medium was again replaced, but this time with antibiotic-free medium (Leibowitz L-15 media and 5% FBS). At day 6-8 after seeding, the cells from these primary cultures were harvested and reseeded onto new substrates.

The harvesting of cells was done by adding 1 ml of trypsin solution to the PBS-rinsed culture flasks. Visual inspection using a phase contrast microscope indicated when all the cells had detached from the bottom of the flask (approximately 5 minutes), at which time the trypsination was terminated. Detachment occurred with the help of gentle manual

agitation. After harvesting, the cell suspension was poured through a cell strainer, into trypsin stop solution (as above). Flasks were rinsed with PBS until all cells were collected. The final cell suspension was centrifuged at 200g for 10 minutes and resuspended in antibiotic-free culture media. Cell density was determined on a haemocytometer. Cell counts were based on viable cells excluding red blood cells (RBCs) and eosin-stained cells. Cells were seeded onto semipermeable Falcon culture inserts (cyclopore polyethylene terephthalate; Becton Dickinson, Franklin Lakes, New Jersey, USA, pore density: 10^8 pores.cm^{-2}, growth surface, 0.83cm^2) at a density of 500 000 cells.cm^{-2}. Prior to seeding, the culture inserts, which sit in companion wells (Becton Dickinson), were soaked in culture media for 1-2 hours to ensure complete wetting. Cells and culture media were added to make a final volume of 0.8 ml in the insert cup (apical side) and 1.0 ml in the companion well in which the insert was suspended (basolateral side). Since the companion well is wider than the insert, these volumes allowed for the height of solution in the insert cup to be slightly higher than that inside the companion well. This arrangement maintained a positive hydrostatic gradient to prevent lifting of the cells.

The insert preparations were placed in the incubator. Media was added to the preparations on the next day so that final volumes were 1.5 ml in the apical insert and 2 ml in the companion well. Bathing solutions were replaced 48 hours after initial seeding with the same volumes of fresh antibiotic-free media, and every 48 hours thereafter.

Double Seeded Insert (DSI) Preparation:

Double-seeding is an alternative means of culturing epithelial cells. This technique is used to establish confluent membranes of brown trout branchial epithelium used for immunocytochemistry and ion fluxes (personal communication from P. Seppo and P. Pärt). Recently, double-seeding culture dishes with branchial cells from rainbow trout produces cultures that contain clusters of mitochondrial-rich cells that appear to be chloride cells (personal communication from V. Thomas and G. McDonald).

The production of an initial primary culture in flasks was omitted in this preparation. Instead, a double-seeding technique with freshly dispersed cells was used to produce confluent cultured membranes on semi-permeable inserts. Fresh viable gill cells were counted using a haemocytometer and seeded onto Falcon culture inserts at a very high density of $2\text{-}2.5*10^{6}$ cells.cm^{-2}. The initial volumes of culture media (0.8 ml on the apical side, 1.0 ml on the basolateral side) were the same as SSI preparations, however at this stage the culture medium was supplemented with antibiotics. The day after seeding, each insert was rinsed with 0.4 ml of supplemented culture media 3 times, or until visual inspection ensured that the mucous layer was removed. At this time, new cells were isolated from a second fish using the same methods as above. These freshly isolated cells were seeded at the same very high density onto the layer of cells already established in the inserts, along with 0.8 ml and 1.0 ml of supplemented media on the apical and basolateral sides respectively. On the day following the second seeding, inserts were rinsed until the mucous layer was once again removed, but this time rinsing was with antibiotic-free

media. Aliquots of 1.5 ml (apical) and 2.0 ml (basolateral) of antibiotic-free media were added to inserts and wells, and the preparations were returned to the incubator. Bathing medium remained antibiotic-free, and was changed every 48 hours.

Analytical Methods for Electrophysiological Measurements:

Transepithelial resistance (TER) was monitored using STX-2 chopstick electrodes connected to a custom-modified EVOM epithelial voltohmeter (World Precision Instruments (WPI), Sarasota, Florida, USA) which would read resistances in the range of 1 - 100 000 ohms.cm^2. Daily measurements of TEP across filter inserts were made starting 48 h after the initial seed of SSI membranes, and 76 h after the initial seed of DSI membranes. TER measurements were taken across a blank insert containing the same solutions as seeded inserts each time the inserts were monitored, and this value was subtracted from the measured values to correct for the resistance of the membrane plus solutions without cells. TER values were obtained in the same manner for inserts with either freshwater (dechlorinated and autoclaved Hamilton tapwater, or Uppsala tapwater diluted 50:50 with double distilled H_2O and sterilized) or seawater (full strength seawater made with a synthetic seawater formulation (Forty Fathoms Crystal Sea Marine Mix, Marine Enterprises International Inc., Baltimore MD, USA) which had [Cl^-]=548 mmol.L^-

^{1}) bathing solutions.

TEP was measured using salt bridges (agar-4% 3M KCl) connected through Ag/AgCl electrodes (WPI) to a pH meter used as a high impedance electrometer (Radiometer pHM 84). All TEP values were expressed relative to the apical side as 0mV, and TEP values for blank inserts with the appropriate apical and basolateral solutions were subtracted from measured values for similar corrections as for TER.

TEP Studies, Uppsala:

TEP measurements were made using the apparatus described above (See previous section). Cultured membranes were bathed in either culture media (L-15 plus 5% FBS) or modified HCO_3^--free Cortland's saline (composition: [NaCl]=124, [KCl]=5, [$CaCl_2.2H_2O$]=1.56, [$MgSO_4.7H_2O$]=0.9, [HEPES]=6, [$NaH_2PO_4.H_2O$]=2.97, [glucose]=5.5 mmol.L^{-1}, pH=7.8) on both sides of the membrane. The metabolic inhibitors: ouabain, iodoacetate, and KCN were dissolved directly in saline, while amiloride, DIDS (4,4'-diisothiocyanostilbene-2,2'-disulphonic acid), and vanadate were dissolved in saline containing dimethylsulfoxide (DMSO, 0.1% final concentration). Inhibitors were added to both apical and basolateral compartments.

Microscopy:

Cells in flasks and on inserts were visualized under a phase contrast microscope to monitor growth and trypsinations. To determine if mitochondrial-rich cells (MRCs) were present in the cultures, 5 ul of Rhodamine 123 (methyl-o-6-amine-3'-imino-3H-xanthen-9-yl benzoate monohydro chloride, Molecular Probes, R302, Eugene OR, USA, $1 mg.ml^{-1}$ of stock in sterile PBS) was added to the apical and/or basolateral media for 30 minutes, at which point the stain was washed away with three consecutive 2 minute rinses prior to viewing. Fluorescence of MRCs was detected using a Zeiss inverted microscope with epifluorescence and automatic exposure control. Densities of fluorescing clusters was determined by using an ocular micrometer and the calculated area of fluorescing clusters in each insert were expressed as a percentage of the total area of the insert ($O.83cm^2$).

Several SSI cultured membranes with TER > 5000 $ohms.cm^2$ were fixed for transmission electron microscopy (TEM). Culture medium was replaced with bicarbonate-free Cortland's saline, and then with 2.5% gluteraldehyde in 100 $mmol.L^{-1}$ sodium cacodylate buffer (pH 7.5) and left overnight at 4°C for fixation. Procedures following fixation were conducted according to Falcon Technical Bulletin 406 (Becton Dickinson Europe, BP 37-38241 Meylan Cedex, France). Dehydrated preparations were embedded in resin (Agar 100, Agar Scientific Ltd. U.K.) and 50nm sections were cut on an ultramicrotome and stained in lead citrate/uranyl acetate. Sections were examined in a Philips CM10 transmission electron microscope.

[^{3}H]PEG-4000 Permeability:

The permeability of cultured epithelia to [^{3}H]polyethylene glycol-4000 (NEN-Dupont) was measured across DSI membranes that were 6 days old. After the measurement of TER, 1uCi of [^{3}H]PEG was added to the basolateral culture media. Its appearance on the apical side as well as TER were monitored at 6 and 12 hours thereafter. On the following day, the apical medium of these same preparations was changed to either freshwater or seawater, and the procedure was repeated. PEG permeability was calculated according to the following equation:

$$(1) \quad P = \frac{\blacktriangle[PEG^*]_{Ap} \cdot Volume_{Ap}}{[PEG^*]_{Bl} \cdot Time \cdot 3600 \cdot Area}$$

where $[PEG^*]_{Bl}$ is mean radioactivity on the basolateral side, $\blacktriangle[PEG^*]_{Ap}$ is the change in radioactivity on the apical side, 3600 converts time from hours to seconds, and area is 0.83cm^2 (area of the insert).

Na^+K^+-ATPase Activity:

Enzyme activities were measured in cells from primary culture flasks (see above for methods). The gill arches were cleared of red blood cells (RBCs) by perfusing the entire

head (Perry and Walsh, 1989). Fish were anaesthetized with tricaine methanesulfonate (MS222, Syndel laboratories, Vancouver, Canada, 0.05g.L^{-1} in aerated dechlorinated Hamilton tapwater), then injected via the caudal blood vessel with 10ul.g^{-1} heparinized saline (composition: 15mg heparin (Sigma) in 5ml PBS) and 1 ul.g^{-1} Rhodamine 123. The fish were left to recover in a well aerated black fish box for 2 hours. After recovery, the fish were sacrificed with an overdose of MS222 and decapitated. The operculae were removed to expose the gills and the ventral flesh between the fins was removed to expose the heart and bulbus arteriosus. The bulbus was catheterized using polyethylene tubing (PE 50, Clay Adams). While continually rinsing externally with dechlorinated freshwater, the gills were perfused via the catheter with dilute heparinized saline (80mg.L^{-1} PBS) for 10 minutes, or until the gills turned white. At this point, the perfused head was taken to the laminar flow hood for the usual isolation procedure.

After isolation, 5 aliquots of 2 million cells suspended in culture media were saved for day 0 activity measurements. The composition of day 0 cells was determined by counting the brightly fluorescent cells (see microscopy). The proportion of RBC's was determined by visual inspection. The remaining cells were seeded in culture flasks (25cm^2 surface) according to the procedure already described, and allowed to grow. Flasks were retrypsinated at day 3 and day 6 until at least 4 samples of 2 million cells were obtained at each day. At day 3 and 6 , cells were restained by adding 1 *u*l.ml^{-1} of R123 to cell suspensions, and rinsing 3 times with fresh media. Observations indicated no RBC's or fluorescing cells at either day. Since cell coverage in the flasks is much lower at day 3,

more flasks were needed at this time to supply the same amount of cells as day 6. Therefore, through trial and error it was determined that a minimum of 8 fish were needed to produce enough samples of cells for accurate Na^+K^+ -ATPase activity measurements. Cell samples were rinsed with 1.5 ml PBS, suspended in 1.0 ml of homogenizing media (composition: 0.25 M sucrose, 6 mM EDTA dissodium) and frozen at -70° C for later analysis.

Experiments in Uppsala, Sweden included measurements of enzyme activity for cells on inserts where TER peaked (see Analytical Methods section for details). The perfusion step was omitted since RBCs are not found after 48 hours in culture. Cells were harvested for samples on day 3 and day 6 with the addition of samples from cultured inserts. Cells from retrypsinated day 6 culture flasks were reseeded onto large Falcon inserts (4.3 cm^2 surface) at a density of 600 000 cells.cm^{-2}. At day 6 on the insert, at the point of peak TER (see Results), cells were removed from the inserts by placing 1 ml of trypsin solutions in both the insert cup and the supporting well. Cell samples were collected and stored as before.

Na^+K^+-ATPase activity of isolated gill cells was determined as the difference between phosphate liberated from adenosine triphosphate (ATP) in the presence of potassium, and in the absence of potassium with ouabain present (Holliday, 1985). The assay, developed by Holliday (1985) to measure Na^+K^+ATPase activity in fiddler crab gills was adjusted to accommodate a smaller protein content in gill cell samples. Protein content was determined using the Bradford reagent (Sigma B-6916) (Bradford, 1976).

Statistical Analysis:

Data has been expressed as means ± 1 (N) where appropriate. N represents the number of filter inserts or cell samples used in the experiment. Regression lines were fitted by the method of least squares, and the significance of the Pearson's correlational coefficient (r) was determined. The statistical significance of differences between means was measured using Student's two-tailed t-test, either paired, unpaired, or one-sample as appropriate. The limit of significance in all statistical analyses was 5%.

Results:

Transepithelial Resistance Growth Curves

Both preparations (SSI and DSI) show similar changes in transepithelial resistance (TER) over time in culture (Figure 2). SSI were previously shown to reach a peak resistance between day 6-9 in culture (Wood *et al.*, 1997, Wood and Part, 1997) therefore all SSI cultured epithelia measured in this study were used for transport or electrophysiological experiments at day 6-7 which was either at their peak TER or just prior to it. The overall range of TER at day 6-7 in culture in different series was 1 250 - 21 000 ohms.cm^2. This large variability was similar to previously measured TER values across the same cultured preparations (Wood *et al.*, 1997). A similar variation is seen in the TER profile of DSI cultured epithelia which range from 1 300 - 33 000 ohms.cm^2 (Figure 2). In general, they reach a greater maximum TER and plateau later at day 7-8.

Transmission Electron Micrograph (TEM) of SSI Preparations:

TEM revealed that cells cultured according to SSI methods at day 6-9 (maximum TER) show several overlapping cell layers connected by putative tight junctions and many desmosomes. Figure 4 illustrates such a preparation at day 7 with a TER of 10 100 ohms.cm^2. As can be seen, the cells have many mitochondria, a well defined rough endoplasmic reticulum, and a prominent glycocalyx. There is also evidence of microridges

Figure 2: Growth curves of SSI membranes measured as an increase in transepithelial resistance (TER) with time. Both apical and basolateral compartments contained culture media (symmetrical conditions). Each curve represents a group of membranes used for a particular experiment. Inserts were seeded at Day 0 and TER was measured for the first time at day 2. Data expressed as means ± 1 SEM.

Series 1: Na^+K^+ATPase activity experiments (n=5)

Series 2: Ca^{2+} Fluxes, Uppsala (n=62)

Series 3: TEP measurements, Uppsala (n=124)

Series 4: TEP measurements, Hamilton (n=8)

Series 5: Growth curve from Wood and Part (1997), (n=14)

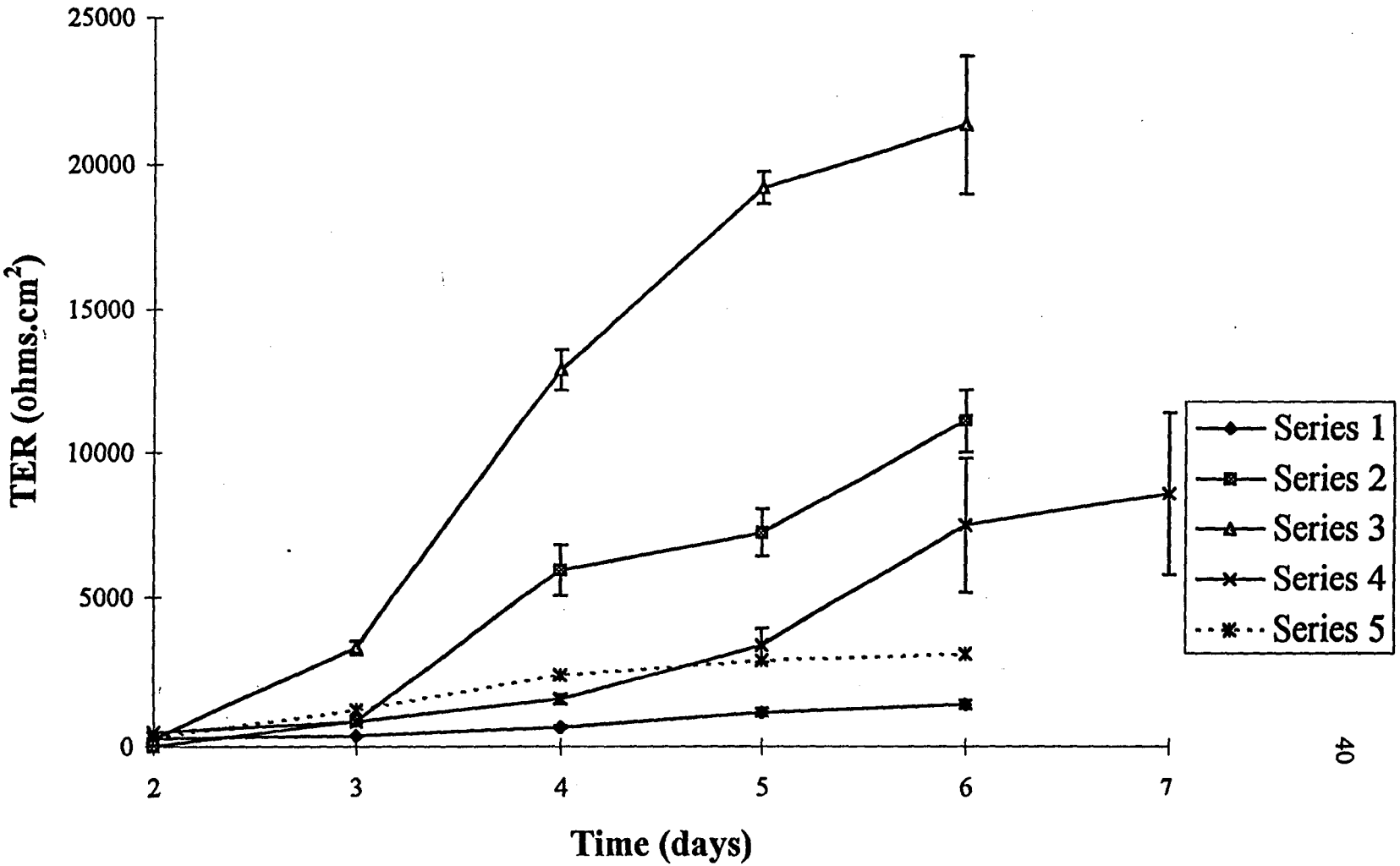

25000
20000
15000
10000
5000
0
TER (ohms.cm2)
2
3
4
5
6
7
Time (days)
Series 1
Series 2
Series 3
Series 4
Series 5

Figure 3: Growth curves for DSI membranes measured as an increase in TER with time. Measurements taken were with culture media on both sides and expressed as means ± 1 SEM. Inserts seeded firstly on day 0 and secondly on day 1. TER was measured every day beginning at day 3. Each series represents a group of membranes used for a particular experiment.

Series 1: Seawater and freshwater exposures, (n=20)

Series 2: Na^+ and Cl^- Fluxes, set 1, (n=28)

Series 3: Na^+ and Cl^- Fluxes, set 2, (n=30)

Series 4: Ca^{2+} fluxes, (n=22)

Series 5: PEG-4000 permeability experiments, (n=22)

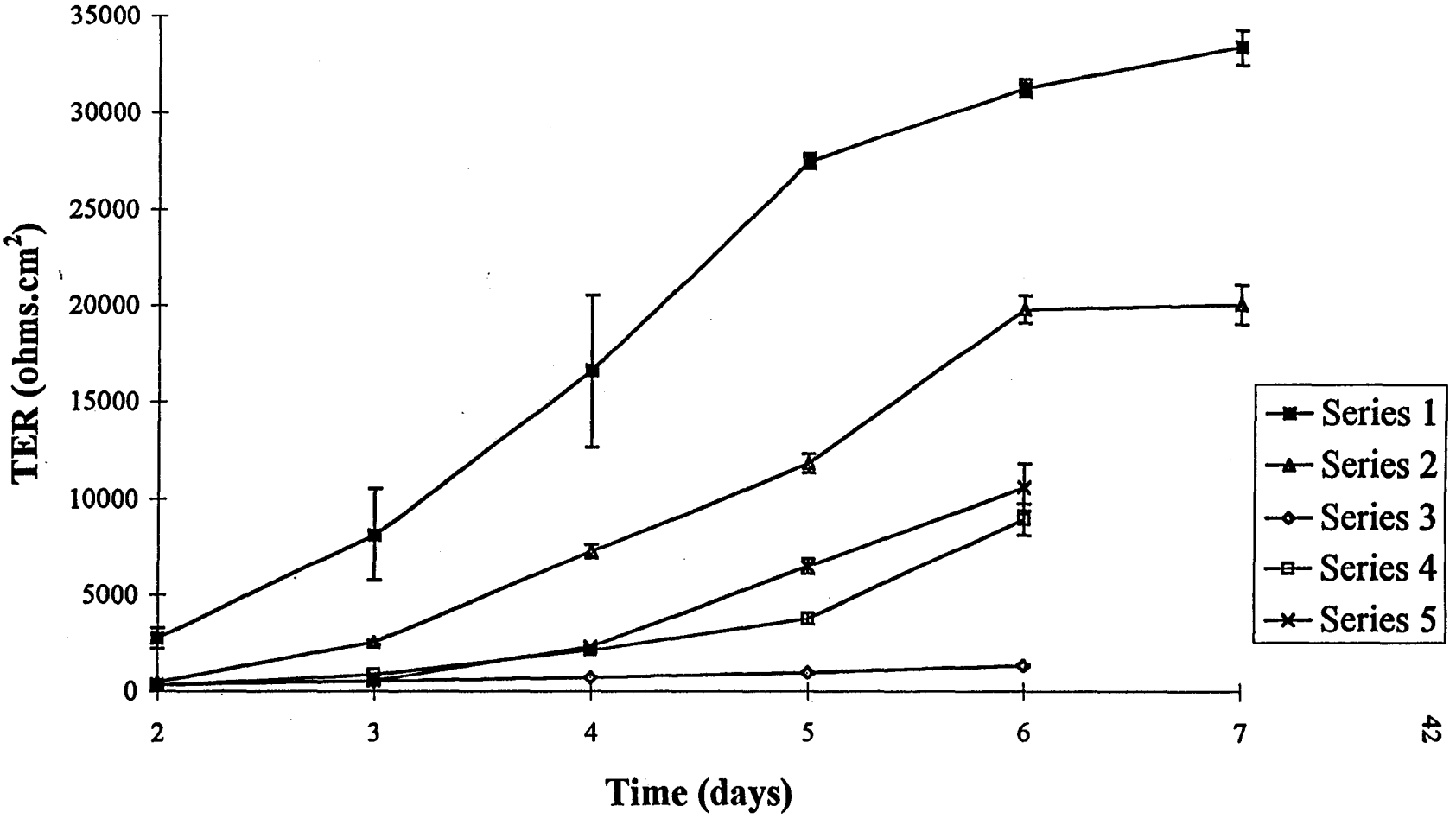
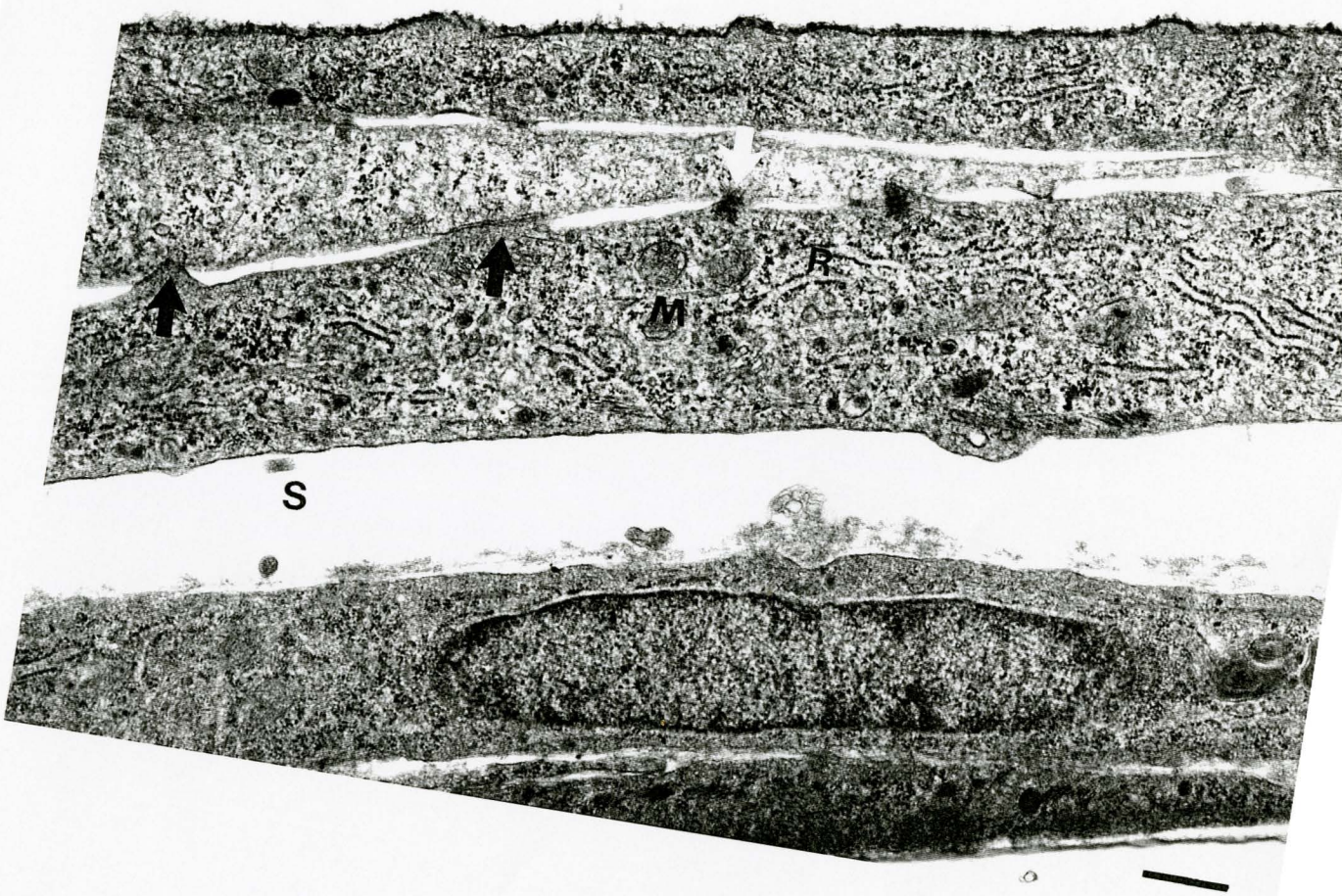

35000
30000
25000
20000
15000
10000
5000
0
TER (ohms.cm^2)
2
3
4
5
6
7
42
Time (days)
Series 1
Series 2
Series 3
Series 4
Series 5

Figure 4: Transmission electron micrograph (TEM) of an SSI cultured membrane on a permeable insert. Cells were grown for 7 days with culture media on both apical and basolateral membranes. The TER prior to fixation was 10 100 ohms.cm^2. The light areas on the upper left and the lower right represent the apical L-15 media and the permeable filter insert respectively. The large intracellular space (S) could be an artefact of the fixation. This TEM clearly shows five overlapping cell layers connected by tight junctions (filled arrows) and desomsomes (open arrow). The apical surface (upper) shows a prominant glycocalyx with apical projections. A few mitochondria (M) and an abundance of rough endoplasmic reticulum (R) are visible in the cultured cells.


R
M
S

on the apical surface of the upper cell layer.

Transepithelial Potential (TEP) Growth Curves:

The DSI cultured epithelia begin developing a small transepithelial potential (TEP) at day 3-4 (Figure 5), serosal positive with culture media on both sides of the insert (symmetrical conditions). This can be compared to SSI cultured epithelia in Hamilton which do not appear to develop a TEP under the same conditions. Prior to these studies, TEPs across SSI cultured membranes under symmetrical conditions were also considered negligible throughout their growth period, including the point at which peak TER was established (Wood *et al.*, 1997). Similar results were found in the present study. SSI cultured membranes mean TEP of 0.44 ± 0.21 (8) at day 7 is not significantly different from zero ($p=0.087$).

The Correlation of TER and TEP:

TEP growth curves were measured for only 2 groups of DSI cultured membranes, but TEP values were obtained for all cultures prior to experimentation. These TEP values correlated positively with TER across the same DSI membranes (Figure 6). The overall mean TEP of 1.87 ± 0.15 (124) mV for DSI cultured membranes was significantly different from zero ($p<0.01$). TEP values for DSI membranes had a range of 0-7 mV, and the slope of the TEP versus TER relationship gives an estimate of the mean current across

Figure 5: Growth curves for SSI and DSI cultured membranes measured as an increase in TEP with time in culture. TEP measurements were taken with culture media on both apical and basolateral sides, and each series represents a group in membranes used for a particular experiment. Cell were first seeded onto filter inserts at day 0, and TEP was first measured on day 2 for SSI and on day 3 for DSI. TEP expressed as means ± 1 SEM.

Series 1: Na^+ and Cl^- Fluxes, DSI membranes (n=58)

Series 2: Ca^{2+} fluxes, DSI membranes (n=22)

Series 3: Microscopy, SSI membranes (n=8)

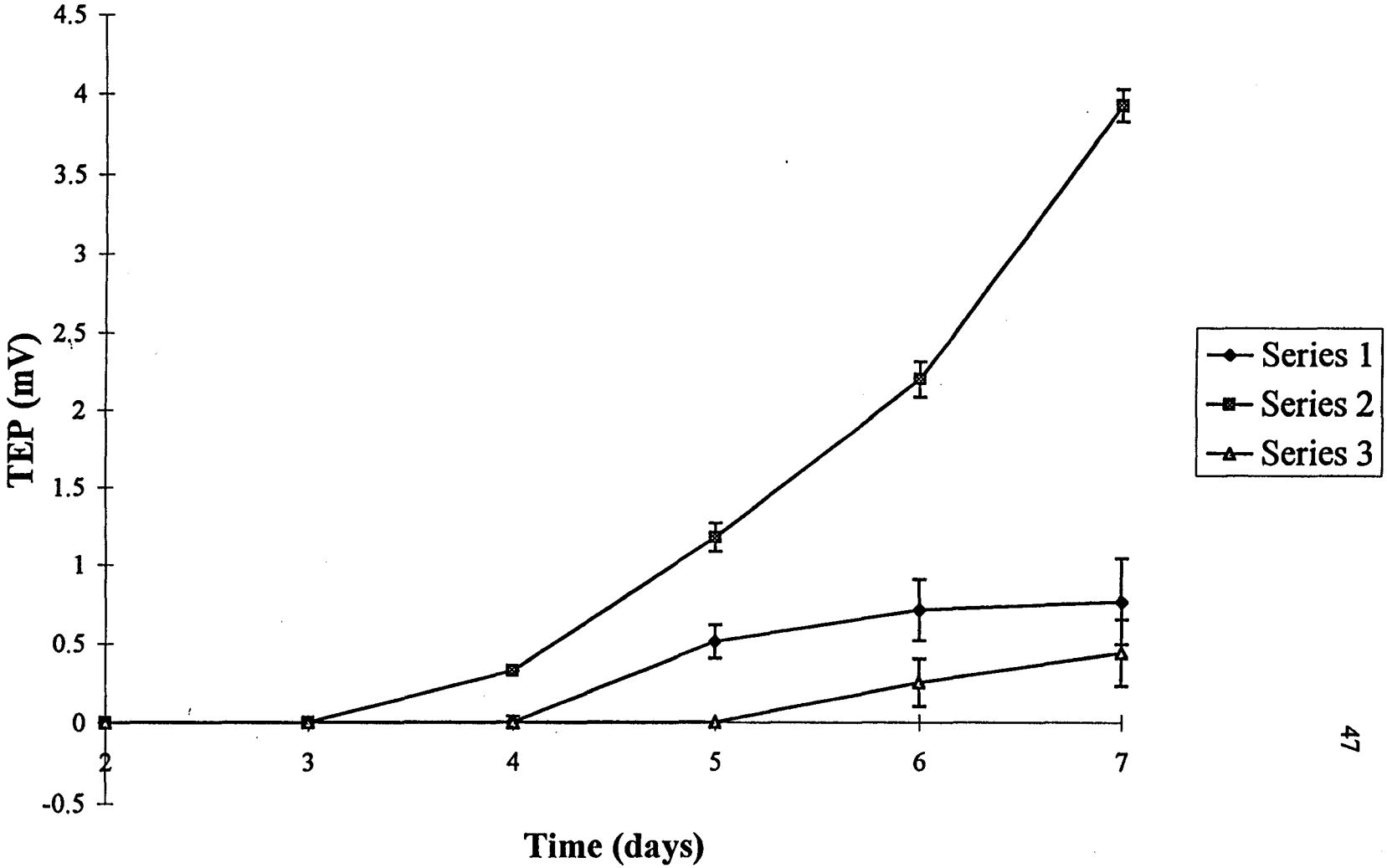
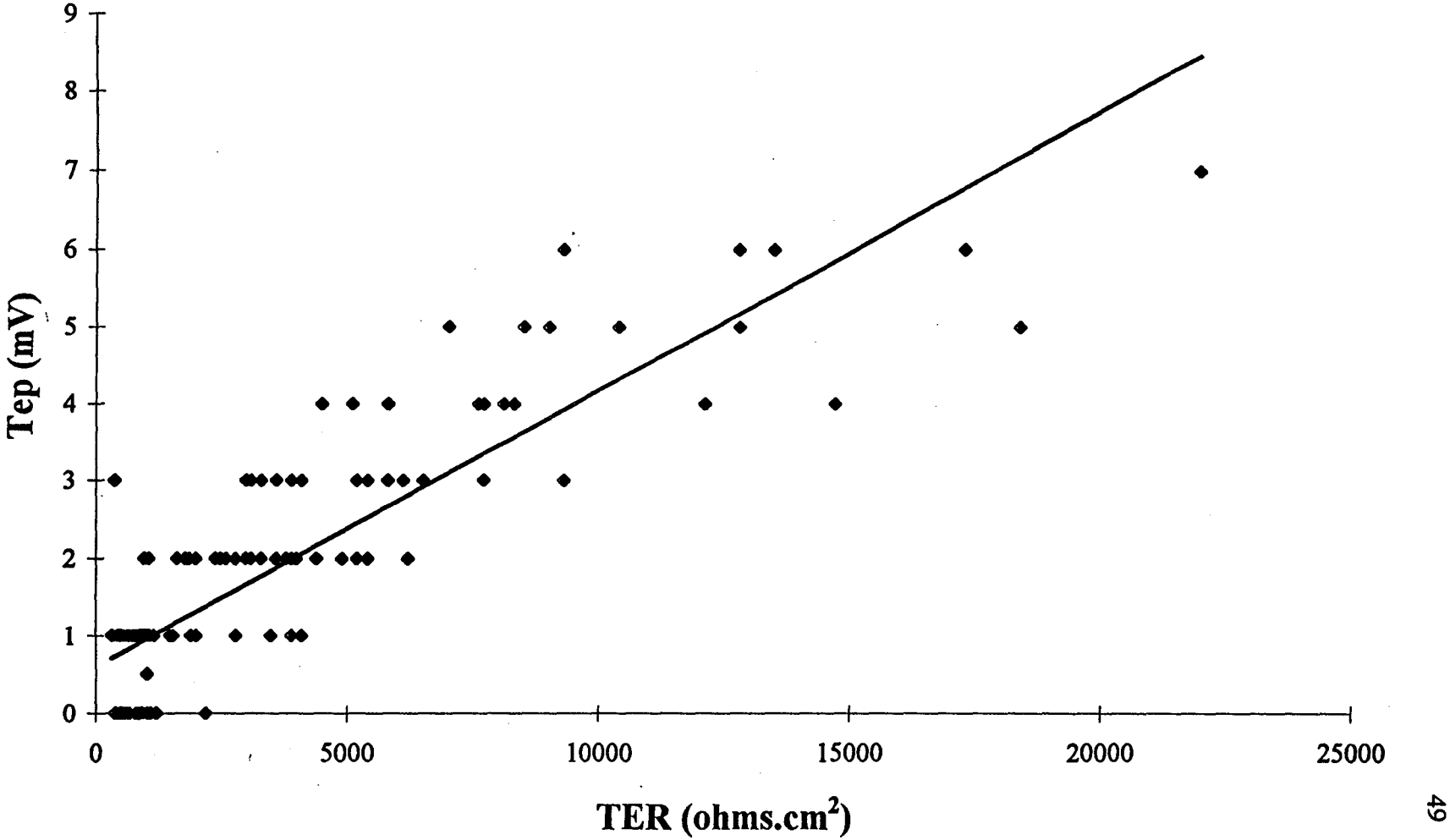

4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
TEP (mV)
2
3
4
5
6
7
Time (days)
Series 1
Series 2
Series 3

Figure 6: Relationship between TEP and TER under symmetrical conditions for all cultured DSI membranes produced in Hamilton.

Regression equation is TEP = 0.0004 (TER) + 0.589, r=0.869, n=124, p<0.0001.


Tep (mV)
9
8
7
6
5
4
3
2
1
0
0
5000
10000
15000
20000
25000
TER (ohms.cm²)

DSI membranes as 0.4 uA/cm^2. A net transepithelial flux of 14.9 nequiv.h^{-1}.cm^{-2} would be necessary to account for this current.

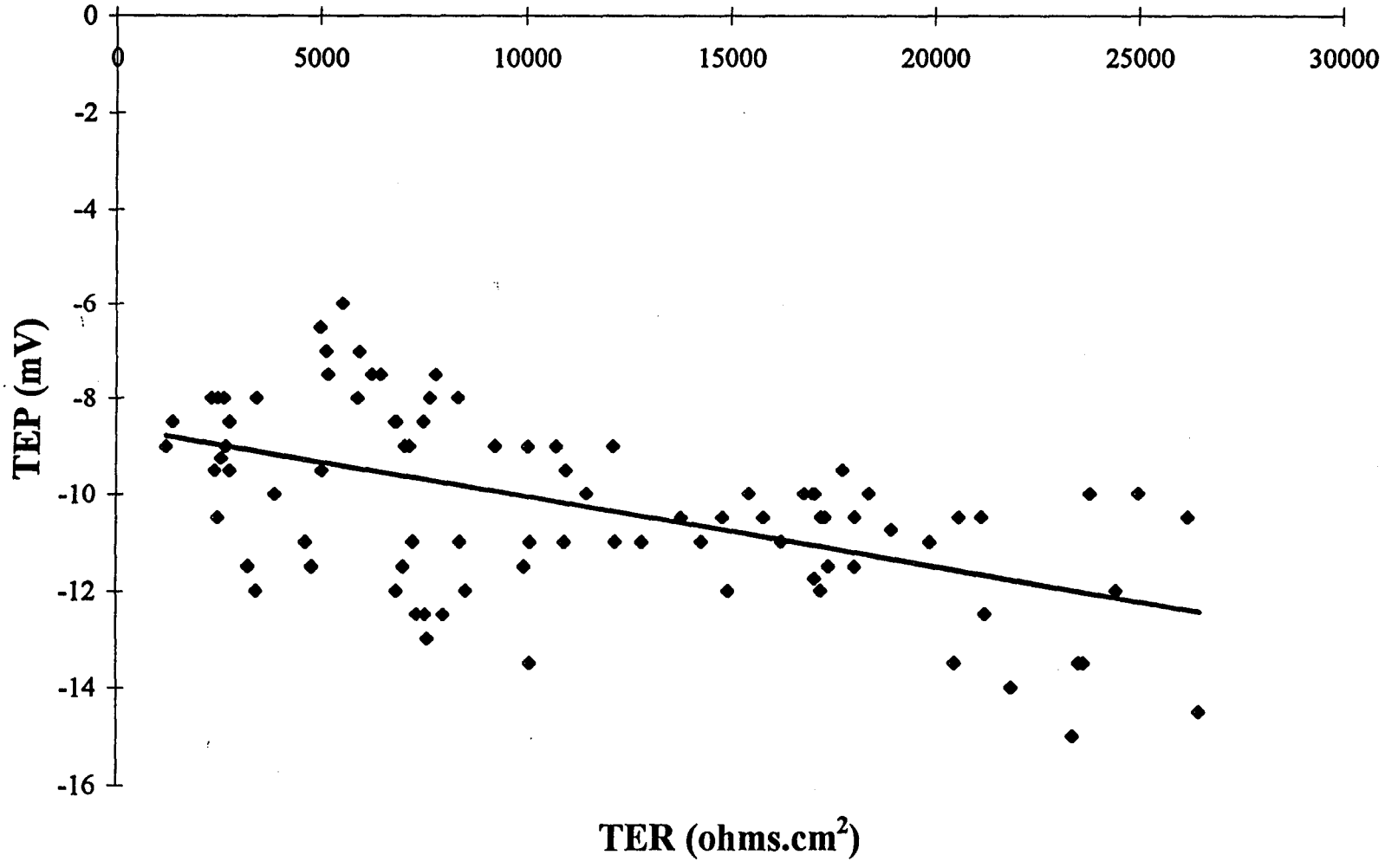
TEP Measurements after Apical Freshwater Exposure:

The DSI cultured membranes established a serosal negative potential (inside negative relative to outside) when exposed to freshwater on the apical side (Figure 7). After 4 hours of apical freshwater exposure the mean TEP was -10.3 ± 0.2 (88) which was not significantly different from mean TEP measurements across SSI membranes exposed to freshwater on the apical side (-10.5 ± 0.7 and -11.1 ± 0.8 mV, Wood *et al.*,1997). The relationship is significance but the slope is smaller than under symmetrical conditions, indicating the mean current across DSI membranes exposed to asymmetrical conditions was 0.1 uA/cm^2 which required a net ionic flux of 3.7 nequiv.cm^{-2}.h^{-1} to produce the current.

Further Transepithelial Potential Studies on Single Seeded Insert (SSI) Preparations:

TEP measurements were conducted on SSI cultures grown in Uppsala, Sweden. The results obtained on those membranes were different from SSI membranes cultured in Hamilton. Membranes from Uppsala with a TER > 20 000 ohms.cm^2 established a TEP of -2.24 ± 0.19 (44) mV with culture media on both sides. This TEP was serosal negative

Figure 7: Relationship between TER and average TEP of DSI cultured membranes after 4 hours of freshwater on the apical side. Regression equations is TEP = -0.0001 (TER) - 8.5987, r=0.543, n=89, p<0.0001.


0
0
5000
10000
15000
20000
25000
30000
-2
-4
-6
-8
-10
-12
-14
-16
TEP (mV)
TER (ohms.cm2)

whereas SSI membranes cultured in Hamilton did not have a significant TEP. However, as with DSI membranes cultured in Hamilton, SSI membranes from Uppsala with TER < 20 000 ohms.cm^2 demonstrated a linear relationship between TER and TEP (Figure 8). The mean TEP of all cultures in Uppsala including those with TER ≥ 20 000 ohms.cm^2 was -1.6 ± 0.2 mV (53)) which was similar in magnitude but opposite in polarity to mean the TEP value of DSI membranes, 1.87 ± 0.15 (124) mV. Despite this similarity in magnitude, the slope of the TEP versus TER relationship gives an estimate of the mean current as 0.04 *u*A/cm^2, and an estimate of the net flux that could account for this current was 1.5 nequiv.cm^{-2}.h^{-1}. These values were 10-fold lower than DSI membranes under symmetrical conditions.

The mean TEP of -1.6 ± 0.2 (53) measured in Uppsala inserts, although small, was significantly different from zero ($p < 0.05$). Two different metabolic poison regimes, 100 uM vanadate and 8mM iodoacetate in combination with 1mM KCN, were found to completely eliminate the TEP when added to both apical and basolateral compartments (Figure 9). Vanadate caused a 95% reduction while Iodoacetate and KCN caused an 88% reduction in TEP. Other pharmaceutical agents including 0.1mM amiloride, 2mM DIDS, 5mM KCN alone, 10mM Iodoacetate alone, and 0.5mM ouabain had no effect on this negative potential over an exposure period of at least 30 minutes.

Apical and Basolateral Freshwater Exposure, SSI Membranes:

Replacing culture media on the apical side with freshwater resulted in a rise in TER

Figure 8: Relationship between TER and TEP of SSI membranes cultured in Uppsala with TER<20 000 ohms.cm^2. Measurements were taken when both apical and basolateral compartments contained culture media (symmetrical conditions). The regression equation is TEP= -0.039 + (4.1 X 10-5) TER, R=0.93, N=18, p<0.0001.

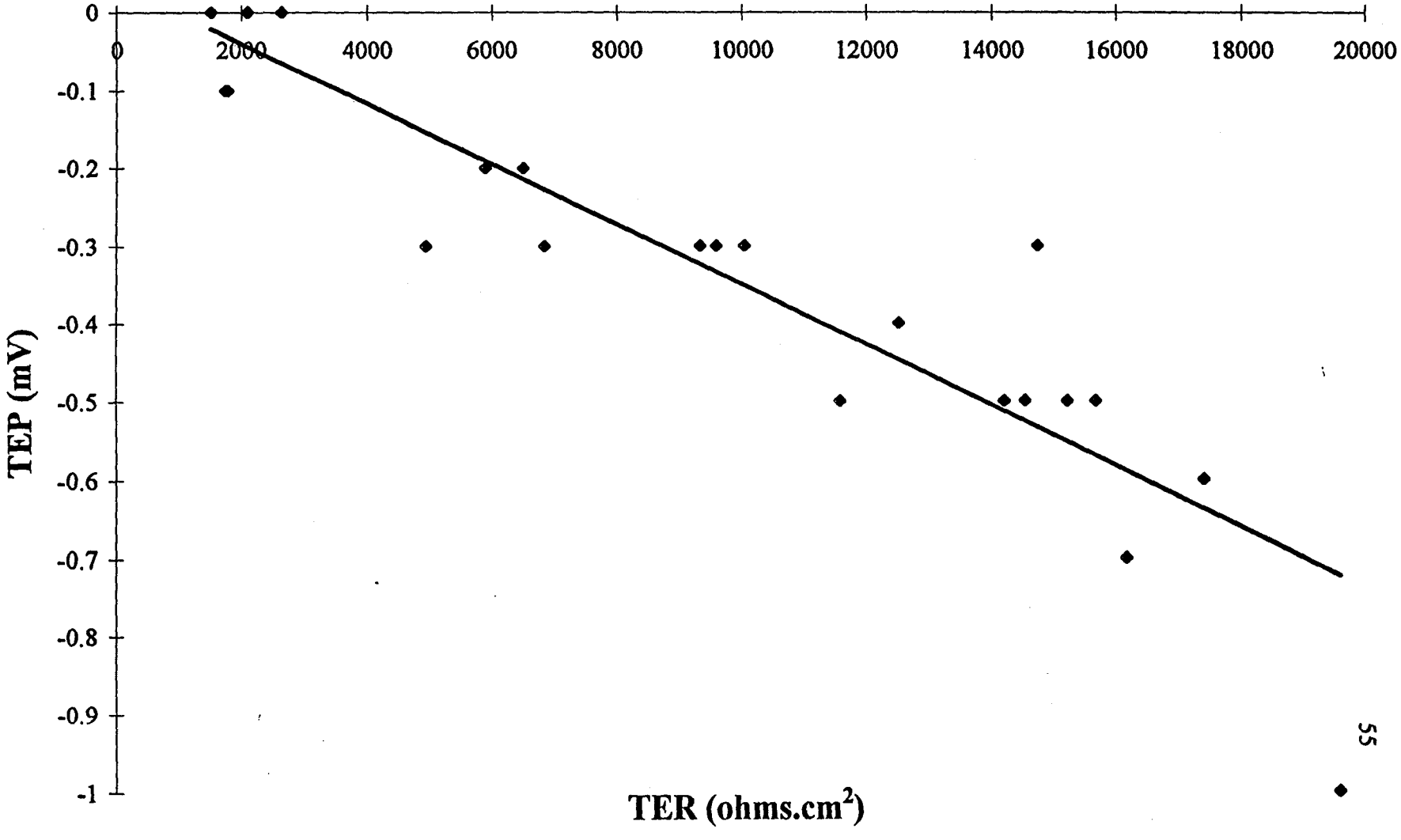
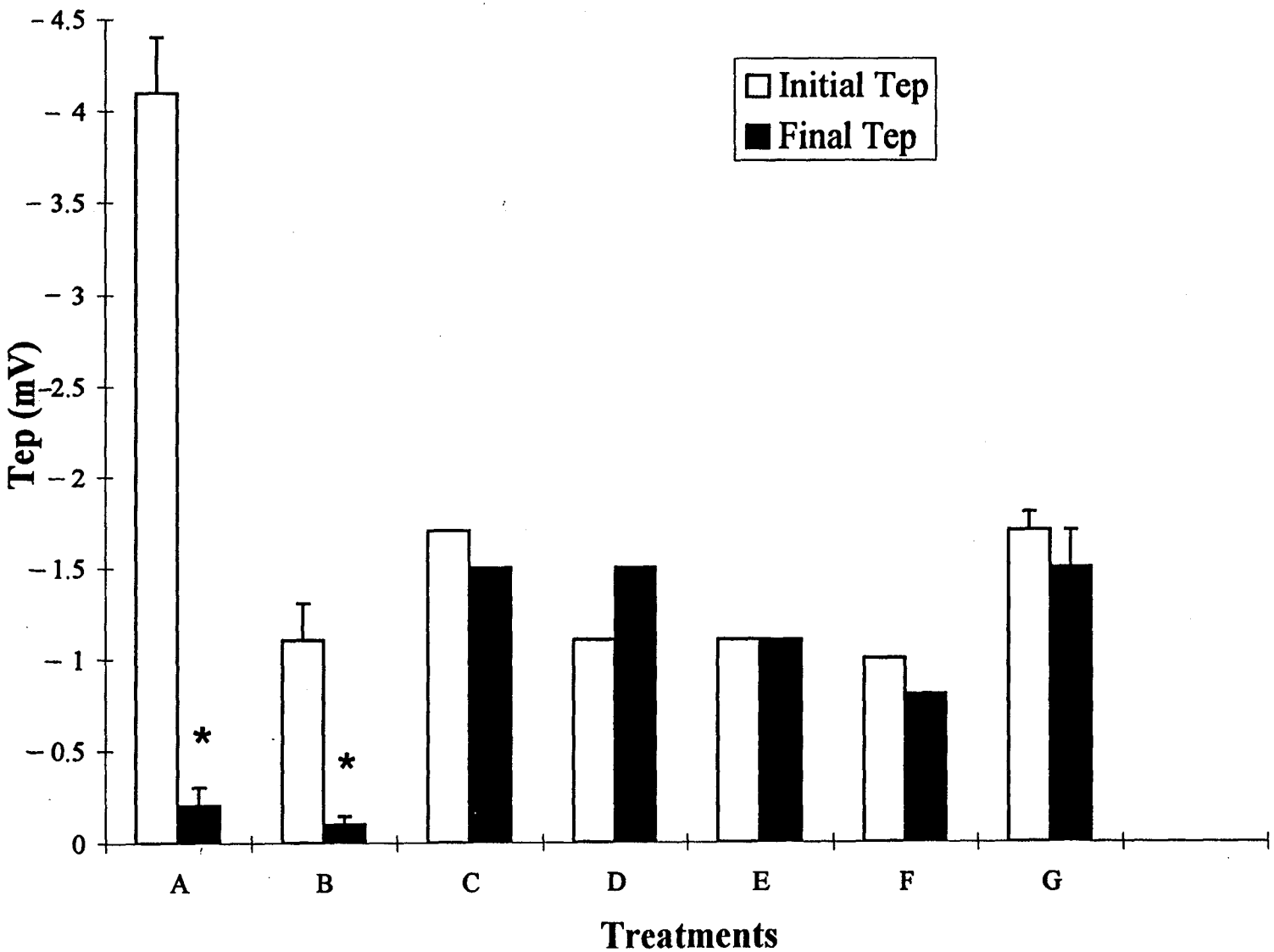

0
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
20000
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
-0.9
-1
TEP (mV)
TER (ohms.cm2)

Figure 9: The effect of exposure to A) vanadate (100umol.L^{-1}, n=5), B) KCN (1mmol.L^{-1}) and iodoacetate (8mmol.L^{-1}) together (n=5), C) amiloride (0.1mmol.L^{-1}, n=1), D) 4,4' diisothiocyanostilbene-2,2'-disulphonic acid (DIDS, 2mmol.L^{-1}, n=1), E) KCN alone (5mmol.L^{-1}, n=5), F) iodoacetate alone (10mmol.L^{-1}, n=5), and G) ouabain (0.5mmol.L^{-1}, n=3) on TEP of SSI membranes with culture media on both apical and basolateral sides. The drug was added to both compartments, and data was expressed as means ± 1 SEM where appropriate. * indicates a significant difference between TEP under control conditions and TEP after exposure to the metabolic poison ($p<0.05$).


Initial Tep
Final Tep
Tep (mV)
-4.5
-4
-3.5
-3
-2.5
-2
-1.5
-1
-0.5
0
*
*
A
B
C
D
E
F
G
Treatments

which remained significantly elevated over a 3 hour exposure period (Figure 10). A drastic drop in resistance was seen when culture medium was returned to the apical side, however the TER recovered within a short period of time to near initial values. Similar findings were seen previously by Wood and Part (1997). This was not the case when freshwater was substituted for culture media on the basolateral side of SSI membranes with similar starting TER values. The initial rise in TER dropped rapidly over the three hour exposure with no recovery seen upon replacing the basolateral freshwater with culture media again.

Apical and Basolateral Freshwater and Seawater Exposure, DSI Membranes:

Apical freshwater and seawater exposure had very different effects on TER (Figure 11A). The large rise in TER seen with apical freshwater in the SSI membranes was not evident with freshwater on the apical side of DSI preparations. However, the initial TER of 34 100 ± 193 (5) was much higher than 3 200 ± 120 (10), the initial TER of SSI exposed to apical freshwater. Even though only a partial recovery of TER was seen when culture media replaced freshwater, the resulting TER of 22 700 ± 777(5) after 24 hours was still very high, higher in fact than the maximum TER measured for SSI membranes. Apical seawater exposure was followed immediately with a decrease in TER which persisted throughout the exposure. After culture media was returned to the apical side, a partial recovery in TER was observed at 24 hours.

As with SSI membranes, basolateral freshwater and seawater exposure resulted in a permanent decline in the TER of DSI membranes (Figure 11B).

Figure 10: The changes in TER of SSI cultured membranes when culture media on the apical side (n=10), or basolateral side (n=10), is replaced with freshwater for 3 hours, then switched back to culture media again. * indicates significant difference from initial TER measurements prior to freshwater exposure ($p<0.05$). Data expressed as means ± 1 SEM.

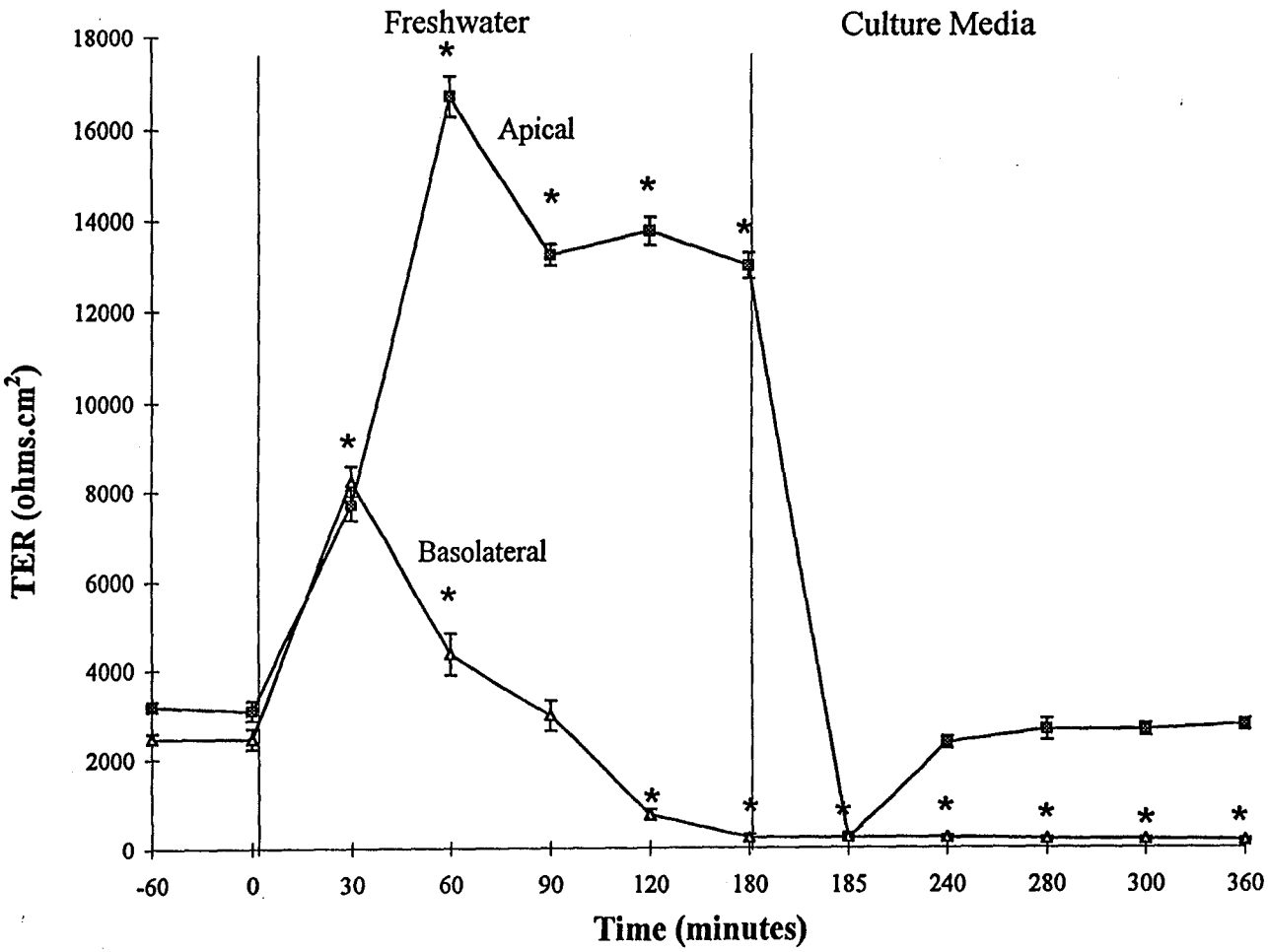

Freshwater
Culture Media
Apical
Basolateral
TER (ohms.cm^2)
Time (minutes)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
-60
0
30
60
90
120
180
185
240
280
300
360

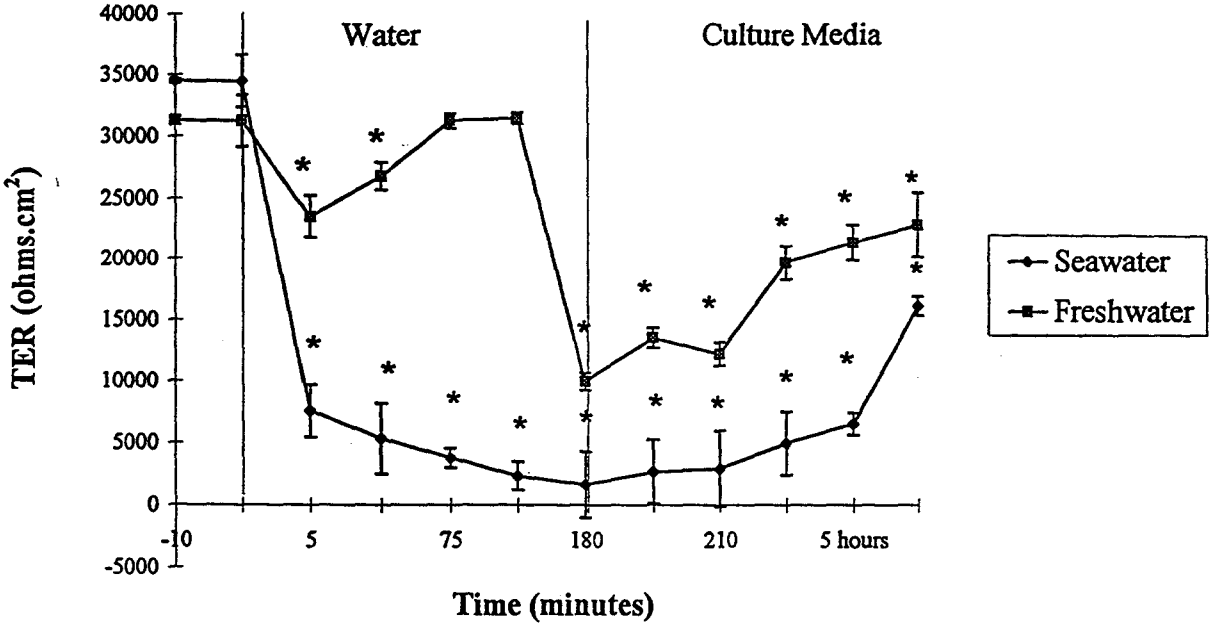
Figure 11:

A:The changes in TER of DSI cultured membranes when culture media on the apical side is changed to seawater (n=5), or freshwater (n=5), for 3 hours, then changed back to culture media again.

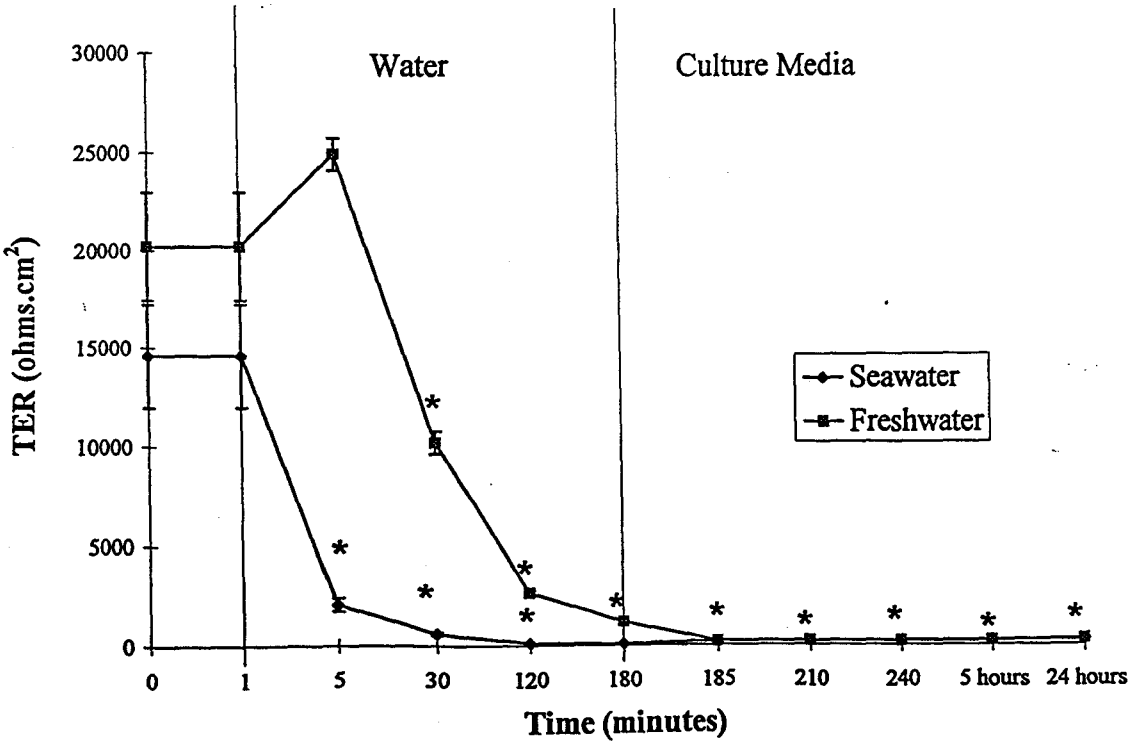
B: The changes in TER of DSI cultured membranes when culture media on the basolateral side is changed to seawater (n=5), or freshwater (n=5), for 3 hours, then switched back again.

* indicates significant difference from initial TER values prior to freshwater of seawater exposure ($p<0.05$). Data expressed as means ± 1 SEM.

A: Apical Exposure



B: Basolateral Exposure



PEG Permeability of DSI membranes:

PEG permeability was measured as an index of paracellular permeability (Munger *et al.*, 1991) during 2 consecutive 6 hour fluxes across membranes with three different apical solutions (Figure 12A). The PEG permeability of membranes with culture media on both sides and with freshwater on the apical side did not significantly change over the entire 12 hour period, however PEG permeability increased 2-fold when cultured membranes were exposed to freshwater. This occurred despite the fact that conductance of cultured membranes exposed to freshwater was more than 2-fold **lower** than conductance of membranes with culture media on both sides (Figure 12B). In contrast, membranes exposed to apical seawater demonstrated a large increase in conductance along with a large increase in PEG permeability. PEG permeability in all three conditions was correlated linearly with conductance (Figure 13A, B, C) but the relationship was most significant with seawater on the apical side (Figure 13C) and least significant with freshwater on the apical side (Figure 13A).

Na^+K^+ -ATPase Activity:

Initial measurements of Na^+K^+ -ATPase activity expressed per milligram protein revealed no significant change in activity over the course of 6 days in primary culture flasks (Figure 14A). This occurred despite the fact that at day 0 there were cell types present which probably have substantial Na^+K^+ -ATPase activity (Table 1) such as Red

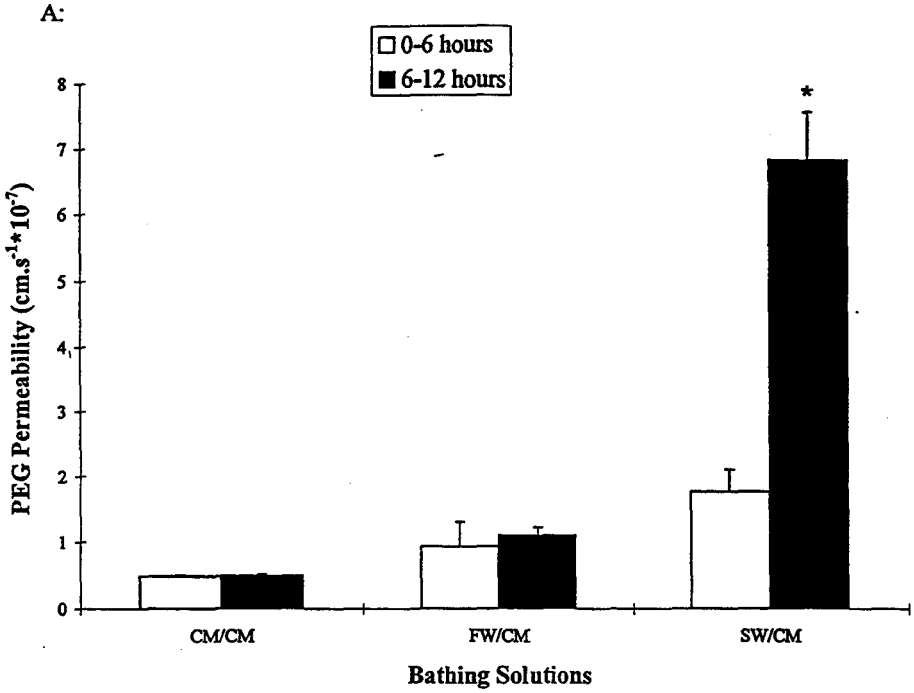
Figure 12:

A: The permeability of DSI membranes to [^{3}H]polyethylene glycol (PEG-4000) when the apical/basolateral compartments contained culture media/culture media (CM/CM, or symmetrical control conditions), freshwater/culture media (FW/CM), and seawater/culture media (SW/CM).

B: The conductance of DSI membranes measured during PEG fluxes under the conditions above.

* indicates a significant difference between the PEG permeability (A) and the conductance (B) during 0-6 hours and 6-12 hours under the same conditions. PEG permeability and conductance measured under both assymmetrical situations (FW/CM and SW/CM) was significantly different from control values during both time periods ($p<0.05$). Data expressed as means $\pm$ 1 SEM.

A:


0-6 hours
6-12 hours
PEG Permeability (cm.s-1*10-7)
0
1
2
3
4
5
6
7
8
*
CM/CM
FW/CM
SW/CM
Bathing Solutions

B:

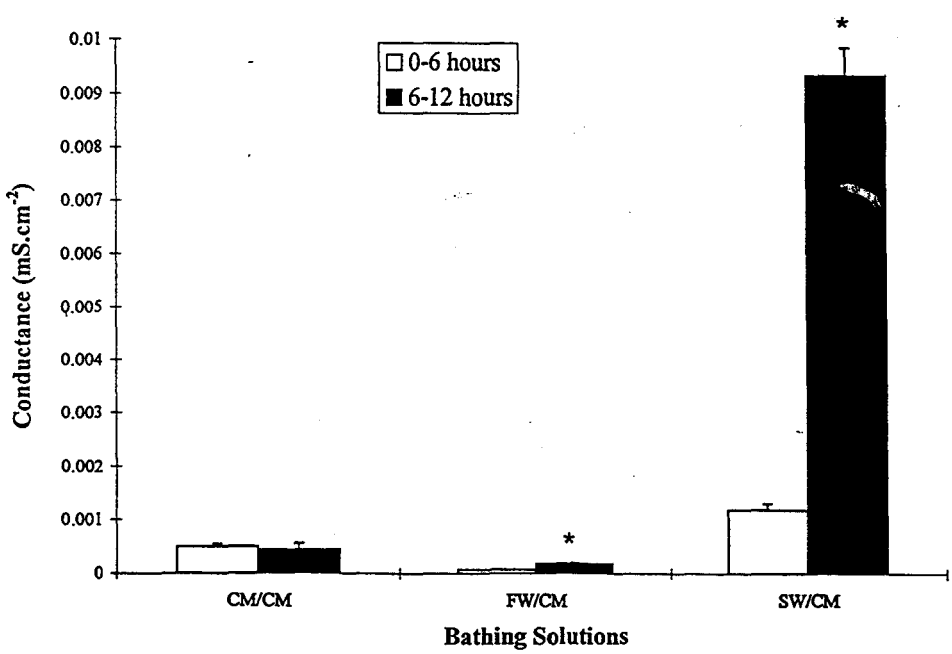

0-6 hours
6-12 hours
Conductance (mS.cm-2)
0
0.001
0.002
0.003
0.004
0.005
0.006
0.007
0.008
0.009
0.01
*
*
CM/CM
FW/CM
SW/CM
Bathing Solutions

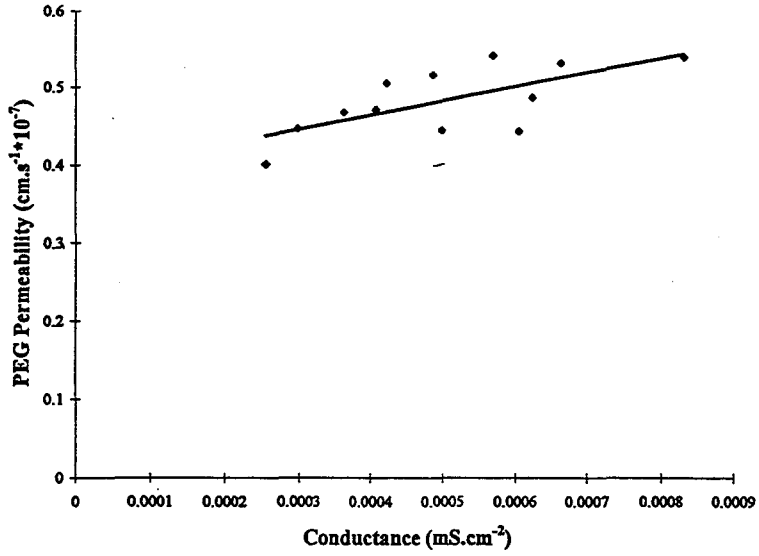
Figure 13: The relationship between PEG permeability and conductance of DSI cultured membranes. The contents of the apical/basolateral compartments and the regression equations for each relationship are:

A:culture media/culture media (control), Permeability = 182.97 (conductance) + 0.3901, $r=0.685$, $n=12$, $p<0.05$.

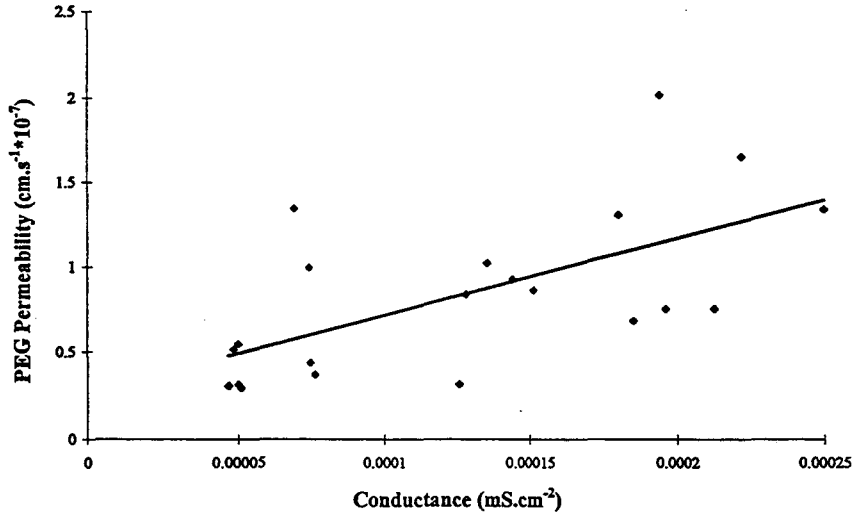
B: freshwater/culture media, Permeability = 4715.3 (conductance) + 0.2648, $r=0.632$, $n=21$, $p<0.05$

C: seawater/culture media, Permeability = 682.21 (conductance) + 0.7186, $r=0.914$, $n=22$, $p<0.001$

A: Control (CM/CM)



B: Freshwater/CM



C: Seawater/CM

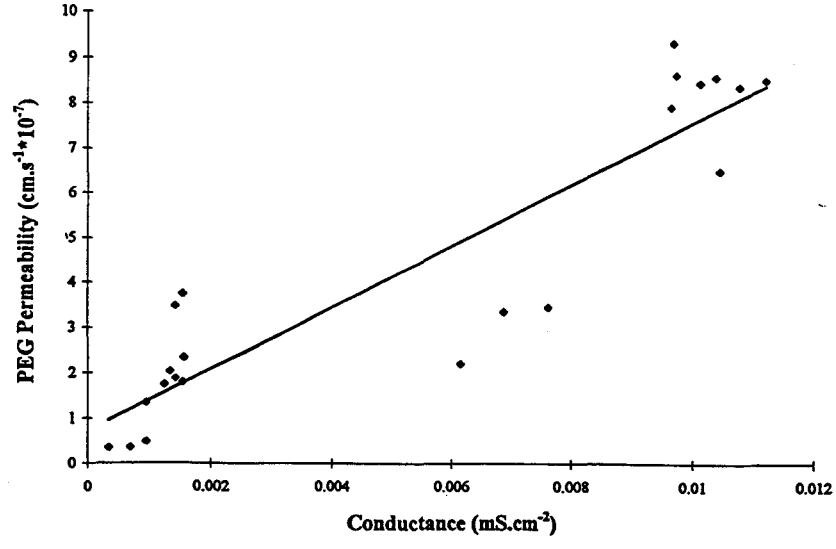
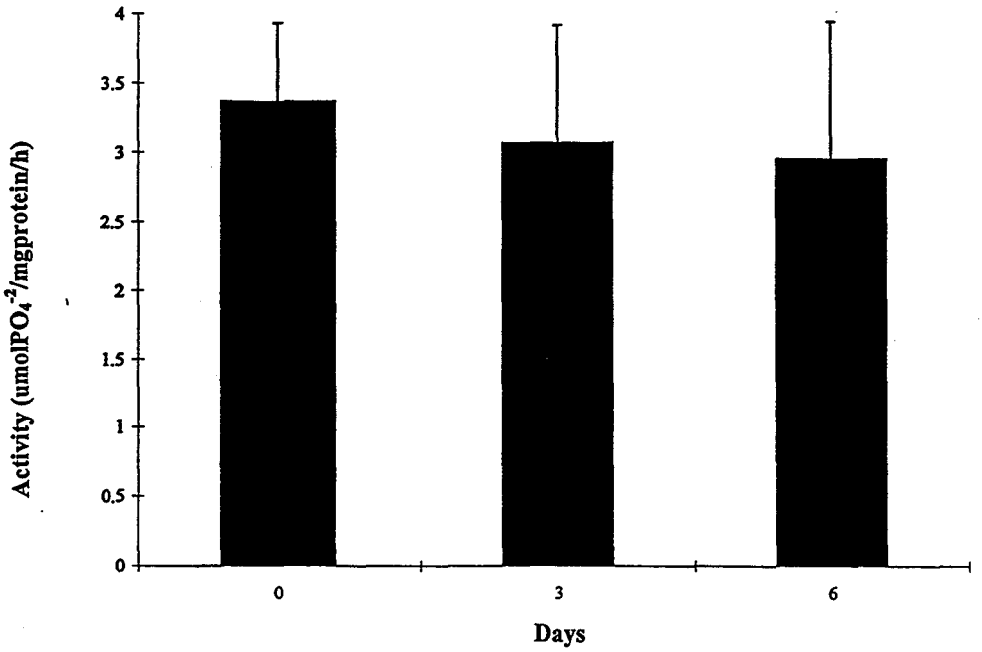


Figure 14: The Na^+K^+ -ATPase activity of cultured branchial cells at various times in culture.

A: Cells were isolated and cultured in Hamilton according to SSI methods, but the cells were not transferred to inserts. The x-axis denotes the number of days in primary culture flasks, where day 0 (n=8) represents freshly isolated cells, day 3 (n=4) and day 6 (n=4) are days in the flasks. No significant differences exist.

B: Cells were cultured in Uppsala according to SSI procedures. Enzyme activity was measured in whole filaments (n=10), at 3 (n=3) and 6 (n=4) days in primary culture flasks, and 6 (n=5) days after seeding onto inserts. * indicates significant difference between cultured cells and filaments ($p<0.05$). Data expressed as means ± 1 SEM, n = number of cell samples where each sample contained approximately 2 million cells.

A: Hamilton



B: Uppsala

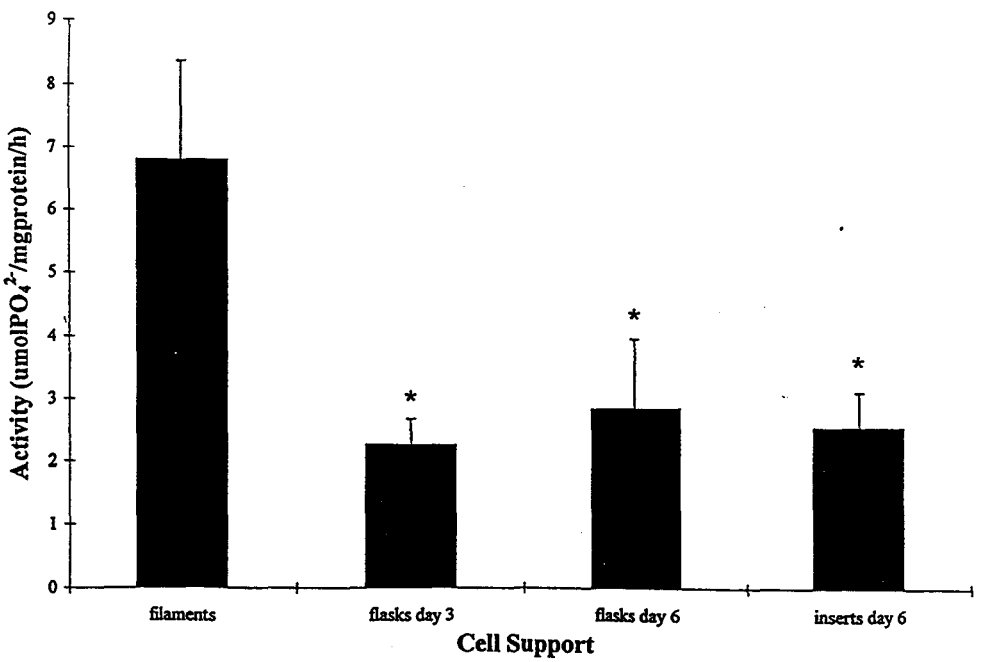


Table 1: The percentage of different cell types in samples of cultured gill cells used for Na^+K^+ -ATPase activity measurements. Cells were stained with mitochondrial-rich stain R123 and visualized under an inverted microscope. The brightly florescent cells and red blood cells (identified by observation) were counted, and expressed as a % of the total cells. All other cells were considered to be pavement cells.

Cell Types	Day 0	Day 3	Day 6
Pavement Cells	85.1± 6.4 (4) %	100 ± 0 (3)%	100 ± 0 (3)%
Mitochondrial-rich Cells	12.8 ± 4.2 (4)%	0 ± 0 (3)%	0 ± 0 (3)%
Red Blood Cells	2.1 ± 0.3(4)%	0.5 ± 0.2 (3)%	0 ± 0 (3)%

Blood Cells (RBCs) (Alberts, 1989) and Mitochondrial-Rich Cells (MRCs) (Perry and Walsh, 1989), which were not present in subsequent days in culture. In Uppsala, Figure 14B shows enzyme activity over a longer course period to include cells that were transferred to inserts via the methods for SSI membranes and a stable TER was established. The activity did not significantly change throughout the culture conditions, both in primary culture flasks (day 3 and day 6) and on inserts. In all culture conditions, R123 indicated no RBCs or mitochondrial rich cells were evident at these times. The activity of cells in culture was significantly lower than whole filaments which is not surprising since the latter contain the other cell types mentioned (Table 1).

Mitochondrial-Rich Cells:

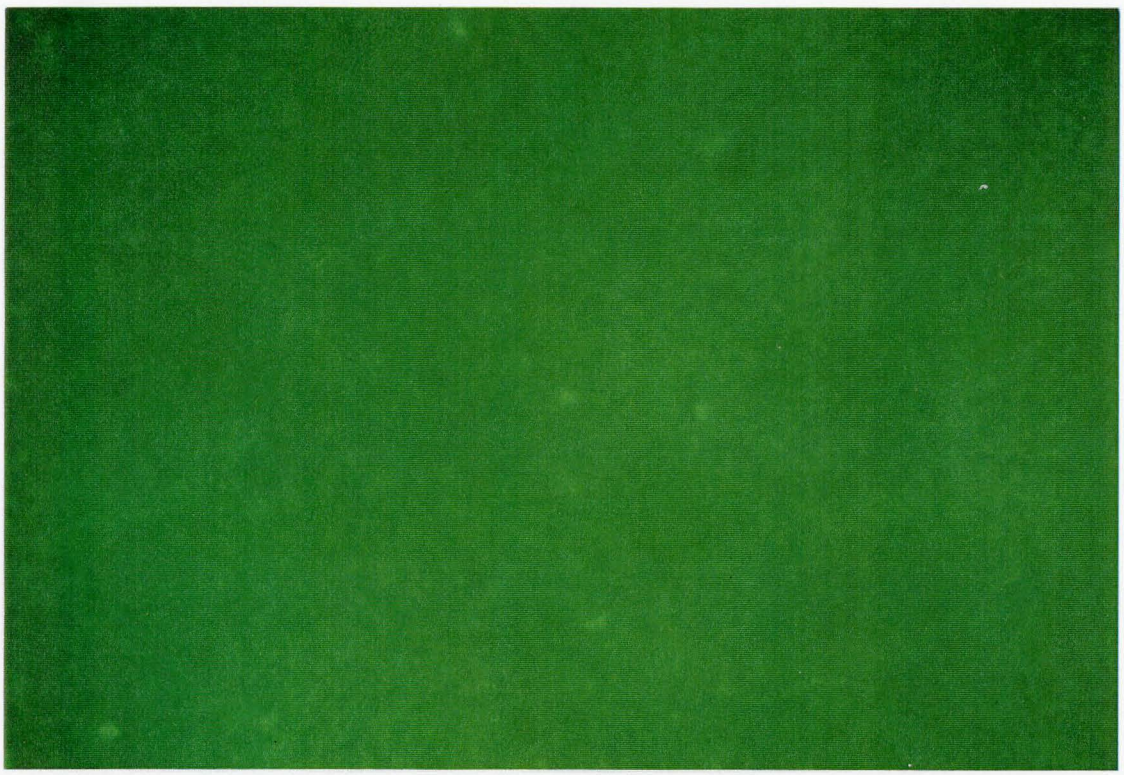
Cells on inserts from both SSI and DSI preparations were stained with Rhodamine 123 (R123) to visualize mitochondrial-rich cells. No brightly fluorescing cells were evident in any of the SSI inserts stained on both apical and basolateral sides at day 7 in culture. Figure 15A shows an example of such an insert which had a TER of 17 100 ohms.cm^2. However, bright fluorescent cells were seen in **all** membranes cultured by the new DSI technique. Some membranes were stained after being used for unidirectional flux experiments (see Chapter 3). R123 applied to both membrane surfaces revealed clusters of fluorescent cells as well as numerous single fluorescent cells (Figure 15B). Figure 16

Figure 15: Cultured SSI and DSI membranes were stained with R123 and visualized within 15 minutes of staining under a Zeiss inverted microscope with fluorescence (blue filter). R123 was added to both apical and basolateral sides of the membranes. Magnification 100X.

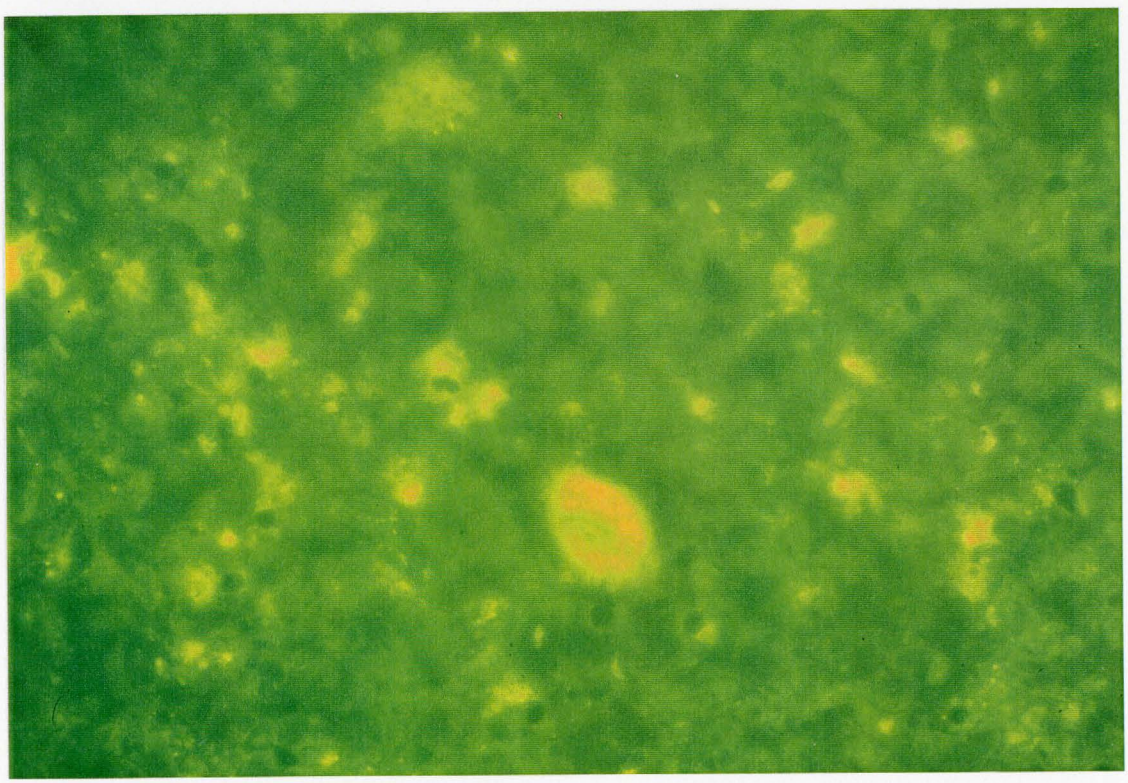
A: A SSI membrane at day 7, TER = 17 100 ohms.cm^2 prior to staining.

B: A DSI membrane at day 6, TER = 9 400 ohms.cm^2 and TEP = 2 mV prior to staining.

A:



B:

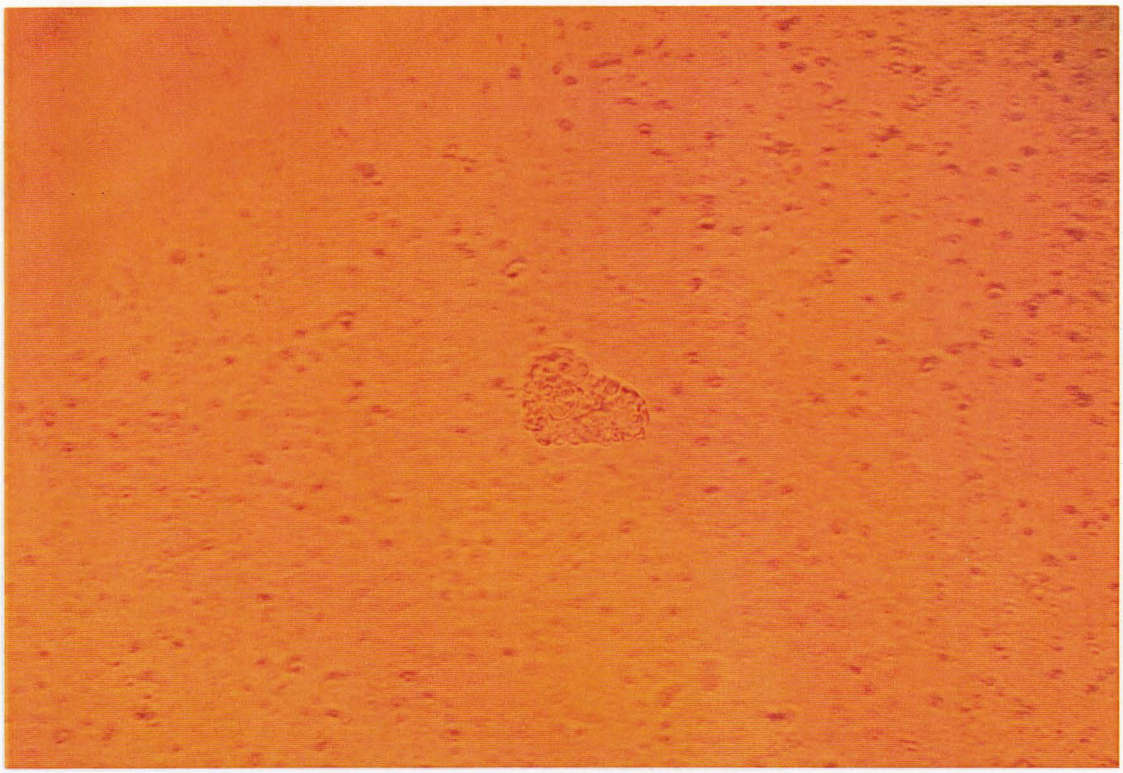


shows one cell cluster under phase and fluorescence. This particular cluster is a tightly packed unit of cells which is distinctive from the surrounding lawn of cells, even under phase (Figure 16A).

The surface area of clustered fluorescing cells was very small, accounting for only 0.03% to 0.44% of the total surface area of the insert. These percentages do not include area covered by single fluorescing cells. This surface area is positively correlated with TER prior to experimentation (initial TER with culture media on both sides) and with TER after 4 hours of freshwater exposure (Figure 17). There was no relationship between surface area and TEP (data not shown).

Figure 16: A Cultured DSI membrane stained with R123 and visualized within 15 minutes of staining under a Zeiss inverted microscope with A) phase and with B) fluorescence (blue filter). R123 was added to only the apical side of the membranes. Magnification 100X. This DSI membrane at day 6 had a TER = 3 500 ohms.cm^2 prior to staining.

A:



B:

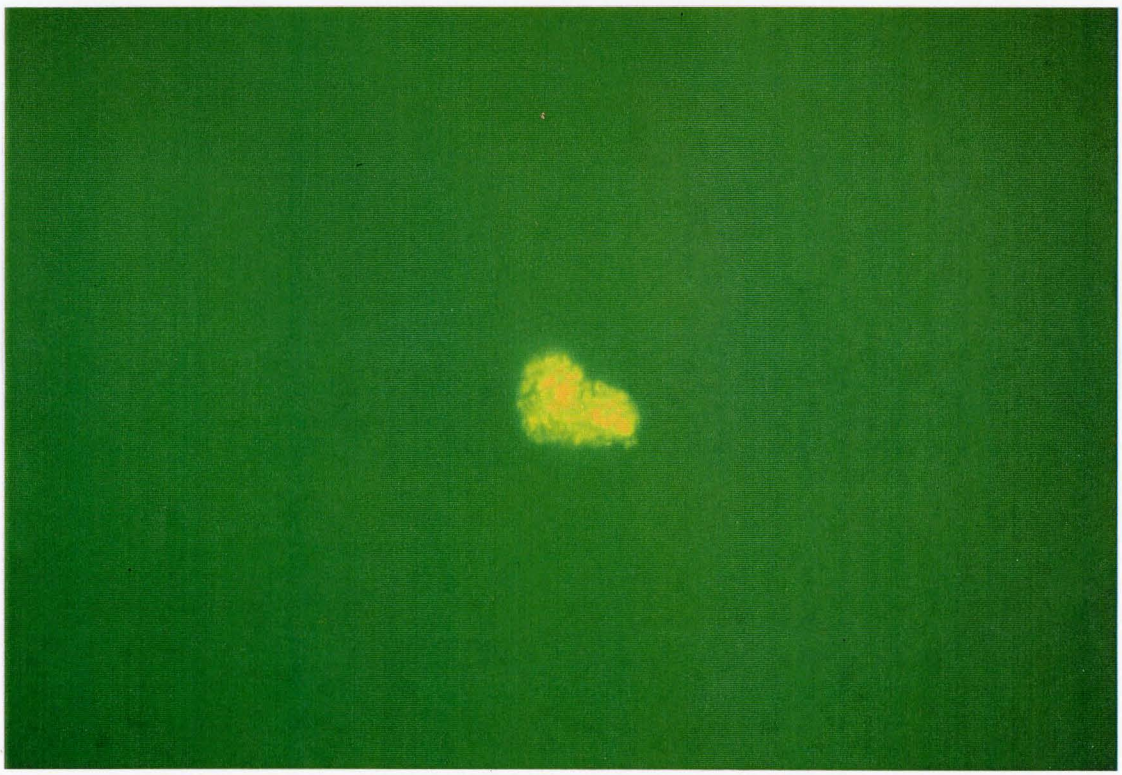
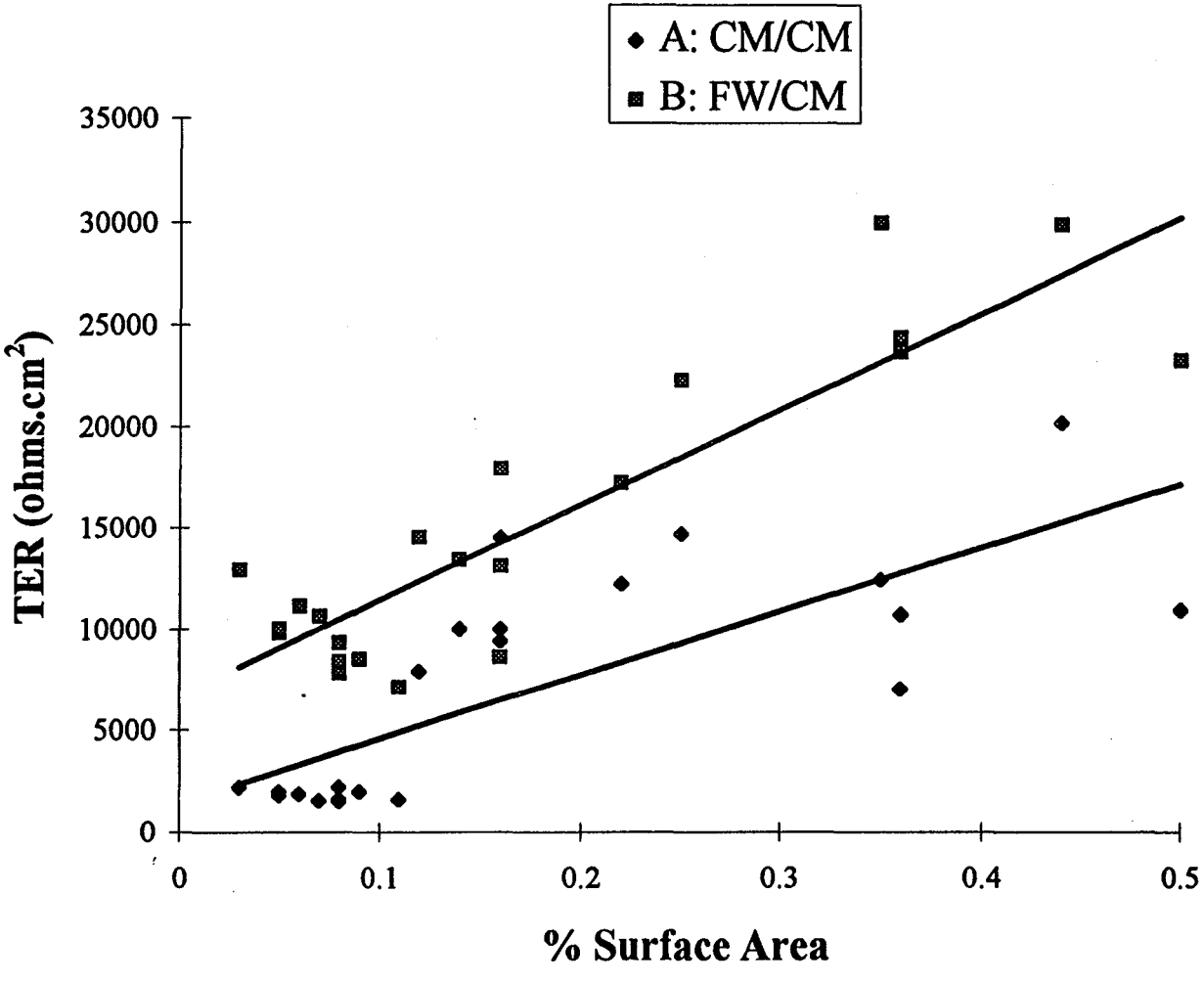


Figure 17: The relationship between chloride cell surface area (SA) and TER. SA expressed as a % of total SA.

A: %SA versus TER with culture media on both apical and basolateral sides. The regression equation is: TER = 31 388 (%SA) + 1438.6, r=0.763, n=24, p< 0.0001.

B: %SA versus TER with freshwater on the apical side and culture media on the basolateral side. The regression equation is: TER = 47 130 (%SA) + 6701, r=0.892, n=24, p<0.0001.


A: CM/CM
B: FW/CM
35000
30000
25000
20000
15000
10000
5000
0
TER (ohms.cm^2)
0
0.1
0.2
0.3
0.4
0.5
% Surface Area

Discussion:

Transepithelial Resistance (TER):

The rise in TER with time in culture (Figure 2 and 3) can be attributed to an increase in epithelial "tightness" as indicated in electron micrographs (Figure 5), showing the formation of desmosomes and tight junctions as the cells become more tightly packed (Wood and Part, 1997). Although the reason for large variations in peak TER values is unknown, the overall thickness of the epithelium (number and cell layers) and the relative numbers of tight junctions may be contributing factors. In general, the number of tight junctions between cells correlates with TER (Cereijido *et al.*, 1981). The mean peak TER values for single-seeded insert (SSI) and double-seeded insert (DSI) preparations were much higher than for "leaky" epithelia such as the gastric mucosa cells from guinea pigs (approximately 400 ohms.cm^2, Rutten, 1992) and the mammalian proximal tubule (5 ohms.cm^2, Lewis, 1997). They were also substantially higher, particularly the new DSI preparations, than typical "tight" epithelia such as cultured A6 kidney cells which can have resistances as high as 6 000 ohms.cm^2 (Candia *et al.*, 1993). The cultured branchial epithelium is also higher than other *in vitro* preparations from fish, such as the opercular epithelium of *Fundulus* (approximately 500 ohms.cm^2, Burgess, 1997), and the cultured flounder kidney cells (approximately 20 ohms.cm^2, Dickman and Renfro, 1986), but

comparable to the sea bass gill epithelium (approximately 12 000 ohms.cm^2, Avella and Ehrenfeld, 1997). It appears then, that both SSI and DSI membranes are tight epithelia.

TER is the combination of transcellular resistance and paracellular resistance, so variability in either or both of these properties would account for large variations in TER, such as those seen in the cultured epithelia (Lewis, 1997). The number of tight junctions and the dimensions of intercellular spaces affect paracellular resistance, while the number of membrane channels effects trancellular resistance. Although no values are available for TER of freshwater fish gills *in vivo*, the abundance of tight junctions in the gill epithelium and the absence of "leaky" junctions between chloride cells and accessory cells (Perry, 1997), since accessory cells are absent in freshwater gills (Laurent and Perry, 1991) would suggest high paracellular resistance. The TEP of freshwater gills *in vivo* (-20mV on average, Potts, 1984) just falls within the range of TEP values for "tight" epithelia (Table 2) (Lewis, 1997), as does the TEP of cultured gill epithelia with freshwater on the apical side (between -10 and -11 mV), therefore a reasonable assumption would be that fish gills *in vivo*, like cultured branchial epithelia, have a high TER.

Transepithelial Potential (TEP):

The most puzzling aspect of this work concerns the TEP across SSI membranes exposed to symmetrical conditions in Hamilton and Uppsala. The preparations in Hamilton had a negligible but slightly serosal positive TEP similar to the TEP across the opercular epithelium of brook trout, which, like the single-seeded cultures of rainbow

Table 2: Characteristics of tight and leaky epithelia (Lewis, 1997).

Type	TER (ohms.cm^2)	TEP (mV)
Leaky	6 - 100	0 - 11
Tight	500 - 70 000	10 - 120

trout gills, lack mitochondrial-rich cells (Marshall, 1985). The same SSI cultured preparations in Uppsala were found to have **significant** TEP values that were **serosal negative** with a mean of -2.24 mV. Not only were the TEP values significant, but they were eliminated with metabolic poisons, suggesting that active (ATP dependent) transport was creating the TEP (Figure 9). The basis of this TEP is uncertain since none of the other treatments, including amiloride, which inhibits Na^+/H^+ exchangers, ouabain which blocks the Na^+K^+ -ATPase, or DIDS which inhibits Cl^-/HCO_3^- exchange, had any effect on the potential.

Transepithelial potentials across freshwater fish gills are thought to be largely due to diffusion potentials created by the differential permeability of the membrane to Na^+ and Cl^- (Potts, 1984). Na^+, being the more permeant ion, has a tendency to leave faster than chloride and therefore causes a serosal negative potential. However, diffusion potentials can be ruled out as a cause of the TEP in SSI cultured membranes prepared in Uppsala because under symmetrical conditions (culture media on both sides) no diffusion potential exists (Potts, 1984). In any event, the elimination of this negative TEP by metabolic poisons suggests an electrogenic potential (Figure 9). There is little evidence of electrogenic potentials in freshwater fish except for gold fish which demonstrates a serosal positive potential in isotonic Ringer's solution (Potts, 1984). While some of the recent *in vitro* models of the gill report the absence of an electrogenic potential (brook trout opercular epithelium, Marshall, 1985), some models such as the cleithrum skin of rainbow trout (Marshall *et al.*, 1992), the opercular epithelium of freshwater killifish (Marshall *et*

al., 1995), and the opercular epithlium of *Tilapia* (Burgess, 1997) report small serosal positive potentials under symmetrical solutions, much like those evident in DSI cultured membranes, but their origin was not determined.

In contrast, the positive potential in cultured sea bass gills under symmetrical solutions is attributed to active Cl^- secretion (ie. efflux) (Avella and Ehrenfeld, 1997). They arrived at this conclusion because net Cl^- fluxes were not significantly different from the current, and the two parameters were correlated. Therefore, in converse, active Cl^- uptake could account for a negative potential like that seen in SSI membranes in Uppsala, but thus far active Cl^- uptake has only been detected in the cultured rainbow trout gills under asymmetrical conditions where they exhibit a negative potential (Wood *et al.*, 1997).

The localization of H^+ -ATPases on the apical membrane of the gill (Lin and Randall, 1995) raises the possibility that the potential could be influenced by these pumps. In the frog skin (*R. esculenta*), the proton pump participates in the generation of a serosal negative potential with freshwater on the apical side since blocking the pump results in an increase in TEP (Ehrenfeld *et al.*, 1985). The same study found that vanadate completely inhibited proton efflux while simultaneously changing the membrane potential. This may represent an explanation for the effects of vanadate on the cultured gill epithelia, and ultimately the role of proton secretion in the TEP. Unfortunately, the effect of bafilomycin, an H+ -ATPase inhibitor was not tested on the TEP of cultured SSI preparations.

Another possibility is that electrogenic calcium transport is contributing to the TEP.

External calcium concentrations have a large influence on the TEP across freshwater fish gills *in vivo* (McWilliams and Potts, 1978). Calcium reduces the permeability of Na^+ and H^+, producing a shift in potential towards positive values. Recently Kirschner, (1994) conducted studies on crayfish gills to assess the dependancy of TEP on calcium. The TEP in Ca^{2+}-free water was characteristic of a diffusional potential based on the differential leakage of Na^+ versus Cl^-. However, if the role of calcium on the TEP is to change the permeabilities, then the resistance across the membrane should correspondently increase, but this was not the case. The TER actually decreased as external Ca^{2+} increased. There was also no corresponding change in the unidirectional flux values for Na^+ and Cl^- as Ca^{2+} increased. Taken together, along with the fact that Ca^{2+} is actively transported in crayfish gills, Kirschner (1994) suggests that the increase in TEP to less negative (more positive) values with Ca^{2+} is due to active calcium uptake. Ca^{2+} transport in the cultured epithelia is investigated in Chapter 3 of this thesis.

A major uncertainty arising from the TEP studies is how the same preparation produced in two different locations (Hamilton and Uppsala) by the same protocol with the same species can result in cultured pavement cells that behave very differently, one which has a serosal negative TEP and one that does not. This discrepancy could be due to many things such as a difference in the fish themselves, or a difference in some specific ingredient in the culturing recipe, such as the quality of the FBS. The more likely explanation is a difference in the fish themselves. As noted above, external Ca^{2+} concentrations influence TEPs (McWilliams and Potts, 1978). Fish were held in freshwater

that was twice as hard in Uppsala than in Hamilton (see Materials and Methods). This difference may have ultimately affected the junctional complexes that formed in between the cells in the cultured membranes and/or active processes, in turn affecting the TEP.

The encouraging result was that double-seeded preparations with mitochondrial-rich cells had a significant TEP that was serosal positive under symmetrical conditions. Its magnitude of 1.87 mV and polarity were similar to previous TEP measurements across the intact gill (Potts, 1984), other in vitro models of the gill that contain chloride cells (opercular epithelium of freshwater-acclimated killifish (approximately 1.1 mV, Marshall *et al.*, 1997, and 1.23 mV, Marshall *et al.*, 1995), and other cultured gill epithelia (sea bass cultured pavement cells, approximately 13 mV, Avella and Ehrenfeld, 1997) under symmetrical conditions. The origin of the TEP in DSI membranes, like SSI membranes from Uppsala, is unknown but may be due to mechanisms such as those described above.

Freshwater and Seawater Exposure:

The recovery of TER measurements after apical freshwater exposure provides solid evidence of the preparations' ability to withstand conditions that mimic those experienced *in vivo* (Figure 10A). Freshwater exposure was accompanied by an immediate increase in TER (from approximately 3 000 to 17 000 ohms.cm^2) which are values similar to previously recorded TERs in other SSI preparations exposed to apical freshwater (Wood and Part, 1997). The natural cleithral epithelium of freshwater-adapted trout, another model of the freshwater gill, also displays high TER values (approximately 11 000

ohms.cm^2, Marshall *et al.*, 1992) which are still considerably lower than SSI preparations in asymmetrical conditions. As well, the *Fundulus* opercular epithelium, although by definition, rather "leaky", demonstrates an approximate 5-fold increase in TER with freshwater adaptation (Wood and Marshall, 1994, Marshall *et al.*, 1997).

The same clear trend seen with changing the media on the apical side of SSI preparations was not evident in DSI membranes. There was not a substantial increase in TER with freshwater on the apical side, however, the initial TER of approximately 32 000 ohm.cm^2 is approximately 10-fold greater than previously recorded values for SSI membranes in symmetrical solutions (approximately 2 500 - 3 500 ohms.cm^2 in this study and Wood and Part, 1997). Instead, TER dropped with freshwater exposure, then regained initial values only to subsequently decrease further when culture media was returned to the apical side. One possible explanation is that the TER was already maximal in symmetrical conditions prior to freshwater exposure, and was unable to increase further. Regardless of the pattern in TER exhibited with apical freshwater exposure, the overall result was that DSI membranes remained extremely "tight" throughout the exposure, and that 24 hours after return to symmetrical conditions following freshwater exposure, the TER was around 20 000 ohms.cm^2 which was still much higher than all values for SSI preparations in symmetrical solutions.

Exposure to seawater on the apical side caused a decrease in TER indicating the epithelium became leaky (see Table 2, Lewis, 1997). Figure 11A gives evidence of a partial recovery after the solution on the apical side was changed back to culture media.

This demonstrates the integrity of the preparation by its ability to withstand a salinity shock opposite to the "normal" *in vivo* situation. This response is not entirely surprising for two reasons. Although the rainbow trout naturally resides in freshwater, it is a euryhaline species that is able to adapt to seawater conditions. Therefore, the cells most likely retain some of their adaptable features. Also, the ability to adapt temporarily to seawater without long term changes in TER may be due to the rearing conditions of the cultured epithelia. Moving from culture media (approximately 150 mM NaCl) to seawater (approximately 550 mM NaCl) on the apical surface represents about a 4-fold increase in salinity whereas moving from culture media to freshwater (<1 mM) is more than a 150-fold decrease in NaCl concentration. It was previously shown that osmotically compensating freshwater with mannitol still results in the same increase in TER with freshwater exposure as seen with normal freshwater (Wood and Part, 1997), so the physiological adaptations are responses to salinity changes and not to osmotic changes.

Both SSI and DSI membranes did not reestablish their original TER after **basolateral** freshwater or seawater exposure. In fact, the TER decreased to values similar to those measured across blank inserts with no cells at all. It appears that some form of irreversible damage occurred, resulting in loss of membrane integrity and "tightness". This indicates the freshwater gill cells were unable to withstand the unnatural condition of freshwater on the basolateral side, providing evidence that the cultured cells were polarized properly such that the apical surface of the cells *in vitro* were oriented the same as *in vivo*, and *vice versa*. In general, cells are polarized if there is an asymmetrical

distribution of membrane-bound ion channels and carriers (Alberts *et al.*, 1989). Cells in epithelia can become polarized in response to external stimuli. In primary cell culture (flasks), cells are believed to orient their basolateral side towards the substrate, although the exact "sorting" signals are unknown (Freshney, 1989). In primary culture on a solid surface, there are no electrophysiological tests to ensure polarity, however on semi-permeable inserts, the direction of the TEP has been the usual indicator of properly polarized epithelia (Avella and Ehrenfeld, 1997, Candia *et al.*, 1993).

The exact mechanism to explain the large increase in TER with apical freshwater exposure is still puzzling. TER was described earlier as a combination of paracellular and transcellular resistance across the membrane. According to Lewis (1997), reducing [Na^+] in the mucosal solution will reduce the rate of Na^+ entry across the apical membrane of an epithelium by two mechanisms: a decrease in the chemical driving force for sodium uptake, and a decrease in apical membrane conductance. Therefore, the TER would increase since it is the inverse of conductance. However, if this were the only contributing factor, then it should be reversible in all instances. Figure 11A as well as PEG-4000 permeability suggest that freshwater causes physiological changes in the epithlium.

PEG-4000 Permeability:

PEG-4000 is a suitable paracellular permeability marker that does not penetrate cell membranes (Munger *et al.*, 1991). Labelled PEG-4000 fluxes were conducted to examine the relationship between paracellular permeability and TER, and to compare the

permeability of DSI membranes to SSI membranes obtained previously by Wood *et al.*, (1997). On an absolute basis, the permeability of SSI membranes to PEG-4000 (5*10-7 $cm.s^{-1}$) was 5 times higher than DSI membranes (1*10-7 $cm.s^{-1}$), indicating DSI membranes had a lower paracellular permeability than SSI. As with SSI preparations, PEG-4000 fluxes across DSI membranes demonstrated a positive linear relationship with conductance (inverse of resistance) indicating that as TER increased (conductance decreased), paracellular permeability decreased. This pattern was evident in all three apical exposures: culture media, freshwater, and seawater, the latter being the strongest relationship. Wood *et al.* (1997) found that in symmetrical conditions with culture media on both sides of SSI membranes, Na^+ and Cl^- effluxes could account for over 80% of the conductance, but that something more complex happens with freshwater on the apical side. The paracellular permeability (ie. PEG-4000 permeability) increases in comparison to symmetrical conditions while the conductance decreases in comparison. This same phenomena was apparent in DSI preparations (Figure 12). Originally it was thought that mechanical swelling and tightening of the epithelia, and hence, a decrease in paracellular permeability accounted for the increase in TER seen with apical freshwater exposure (Wood and Part, 1997) however, this is apparently not the case if PEG-4000 permeability increases in comparison to the permeability across the same preparations exposed to apical culture media. One possibility is that **transcellular** permeability decreases in response to apical freshwater, hence the increase in TER. Wood *et al.* (1997) proposes the argument that, like other transporting epithelia such as the opercular epithelia of killifish (Wood and

Marshall, 1994), transferring epithelia to a dilute media closes apical ion channels which decreases transcellular permeability. Since conductance depends on both the availability of the ions (Na^+ and Cl^-) and the number of open ion channels in the apical membrane (Lewis, 1997), it is likely that both chemical and physiological changes account for the change in the conductive behaviour of the membrane with freshwater exposure. It should be noted that SSI membranes were monitored under prolonged apical freshwater exposure(48 hours) (Gilmour *et al.*, 1997). The initial increase in paracellular permeability continues throughout while the conductance, which decreases during acute exposure, eventually increases until the epithelium is by definition, "leaky". This seems to indicate the suggested increase in transcellular permeability with apical freshwater is a transient response.

Substituting culture media for seawater on the apical side of DSI membranes resulted in an increase in paracellular permeability and an increase in conductance, again emphasizing the "leakiness" that resulted from seawater exposure. Although partial recovery of TER was seen with 3 hours of apical seawater exposure (Figure 11B), after 12 hours none of the cultured preparations demonstrated a recovery in TER within 24 hours of the exposure, whereas all of the membranes exposed to freshwater for 12 hours recovered. Therefore, despite the salinity shock, it seems that long term exposure to freshwater is better tolerated than long-term exposure to seawater. This demonstrates the ability of the cultured epithelia to retain its freshwater "program" even though it was: a) cultured in isotonic media, and b) the species is capable of adapting to seawater.

Na^+K^+ -ATPase Activity

The Na^+K^+ -ATPase activity of cultured gill cells gives an indication of the cells' ability to function normally since the sodium pump is required to maintain ionic and electrical gradients. This pump is located on the basolateral membrane of ion transporting cells in the gill, and is responsible for directly excreting excess Na^+ and indirectly excreting Cl^- from the cell into the blood (McCormick, 1995). Both cultured pavement cells and whole filaments have activity values (2-7 uM PO_4^{2-}.mgprotein^{-1}.h^{-1}) that correspond well with previously measured Na^+K^+ -ATPase activities in gill filaments (approximately 2.76 uM.mg^{-1}.h^{-1} in juvenile coho salmon (McCormick and Bern, 1989), and 1.0-1.5 uM.mgprotein^{-1}h^{-1} in rainbow trout (Morgan *et al.*, 1997)). Studies with organ cultures of coho salmon gills showed a 66% decrease in Na^+K^+ -ATPase activity with time in culture (McCormick and Bern, 1989). This most likely reflects their decrease in viability since the organ cultures only survived for 4 days, unlike cultured rainbow trout gill cells which have been known to survive more than 90 days in culture dishes (V. Thomas and D.G. McDonald, personal communication). Cultured branchial epithelia from rainbow trout maintained their Na^+K^+ -ATPAse activity up to 12 days. Most importantly, activity was maintained after the cells were transferred and cultured on semi-permeable inserts (Figure 14).

Na^+K^+ -ATPase pumps are more abundant in chloride cells, the primary ion transporting cells in seawater (McCormick, 1995). Witters *et al.*(1996) have localized

Na^+K^+ -ATPase pumps in primary cultures of freshwater rainbow trout branchial epithelium, however these cultures appear to also contain chloride cells. Despite this, immunolocalization studies with a Na^+K^+ -ATPase antibody (*Drosophila B*) have detected the presence of this enzyme in cultured SSI type epithelia in Hamilton (K.M. Gilmour, S. Alper, C.M. Wood, unpublished results). The activity measurements and the localization of this enzyme in SSI membranes in Hamilton are enough to suggest that pavement cells may have some role in ion transport in freshwater fish. Although the activity measurements in this study were within the range of other studies, the significant difference between whole filaments and cultured cells in Figure 14B could be due in part to the different environments of the filaments and cells. The filaments were freshly obtained from live fish whereas cultured cells were exposed to artificial symmetrical conditions for various periods of time. It would be useful to measure the Na^+K^+-ATPase in cultured cells after a period of freshwater exposure to measure the influence of this treatment on the activity of the Na^+K^+ -ATPase pump. It would also be useful to measure Na^+K^+ -ATPase activity in DSI membranes. Since chloride cells have high Na^+K^+ -ATPase activity relative to other gill cells (McCormick, 1995), then DSI membranes would be expected to have higher activity the SSI membranes if the MRCs are indeed chloride cells.

"Mitochondrial-Rich" Cells on Permeable Inserts:

Rhodamine 123 (methyl-o-6-amine-3'-imino-3H-xanthen-9-yl benzoate monohydrochloride) is a positively charged dye which is sequestered by actively respiring

mitochondria (Shinomiya *et al.*, 1992). This dye accumulates in mitochondrial-rich cells in the gill such as chloride cells (Laurent, 1984, Perry, 1997, Li *et al*, 1997). Single seeded cultures exhibited no brightly fluorescing cells, consistent with previous findings from pure pavement cell cultures (Part *et al.*, 1993, Part and Bergstrom, 1995, and Wood and Part, 1997). The appearance of cultured SSI cells by TEM (Figure 4) is also suggestive of pavement cells, the apical microridges being a feature consistence with these cell types (Laurent, 1984). On the other hand, the double-seeded preparation contained many fluorescent cells, both in clusters and as single cells indicating the existence of cell types other than pavement cells in the cultured epithelia. The double-seeding technique allows for mitochondrial-rich cells to exist in primary culture of branchial cells on solid supports (D.G. McDonald. unpublished results), however this is the first time they appear with pavement cells on permeable inserts. This discovery produces the first *in vitro* model of the freshwater gill epithelium from cells directly isolated from gill tissue. The cultures produce a heterogeneous epithelium of both pavement cells and MRCs, much like the intact gill, which can now hopefully be used to establish a more concrete theory of ion transport mechanisms in freshwater gills.

The positive correlation between the surface area of chloride cells and resistance may seem counterintuitive since chloride cells form "leaky" junctions with accessory cells in seawater gills (Perry, 1997). However, these accessory cells appear to be absent in freshwater fish (Laurent and Perry, 1991) so the relationship may not be unreasonable. One consideration is that the values for chloride cell surface area are an underestimation of

their real surface area since only clusters of fluorescing cells were included in the calculation. There were many single cells not included because it would have been too tedious to count each individual cell under a microscope. A more accurate way to estimate chloride cell coverage might be to harvest the cells from the permeable support, stain several aliquots with R123 and count the cells with a haemocytometer. This would incorporate clustered and single fluorescing cells in the calculations, and might produce a value more similar to *in vivo* conditions where freshwater gills usually contain around 15% chloride cells, although with considerable variability (Perry, 1997). For example, the eel (*Auguilla anguilla*) is known to have a very low chloride cell fractional area of about 1% (Perry *et al.*, 1992b) which is still higher than any of the values (0.03 - 0.44%) calculated for the cultured rainbow trout cells (Figure 17).

Conclusion

Many of the relationships between electrical and physiological parameters measured in SSI preparations were evident in DSI membranes such as the increase in TER as the membranes grow, the increase in TER with freshwater on the apical surface of the cultured membranes along with a subsequent increase in PEG-4000 permeability, and the development of a negative potential. For the first time it was demonstrated that SSI and DSI membranes cannot withstand freshwater or seawater on their basolateral sides, suggesting they are polarized as *in vivo*. DSI preparations have two unique features: they develop a significant positive potential in symmetrical conditions and most importantly,

they are comprised of two cell types, pavement cells and cells rich in mitochondria. Analysis of ion transport functions is required to provide a more detailed comparison of the two cultured branchial preparations.

Chapter 3: Unidirectional Na^+, Cl^-, and Ca^{2+} Fluxes.

Introduction:

Studies of ion transport by freshwater fish are hampered by the structural and physiological complexity of the gill. Cultured branchial epithelia provide an *in vitro* model of the freshwater gill which has been used in the hopes of revealing sites and mechanisms of Na^+, Cl^-, and Ca^{2+} uptake (see Chapter 1). Results to date indicate that Cl^- is actively taken up from apical freshwater by cultured preparations, while Na^+ transport is passive (Wood *et al.*, 1997). However, given that these cultures consist entirely of pavement cells, the results contradict the current understanding that chloride cells are responsible for chloride uptake. Cultured epithelia from sea bass gills which also contain only pavement cells also secrete chloride, again thought to be a function of the chloride cells in gills of seawater fish (Avella and Ehrenfeld, 1997). It appears, therefore that the role of each cell type in the gill is not clearly defined and further study with the cultured preparations of the freshwater gill is warranted.

Chloride cells are also thought to be the site of transcellular calcium uptake (Flik et al., 1995). Proof of this comes from many correlations *in vivo* between chloride cell surface area and chloride influx. Similar relationships between chloride cell composition and calcium uptake are evident in *in vitro* models of the gill, such as the opercular epithelium of killifish (Marshall *et al.*, 1995). Na^+K^+ -ATPase is localized to the basolateral membrane

of chloride cells in the gill, and is also associated with Ca^{2+} -ATPase, and Na^{+}/Ca^{2+} exchangers. Thus far, Ca^{2+} transport has not been investigated in the cultured freshwater branchial epithelium.

This chapter examines ion transport in two cell culture preparations. The first single-seeded insert (SSI) preparation was devised by Wood and Part (1997) and consists of one cell type only, pavement cells. The second preparation is a new double-seeded insert (DSI) cultured membrane that contains both pavement cells and mitochondrial-rich cells (MRCs)(see Chapter 2). Unidirectional Na^{+} and Cl^{-} flux measurements were performed on DSI membranes so that comparisons could be made with previous results from SSI membranes, and Ca^{2+} transport was assessed in both preparations for the first time.

Materials and Methods:

Experiments were performed in both Uppsala, Sweden and Hamilton, Canada. Information on cell culture procedures, analytical methods, and microscopy, is provided in detail in the Materials and Methods section of Chapter 2. The cultured membranes were prepared and grown according to methods for SSI and DSI membranes. SSI membranes were cultured and used for experiments in Uppsala, Sweden whereas all DSI membranes were prepared and used for experiments in Hamilton, Canada. Due to limitations in the EVOM epithelial voltohmeter (World Precision Instruments, U.S.A.), TER measurements made in Uppsala could be determined only for membranes with TER $\leq$ 20 000 ohms.cm^2. In Hamilton, a custom modified EVOM voltohmeter was employed measuring TER values

from 1 - 100 000 ohms.cm^2.

Unidirectional Ion Fluxes

Unidirectional Na^+, Cl^-, and Ca^{2+} flux experiments were conducted across cultured membranes while simultaneously recording transepithelial resistance (TER) using the chopstick electrode/EVOM method, and transepithelial potential (TEP) using Ag/AgCl electrodes attached to a pH meter (see Chapter 2 for details). TEP was monitored for unidirectional fluxes performed in Hamilton.

With culture media in both apical and basolateral compartments, unidirectional fluxes were conducted by adding 1 uCi of the appropriate isotope (^{22}Na, ^{36}Cl, ^{45}Ca, all NEN-Dupont) to one side of the membrane and monitoring it's appearance on the other side. For influx studies, the radioisotope was added to the apical side and for efflux, it was added to the basolateral side. Each flux began with an initial volume of 1.5 ml of culture media on the apical side (insert) and 2 ml on the basolateral side (in the well). TEP (Hamilton), TER, and solution samples were obtained at the beginning and end of each flux period which varied from 5 to 9 hours.

Unidirectional fluxes on cultured preparations with freshwater on the apical side followed a slightly different procedure. In Hamilton, sterile freshwater was prepared by autoclaving dechlorinated tapwater. In Sweden, running tapwater was diluted 50:50 with ddH2O to match the hardness of Hamilton tapwater, and then sterilized by passing through an Acrodisc (0.2um syringe filter, Ann Arbor, USA). Before the isotope was

added, apical culture medium was replaced with freshwater for a four hour period of acclimation. To ensure the freshwater was not contaminated with remnants of media, the membrane was rinsed four consecutive times with 1 ml of freshwater before the final 1.5 ml was added to the apical compartment. At the four hour mark, freshwater was replaced by the same procedure and isotope was added to the "hot side" for either influx or efflux, and TEP, TER, and solution samples were taken at this time and at the end of the flux period. As with symmetrical conditions, the flux was recorded by the appearance of radioactivity on the "cold side".

The cultured epithelia used in flux experiments were chosen based on the stability of TER throughout the flux procedures. Only those membranes for which TER was initially above 1 000 ohms.cm^2, and declined < 50% 24 hours following a flux experiment were used (Wood and Part, 1997). This protocol excludes preparations that may have suffered from mechanical damage or microorganism contamination during the flux procedures.

Analysis:

TEP and resistance of blank inserts was subtracted from measured values for correction (see Chapter 2). Na^+ and Ca^{2+} concentrations were measured on a Varian AA1275 atomic absorption unit in Hamilton. In Uppsala, the Ca^{2+} concentration of samples was measured using a colorimetric assay (Sigma KC308). All Cl^- concentrations were determined by a colorimetric assay (Zall *et al.*, 1956). Radioactivity was determined by scintillation counting using either a Packard Tricarb 1900 CA (Uppsala) or LKB 1217 Rackbeta (Hamilton).

Calculations:

Unidirectional ionic fluxes (J) (influx and efflux) were measured according to the following example equation:

(1) Na^+ influx = $\blacktriangle[Na^{+*}]_{cold} * SA^{-1}_{hot} * Volume_{cold} * time^{-1} * area^{-1}$

where $\blacktriangle[Na^{+*}]_{cold}$ is the change in radioactivity on the cold side (apical for effluxes and basolateral for influxes), SA is the mean specific activity on the hot side (apical for influxes and basolateral for effluxes). Net flux was calculated as the difference between influx and efflux:

(2) Net flux = Influx-efflux

Each insert preparation could be used for influx or efflux experiments, but not both. Therefore, in order to calculate net flux, it was necessary to pair cultured membranes that underwent opposite procedures. Pairs were matched according to the criteria listed in Table 1. Net fluxes were calculated based on the best match for each criterion, then all the net fluxes from all the criteria were combined to calculate mean net flux. This gave an inflated N number (number of fluxes) for net flux calculations.

Table 1: Criteria used for matching cultured preparations that underwent opposite experiments. Influx and efflux pairs were created based on the best match for each criterion.

Matching Criteria for Influx-Efflux Pairs
1. Initial TER, culture media both sides
2. Initial TEP, culture media both sides
3. TER, after 4 hours of apical freshwater acclimation
4. TEP, after 4 hours of apical freshwater acclimation
5. Mean TER, calculated from initial and final measurements in symmetrical conditions
6. Mean TEP, calculated from initial and final measurements in symmetrical conditions

The Ussing Flux Criterion (Ussing, 1949) was used to determine if the ion movements were active or passive. This criterion is based on the difference between the measured flux ratio (influx/efflux) and the predicted flux ratio which is calculated according to the following equation:

$$(3)\ \frac{\text{influx}}{\text{efflux}} = \frac{A_{Ap}}{A_{Bl}} \cdot e^{(zFV/RT)}$$

where A_{Ap} and A_{Bl} are the activities of the ions (Na^+, Cl^-, or Ca^{2+}) on the apical and basolateral sides, z is the ionic valence, V is the measured TEP in volts, and F, R, and T have their usual thermodynamic values. It was previously determined using microelectrodes that the A_{Na+} and A_{Cl-} in culture media was 75% of the total concentration (Wood *et al.*, 1997). Similar methods determined that the A_{Ca2+} in L-15 media supplemented with 5% FBS was 1.5 ± 0.05 (5) $mmol.L^{-1}$. For freshwater, the activities of all three ions were taken as equal to their measured concentrations.

Statistical Analysis:

Data was expressed as means ± 1 SEM (N) where appropriate, N being the number of cultured membranes used in the calculation. Regression lines were fitted by the method of least squares, and the significance of Pearson's correlation coefficient (r) was determined.

The statistical significance between means was calculated using Student's two-tailed paired, unpaired, or one-sample test as needed. The limit for statistical significance was 5%.

Results:

Unidirectional Na^+ and Cl^- Fluxes

Electrical parameters of cultured membranes prepared according to DSI methods for Na^+ and Cl^- unidirectional fluxes are listed in Table 2. The range of TER and TEP values was 1 300 to 20 600 ohms.cm^2 and 0 to 7 mV respectively, at or around their point of plateau (see Chapter 2). These measurements were taken with culture media in both apical and basolateral compartments, or in other words, under symmetrical conditions. Unidirectional Na^+ and Cl^- flux measurements across membranes in symmetrical solutions demonstrated an approximate balance in the two directions (Figure 1). No significant difference existed between both absolute influx and efflux values for the same ion, and corresponding unidirectional or net flux values for Na^+ versus Cl^-.

Unidirectional Na^+ and Cl^- fluxes were also performed across DSI cultured membranes under asymmetrical conditions with freshwater in the apical compartment and culture media in the basolateral compartment. As seen previously in Chapter 2, Wood *et al.*, (1997), and Gilmour *et al.* (1997), TER values increased and TEP became negative with freshwater on the apical side (Table 3). Under these conditions Na^+ and Cl^- fluxes were greatly altered (Figure 2). Large Na^+ and Cl^- effluxes occurred which were significantly greater than effluxes observed under symmetrical conditions (Figure 1). Conversely, large reductions in influxes also resulted in significant differences between these values and

Table 2: Mean transepithelial resistance (TER) and potential (TEP) of cultured DSI membranes used for unidirectional Na^+ and Cl^- fluxes under symmetrical conditions. Preparations for both influx and efflux mesasurements were pooled for calculations, and measurements of TER and TEP were taken prior to flux experiments. Data expressed as mean ± 1 SEM.

Na^+	Cl^-
TER = 8 514 ± 1 443 ohms.cm^2	TER = 7 896 ± 1 368 ohms.cm^2
TEP = 2.9 ± 0.5 mV	TEP = 2.6 ± 0.6 mV
n = 28	n = 30

Figure 1: Unidirectional Na^+ and Cl^- influx (apical to basolateral), efflux (basolateral to apical), and net flux (difference between influx and efflux) in DSI cultured membranes with culture media on both apical and basolateral sides (symmetrical conditions). Means ± 1 SEM. (N=14 for Na^+, N=15 for Cl^-). No significant differences exist between influx and efflux values for the same ion, and corresponding fluxes for different ions.

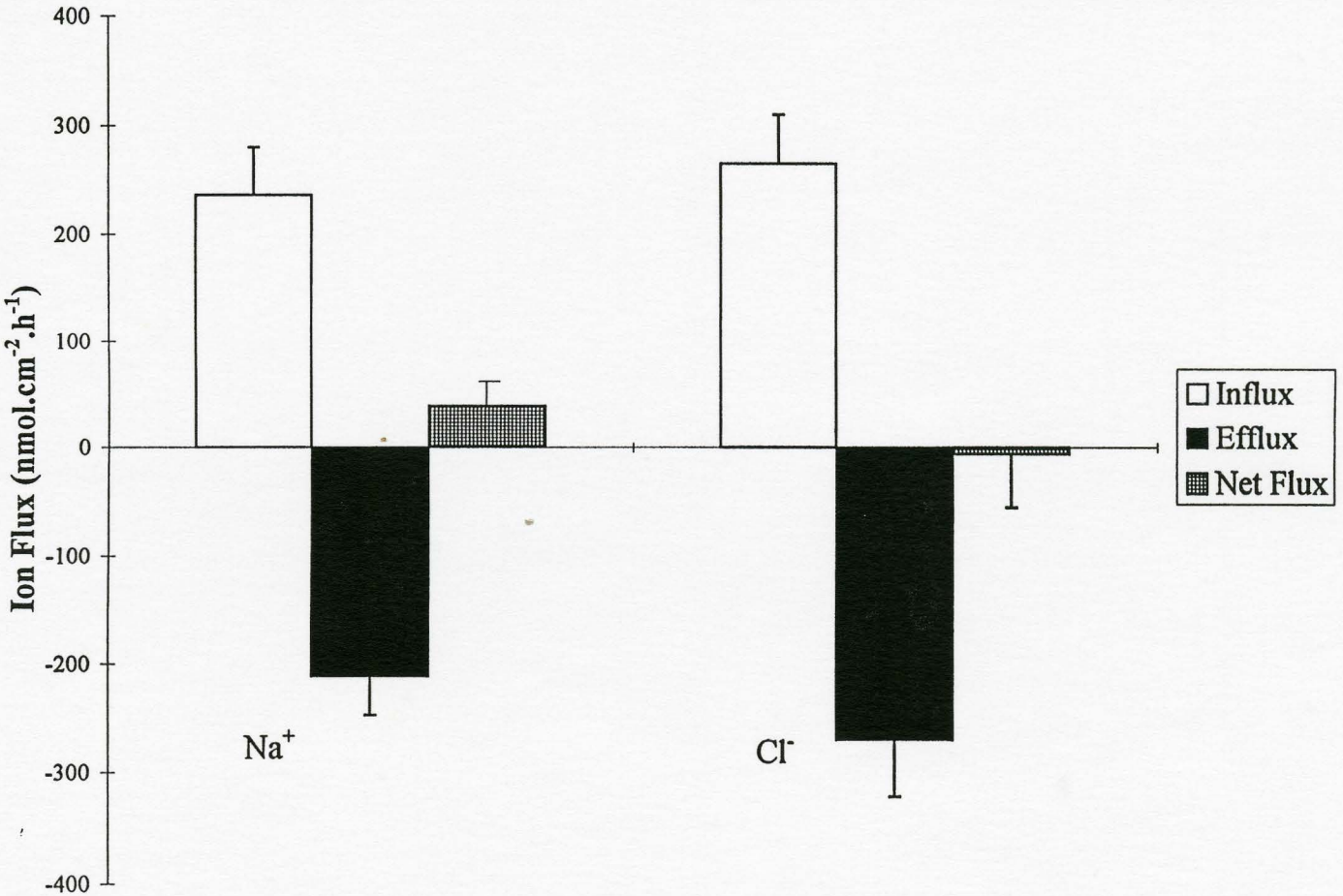
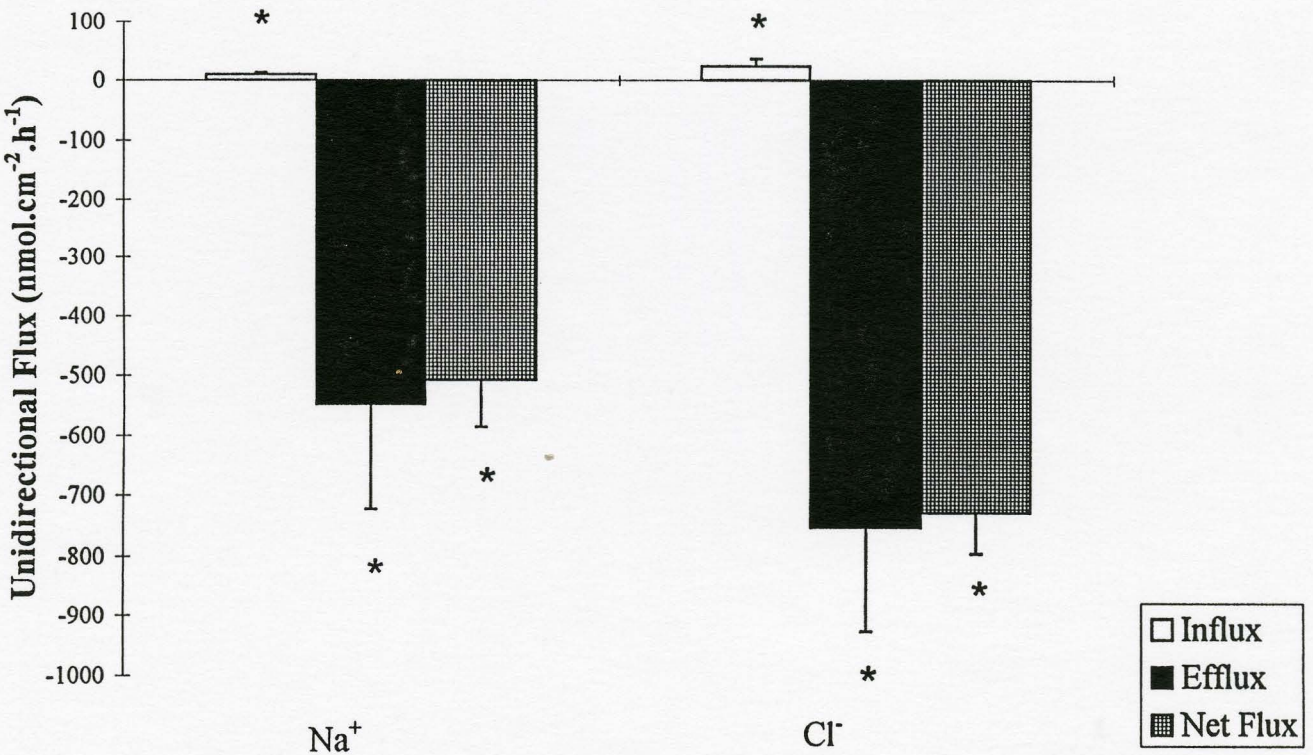

400
300
200
100
0
-100
-200
-300
-400
Ion Flux (nmol.cm-2.h-1)
Na+
Cl-
Influx
Efflux
Net Flux

Table 3: Mean transepithelial resistance (TER) and potential (TEP) of cultured DSI membranes used for Na^+ and Cl^- fluxes under asymmetrical conditions. Preparations for both influx and efflux measurements were pooled for calculations, and measurments of TER and TEP were taken at two separate times: initally with culture media on both apical and basolateral surfaces (TER1 and TEP1) , and after 4 hours of acclimation to freshwater on the apical side of the cultured epithelium (TER2 and TEP2). Data expressed as mean ± 1 SEM, and * indicates TER2 and TEP2 are significantly different from TER1 and TEP1 respectively.

Na^+	Cl^-
TER1 = 5 705 ± 999 ohms.cm^2	TER1 = 4 750 ± 771 ohms.cm^2
TER2 =*12 359 ± 1379 ohms.cm^2	TER2 =*11 167 ± 1219 ohms.cm^2
TEP1 = 0.8 ± 0.2 mV	TEP1 = 0.9 ± 0.2 mV
TEP2 =*-10.9 ± 0.3 mV	TEP2 =*-10.7 ± 0.3 mV
n = 30	n = 32

Figure 2: Unidirectional Na^+ and Cl^- influx, efflux, and net flux in DSI membranes under asymmetrical conditions (freshwater in the apical compartment, culture media in the basolateral compartment). Means ± 1 SEM (N=15 for Na^+, N=16 for Cl^-). Astericks (*) indicates mean values are significantly different ($p<0.05$) from original mean values measured under symmetrical conditions (see Figure 1).


Unidirectional Flux (nmol.cm-2.h-1)
100
0
-100
-200
-300
-400
-500
-600
-700
-800
-900
-1000
Na+
Cl-
Influx
Efflux
Net Flux

influx rates under symmetrical conditions.

Influx and efflux values for Na^+ and Cl^- in symmetrical and asymmetrical conditions varied inversely with TER such that at high TER ion flux was small and vice versa (Figure 3). Therefore the same data varied linearly with conductance, the inverse of TER. Figure 4 shows that a stronger linear relationship existed between flux rates and conductance under asymmetrical conditions than under symmetrical conditions.

The Ussing Flux Ratio Criteria revealed contrasting results between ion fluxes in symmetrical and asymmetrical conditions. With the same culture media in both compartments, the predicted ratio for Na^+ influx/efflux was significantly smaller than the measured ratio obtained from unidirectional flux experiments (Figure 5). This difference is indicative of active Na^+ uptake from apical culture media to basolateral culture media in the absence of an electrochemical gradient favouring Na^+ movement. There was no significant difference between the actual and predicted ratios for Cl^- transport suggesting that Cl^- movements are passive.

The opposite results were seen for fluxes measured with freshwater on the apical side (Figure 6). This time the predicted ratio for Cl^- transport was significantly smaller than the measured ratio indicating active Cl^- uptake from apical freshwater to basolateral culture medium. There was no evidence of active Na^+ movements under asymmetrical conditions.

Ca^{2+} Transport:

Unidirectional Ca^{2+} fluxes were performed on SSI membranes cultured in Uppsala,

Figure 3: The relationship between unidirectional ion flux (Na^+ or Cl^-) and transepithelial resistance (TER) with culture media on both apical and basolateral sides (symmetrical) and freshwater on the apical side, culture media on the basolateral side (assymetrical). Fluxes in both directions are included in each data set.

1: Chloride Fluxes - asymmetrical solutions (N=31)

2: Sodium Fluxes - asymmetrical solutions (N=35)

3: Chloride Fluxes - symmetrical solutions (N=31)

4: Sodium Fluxes - symmetrical solutions (N=35)

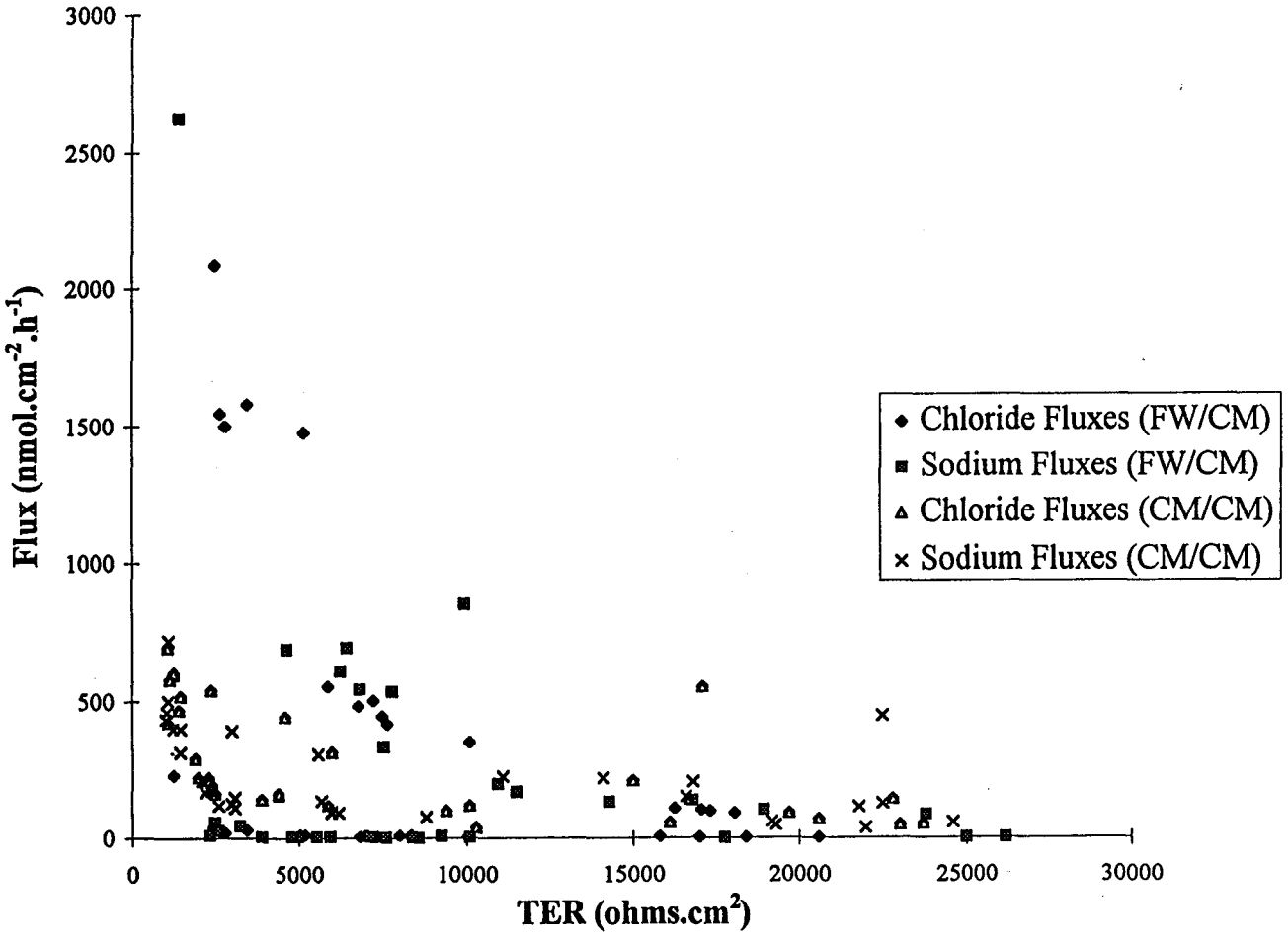

3000
2500
2000
1500
1000
500
0
Flux (nmol.cm-2.h-1)
0
5000
10000
15000
20000
25000
30000
TER (ohms.cm2)
Chloride Fluxes (FW/CM)
Sodium Fluxes (FW/CM)
Chloride Fluxes (CM/CM)
Sodium Fluxes (CM/CM)

Figure 4: The relationship between ion flux (Na^+ or Cl^-) and transepithelial conductance across DSI membranes exposed to apical freshwater or apical culture media.

A) Fluxes under symmetrical conditions. Each data set includes fluxes in both directions. Linear regression equations are:

Na^+ flux = 399 108 (conductance) + 101.7, r=0.66925, N=33, $p<0.01$

Cl^- flux = 496 948 (conductance) + 118, r=0.6849, N=33, $p<0.01$

B) Influx values under asymmetrical conditions. Equations are:

Na^+ influx = 94 046 (conductance) - 8.1387, r=0.70799, N=18, $p<0.0001$

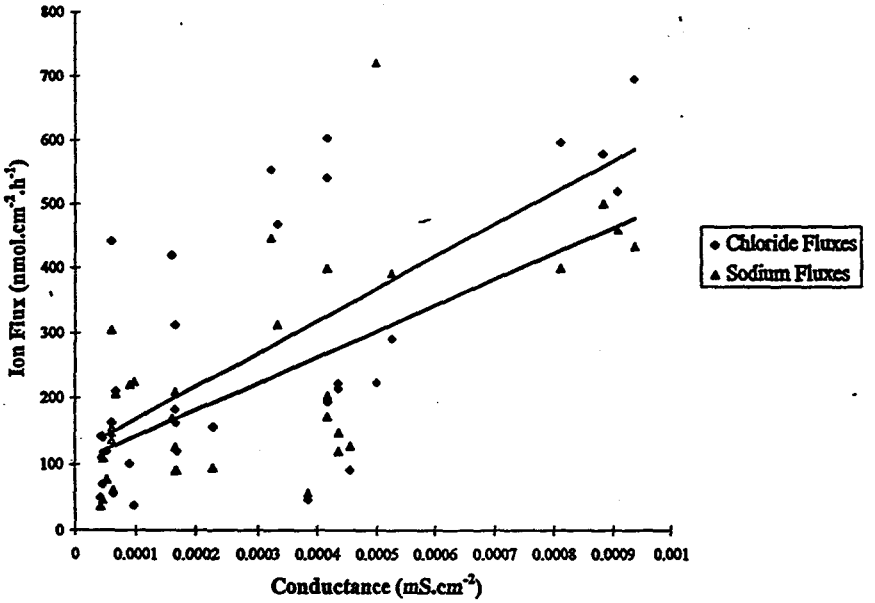
Cl^- influx = 252 289 (conductance) - 29.24, r=0.88896, N=18, $p<0.0001$

C) Effluxes under asymmetrical conditions. Equations are:

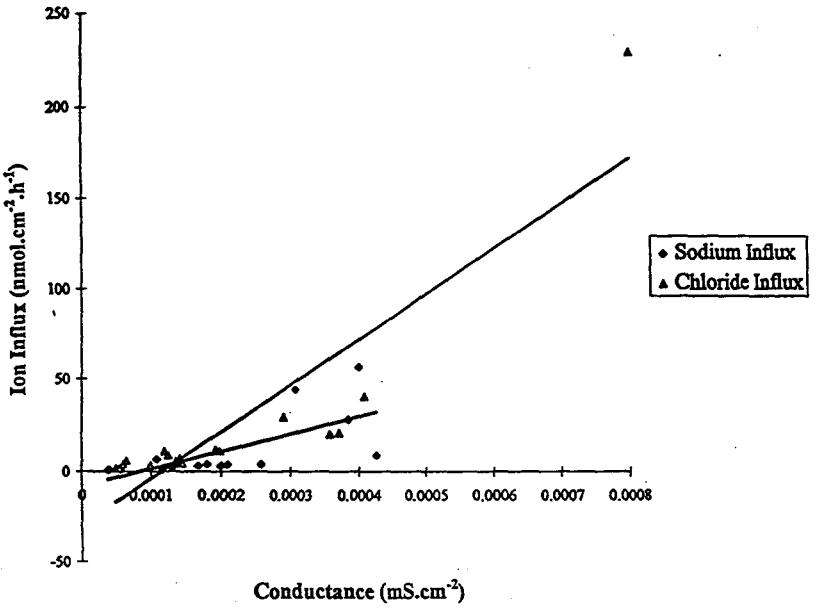
Na^+ efflux = $4*10^6$ (conductance) - 26, r=0.96696, N=15, $p<0.00001$

Cl^- efflux = $5*10^6$ (conductance) - 190.19, r=0.94833, N=15, $p<0.0001$

A


Ion Flux (nmol.cm-2.h-1)
Conductance (mS.cm-2)
Chloride Fluxes
Sodium Fluxes

B


Ion Influx (nmol.cm-2.h-1)
Conductance (mS.cm-2)
Sodium Influx
Chloride Influx

C

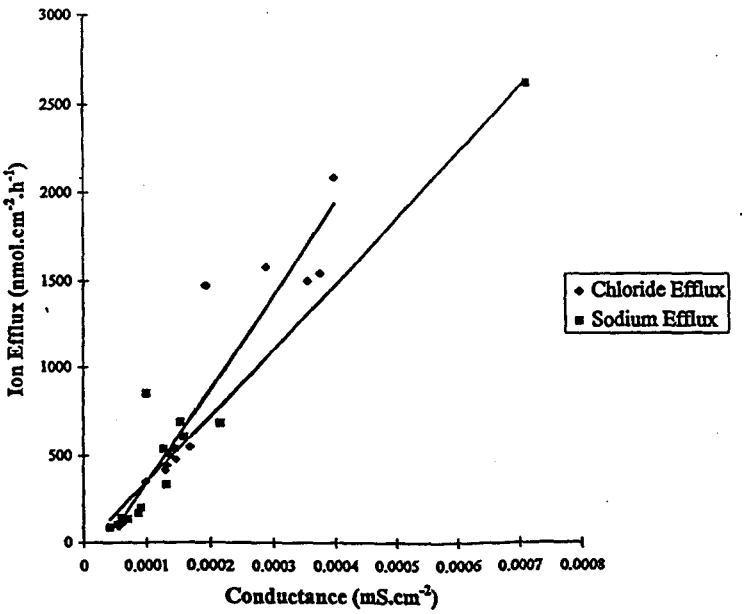

Ion Efflux (nmol.cm-2.h-1)
Conductance (mS.cm-2)
Chloride Efflux
Sodium Efflux

Figure 5: A comparison between actual and predicted Ussing flux ratios for Na^+ and Cl^- under symmetrical conditions. Means ± 1 SEM. (N=53 for Na^+, N=55 for Cl^-). Asterisk (*) indicates a significant difference ($p<0.05$) between actual and predicted flux ratio for the same ion. Flux ratios are expressed as influx/efflux, and values were obtained from matched pairs according to a set of criteria (see Materials and Methods).

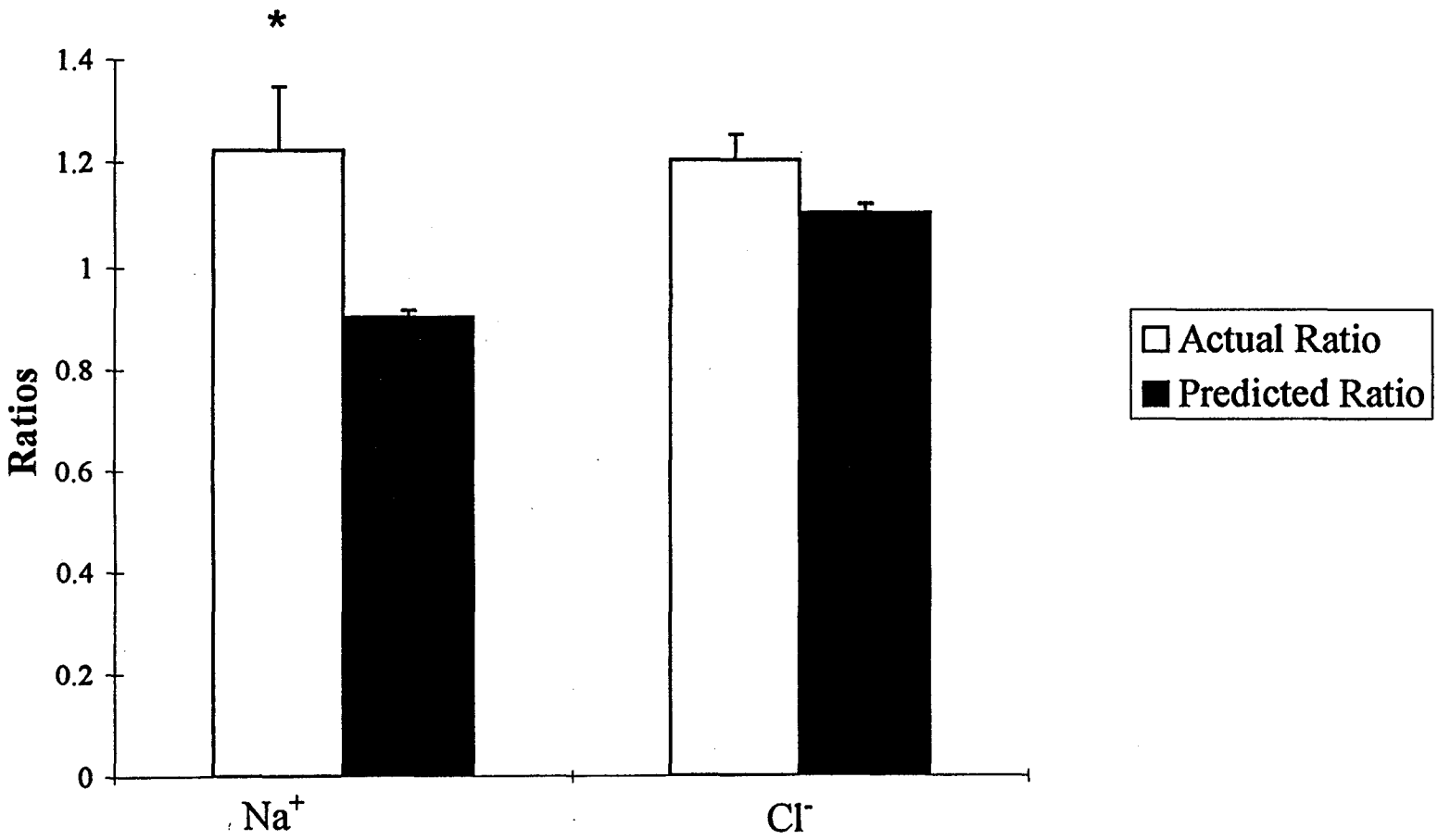
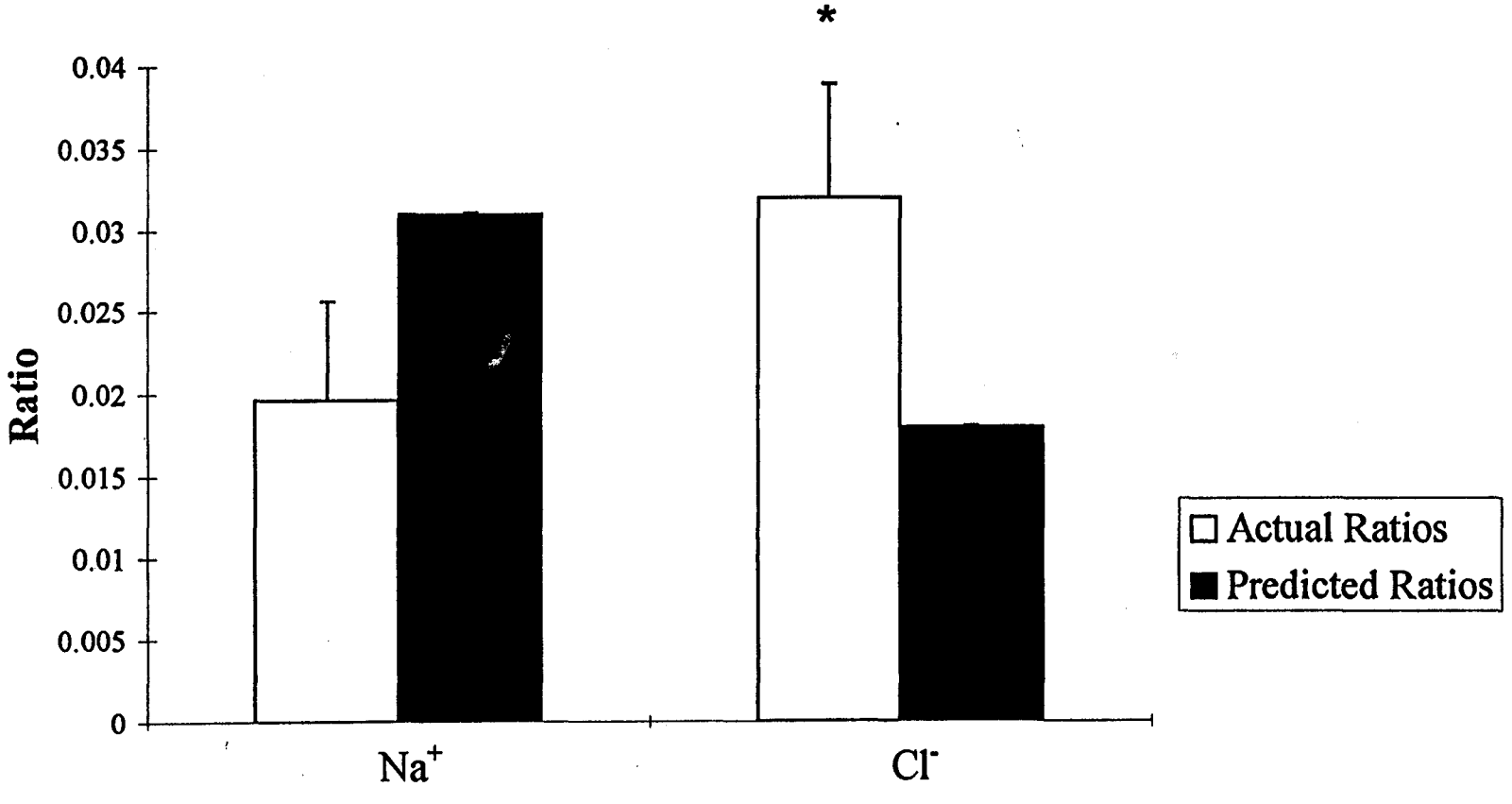

*
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ratios
Na+
Cl-
Actual Ratio
Predicted Ratio

Figure 6: A comparison between actual and predicted Ussing flux ratios for Na^+ and Cl^- under asymmetrical conditions (freshwater on the apical side). Means ± 1 SEM. (N=85 for Na^+, N=91 for Cl^-). Asterisks (*) indicates significant difference ($p<0.05$) between actual and predicted ratio for the same ion. Ratios are expressed as influx/efflux, and value were obtained from matched pairs according to a set of criteria (See Materials and Methods).


*
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
Ratio
Na+
Cl-
Actual Ratios
Predicted Ratios

Sweden. Table 4 lists the TER and TEP measurements for these preparations. With culture media on both apical and basolateral sides, the TER ranged from 1 250 to 20 000 ohms.cm^2. TEP was not monitored during these fluxes but instead was taken to be -2.24 mV based on values obtained during a study of TEP in Uppsala, Sweden (see Chapter 2). The SSI membranes exhibited a significant increase in TER upon replacing the apical media with freshwater. Again, since TEP measurements were not taken, a previously recorded mean value of -10.8 mV (Wood *et al.*, 1997) measured under asymmetrical conditions was used for Ussing Flux Ratio calculations.

In relation to absolute values of Na^+ and Cl^- unidirectional fluxes, Ca^{2+} fluxes were extremely small. During both symmetrical and asymmetrical conditions there was a small net calcium efflux, the value being significantly greater with cultures in asymmetrical solutions (Figure 7). In fact, all three parameters, influx, efflux, and net flux were significantly increased with apical freshwater exposure.

The TER and TEP values for DSI membranes prepared in Hamilton and used for Ca^{2+} flux experiments are listed in Table 5. In this case, TEP was directly measured using Ag/AgCl electrodes. Again, TER significantly increased and TEP became serosal negative when apical culture media was substituted for freshwater. Unidirectional calcium fluxes across DSI membranes revealed similar patterns to SSI membranes under symmetrical situations, but not under apical freshwater conditions (Figure 7). Influx, efflux, and net flux across DSI membranes exposed to apical freshwater were approximately 2-fold smaller than SSI membranes.

Table 4: Mean TER and TEP of cultured DSI and SSI membranes used for unidirectional Ca^{2+} fluxes. Influx and efflux preparations were pooled for the calculations, and data is expressed as mean ± 1 SEM. Measurements were taken prior to flux experiments with culture media on both apical and basolateral sides of the membrane. § indicates the value was obtained from previous results, and was not measured prior to flux experiments (see text).

SSI	DSI
TER = 9623 ± 1452 ohm.cm^2	TER = 9854 ± 993 ohms.cm^2
TEP = §-2.24 mV	TEP = 4.3 ± 0.3 mV
n = 24	n = 24

Figure 7: Calcium influx (apical to basolateral), efflux (basolateral to apical), and net flux (difference between influx and efflux) for both SSI and DSI cultured membranes under symmetrical (apical culture media) and asymmetrical (apical freshwater) conditions. Mean ± 1 SEM (N=12 for SSI symmetrical, N=19 for SSI asymmetrical, N=11 for DSI symmetrical, N=11 for DSI asymmetrical). 1. indicates significant difference ($p<0.05$) between fluxes under different conditions from the same preparation, and 2. indicates significant difference ($p<0.05$) between SSI and DSI under the same conditions.

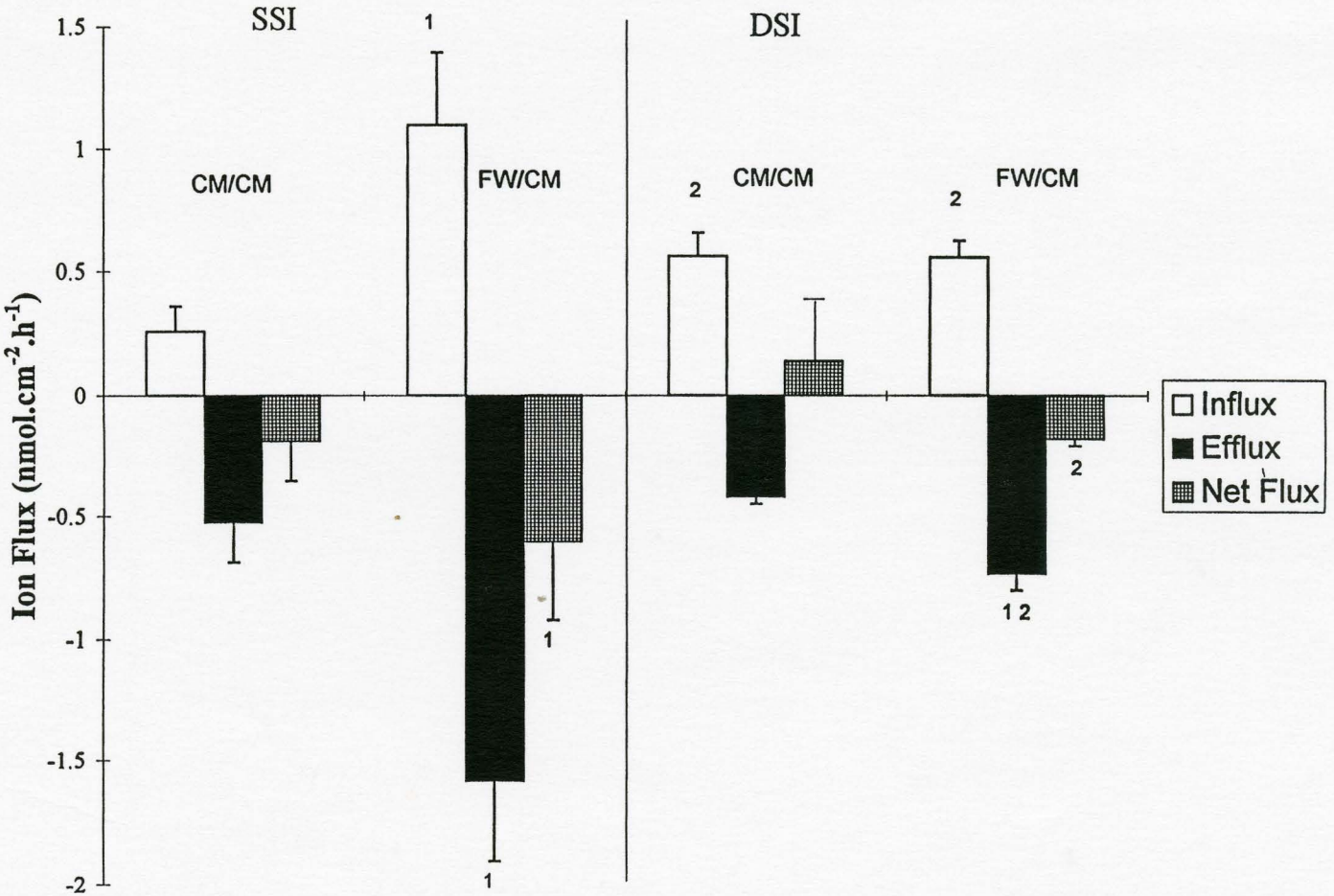

SSI
DSI
CM/CM
FW/CM
CM/CM
FW/CM
1
2
2
1
1
1 2
2
Influx
Efflux
Net Flux
Ion Flux (nmol.cm-2.h-1)
1.5
1
0.5
0
-0.5
-1
-1.5
-2

Table 5: TER and TEP of cultured DSI and SSI membranes used for unidirectional Ca^{2+} fluxes. Membranes used for influx and efflux were pooled for the calculations, and data expressed as mean ± 1 SEM. Measurements were taken at two different times: prior to flux experiments with culture media on both apical and basolateral surfaces (TER1 and TEP!) and after 4 hours of acclimating the cultured membranes to freshwater on their apical surfaces (TER2 and TEP2). § denotes values that were obtained from previous measurements and not prior to flux experiments, and * indicates that TER2 and TEP2 and significantly different from TER1 and TEP1 respectively.

SSI	DSI
TER1 = 13706 ± 1197 ohms.cm^2	TER1 = 10679 ± 993 ohms.cm^2
TER2 = *18250 ± 631 ohms.cm^2	TER2 = *25716 ± 1338 ohms.cm^2
TEP1 = §-2.24 mV	TEP1 = 4.7 ± 0.3 mV
TEP2 =*§-10.5 mV	TEP2 = *-12.3 ± 0.3 mV
n = 38	n = 22

As with Na^+ and Cl^- fluxes, Ca^{2+} fluxes across DSI membranes were inversely related to TER (Figure 8) and linearly correlated with conductance (Figure 9). Fluxes performed across membranes with culture media on both sides had a stronger significant relationship with TER and conductance than did those under asymmetrical conditions.

To determine whether calcium movements were active or passive, the Ussing flux criteria was applied to the flux measurements. Results revealed no significant difference between the predicted and actual influx/efflux ratios for SSI membranes from Uppsala, indicating calcium movements were passive with both culture media and freshwater on the apical side (Figure 10). However, differences existed between predicted and actual flux ratios calculated for DSI membranes. In symmetrical conditions, the actual ratio was more than 3-fold **higher** than the predicted ratio, indicating active uptake of calcium (apical to basolateral transport). In contrast, the actual influx/efflux ratio is approximately 3-fold **lower** than the predicted ratio when membrane solutions are asymmetrical. According to the Ussing flux criteria, this indicates calcium was actively extruded from the basolateral culture medium to the apical freshwater, which is in the opposite direction to transport under symmetrical conditions.

Figure 8: The relationship between unidirectional calcium flux and TER with culture media on the apical and basolateral sides (symmetrical) and freshwater on the apical side, culture media on the basolateral side (asymmetrical) of DSI membranes. Fluxes in both directions are included in each data set. (N= 24 for apical culture media (symmetrical), N= 24 for apical freshwater (asymmetrical)).

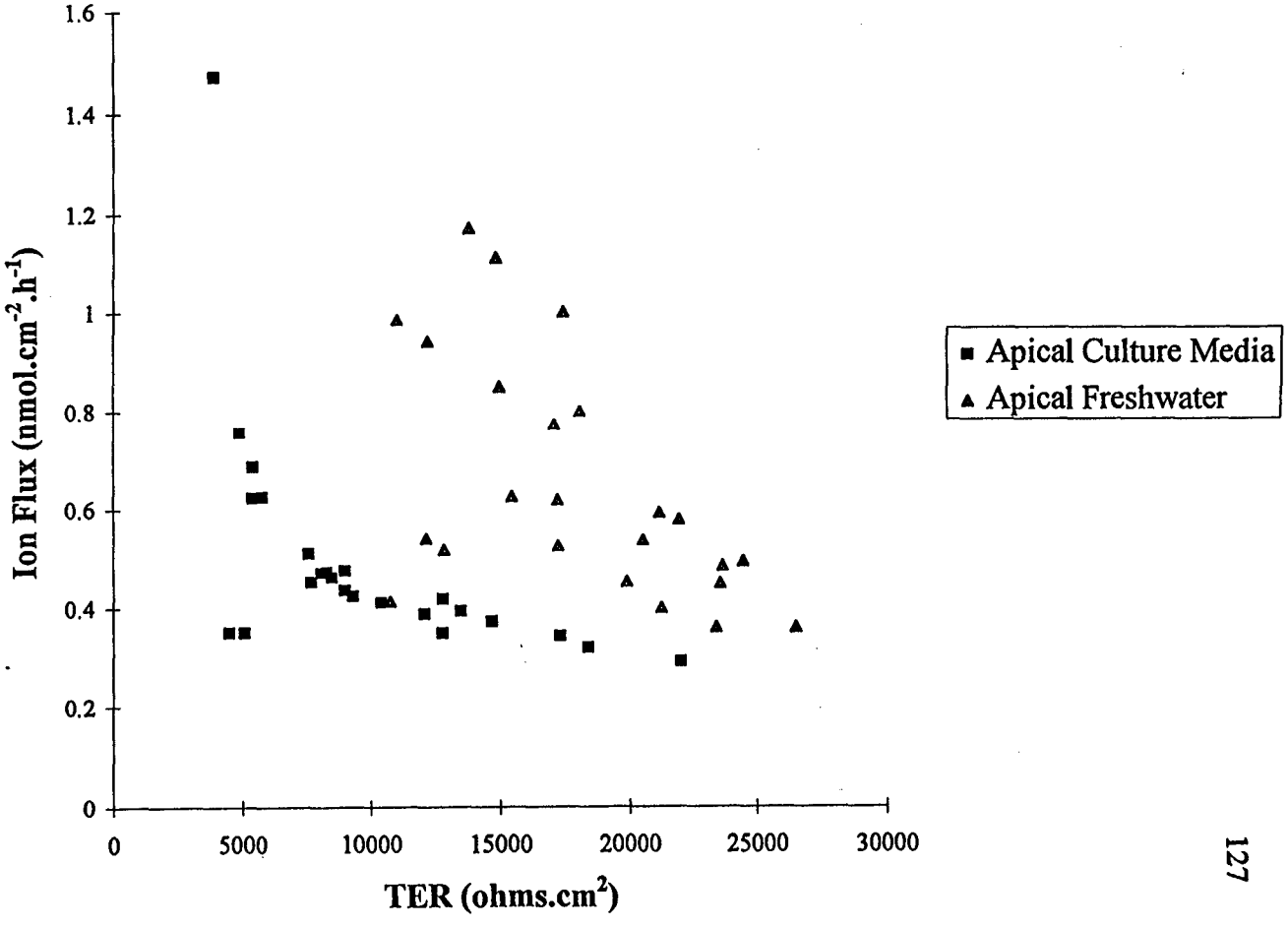

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ion Flux (nmol.cm-2.h-1)
0
5000
10000
15000
20000
25000
30000
TER (ohms.cm2)
Apical Culture Media
Apical Freshwater

Figure 9: The relationship between calcium flux and transepithelial conductance in DSI cultured membranes either exposed to apical freshwater or apical culture media. Both influx and efflux measurements are included in the data set. The regression equations are:

Ion flux = 6 925.7 (conductance) + 0.2366, r=0.4657, N=24, $p < 0.05$ for apical culture media ($p=0.022$).

Ion flux = 2 866.4 (conductance) + 0.1344, r=0.6934, N=24, $p < 0.0005$ for apical freshwater ($p=0.0002$).

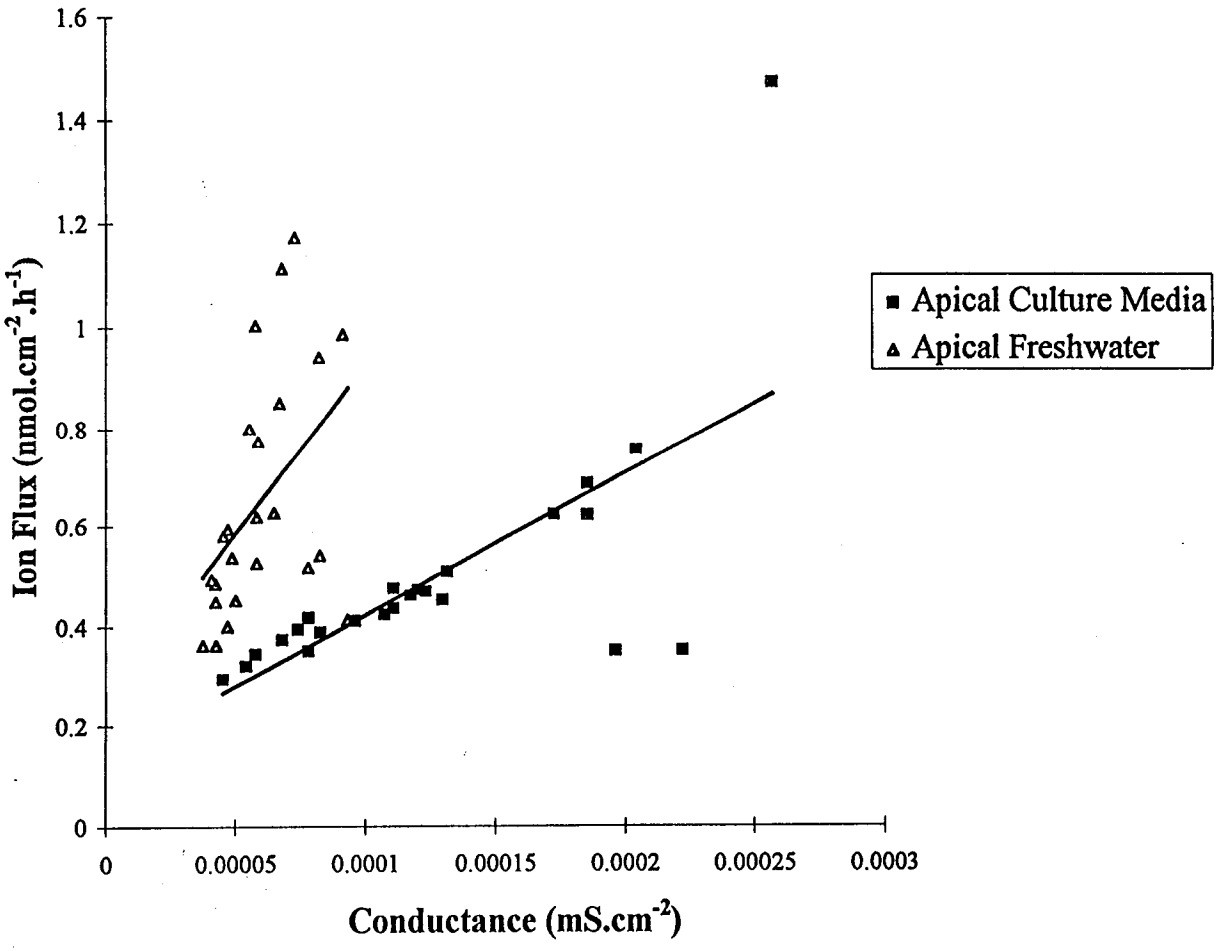
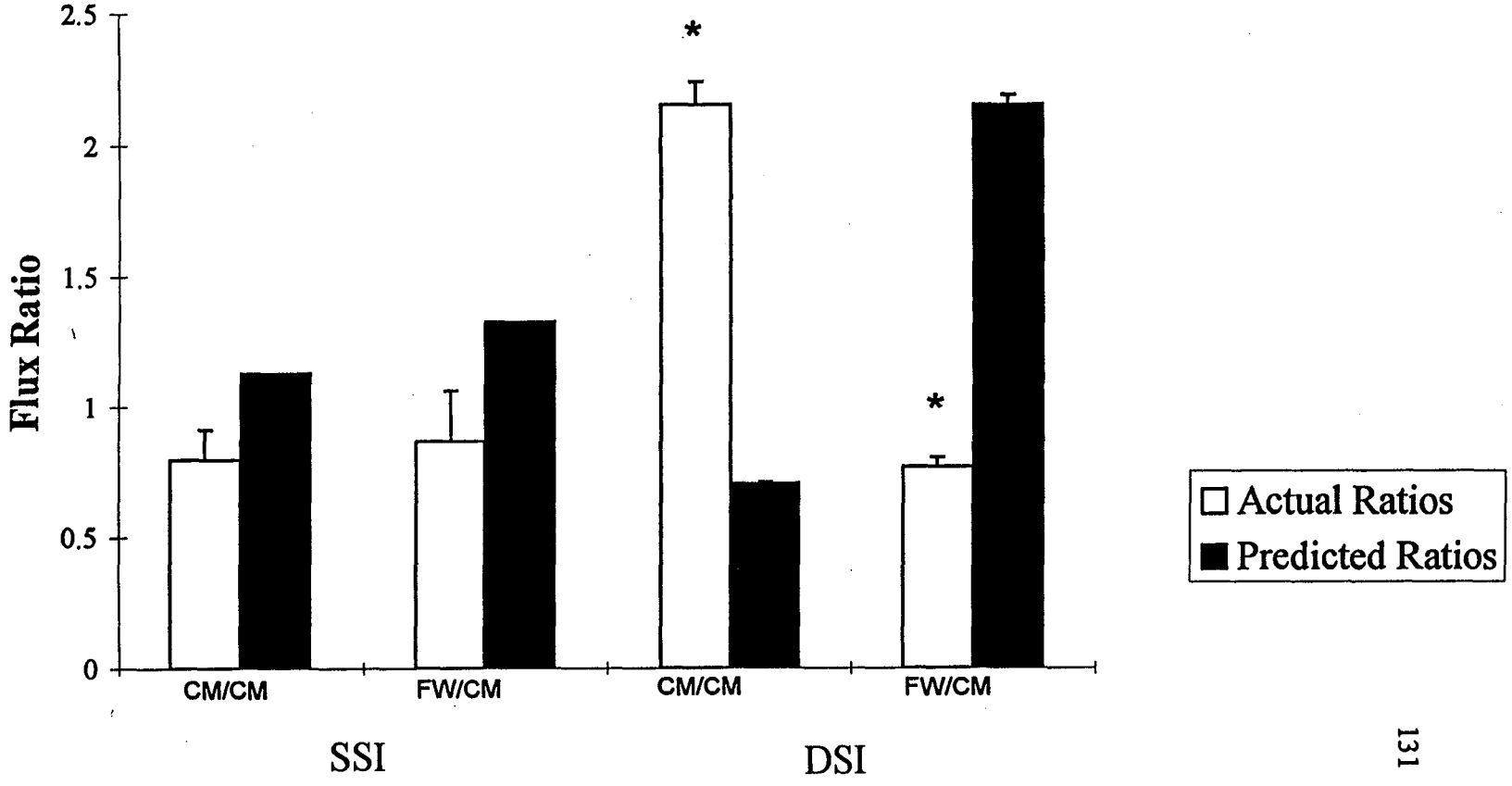

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Ion Flux (nmol.cm-2.h-1)
0
0.00005
0.0001
0.00015
0.0002
0.00025
0.0003
Conductance (mS.cm-2)
Apical Culture Media
Apical Freshwater

Figure 10: A comparison of Ussing flux ratios for calcium fluxes performed across SSI and DSI membranes exposed to apical freshwater or apical culture media. Means ± 1 SEM. (N =12 SSI symmetrical, N=19 SSI asymmetrical, N=37 for DSI symmetrical, N=76 DSI asymmetrical). * indicates significant difference ($p<0.05$) between actual and predicted ratio for the same preparation and apical solution. Flux ratios expressed as influx/efflux and values obtained from matched pairs according to various criteria (see Materials and Methods).


Flux Ratio
2.5
2
1.5
1
0.5
0
*
*
CM/CM
FW/CM
CM/CM
FW/CM
SSI
DSI
Actual Ratios
Predicted Ratios

Chloride Cell Surface Areas versus Flux Rates

Rhodamine 123 was used to make estimates of clustered chloride cell surface area in DSI membranes after unidirectional fluxes were performed (see Chapter 2). Results showed no evidence of relationships between % chloride cell coverage and flux rates for all 3 ions (Figure 11). Although linear relationships are suggested by the figure, none of them are significant.

Figure 11: Chloride cell surface area expressed as % of total area (%SA) versus ion fluxes for Na^+, Cl^- and Ca^{2+} across DSI membranes under asymmetrical conditions.

A) Na^+ and Cl^- influxes versus %SA, equation is:

Ion flux = -12.437 (%SA) + 6.5718, r = 0.523, n = 17, p=0.12, not significant.

B) Na^+ and Cl^- effluxes versus %SA, equation is:

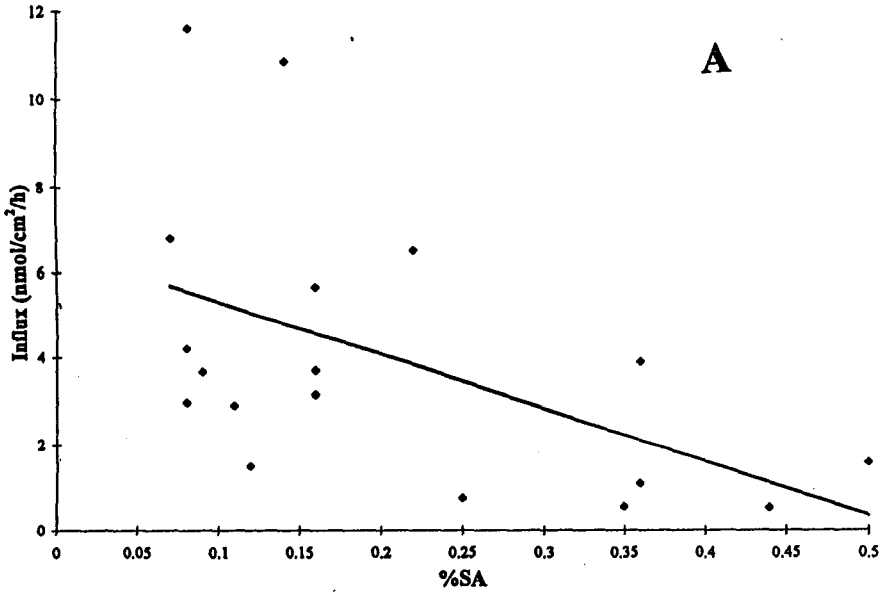
Efflux = -527.91 (%SA) + 580.21, r = 0.097, n=6, p=0.1, not significant.

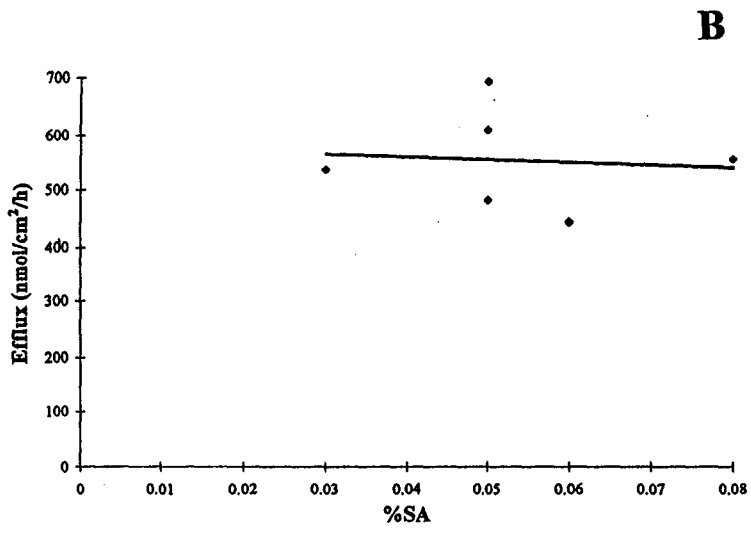
C) Calcium influx versus %SA, equation is:

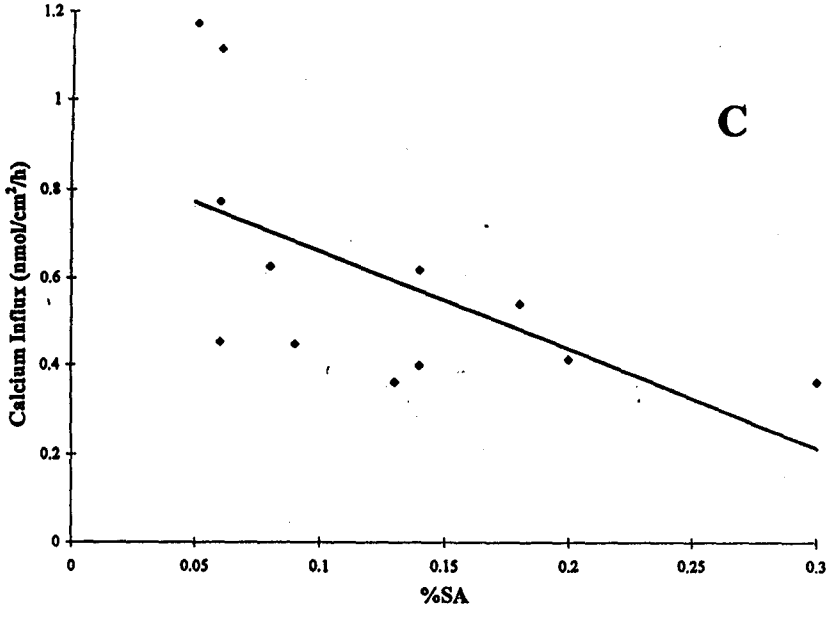
Influx = -2.228 (%SA) + 0.883, r=0.59, n=11, p=0.06, not significant.

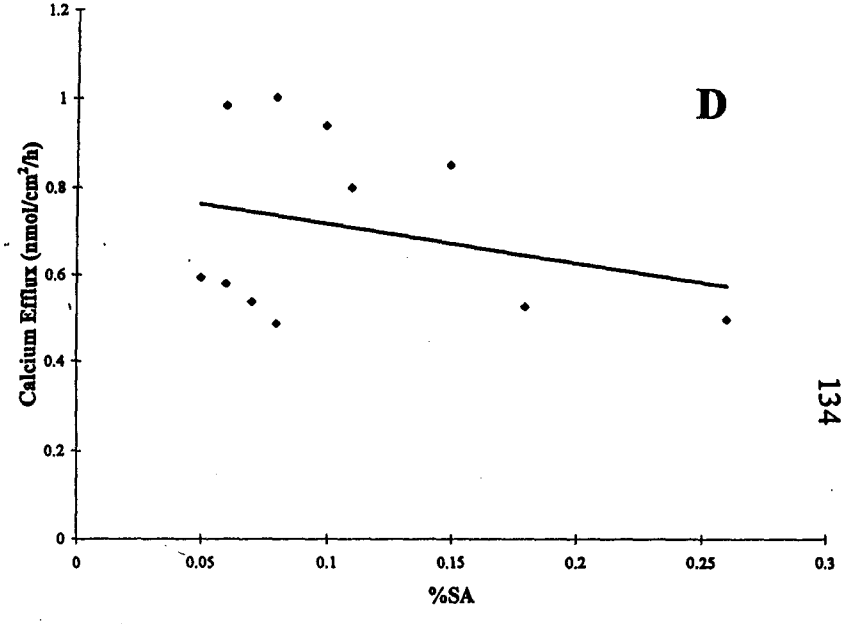
D) Calcium efflux versus %SA, equation is:

Efflux = -0.91 (%SA) + 0.8072, r=0.279, n=10, p=0.07, not significant.


A
Influx (nmol/cm²/h)
%SA
0
2
4
6
8
10
12
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
0.5


B
Efflux (nmol/cm²/h)
%SA
0
100
200
300
400
500
600
700
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08


C
Calcium Influx (nmol/cm²/h)
%SA
0
0.2
0.4
0.6
0.8
1
1.2
0.05
0.1
0.15
0.2
0.25
0.3


D
Calcium Efflux (nmol/cm²/h)
%SA
0
0.2
0.4
0.6
0.8
1
1.2
0.05
0.1
0.15
0.2
0.25
0.3

Discussion:

TER and TEP:

In all preparations, TER increased significantly when freshwater replaced culture media on the apical surface of cultured membranes (Tables 3 and 5). As discussed in Chapter 2, this same phenomena is also apparent in other epithelia exposed to freshwater (Marshall, 1985, Marshall et *al.*, 1992, Wood and Marshall, 1994). Although the cause of this increase is still unknown, it is thought to be more complex than simple cell swelling or epithelial tightening (Marshall, 1985), and instead may involve increases in paracellular permeability compensated for by decreases in transcellular permeability due to closing of apical channels (Wood *et al.*, 1997). This situation was further investigated by Gilmour *et al* (1997) over a prolonged period of freshwater exposure. Again the TER rose to much higher values, but slowly deteriorated over 48 hours. However, the same conclusions as Wood *et al.*, (1997) were suggested, with the addition that paracellular conductance continued to increase throughout the freshwater exposure.

The increased TER is accompanied by the development of a serosal negative potential (Table 3 and 5). Similar potentials are evident across branchial epithelium of rainbow trout *in vivo* (-5mV, Perry and Flik, 1988, and -1 to -10 mV, Perry and Wood, 1985), across opercular epithelium of freshwater-adapted brook trout (-8 to -12 mV, Marshall , 1985), and freshwater adapted killifish opercular epithelium (-15 to -50 depending on external

Ca^{2+} concentrations, Marshall *et al.*, 1995). As discussed in Chapter 2, the TEP is most likely a diffusion potential resulting from the higher gill permeability to Na^+ than to Cl^- (Potts, 1984). Although TEP is influenced by external Ca^{2+} levels (Perry and Wood, 1985), calcium activities are the same with culture media on both sides (symmetrical) and virtually identical with freshwater on the apical side and culture media on the basolateral (1-1.2 and 1.05 respectively). Changes in external Ca^{2+} activities therefore are unlikely to contribute to the TEP changes with freshwater exposure.

Unidirectional Na^+ and Cl^- fluxes:

In agreement with previous studies of SSI membranes in symmetrical conditions (Wood *et al.*, 1997), Na^+ and Cl^- fluxes in DSI membranes were inversely related to TER and directly related to conductance (Figure 4A). Although SSI membranes were found to have more variable fluxes per unit conductance when conductance is high (TER low), Figure 4A suggests that fluxes across DSI membranes were more variable when conductance was low (TER high). A 1:1 correlation between conductance and ion flux, such as that seen in brook trout opercular epithelium (Marshall, 1985), indicates that ion fluxes follow paracellular conductive pathways. Like the brook trout opercular epithelium, SSI membranes lack mitochondrial-rich cells (MRCs), and they exhibit a stronger linear relationship between conductance and ion flux in symmetrical conditions than DSI membranes. The y-intercept was clearly different from 0 in the conductance versus ion flux relationship for DSI preparations (Figure 4A). This was similar to the

brook trout urinary bladder which showed a non-zero y-intercept in the same relationship suggesting that a portion of the ion flux was not due to conductive pathways (Marshall, 1988). The y-intercept approaches 0 in SSI membranes (Wood *et al.*, 1997), and in brook trout opercular epithelium (Marshall, 1985), however the non-zero y-intercept in the present DSI preparations suggests that a smaller proportion of ion fluxes in DSI membranes follow conductive pathways than in SSI membranes.

As with SSI membranes and the opercular epithelium of tilapia and killifish (see Table 6 for comparisons), DSI membranes under symmetrical conditions exhibited no significant net flux for either Na^+ or Cl^- (Figure 1). The relationship with conductance, however suggests non-conductive transport, and the Ussing criterion analysis of the ion fluxes indicates that active Na^+ uptake occurs across DSI membranes in symmetrical conditions (Figure 5). Active Na^+ uptake has not been demonstrated in any of the *in vitro* models of the gill prior to this (Marshall, 1985, Marshall *et al.*, 1992, Wood and Marshall, 1994, Wood *et al.*, 1997), despite the fact that freshwater gills *in vivo* take up sodium (Marshall, 1995). This suggests a possible role for chloride cells in sodium uptake since SSI membranes consisting of only pavement cells did not exhibit active Na^+ uptake (Wood *et al.*, 1997). Although recent evidence has implicated pavement cells in sodium uptake (Morgan *et al.*,1994), correlations between chloride cell surface area and sodium influx have been reported in earlier studies (Avella *et al.*, 1987, Perry and Laurent, 1989).

However, if chloride cells on the cultured preparations were involved in sodium uptake, why was active Na^+ transport not evident with freshwater on the apical surface of

Table 6: Unidirectional Na^+ and Cl^- influxes and effluxes ($nmol.cm^{-2}.h^{-1}$) across freshwater epithelia under symmetrical conditions.

	References	Na^+ influx	Na^+ efflux	Cl^- influx	Cl^- efflux
Opercular Epithelium - *Oreochromis*	Burgess, D. (1997)	5000-8000	5000-10000	8000-12000	6500-14000
Opercular Epithelium - *Fundulus*	Wood and Marshall (1994)			1000-1200	1300-1500
Cultured SSI Membranes - *Oncorhynchus*	Wood *et al.*, (1997)	500-600	580-660	700-800	780-830
Cultured DSI Membranes - *Oncorhynchus*	present study	200-280	180-280	200-300	250-325

DSI preparations (Figure 6)? This seems unreasonable since in vivo freshwater gills take up Na^+ from their hypoosmotic environment. The sodium transport evident in symmetrical conditions implies that the cellular structures to support sodium transport are there, but perhaps they are not receiving the proper signals to take up sodium in freshwater. The cultured preparations lack the hormones that naturally influence the intact gill, such as prolactin and cortisol which are involved in adaptation to different salinities (McCormick, 1995). Prolactin plays a role in freshwater acclimation, and may be a necessary factor in sodium uptake *in vivo*. This lack of hormonal support will be discussed further in the section dealing with calcium transport.

The mechanisms for sodium uptake across the gill include either a $Na^+/H^+(NH_4^+)$ exchanger and/or the combinations of a sodium channel and an H^+-ATPase on the apical membrane, and Na^+/K^+ ATPase on the basolateral membrane (see Chapter 1). So far, evidence exists for 2 of the 3 components in cultured branchial epithelia. The Na^+K^+ - ATPase has been localized to cultured gill cells (Witters *et al.*, 1996) and the activity of this pump remains constant throughout culture conditions (see Chapter 2). Also, Part and Wood (1996) identified a Na^+/H^+ exchanger in the cultured gill cells, however, they postulated that it was located on the basolateral membrane and not the apical as *in vivo*, and its role was primarily for pH regulation and not transepithlial sodium transport in the cultured cells. However, these cells were not cultured on permeable supports, which suggests that perhaps with a solid substrate on the basolateral surface and culture media on their apical surface, their normal *in vivo* polarity was disrupted. It is possible that the

Na^+/H^+ antiport, being exposed to unnatural conditions, was not demonstrating normal site specificity, and would be located on the apical membrane *in vivo*. This would provide the basis for a Na^+ uptake apparatus, Na^+/H^+ exchange on the apical membrane and Na^+K^+ ATPase on the basolateral which could account for the active uptake seen in DSI preparations. The same study by Part and Wood (1996) ruled out the existence of a H^+-ATPase in the cultured SSI-type cells.

Both Na^+ and Cl^- are lost to the apical freshwater across DSI membranes (Figure 2). Effluxes increased 2-3 fold for both ions upon apical freshwater exposure which is similar to SSI membranes (Wood *et al.*, 1997). Linear relationships with conductance were maintained (Figure 4B and C). As noted with SSI membranes, these results are opposite to the opercular epithelium of trout which demonstrates an increase in conductance, but a decrease in effluxes during freshwater exposure (Marshall, 1985). Cl^- fluxes in asymmetrical conditions were generally larger than Na^+, possibly reflecting the higher mobility of Cl^- compared to Na^+ (Potts, 1984). Unidirectional flux rates were similar to those of other *in vitro* models such as the opercular epithelium of *Oreochromis* (Burgess, 1997) and the cleithrum skin of rainbow trout (Marshall *et al.*, 1992) (see Table 7 for comparisons).

Although the demonstration of Na^+ transport in cultured branchial epithelia is new, active Cl^- **uptake** has been reported in SSI cultured membranes under asymmetrical conditions (Wood *et al.*, 1997), and cultured sea bass gills under symmetrical conditions demonstrate active Cl^- **secretion** (Avella and Ehrenfeld, 1997). The Ussing flux ratios in

Table 7: Unidirectional Na^+ and Cl^- influxes and effluxes ($nmol.cm^{-2}.h^{-1}$) in freshwater epithelia measured under asymmetrical conditions.

	References	Na^+ influx	Na^+ efflux	Cl^- influx	Cl^- efflux
Cleithrum Skin - *Oncorhynchus*	Marshall *et al.* (1992)	0.1-0.2	100-160	2.5-5.0	250-400
Opercular Epithelium - *Fundulus*	Burgess, D. (1997)	120- 160	1200-1500	75-125	1000-1100
Opercular Epithelium - *Oreochromis*	Burgess, D. (1997)	5-25	500-600	25-30	600-750
Opercular Epithelium - *Fundulus*	Wood and Marshall (1994)	900-1000	1000-1100	30-40	150-250
Cultured SSI Membranes - *Oncorhynchus*	Wood *et al.*, (1997)	1.2-1.5	250-300	1.6-2.0	100-120
Cultured DSI Membranes- *Oncorhynchus*	present study	10-30	400-700	20-50	600-900

this study indicate the third instance where Cl^- transport is evident in cultured gill preparations (Figure 6). Both SSI and sea bass membranes are thought to consist entirely of pavement cells (Part *et al.*, 1993, Part and Bergstrom, 1995, Avella and Ehrenfeld, 1997). These cells have not been implicated in chloride transport by any previous theory. Chloride is thought to be taken up in exchange for HCO_3^- across the apical membrane of chloride cells (reviewed by McDonald *et al.*, 1989, Goss *et al.*, 1995, and Perry, 1997). Unfortunately, since active Cl^- uptake occurs in DSI membranes and SSI membranes, the results do not further our understanding of which cell type is responsible for Cl^- transport. In SSI membranes, the actual ratio of Cl^- influx/efflux is 3-fold higher than the predicted flux ratio (Wood *et al.*, 1997), whereas in DSI membranes the actual ratio is only 2-fold higher than the predicted ratio. Since DSI membranes consist mainly of pavement cells (see Chapter 2) there is nothing to distinguish the site of Cl^- uptake in the cultured preparations.

Ca^{2+} Fluxes:

Due to limitations in the available electrophysiological equipment, TEP was not measured for Ca^{2+} fluxes across SSI membranes prepared in Uppsala, Sweden. For purposes of calculating flux ratios, TEP values were chosen from other studies of the same preparation. The TEP under symmetrical conditions was taken as -2.24 mV, the mean TEP value obtained during a TEP analysis of SSI preparations in Uppsala (see Chapter 2). Wood *et al.*, (1997) previously reported a TEP of 0 mV in SSI membranes recorded in

Hamilton, and Chapter 2 of this thesis indicates a significant difference between TEP in SSI membranes measured in the two locations. Therefore, the mean value measured in Uppsala was used. The TEP value for flux calculations in SSI membranes under asymmetrical circumstances was selected as -10.8mV. This value represents the mean TEP value obtained during previous flux experiments with SSI membranes in Hamilton (Wood *et al.*, 1997). As well, very similar values were recorded in Uppsala, Sweden upon replacing the apical culture media with freshwater (Wood and Part, 1997). It seems reasonable to assume that -10.8 mV would approximate the TEP in these studies, especially since the TER, which was measured in all flux experiments, demonstrated the same increase with freshwater exposure as the aforementioned studies.

Compared to previous values, Ca^{2+} influxes across cultured SSI and DSI membranes under symmetrical and asymmetrical conditions were very small (Figure 7). Generally, the Ca^{2+} influxes across intact gills are within the range of values for cultured branchial epithelium, whereas the opercular epithelium of killifish exhibits flux rates in the range of 30 $nmol.cm^{-2}.h^{-1}$ for Ca^{2+} influx in asymmetrical solutions, and 50-70 $nmol.cm^{-2}.h^{-1}$ in symmetrical conditions (Marshall *et al.*, 1995) which is much higher than 0.25-1.0 $nmol.cm^{-2}.h^{-1}$ demonstrated by the cultured epithelia in both conditions (see Table 8 for comparisons). However, measured efflux rates of approximately 0.5-1.5 $nmol.cm^{-2}.h^{-1}$ in both cultured preparations in either conditions was comparable to calcium efflux across the opercular epithelium of killifish, 0.5 $nmol.cm^{-2}.h^{-1}$ (Marshall *et al.*1995), and the cleithrum skin of trout, 0.2-0.4$nmol.cm^{-2}.h^{-1}$ (Marshall *et al.*, 1992). This small flux is not

Table 8: Unidirectional Ca^{2+} fluxes ($nmol.cm^{-2}.h^{-1}$) across freshwater epithelia. Apical/Basolateral solutions are A) Freshwater/Culture media (FW/CM), and B) Culture Media/Culture Media (CM/CM).

A. Freshwater/Culture media (FW/CM)	References	Ca^{2+} influx	Ca^{2+} efflux
Opercular Epithelium - *Oreochromis*	Burgess (1997)	7.0-8.5	4.0-5.0
Opercular Epithelium - *Fundulus*	Marshall *et al.* (1995)	28-30	0.25-0.5
Cleithrum Skin - *Oncorhynchus*	Marshall *et al.* (1992)	0.03-0.04	0.2-0.3
in vivo - *Oncorhynchus*	Perry and Wood (1985)	7.0-8.0	3.5-4.5
in vivo - *Oncorhynchus*	Perry and Flik (1988)	7.0-7.5	
Cultured SSI Membranes - *Oncorhynchus*	present study	0.75-1.75	0.75-2.0
Cultured DSI Membranes - *Oncorhynchus*	present study	0.4-0.6	0.6-0.8
B. Culture media/Culture media(CM/CM)			
Opercular Epithelium - *Fundulus*	Marshall *et al.* (1995)	50-70	1.5-4.0
Cultured SSI Membranes - *Oncorhynchus*	present study	0.2-0.4	0.4-0.7
Cultured DSI Membranes - *Oncorhynchus*	present study	0.4-0.7	0.3-0.5

surprising since Na^+ and Cl^- fluxes measured in SSI membranes can account for all of the conductive transport, indicating that contributions of other ions would be negligible (Wood *et al.*, 1997). Although the cultured gill epithelia, like the intact gill, is a "tight" epithelium, the intact gill exhibits passive calcium efflux (Flik *et al.*, 1995) while uptake is thought to occur strictly through transcellular pathways.

The Ussing flux criteria indicated that none of the calcium fluxes in SSI membranes were active, whereas in DSI membranes under both asymmetrical and symmetrical conditions, active transport of Ca^{2+} took place (Figure 10). Ca^{2+} is thought to enter the gill cells through a lanthanum sensitive apical channel (Flik *et al.*, 1995, Perry and Flik, 1988), and to enter the blood by either a Ca^{2+}-ATPase, or a Na^+/Ca^{2+} exchanger. Both basolateral extrusion mechanisms play a role in calcium uptake *in vivo*, and their relative importance is still under debate (Verbost *et al.*, 1994). It is not surprising that active calcium transport is absent in SSI membranes for two reasons: 1) these cultured preparations do not contain mitochondrial rich cells which are thought to be the primary site up calcium uptake in vivo (Flik *et al.*, 1995) and 2) it was previously concluded that the cultured pavement cells do not have a lanthanum-sensitive apical calcium channel (Block and Part, 1992).

It is encouraging that DSI membranes demonstrate active Ca^{2+} transport since DSI membranes contain mitochondrial-rich cells which are thought to be the site of Ca^{2+} transport in the gill (Perry *et al.*, 1992a, Marshall, 1995, Flik *et al.*, 1996). DSI membranes with culture media on both sides (symmetrical) behaved like an intact gill by

demonstrating greater influx than efflux (Figure 7), and active calcium uptake from the apical to the basolateral side. Since both freshwater and seawater fish take up calcium (Perry and Flik, 1988) whereas sodium and chloride which are taken up by freshwater gills but secreted by seawater gills (Marshall, 1995), it is unlikely that salinity acts as a cue for calcium uptake. In other words, it is not surprising that cultured gill cells took up calcium in conditions that did not mimic their external environment.

Active Ca^{2+} transport in the cultured epithelia is also promising because it indicates the machinery for Ca^{2+} uptake was present in DSI membranes. If calcium uptake occurs transcellularly as *in vivo*, then the preparation must have contained both apical calcium channels and a basolateral Ca^{2+}-ATPase pump and/or a Na^{+}/Ca^{2+} exchanger. However, it was surprising to witness active calcium secretion with freshwater on the apical side since DSI membranes were found to take up calcium in isotonic conditions. A few considerations can be ruled out. Low external calcium concentrations stimulate calcium influx (Perry and Wood, 1985), yet the cells were exposed to a fairly similar activity throughout (1.05mM in culture media, 1-1.2mM in freshwater). Similarly, incubating cells in high external calcium and then transferring to normal levels is know to inhibit calcium influx, yet moving from culture media to freshwater, as just stated, does not sufficiently alter calcium concentrations.

It is worth noting a possible connection between Ca^{2+} uptake and Na^{+} transport. It is known that fish gills take up calcium across the basolateral membrane of chloride cells by Ca^{2+} ATPase and/or by Na^{+}/Ca^{2+} exchanger (see Chapter 1). Although both mechanisms

are thought to be involved in transepithelial transport and calcium homeostasis (Verbost *et al.*, 1994), most studies have concentrated on the role of Ca^{2+} ATPase (Flik, 1997). An unpublished study by Verbost *et al.* (1997) (reviewed by Flik, 1997) puts forth the hypothesis that increased Na^+ traffic, like that seen in seawater fish, may increase the role of Ca^{2+}/Na^+ exchangers in calcium uptake. However, their results found the opposite, that despite the increase in sodium efflux, opercular epithelium from seawater-adapted fish exhibited a lower amount of sodium-dependent calcium transport than freshwater-adapted fish. As with seawater gills, cultured branchial epithelia displayed a large efflux of both Na^+ and Cl^- in response to freshwater exposure (Figure 2). Therefore, a similar phenomena may have occurred in the cultured gills as in the seawater-adapted opercular epithelium. Perhaps Na^+ dependent calcium transport predominated in symmetrical conditions, but decreased with freshwater exposure. This assumes of course, that Ca^{2+}-ATPase plays a minimal role in transepithelial transport. Although highly unlikely, this assumption cannot be ruled out until Ca^{2+} ATPase has been localized to the cultured gill cells.

Nevertheless, it is very likely that the cultured branchial epithelia have Ca^{2+} -ATPases. The Ca^{2+} pump is active in most gill tissue (Flik *et al.*, 1995) and the cultured cells are still equipped with Na^+K^+ ATPase activity (see Chapter 2). However, it could be that the structures are there but the regulating hormonal support is absent. The pituitary hormone, prolactin, is known to activate ATP dependent Ca^{2+} transport in eels and tilapia (reviewed by Flik *et al.*, 1995). In tilapia, prolactin treatments increase chloride cell density in a

dose-dependent fashion along with calcium influx, and the density of Ca^{2+} -ATPase also increases. The steroid hormone cortisol also increases branchial calcium influx (Perry and Wood, 1985).The lack of hormonal support may lead to the Na^{+}/Ca^{2+} exchanger playing the more dominant role in calcium uptake across the basolateral membrane, much like the intestine of tilapia (Schoenmakers *et al.*, 1993). The presence of $Na^{+}K^{+}$-ATPase indicates the gradient can be established for the exchanger (Verbost *et al.*, 1994).

Hormones are essential for adjustments in the physiology of fish that are transferred from seawater to freshwater (McCormick, 1995). Specifically, prolactin levels rapidly increase when fish are placed in freshwater. Other hormones such as growth hormone and cortisol also have roles in this adaptation. Previously, SSI membranes were exposed to prolactin and growth hormone to test the ability of the hormones to sustain the integrity of the preparations with apical freshwater (Gilmour *et al.*, 1997). It was found that hormonal supplementation had no effect on the deterioration of TER, PEG-4000 permeability, or ion fluxes during 48 hours of apical freshwater exposure. This, however, does not rule out the possibility that DSI membranes with hormonal treatment will exhibit benefits, especially since prolactin, growth hormone, and cortisol exhibit their effects on chloride cells which the DSI membranes possess. As well, the effect of hormones on calcium transport in SSI membranes was not investigated.

Despite the demonstration of active calcium transport in DSI preparations, as with Na^{+} and Cl^{-}, there was no correlation between flux rates and chloride cell surface area. Much of the evidence suggesting chloride cells are the site of calcium uptake comes from

circumstances in which calcium uptake correlates with chloride cell surface area (Marshall *et al.*, 1992, McCormick *et al.*, 1992, Perry and Wood, 1985), and the surface area correlates with the activity of Ca^{2+}-ATPase (reviewed by Flik, 1997). There are two reasons which may account for this observed relationship between MRCs and Ca^{2+} transport; 1) an underestimation of chloride cell surface area in DSI preparations (see Discussion of Chapter 2), and 2) the lack of hormonal support mentioned previously since prolactin and cortisol are thought to play a role in both calcium transport and chloride cell surface area (Flik *et al.*, 1995).

Conclusion:

DSI membranes demonstrate active Na^+ uptake and Ca^{2+} uptake in symmetrical conditions, whereas SSI membranes do not demonstrate any active transport in similar conditions. Like SSI preparations, active Cl^- uptake was witnessed in DSI membranes under asymmetrical conditions. For the first time, active transport of both Ca^{2+} and Na^+ was demonstrated in the cultured preparations.

Chapter 4: Conclusions and Future Directions

Conclusions:

The following is a summary of the main conclusions obtained from Chapter 2 and Chapter 3 of this thesis.

1. A major goal for quite some time has been to develop a cultured model of the intact gill that contains chloride cells as well as pavement cells. A heterogenous model on permeable supports would better represent the morphology of the intact gill as well as provide a preparation in which experiments could be performed under asymmetrical conditions that mimic those *in vivo*. That goal was accomplished in this thesis. It was found that the new double-seeded (DSI) membranes contained two cell types, one consistent with pavement cells seen in other single-seeded (SSI) preparations (Part and Bergstrom, 1995, Part *et al.*, 1993) and one rich in mitochondria. These latter cells were most likely chloride cells. This was the first time in which two cell types were observed in cultured branchial epithelium on permeable supports.

2. Transepithelial resistance (TER) was similar in both SSI and DSI membranes showing high values that were characteristic of "tight" epithelia. TER rose in SSI preparations and

was relatively unchanged in DSI membranes when apical culture media was replaced with freshwater. Both preparations retained their characteristic "tightness" and gave no indication of damage.

3. TER decreased when freshwater was placed on the basolateral side of SSI and DSI membranes. This change was irreversible. Both DSI and SSI preparations appear to be polarized as *in vivo* since TER was maintained with apical freshwater (see above) but not when freshwater was placed on the basolateral side (opposite to *in vivo*).

4. Transepithelial potential (TEP) in SSI membranes was not consistent. Measurements taken in Hamilton during these studies as well as others (Wood and Part, 1997, Wood *et al.*, 1997) under symmetrical conditions show the TEP to be absent (0 mV). However, TEP measurements taken in Uppsala, Sweden demonstrate a significant serosal **negative** TEP that appears to be ATP-dependent. In contrast, DSI membranes exhibit a serosal **positive** TEP. This TEP is more similar to the TEP across intact gills (Potts, 1984) and other *in vitro* preparations of the gill (Marshall *et al.*, 1992, Marshall *et. al.*, 1995).

5. The Na^+K^+ -ATPase activity of cultured gill cells remained stable throughout primary culture in flasks, and after culture of the epithelial membrane on permeable inserts via SSI methods.

6. Paracellular permeability, measured as the permeability to radiolabelled PEG - 4000 increased with freshwater on the apical side compared to culture media on both apical and basolateral sides (control conditions). This increase in permeability was accompanied by a decrease in conductance. The same trend was seen previously in SSI membranes (Wood *et al.*, 1997). In contrast, seawater on the apical side of DSI membranes caused an increase in PEG-4000 permeability and an increase in conductance to levels characteristic of "leaky" epithelia.

7. DSI and SSI membranes demonstrated similar unidirectional fluxes of Na^+ and Cl^-. Influx and efflux values were comparable resulting in small net fluxes for both ions under symmetrical conditions. By contrast, large net effluxes to the apical freshwater were evident for both ions under asymmetrical conditions. However, DSI membranes demonstrated active Na^+ uptake and passive Cl^- transport in symmetrical conditions, and active Cl^- uptake and passive Na^+ transport in asymmetrical conditions. While active Cl^- uptake was demonstrated across SSI membranes previously (Wood *et al.*, 1997), this is the first time that active Na^+ transport has been seen in a model of the freshwater gill. This suggests that the mitochondrial-rich cells present in the DSI membranes may play a role in Na^+ transport.

8. Unidirectional Ca^{2+} fluxes were very small across both SSI and DSI membranes. Ca^{2+} transport was passive in SSI membranes under both symmetrical and asymmetrical

conditions, however, Ca^{2+} transport was active in DSI membranes under both these conditions. These results indicate that mitochondrial-rich cells in DSI membranes may be involved in Ca^{2+} transport.

9. In addition to the differences outlined above, there are two practical advantages to the double-seeding technique: 1) Cultured membranes on permeable supports with stable TER values are obtained much more quickly, 5-7 days after isolating gills cells as opposed to 12-14 days after cell isolation with the SSI technique. 2) The DSI technique yields twice as many insert preparations as the SSI technique with the same number of fish. Although the reasons for this are not entirely clear, it may reflect the fact that SSI methods require gill cells to undergo two sets of trypsinations while the DSI methods require only one set.

Future Directions:

The following approaches could be used to further characterize cultured gill cells and their validity as an *in vitro* model:

1. Transmission electron micrographs (TEM) are needed to positively identify the

mitochondrial-rich cells in the DSI membranes as chloride cells. Although Rhodamine 123 sequestration is a reliable indication that the cells are rich in mitochondria (Shinomiya *et al.*, 1992), other evidence of cell morphology is needed to verify their identity (P. Laurent, personal communication). TEMs of osmium-stained cultures that contain fluorescent clusters of cells in culture dishes indicate that not all of the fluorescent clusters were identified as chloride cells (V. Thomas, personal communication). However, those cultures did not appear to contain single fluorescing cells as the membranes did. Also the surface area of mitochondrial-rich cells in DSI membranes needs to be recalculated by an alternative means to yield a value that includes single cells as well as clustered cells. The discussion section of Chapter 2 outlines one possible method for this which involves retypsinating cultured membranes, staining the isolated cells, and counting the proportion of fluorescent cells. Therefore, further analysis of the morphology of cultured fluorescent cells on DSI membranes is warranted.

2. The effects of hormonal stimulation on DSI membranes should be investigated. Previously, prolactin and growth hormone did not affect the ability of SSI membranes to withstand prolonged freshwater exposure (Gilmour *et al.*, 1997). However, this may not prove to be the case for DSI membranes since these hormones are known to influence chloride cells which may be present in DSI membranes but not SSI membranes. The following summarizes the possible effects that various major hormones may elicit on DSI membranes (reviewed by McCormick, 1995):

a) Cortisol is known to stimulate Na^+K^+ -ATPase activity in the gill. DSI membranes with or without cortisol may exhibit increased Na^+K^+ -ATPase activity in comparison to SSI membranes since they contain mitochondrial-rich cells that have high activity (Perry and Walsh, 1989). Cortisol also stimulates whole body calcium uptake in rainbow trout (Perry and Wood, 1985, Flik and Perry, 1989), and may alter calcium fluxes across cultured gill epithelia as well.

b) Growth hormone (GH) plays a role in salinity tolerance. GH is known to increase chloride cell surface area during salinity changes, thereby improving ionoregulatory performance by altering Na^+ and Cl^- transport. Although the main role of GH is thought to be in seawater fish, changes in ion transport in DSI membranes may occur.

c) Prolactin helps maintain ion homeostasis in freshwater fish. Prolactin levels increase in euryhaline fish transferred from seawater to freshwater, and decrease when transferred from freshwater to seawater. Its role in acclimation to different environments involves changing the morphology and density of chloride cells. This hormone, like cortisol, is found to increase calcium transport across the intact gills through an increase in Ca^{2+}-ATPase activity (Flik *et al.*, 1995). The effects of prolactin on calcium transport are worth investigating.

3. The cultured epithelia would be of greater use if the tolerance of prolonged freshwater exposure could be extended. The hormonal support discussed above may lead to increased tolerance, but there are other ideas worth mentioning. For instance, gradually

changing the culture media to freshwater with a series of dilutions rather than all at once may improve the cells' ability to adapt to changing salinity. In addition, manipulations of the culture conditions may be worthwhile. Avella and Ehrenfeld (1997) found that sea bass gill cultures grown in media supplemented with sea bass serum instead of fetal bovine serum demonstrated a higher degree of differentiation and increased ion transport. Rainbow trout serum may exert similar effects on rainbow trout gill cultures. Also, supplementing the culture media with different metabolic fuels that are known to be used by gill cells, such as lactate and fatty acids (Mommsen, 1984) may improve their ability to withstand apical freshwater exposure.

4. Theories of Na^+ and Cl^- transport suggest these ions are taken up in exchange for acid-base equivalents (McDonald *et al.*, 1989). Variations in the acid-base composition of the media may therefore influence Na^+ and Cl^- transport in the cultured preparations. By current theories, basolateral acidosis should stimulate Na^+ uptake and inhibit Cl^- uptake, and basolateral alkalosis should have the opposite effects. Since DSI membranes exhibited active ion transport, pharmacological studies of ion fluxes and TEP in DSI membranes may reveal more information about the regulation of this transport.

5. Antibodies could be used for immunolocalization of ion pumps such as H^+ -ATPase (Lin and Randall, 1995) and Ca^{2+} -ATPase (Perry, 1997) as well as other transporters such as Cl^-/HCO_3^- exchangers (Sullivan *et al.*, 1996). Measurments of the activities of

energetic pumps such as Ca^{2+} -ATPase and H^{+} -ATPase would be useful in characterizing the transport functions of the cultured gill epithelia.

The production of DSI membranes is a positive step towards producing a model of the intact freshwater gill. Hopefully, this method will provide many more opportunities for investigating the role of not only the gill in ion transport, but the individual roles of the different cell types in the gill.

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