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CHLORIDE CELLS AND THE HORMONAL CONTROL OF TELEOST FISH OSMOREGULATION

BY J. KEVIN FOSKETT*,†,‡, HOWARD A. BERN*, TERRY E. MACHEN† AND MARILYN CONNER*

**Department of Zoology and Cancer Research Laboratory and*

† Department of Physiology-Anatomy, University of California, Berkeley, California 94720, U.S.A.

SUMMARY

Teleost fish osmoregulation is largely the result of integrated transport activities of the gill, gut and renal system. The basic 'epithelial fabric' in each of these tissues is adapted to provide the appropriate transport mechanisms depending upon whether the fish is in fresh water or sea water. Net NaCl transport by the branchial epithelium reverses direction when euryhaline species migrate between the two media, providing a useful focus in experiments designed to elucidate mechanisms of differentiation and integration of transport function. Isolated opercular membranes and skins from certain seawater-adapted species are good models to study branchial salt extrusion mechanisms. These heterogeneous tissues generate short-circuit currents equal to net chloride secretion. The vibrating probe technique has allowed localization of all current and almost all conductance to the apical crypt of chloride cells. Area-specific surface current and conductance of chloride cells are $18\,mA\,cm^{-2}$ and $580\,mS\,cm^{-2}$ ($1{\cdot}7\,\Omega cm^2$), ranking them as one of the most actively transporting and conductive cells known. There is no net sodium transport under short-circuit conditions but the chloride secretion process is sodium-dependent and ouabain and 'loop'-diuretic sensitive. Sodium fluxes through chloride cells are large ($P_{Na} = 5{\cdot}2 \times 10^{-4}\,cm\,s^{-1}$) and appear passive and rate-limited by a single barrier. A link may exist between the active transport and leak pathways since sodium fluxes always account for 50% of chloride cell conductance. The sodium pathway is probably the chloride cell-accessory cell tight junction, although this is still unresolved. Chloride secretion can be rapidly modulated by several hormones, including catecholamines, somatostatin, glucagon, vasoactive intestinal polypeptide and urotensins I and II. Regulation by these hormones may be by rapid alterations of cellular cAMP levels. Differentiation of chloride cells and chloride secretion may be controlled by cortisol and prolactin. Cortisol stimulates chloride cell proliferation and differentiation and appears to interact with NaCl to initiate salt secretion. Prolactin appears to cause chloride cell dedifferentiation by reducing both the active-transport and leak pathways proportionately. Prolactin and cortisol also affect epithelial cell proliferation and differentiation in the other osmoregulatory tissues in fish, suggesting that these hormones are primary agents in the integration of transport activities to achieve whole animal osmoregulation.

‡ Present address: Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung and Blood Institute, Bldg. 10, Rm. 6N-307, National Institutes of Health, Bethesda, Maryland 20205.

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INTRODUCTION

Teleost fish maintain the ionic composition and osmolarity of their body fluids at levels significantly different from the external environment. Despite the fact that respiratory demands necessitate exposure of large areas of permeable body surface (i.e., the gills), the blood of freshwater-adapted teleosts is markedly hypertonic and that of seawater fish markedly hypotonic compared with their respective external milieux. Studies over the past 50 years have provided a general scheme of the osmoregulatory problems faced and the solutions employed by teleosts. Briefly, active uptake of salts from fresh water by the gills, along with a low body-surface ionic permeability, enables the freshwater-adapted fish to maintain blood ion levels far more concentrated than the dilute milieux (typically ~150 mM *vs* <1 mM-NaCl). The renal system aids in this respect by maintaining a high glomerular filtration rate along with tubular and bladder reabsorption of filtered ions, producing a copious dilute urine to compensate for the high water permeability of the whole fish. Generally, freshwater-adapted fish drink less than seawater-adapted ones, in which increased drinking of the concentrated medium and subsequent absorption by the gut serve to replace the water lost osmotically. Water conservation is enhanced by reduced glomerular filtration, the kidney serving principally as a divalent-ion secretory organ. Excess monovalent ions derived from both swallowed sea water and passive entry across the body surfaces are excreted extrarenally, nearly entirely by the gills (reviewed by Maetz, 1974; Kirschner, 1979*a*; Evans, 1979, 1980*a*).

Thus, the basic NaCl transport mechanisms in the gill, gut and renal system depend on the adaptational state of the fish. That is, the basic 'epithelial fabric' in each of these tissues is adapted to provide the appropriate transport mechanisms for the particular environmental condition. Equally important, the transport activities of these tissues are integrated in each environment to provide the appropriate coordinated osmoregulatory response. The agents which control differentiation and integration of transporting epithelia generally require a sustained perturbation (environment, diet, etc.) to be elucidated. Fortunately for physiologists, euryhaline fish can be adapted to both fresh water and sea water, and thus provide a natural way to perturb the epithelial transport processes and the trophic, regulatory mechanisms. Of all the transporting epithelia, the perturbation of transport function in the gills is most radical since the direction of net NaCl transport is reversed following transfer from one environment to the other. Consequently, the branchial transport mechanisms provide a useful focus for experiments designed to uncover the agents and mechanisms of differentiation and integration of transport function. The present paper discusses the branchial transport mechanisms and their control and differentiation in an attempt to elucidate those factors involved in integration of the several osmoregulatory surfaces in fish.

THE ISOLATED OPERCULAR MEMBRANE AND SKIN AS MODELS FOR BRANCHIAL TRANSPORT MECHANISMS

Early work by Smith (1930), Keys (1931) and Krogh (1939) demonstrated that the branchial epithelium was the site of important ion and water exchanges in both

[fres]hwater- and seawater-adapted teleosts. Subsequent studies of the roles of the gills in teleost osmoregulation have employed a number of experimental approaches, including intact whole animals, perfused heart-gill preparations, isolated perfused heads and isolated perfused gills. As a result, there now exists a wide literature concerning the roles of the gills in osmoregulation and the nature of the transport processes. This field has been extensively reviewed (Maetz, 1970, 1973, 1974, 1976; Motais & Garcia-Romeu, 1972; Evans, 1975, 1980*a,b,c*; Maetz & Bornancin, 1975; Maetz, Payan & de Renzis, 1976; Potts, 1977; Kirschner, 1973, 1979*a*; Karnaky, 1980; Girard & Payan, 1980; Evans *et al.* 1982), and so will not be discussed here. The advantages and disadvantages of each of these approaches have also been previously discussed (Karnaky, 1980; Degnan & Zadunaisky, 1982; Evans *et al.* 1982). The geometrical and vascular complexities of these preparations have not permitted precise measurement or control of the transepithelial voltage, control or determination of the perfused surface area, or access with microelectrodes to specific transporting cell types, all necessary for a rigorous analysis of transport mechanisms. These limitations have been overcome with the utilization of the isolated opercular membranes from the minnow *Fundulus heteroclitus* (Degnan, Karnaky & Zadunaisky, 1977; Karnaky, Degnan & Zadunaisky, 1977) and subsequently from the tilapia *Sarotherodon mossambicus* (Foskett *et al.* 1981), and of skin from the goby *Gillichthys mirabilis* (Marshall, 1977; Marshall & Bern, 1980). These tissues appear to contain the same cell types as the branchial epithelium (Karnaky & Kinter, 1977; Marshall & Nishioka, 1980; Foskett *et al.* 1981), especially the chloride cells, and are flat-sheet epithelia which can be mounted *in vitro* in Ussing-style chambers, allowing precise measurement and control of all chemical and electrical gradients, thereby permitting a rigorous biophysical analysis of the mechanisms of ion transport.

TRANSPORT MECHANISMS IN OPERCULAR MEMBRANES AND CHLORIDE CELLS FROM SEAWATER-ADAPTED FISH

Table 1 summarizes the basic electrophysiological and ion-flux data obtained from the seawater-adapted tilapia opercular membrane. The essential features, shared by

Table 1. *Electrophysiology and ion fluxes across seawater-adapted tilapia opercular membranes*

	Cl	Na
J_{sm}	3·25 ± 0·28 (13)	0·97 ± 0·15 (7)
J_{ms}	0·46 ± 0·04 (13)	0·98 ± 0·11 (7)
J_{net}	2·79 ± 0·28 (13)*	−0·01 ± 0·05 (7)**
I_{sc}	2·91 ± 0·26 (13)	2·38 ± 0·37 (7)
$\bar{G}_t$	3·26 ± 0·33 (13)	2·54 ± 0·34 (7)
PD	28·8 ± 1·2 (48)	27·6 ± 1·1 (34)

J is unidirectional flux (μequiv cm^{-2} h^{-1}; sm = serosa to mucosa; ms = mucosa to serosa) under short-circuit conditions, I_{sc} is short-circuit current (μequiv cm^{-2} h^{-1}), G_t is transepithelial conductance (mS cm^{-2}) and PD is the open-circuit potential (mV). Numbers in parenthesis = N. Fluxes were measured on paired tissues with conductances within 80 % of each other.

* Not significantly different from I_{sc} ($P > 0·30$).

** Not significantly different from 0 ($P > 0·40$).

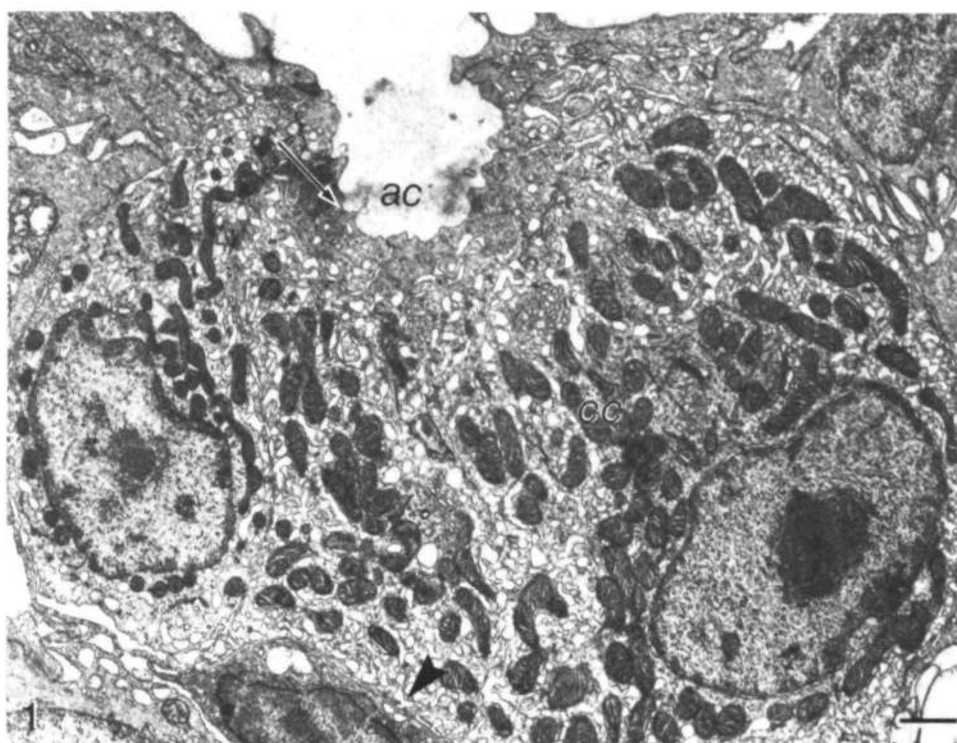
the *Fundulus* and *Gillichthys* preparations, are the following. When bathed in a Ussing-style chamber with identical oxygenated fish Ringer solution on both sides, the tilapia opercular membrane generates a serosa-positive transepithelial voltage ranging from 10 to 60 mV. The unidirectional efflux (serosa to mucosa) of chloride is typically about seven times greater than the influx (mucosa to serosa), which results in a large net chloride secretory flux. Chloride secretion appears to be entirely responsible for the spontaneous transepithelial potential difference and the short-circuit current since (i) the net flux of chloride is not significantly different from the short-circuit current, (ii) there is no significant net flux of sodium under these conditions, and (iii) removal of chloride abolishes the short-circuit current. The rate of chloride secretion, measured as the short-circuit current, is linearly related to the transepithelial conductance in the tilapia opercular membrane (Foskett, Machen & Bern, 1982*b*), reflecting the conductive nature of the secretory transport process. The basis of the branchial salt extrusion mechanism for most (but not all) species examined is also an active chloride secretion process (Kirschner, 1977).

Morphological studies (Karnaky & Kinter, 1977; Marshall & Nishioka, 1980; Foskett *et al.* 1981) have provided further support for the idea that opercular membranes and skin are excellent models for branchial salt secretion. Thus, both the opercular membrane and the gills are thin, stratified epithelia which contain the same cell-types: mucous cells, non-differentiated 'support' cells, a thin layer of surface 'pavement' cells which constitute the external surface (see Figs 1 and 2), and so-called chloride cells. The tilapia opercular membrane also possesses a layer of basal cells (Foskett *et al.* 1981), as observed in the gill (Conte & Lin, 1967).

Chloride cells were originally described and named by Keys & Willmer (1932) in seawater eel gills following Keys's discovery (1931) of the chloride-secretory capacity of the fish gill. Subsequent ultrastructural, enzymatic and kinetic data have provided indirect support for the idea that chloride cells are the sites of branchial NaCl secretion (reviewed by Maetz & Bornancin, 1975; Kirschner, 1977; Karnaky, 1980; Philpott, 1980). For example, the chloride cell, more than any other in the gill, has ultrastructural characteristics suggestive of specialized transport activity (Fig. 1). These cells are characterized typically by the presence of large, well-developed mitochondria in close association with a greatly amplified membranous system. This membranous system is continuous with the basolateral membrane (reviewed by Philpott, 1980), which is so elaborate that it fills nearly all the cell, up to a narrow band of cytoplasm just beneath the apical membrane. As a result, much of the 'interior' of the chloride

Fig. 1. Parasagittal section of a chloride cell (*cc*) in the opercular membrane isolated from a seawater-adapted tilapia. The well-developed apical crypt (*ac*) is filled with amorphous material (probably mucous). An extensive tubular system continuous with the basal and lateral plasma membrane (short arrow) fills the entire cell except for a clear zone of cytoplasm near the apical crypt and a perinuclear region containing Golgi membranes. Mitochondria are prominent. Portions of an accessory cell are connected by shallow junctions (long arrow) in the apical crypt region. A second cell, associated with the chloride cell and sharing the apical pit, may be an accessory cell or another chloride cell. Bar is 1 μm × 7500. (From Foskett *et al.* 1981.)

Fig. 2. Scanning electron micrograph of the apical surface of an opercular membrane isolated from a seawater-adapted tilapia. 'Pavement' cells, with characteristic microridges, form a continuous surface layer interrupted by occasional contacts of underlying chloride cells with the external milieux *via* small apical crypts (arrow). Bar is 5 μm × 2000.



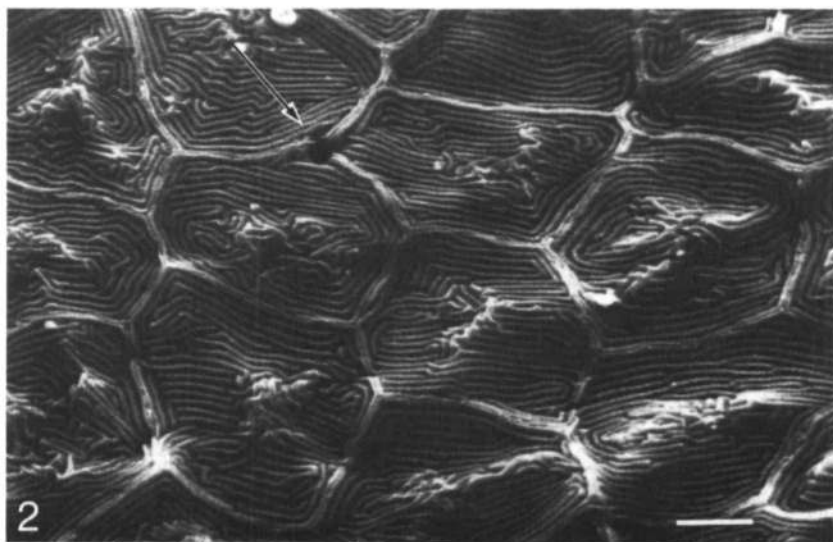


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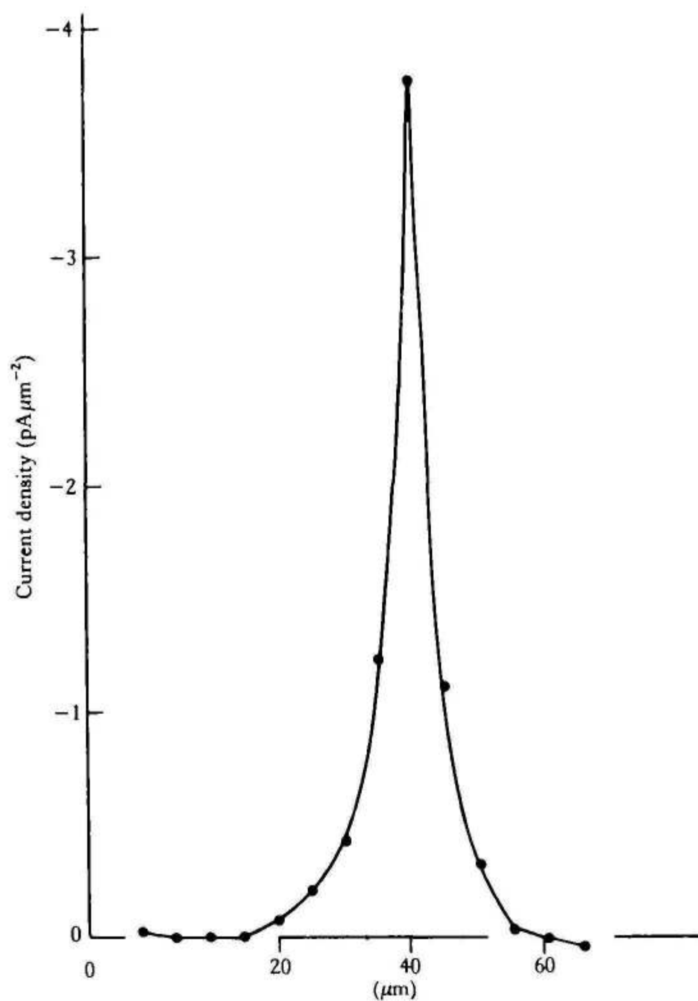
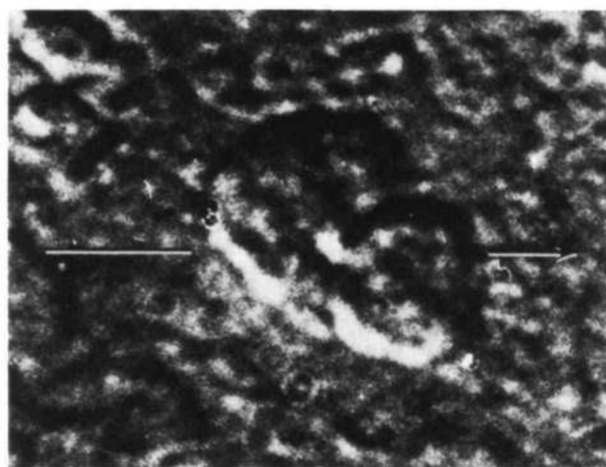
Fig. 3





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is in fact extracellular space. A high level of Na/K-ATPase activity (often correlated with transport activity in epithelia) has been specifically localized to the basolateral tubular membrane system of chloride cells in autoradiographic and cytochemical studies (Karnaky, Kinter, Kinter & Stirling, 1976*b*; Hootman & Philpott, 1979) and in isolated chloride cells (Kamiya, 1972*a*; Sargent, Thomson & Bornancin, 1975). Seawater adaptation elicits branchial salt secretion and concomitantly causes increases in the size and/or number of chloride cells (Liu, 1942, 1944; Getman, 1950; Vickers, 1961; Virabhadrachari, 1961; Jozuka, 1966; Ahuja, 1970; Olivereau, 1970; Shirai & Utida, 1970; Utida, Kamiya & Shirai, 1971*b*; Doyle & Epstein, 1972; Karnaky, Ernst & Philpott, 1976*a,b*; Thomson & Sargent, 1977; Hootman & Philpott, 1978; Tay & Garside, 1978, 1979; Pisam, 1981) and an increase (in most, but not all, species) in gill levels of Na/K-ATPase (Epstein, Katz & Pickford, 1967; Epstein, Cynamon & McKay, 1971; Epstein, Silva & Kormanik, 1980; Kamiya & Utida, 1968; Jampol & Epstein, 1970; Motais, 1970; Zaugg & McLain, 1970; Milne, Ball & Chester Jones, 1971; Utida *et al.* 1971*b*; Bornancin & de Renzis, 1972; Butler & Carmichael, 1972; Kamiya, 1972*b*; Forrest, Cohen, Schon & Epstein, 1973; Utida & Hirano, 1973; Evans & Mallery, 1975; Karnaky *et al.* 1976*a,b*; Scheer & Langford, 1976; Thomson & Sargent, 1977; Hootman & Philpott, 1979; Hossler, Ruby & McIlwain, 1979). Finally, the chloride cells also undergo marked ultrastructural changes associated with development of branchial salt secretion, including the development of the tubular system (Shirai & Utida, 1970; Doyle & Epstein, 1972; Karnaky *et al.* 1976*a*; Pisam, 1981) and an increase in the development of mitochondria (Shirai & Utida, 1970; Sargent *et al.* 1975).

Localizing sites of active transport and conductance

Despite this wealth of correlative data implicating the chloride cells as the cell-type responsible for branchial salt secretion, the gill, opercular membrane and skin are heterogeneous epithelia, and each of the four principal cell-types has at one time or another been implicated as the salt-secretory cell type (Keys & Willmer, 1932; Bevelander, 1935; Munshi, 1964).

Recently, Foskett & Scheffey (1982) employed the vibrating probe technique in an attempt to localize the current and conductance pathways in the tilapia opercular membrane. This technique allows low drift measurements of extracellular current density immediately above the epithelial surface. Under short-circuit conditions, the vibrating probe detected peak current densities only over chloride cells. The polarity of the current was consistent with the known chloride-secretory capacity of this tissue. Current-voltage relations determined for individual chloride cells and pavement cells revealed that chloride cells are localized sites of high conductance as well. Conductance of pavement cells appears to be two to three orders of magnitude less than for individual chloride cells. These data provided the first direct evidence that chloride

Fig. 3. Vibrating probe measurements of current density obtained as the tip of the probe was moved at a height of 8 μm above the apical surface in a straight line from left to right over an apical crypt shared by two chloride cells in the tilapia opercular membrane. Peak negative current density is over the crypt; current density away from the chloride cell is not different from zero. Similar peaks of current density are associated with almost all chloride cells in the tilapia opercular membrane.

cells are significant electrogenic and conductive elements in the tilapia opercu[illegible] membrane and, by inference, the gill as well, establishing these cells as the extrarenal salt secretory cells in teleost fish. The rest of the epithelium does not appear to be engaged in electrogenic ion transport and probably serves as a high resistance barrier to dissipative conductive ionic flow.

In a subsequent study (Scheffey, Foskett & Machen, 1983), Nomarski optics and improved probe spatial resolution allowed localization of the tissue current and conductance specifically to the chloride cell apical crypt (Fig. 3), the approximately 3 μm diameter opening at the surface where the chloride cell makes limited contact with the external medium (see Figs 1 and 2). As a result of these studies, it is now clear that all electrogenic ion transport and nearly all tissue-specific conductance of the tilapia opercular membrane are localized to chloride cells. Ussing chamber studies, therefore, will largely represent studies of chloride cell biology, to the extent that nonspecific conductance (i.e., edge damage) is minimized or taken into account (see Scheffey *et al.* 1983).

The achievement of high probe spatial resolution allows study of individual chloride cells without the detection of current and conductance through other chloride cells and edge damage. In a series of 'cell-sampling' experiments (Scheffey *et al.* 1983; J. K. Foskett & T. E. Machen, in preparation), of 315 chloride cells examined in 10 opercular membranes, only five cells failed to generate detectable current. A great deal of variability exists among chloride cells in their transport rates: cell currents ranged from 0 to 14 nA and individual cell conductances from 0 to 697 nS. Using the average chloride cell current ($2{\cdot}5 \pm 0{\cdot}1$ nA) and conductance ($82{\cdot}4 \pm 4{\cdot}1$ nS) and assuming a hemispherical apical membrane with a diameter of 3 μm, it is possible to calculate area-specific surface current and conductance for chloride cells of 18 mA cm^{-2} and 580 mS cm^{-2} ($1{\cdot}7\ \Omega$cm^2), respectively. Certainly, the chloride cell must rank as one of the most actively transporting and conductive cells known! Since the current obviously traverses the apical membrane, the conductance of this membrane must therefore be tremendous. The total chloride cell conductance, however, cannot be partitioned into its cellular and paracellular components associated with the apical crypt using the vibrating probe technique.

Importance of sodium for chloride secretion

The mechanisms involved in chloride secretion by the chloride cell appear to resemble those observed in other chloride-secreting epithelia, including involvement of Na/K-ATPase, basolateral NaCl co-transport and dependence on sodium.

As already mentioned, high levels of Na/K-ATPase have been localized in chloride cells, and changes in branchial levels are associated with changes in salinity. Na/K-ATPase appears to be involved in chloride secretion by the opercular membranes and skin since ouabain addition to the basolateral side results in a rapid inhibition of the short-circuit current as a result of a decreased chloride efflux and increased influx (Degnan *et al.* 1977; Karnaky *et al.* 1977; Marshall, 1977, 1981*a*; Mayer-Gostan & Maetz, 1980; J. K. Foskett, unpublished observations). In addition, 70–90% of the short-circuit current depends on serosal potassium (Marshall, 1977; Degnan & Zadunaisky, 1980).

Replacement of sodium with an impermeant cation reduces the short-circ[illegible]

rent to nearly zero (Degnan *et al.* 1977; Marshall, 1977, 1981*a*; Mayer-Gostan & Maetz, 1980; J. K. Foskett, unpublished observations). This sodium dependency appears to be at the basal membrane in the tilapia opercular membrane (J. K. Foskett, unpublished observations) and *Gillichthys* skin (Marshall, 1977), although Degnan & Zadunaisky have reported that the sodium dependency in the *Fundulus* opercular membrane is predominantly (1980) or equally (1982) apical. There may be problems with this latter interpretation since unilateral sodium removal results in a substantial sodium diffusion potential which was not considered and which will suggest an apical sodium dependency when none exists and, similarly, a lack of basal dependency when in fact one is present. Also, precautions were not taken to control for junction potentials arising at the agar-Ringer bridges during asymmetric changes of solutions. Thus, at present, the data indicate that the sodium dependence of chloride secretion is exerted solely at the basal side of chloride cells.

The sensitivity of the chloride secretion process to 'loop' diuretics (often assumed to inhibit Na-Cl or Na-K-Cl co-transport systems) has been examined. 1 mM-furosemide on both sides inhibits the short-current in the *Fundulus* opercular membrane by ~90% within a few minutes following exposure (Karnaky *et al.* 1977; Degnan *et al.* 1977; Mayer-Gostan & Maetz, 1980) as a result of an 80% decrease in chloride efflux (Mayer-Gostan & Maetz, 1980). Recent studies have demonstrated that furosemide may interact non-specifically with the plasma membrane since Na/K-ATPase activity, Na-dependent amino acid uptake and anion self-exchange are all inhibited in erythrocytes by 1 mM-furosemide (Palfrey, Feit & Greengard, 1980). Bumetanide, a derivative of furosemide which is two orders of magnitude more potent than furosemide and appears to be specific for NaCl cotransporters (Palfrey *et al.* 1980), on the basal side only rapidly inhibits the short-circuit current (−93%), net flux of chloride (−91%) and conductance (−39%) of the tilapia opercular membrane (J. K. Foskett, in preparation). Inhibition is primarily due to reduction of the secretory flux; whether or not bumetanide also blocks a cellular component of the backflux is unclear since it is an effect which could not be seen consistently because of a separate effect of bumetanide on the permeability of an extracellular pathway (as determined from mannitol fluxes).

The sodium-dependency and inhibition by 'loop' diuretics are consistent with a NaCl cotransport mechanism on the basolateral membrane as the chloride entry mechanism. An alternative mechanism for NaCl entry is the operation of parallel Na-H and Cl-HCO_3 exchangers (reviewed by Warnock & Eveloff, 1982) and, indeed, chloride transport by the *Fundulus* opercular membrane is dependent on the bath HCO_3 concentration (Degnan *et al.* 1977; Karnaky *et al.* 1977). However, there has been no demonstration of amiloride (to block Na-H exchange) or SITS (to block Cl-HCO_3 exchange) sensitivity of the short-circuit current of opercular membranes or skin. Carbonic anhydrase activity is not restricted to or more highly concentrated in chloride cells (see Haswell, Randall & Perry, 1980) and inhibition of carbonic anhydrase with acetazolamide has no effect on the short-circuit current in the *Fundulus* (Degnan *et al.* 1977) or tilapia (J. K. Foskett, unpublished observations) opercular membranes. However, the chloride secretory current in the *Gillichthys* skin is largely inhibited by serosal 5 mM-acetazolamide (Marshall, 1977). Since it is unlikely t the chloride secretion mechanism in skin chloride cells differs from that observed

in opercular membranes, it may be that at the concentration employed (5 mM), the drug interacts with the coupled NaCl transport mechanism to inhibit it competitively, as observed in rabbit ileum (Frizzell *et al.* 1973).

The sodium dependency and ouabain and 'loop' diuretic sensitivities of the chloride secretion process are consistent with a coupled NaCl entry mechanism at the basolateral membrane which is driven by the favourable sodium gradient maintained by the Na/K-ATPase. Unknown, however, are the nature of the sodium dependence, the involvement of other ions (e.g., potassium), or the stoichiometry, kinetics, electrogenicity and magnitudes of the driving forces. Vesicles prepared from basolateral membranes and measurements of intracellular ion activities may aid in answering these questions.

In spite of the sodium dependence of chloride secretion, there is no net sodium transport under short-circuit conditions in the opercular membranes or in skin (Table 1; Degnan *et al.* 1977; Mayer-Gostan & Maetz, 1980; Marshall, 1981*a*), suggesting that these fluxes are passive. These data are consistent with a model whereby following entry into the cell, sodium is recycled across the basolateral membrane by the Na/K-ATPase and chloride leaves the cell by pathways in the apical membrane (see Fig. 5). Under open-circuit conditions, when the serosal solution becomes positive by 20–30 mV, sodium efflux increases and influx decreases, yielding a net efflux (Mayer-Gostan & Maetz, 1980; Marshall, 1981*a*). The sodium flux-ratio under these conditions does not differ from that predicted for a passive, independently moving ion (Marshall, 1981*a*; data from Mayer-Gostan & Maetz, 1980). Similarly, sodium fluxes conform to the flux-ratio equation in open circuit in the presence of a high mucosal NaCl concentration and following subsequent inhibition by ouabain (Degnan & Zadunaisky, 1979). Evidence has also been presented that unidirectional sodium fluxes respond to voltages as predicted for independent, diffusive ion movement through a single rate-limiting barrier (Degnan & Zadunaisky, 1980). However, it was not determined whether the partial ionic conductance of sodium was independent of voltage, as required for this analysis (Schultz & Zalusky, 1964). Voltage clamping changes the conductance of tilapia opercular membrane (Foskett *et al.* 1982*b*) and *Gillichthys* skin (Marshall, 1981*b*), so it is not clear that the necessary criteria for this analysis were fulfilled. However, the flux-ratio data do suggest that sodium fluxes are passive and diffusive.

The magnitude of the unidirectional sodium fluxes is large, and removal of sodium from the bathing media results in large decreases in tissue conductance in all three tissues (Degnan & Zadunaisky, 1980; Marshall, 1981*a*; J. K. Foskett, unpublished observations), as expected for a diffusive flux. As this manipulation also abolishes chloride secretion, these data do not permit evaluation of the contribution of sodium to a leak conductance, since changes in cellular active pathway conductance are also likely. In the tilapia opercular membrane, tissue-to-tissue variability describes indistinguishable linear relationships between each unidirectional sodium flux J_{Na} under short-circuit conditions and the tissue conductance G_t, suggesting that influx and efflux are by the same pathway (J. K. Foskett, in preparation). The pooled data are defined by $J_{Na} = 0{\cdot}52G_t - 0{\cdot}28$. For a monovalent ion at equilibrium whose movements are passive and independent, the unidirectional flux is a measure of its partial ionic conductance (Hodgkin, 1951). Thus sodium appears to contribute an aver-

~50 % to the total tissue conductance. A nearly identical relationship has been observed in the *Fundulus* membrane (Degnan & Zadunaisky, 1980). This linear relationship suggests that the sodium fluxes are not associated with constant non-tissue-specific pathways (i.e., edge damage); the sodium leak conductance must, therefore, be associated with the chloride cells since these are the only sites of appreciable conductance in the epithelium (Foskett & Scheffey, 1982). Thus, sodium appears to move conductively through a leak pathway associated specifically with chloride cells, the magnitude of which represents ~50 % of the chloride cell conductance. Since the conductance determined with the vibrating probe for an average chloride cell is ~580 mS cm^{-2} (see above), the sodium conductance associated with a typical chloride cell is ~290 mS cm^{-2}, representing a flux of nearly 300 μequiv cm^{-2} h^{-1}, or a sodium permeability of the chloride cell $P = 5{\cdot}2 \times 10^{-4}$ cm s^{-1}.

The anatomical location of this prodigious leak pathway is presumed to be the chloride cell tight-junctional pathway. Freeze-fracture observations indicate that chloride cells make two different kinds of tight junctions, depending on the cell type with which it is made: junctions with surface pavement cells are deep, multi-stranded and resemble the tight junctions between pavement cells; tight junctions between adjacent chloride cells or between chloride cells and so-called accessory cells are shallow and single or double-stranded (Sardet, Pisam & Maetz, 1979; Ernst, Dodson & Karnaky, 1980; Laurent & Dunel, 1980; Kawahara, Sasaki & Higashi, 1982). Accessory cells closely resemble chloride cells, although there are subtle ultrastructural differences and accessory cells do not show Na/K-ATPase activity (reviewed by Hootman & Philpott, 1980 and by Laurent & Dunel, 1980). These thin and sheet-like or ovoid cells, closely apposed to the sides of nearly every chloride cell (see Sardet *et al.* 1979), send slender cytoplasmic processes into the apical crypt, resulting in a greatly amplified perimeter of shallow tight junctions associated with the chloride cell apical crypt (Sardet *et al.* 1979; Dunel-Erb & Laurent, 1980; Ernst *et al.* 1980; Hootman & Philpott, 1980; Laurent & Dunel, 1980).

Correlations observed in other epithelia between tight-junctional structure and permeability (Claude & Goodenough, 1973; Claude, 1978) have led to the speculation that the chloride cell-accessory cell junctions represent the chloride cell leak pathway. That the junctions between pavement cells are indeed electrically tight is supported by conductance measurements of these cells with the vibrating probe (Foskett & Scheffey, 1982; Scheffey *et al.* 1983). Thus, the question becomes whether the chloride cell-accessory cell tight junction is electrically leaky and represents the chloride cell leak pathway. As pointed out by Degnan & Zadunaisky (1980), the sodium flux data are also consistent with sodium movement through the chloride cell if one membrane has a much higher permeability than the other. Since intracellular voltage and ion activity are unknown, this remains a possibility. Degnan & Zadunaisky (1980) interpreted lack of effects of amiloride and amphotericin B on unidirectional sodium fluxes as evidence against a cellular route for sodium. However, cellular sodium conductance is amiloride-insensitive in fish urinary bladder (Loretz & Bern, 1983) and other tissues (Will, Lebowitz & Hopfer, 1980; Thomas, Jallageas, Munck & Skadhauge, 1980) and a lack of effect of amphotericin B may simply suggest no plasma membrane penetration. Inhibition of a substantial fraction of the unidirec-nal sodium fluxes in the *Fundulus* opercular membrane by triaminopyrimidine

(TAP) has also been interpreted as evidence for a paracellular pathway for sodium (Degnan & Zadunaisky, 1980; Ernst *et al.* 1980; Evans *et al.* 1982), but this conclusion may not be completely warranted. Although TAP was originally (Moreno, 1975) proposed as a specific blocker of cation-selective paracellular pathways in leaky epithelia, it may also have a multiplicity of other effects, including an amiloride-like inhibition of membrane sodium conductance (Lewis & Diamond, 1976; Balaban *et al.* 1979; Fanestil & Vaugh, 1979), inhibition of membrane potassium conductance (Reuss & Grady, 1979) and inhibition of NaCl co-transport (Frizzell, Smith & Field, 1981). The observed effect of TAP on the opercular membrane may reflect one or more of these effects. Whereas TAP did reduce sodium fluxes, the short-circuit current was also greatly reduced, the tissue conductance was reduced by more than the partial ionic conductance of sodium, and the percent contribution of sodium to the tissue conductance was unaltered, all unexpected if TAP was blocking only a paracellular sodium conductance. These inhibitory effects are not observed when TAP is placed only on the apical side (J. K. Foskett, unpublished observations), suggesting that TAP may interact with the basolateral potassium conductance and/or the coupled NaCl entry step.

Some other data may not be consistent with a highly permeable paracellular pathway for sodium. Bumetanide and ouabain inhibit similarly both unidirectional sodium fluxes while simultaneously increasing the unidirectional fluxes of the extracellular space marker mannitol (J. K. Foskett, in preparation). Ouabain affects only one unidirectional sodium flux in the *Fundulus* opercular membrane (Degnan *et al.* 1977; Mayer-Gostan & Maetz, 1980). There is a linear relationship between sodium conductance and tissue conductance but there is no relationship between urea (Degnan & Zadunaisky, 1980) or mannitol (J. K. Foskett, in preparation) fluxes and the tissue conductance, or between simultaneously measured sodium and mannitol fluxes (J. K. Foskett, in preparation). These data may have more to do with the selectivity of the sodium pathway than its location.

Our present ideas about the movements of sodium across the opercular membrane can be summarized as follows: passive, conductive fluxes are associated specifically with the chloride cells and they are rate-limited by a single membrane. Although our intuition tells us that sodium probably traverses paracellular, junctional pathways, the specific anatomical location of the chloride cell leak pathway remains an unresolved and important question.

RAPID HORMONAL REGULATION OF CHLORIDE SECRETION

The use of the chloride cell-containing skin and opercular membrane preparations has been especially advantageous for examining the direct effects of rapidly-acting hormones since they can be studied free of possible haemodynamic and other effects. It now appears that chloride cells are under multiple hormonal control. The list of hormones and possible intracellular messengers now known to affect rapidly chloride secretion has grown to include catecholamines, somatostatin, glucagon, vasoactive intestinal polypeptide (VIP), urotensins I and II and cAMP.

Epinephrine and norepinephrine rapidly inhibit the short-circuit current in the opercular membranes and skin as the result of specific reduction in the chloride

secretory flux (Degnan *et al.* 1977; Marshall & Bern, 1980; Foskett, Hubbard, Machen & Bern, 1982*a*; Mendelsohn, Cherksey & Degnan, 1982). Vibrating probe measurements suggest that all chloride cells in the tilapia opercular membrane respond to epinephrine, and with a time-course identical to the time-course of inhibition of tissue short-circuit current (J. K. Foskett & T. E. Machen, in preparation). Epinephrine inhibition is by α-receptors and is dose-dependent with a minimum effective dose of ~1 nM, a half-maximum effect at ~200 nM and a maximum inhibition at 1–10 μM of nearly 80% (Marshall & Bern, 1980; Foskett *et al.* 1982*a*), suggesting that epinephrine may regulate chloride cells *in vivo*. The intracellular second messenger is unknown. The tilapia opercular membrane appears to transport normally at maximum rates and cannot be further stimulated by phosphodiesterase inhibition (Foskett *et al.* 1982*b*). However, phosphodiesterase inhibition almost completely reverses the inhibitory effects of epinephrine (Foskett *et al.* 1982*a*), suggesting that epinephrine may act by lowering cAMP levels. Mendelsohn *et al.* (1982) reported no effect of α-adrenergic activation on cAMP levels in the *Fundulus* opercular membrane. However, their tissue incubations were always in the presence of a phosphodiesterase inhibitor which can reverse α-inhibition; so it remains possible that epinephrine inhibits chloride secretion by lowering cAMP levels. Ca^{2+} and cGMP may not be involved since epinephrine is equally effective in Ca^{2+}-free media (Mendelsohn *et al.* 1982; J. K. Foskett, unpublished observations) and in the presence of 0·1 mM-trifluoperazine to block calmodulin (Mendelsohn *et al.* 1982), and since exogenous cGMP is without effect on short-circuit current (Mendelsohn *et al.* 1982).

The teleost caudal neurosecretory system synthesizes two peptides, urotensins I and II (UI and UII). Marshall & Bern (1979) discovered that UII, a dodecapeptide resembling somatostatin (Pearson *et al.* 1980), inhibits the short-circuit current by ~30% and increases the resistance of the *Gillichthys* skin, as a result of a reduction in the chloride efflux (Marshall & Bern, 1981). Inhibition can be reversed by phosphodiesterase inhibitors and UI (Marshall & Bern, 1979, 1981). The tilapia opercular membrane can also be inhibited by UII, but at much higher concentrations (Loretz, Bern, Foskett & Mainoya, 1981).

Somatostatin rapidly inhibits the short-circuit current and reduces tissue conductance in the tilapia opercular membrane in a dose-dependent fashion with a minimum effective dose of 10 nM and 65% inhibition of the current at 1 μM (Foskett & Hubbard, 1981). *Gillichthys* skin responds similarly except that the inhibited current spontaneously reverses (J. K. Foskett, unpublished observations). Somatostatin does not block glucagon and VIP stimulation of epinephrine-inhibited tissues, in contrast to its effects on VIP-stimulated chloride secretion in the shark rectal gland (Stoff *et al.* 1979). Somatostatin-inhibited short-circuit current can be reversed by phosphodiesterase inhibition and also by glucagon, suggesting that somatostatin and epinephrine may act *via* a final common pathway (reduction of cAMP levels?) to inhibit chloride secretion.

Stimulation of chloride secretion by increased levels of cAMP appears to be a characteristic response of all chloride-secreting epithelia (see review by Frizzell, Field & Schultz, 1979). Phosphodiesterase inhibition increases intracellular cAMP levels in the *Fundulus* opercular membrane (Mendelsohn *et al.* 1982) and stimulates the

short-circuit current in the *Fundulus* opercular membrane and *Gillichthys* s[illegible] (Degnan *et al.* 1977; Marshall & Bern, 1979, 1981) and in epinephrine-inhibited tilapia opercular membranes (Foskett *et al.* 1982*a*). Chloride secretion in the *Fundulus* opercular membrane (Degnan & Zadunaisky, 1979) and *Gillichthys* skin (Marshall & Bern, 1980), but not in the tilapia opercular membrane (Foskett *et al.* 1982*a*), is also stimulated by β-adrenergic agonists which increase exclusively the chloride efflux (Degnan & Zadunaisky, 1979) and is associated with elevated cAMP levels (Mendelsohn *et al.* 1982). The physiological significance of a β-adrenergic stimulation is unclear since the dominant catecholamine in fish is epinephrine (see Foskett *et al.* 1982*a* for references) which inhibits chloride secretion via α-receptors. However, several naturally-occurring hormones do stimulate chloride secretion by chloride cells. *Gillichthys* skins previously inhibited with epinephrine or with spontaneously low short-circuit currents respond to urotensin I (UI), a 41-amino acid peptide from the caudal neurosecretory system (Lederis *et al.* 1982), with rapid increases in short-circuit current (Marshall & Bern, 1981). UI raises cAMP in other tissues (Gerritsen & Lederis, 1976; Gerritsen, Mathison & Lederis, 1977), and the response of the *Gillichthys* skin is similar to the response to phosphodiesterase inhibition, suggesting that UI acts *via* elevated cAMP levels. Interestingly, UI inhibits chloride secretion by the tilapia opercular membrane, demonstrating the possibility of species differences in the response of chloride cells to hormones and other perturbations (Loretz *et al.* 1981).

Glucagon and VIP stimulate chloride secretion in tilapia opercular membranes previously inhibited with epinephrine (Foskett *et al.* 1982*a*). Both hormones stimulate cAMP in other tissues (see Foskett *et al.* 1982*a*), and the effects of both on the opercular membrane are mimicked and optimized, respectively, by high and low doses of phosphodiesterase inhibitors, suggesting that VIP and glucagon stimulate chloride secretion by elevating intracellular cAMP. The blood concentration of glucagon is unknown in teleosts but plasma levels of immunoreactive-VIP concentrations are similar to the high levels measured in dogfish (Stoff *et al.* 1979; Holstein & Humphrey, 1980). The minimum effective dose of glucagon is 1 nm, and the dose-dependency is similar to that for VIP-stimulated chloride secretion in the shark rectal gland (Stoff *et al.* 1979) suggesting that these hormones may be physiological regulators of chloride cell secretion, as has been suggested for VIP in the regulation of rectal gland chloride secretion (Stoff *et al.* 1979).

It has been proposed that cAMP stimulates chloride secretion in epithelia by a specific enhancement of apical membrane chloride conductance (reviewed by Frizzell *et al.* 1979). In support of this, phosphodiesterase inhibition (Degnan *et al.* 1977) and β-adrenergic agonists (Degnan & Zadunaisky, 1979) cause conductance of the *Fundulus* opercular membrane to increase. Foskett *et al.* (1982*a*) have proposed that cAMP may also stimulate chloride secretion by an effect on an electrically neutral mechanism. Analysis of the time-courses of current and conductance following epinephrine, phosphodiesterase inhibitors, or glucagon demonstrated that large changes in current and conductance are not always coupled. It was proposed that epinephrine may inhibit neutral NaCl entry and that increased cAMP following hormone (VIP, glucagon) treatment and phosphodiesterase inhibition may stimulate chloride secretion by acting on the same entry mechanism. It may be that alteratio[illegible]

ellular cAMP can have effects at both the basolateral entry step (presumably neutral and therefore nonconductive) and apical chloride conductance channel (Fig. 5).

DIFFERENTIATION OF THE CHLORIDE EXTRUSION MECHANISM

Time course of development

In contrast to the situation in sea water, the gills in freshwater-adapted fish absorb NaCl (albeit at low rates compared with the secretion rates in sea water). NaCl uptake appears to be the result of parallel amiloride-sensitive Na-H and/or Na-NH_4 and SITS-sensitive Cl-HCO_3 exchangers which reside in the gill secondary lamellae (reviewed by Evans, 1980*a,b,c*). Kirschner (1979*b*) has suggested that these uptake exchangers are ancient mechanisms for life in fresh water but appear to have originated in marine ancestors as acid-base regulatory mechanisms. Recently it has become apparent that the Na-H, Cl-HCO_3 exchangers continue to operate in seawater-adapted fish (Evans, 1980*b*, 1982). It can be argued, therefore, that the main difference in branchial function between freshwater and seawater fish is the transport activity of one cell type, the chloride cell. One requirement for euryhalinity, therefore, may be the ability to turn the chloride cell on and off. This may be by some of the rapidly-acting hormones (discussed above) for fish which make short excursions into different salinities. For euryhaline fish which spend prolonged periods in each environment, however, turning the chloride cell on and off appears to involve differentiative processes.

Morphological alterations in the branchial epithelium during seawater adaptation appear to be restricted to changes in the chloride cells. In most species examined, salt adaptation includes augmentation of the chloride cell population and/or chloride cell hypertrophy (as cited above). Chloride cell hypertrophy is associated with increased synthesis and incorporation of plasma membrane into the basolateral membrane tubular system which is paralleled by increases in the activity of Na/K-ATPase localized to the basolateral membrane (as described and cited above). The mitochondria become increasingly differentiated, as evidenced by increased activity of mitochondrial enzymes (Shirai & Utida, 1970; Sargent *et al.* 1975). The apical membrane forms a crypt which becomes associated with the interdigitating arms of accessory cells (Sardet *et al.* 1979; Laurent & Dunel, 1980).

The isolated opercular membrane from tilapia has proved to be an excellent model for studying the electrophysiological correlations of differentiation of the teleost salt extrusion mechanism. Tilapia is a broadly euryhaline species, but unlike *Fundulus* and *Gillichthys*, which only periodically make short excursions into dilute water, normally lives in fresh water. In contrast to the situation in sea water, opercular membranes removed from freshwater-adapted tilapia have a high transepithelial resistance ($>3000\,\Omega cm^2$: in the range of so-called 'tight' epithelia), a negligible short-circuit current and small transepithelial voltage (Foskett *et al.* 1981). Unidirectional chloride influx and efflux are only 1/3 and 1/13, respectively, of those in seawater membranes, with the result that there is no net chloride flux. Similar differences in ion permeability between freshwater and seawater fish have been consistently observed in whole animal studies (see Maetz, 1974).

Differences in chloride cell ultrastructure are the only obvious morphological differences between opercular membranes from tilapia in each media (Foskett *et al.* 1981). The chloride cells in the opercular membrane in fresh water lack all the characteristics of seawater chloride cells. They are small and poorly developed; there are fewer, smaller, and less developed mitochondria; the tubular system appears rudimentary; contact with the surface is absent or minimal – an apical crypt is never present; and associations with accessory cells are never observed.

Following transfer to sea water, the chloride current is activated within 24 h and continues to increase to fully-adapted levels after 1–2 weeks (Fig. 4; Foskett *et al.* 1981). This first clear demonstration of changes in net branchial salt secretion during teleost seawater adaptation reflects similar changes in unidirectional ion fluxes measured in several species *in vivo* (see Maetz, 1974). Changes in the number and size of the mitochondria-rich chloride cells were followed concomitantly during seawater adaptation, using a fluorescent probe specific for mitochondria (see Fig. 4; Foskett *et al.* 1981). Interestingly, the number of chloride cells appears to increase only during the first 3 days in sea water (Fig. 4). These cells appeared to be 'immature' chloride cells since their size did not increase during this time (Fig. 4). Subsequent chloride cell hypertrophy after 3 days in sea water, which appears to be due to increased basolateral membrane elaboration, accompanied by increased levels of transport enzymes, is highly correlated with the amount of chloride secretion by each cell (Foskett *et al.* 1981). Thus, it appears that development of chloride extrusion involves two phases: first, an activation and augmentation of the 'immature' chloride cell population; subsequently, after 3 days in sea water, increased differentiation of these cells appears to be responsible for enhancement and maintenance of high chloride secretion rates. The result is the transformation of the epithelium from a

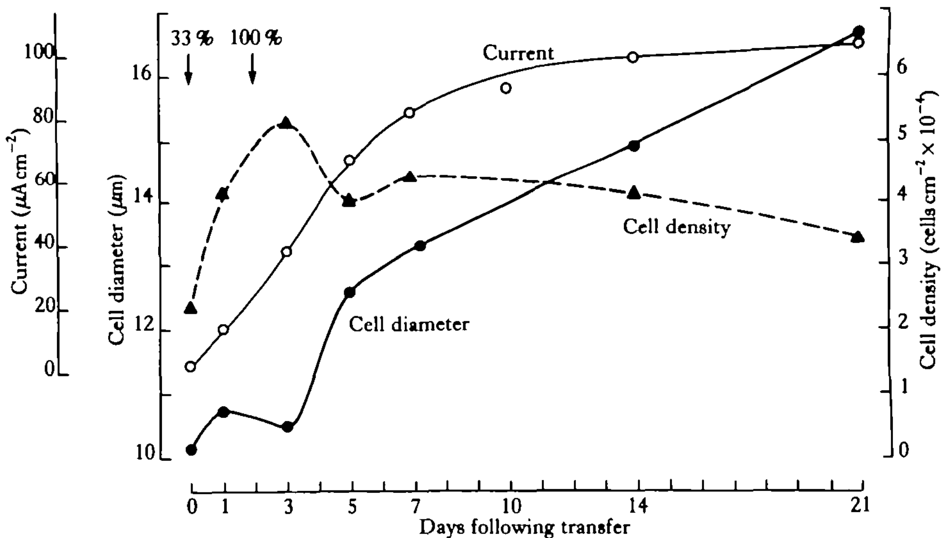

Fig. 4. Time-course of changes in chloride secretion (measured as the current; ○), chloride cell density (▲) and chloride cell diameter (●) during the 3 weeks following transfer of freshwater-adapted tilapia to sea water. The fish were transferred to 33 % seawater on day 0 and subsequently transferred to full-strength (100 %) seawater on day 2 (from Foskett *et al.* 1981).

tively impermeable, non-transporting tissue in fresh water to one in sea water dominated by cells with some of the highest ionic permeabilities and transport rates ever recorded.

Roles for cortisol and prolactin in controlling chloride cell differentiation

The nature of the stimuli which induce these morphological and physiological changes following seawater transfer is largely unknown. Hormonal (Doyle & Epstein, 1972; Kamiya, 1972*a*) and neural (Mayer-Gostan & Hirano, 1976) initiation has been proposed, as well as direct induction by increased blood ionic or osmotic concentrations (Mayer & Nibelle, 1970; Olivereau, 1970) or by osmotically active substances in the environment (Liu, 1944).

Interrenalectomy reduces branchial sodium efflux (Mayer *et al.* 1967; Maetz, 1969) and hypophysectomy reduces branchial sodium efflux and Na/K-ATPase activity in seawater fish, and cortisol, the dominant corticoid in fish (Henderson, Chan, Sandor & Chester Jones, 1970), can restore both towards normal (Maetz, 1969; Pickford *et al.* 1970*a*; Milne *et al.* 1971; Butler & Carmichael, 1972; Kamiya, 1972*b*). Cortisol might, therefore, play an important role in chloride cell differentiation. A series of cortisol injections into freshwater-adapted tilapia results in a nearly three-fold increase in the size of the population of 'immature' chloride cells in the opercular membrane (Foskett *et al.* 1981). Cortisol also stimulates chloride cell differentiation (Doyle & Epstein, 1972) and Na/K-ATPase activity (Epstein *et al.* 1971; Forrest *et al.* 1973; Kamiya, 1972*b*) in the branchial epithelium in fresh water. Interestingly, although cortisol induced more chloride cells in the tilapia opercular membrane, the electrophysiological properties of the tissue are unchanged and chloride secretion is not initiated (Foskett *et al.* 1981). Similarly, cortisol treatment of the freshwater eel enhances gill Na/K-ATPase levels but not sodium efflux (Forrest *et al.* 1973). Why salt secretion is not initiated is unknown but may reflect incomplete development of specific transport pathways (e.g., NaCl co-transporter, apical membrane chloride permeability) or lack of apical exposure to the epithelial surface (Doyle & Epstein, 1972; Foskett *et al.* 1981).

Clearly, these data indicate that other factors besides cortisol must be involved in the complete differentiation of the functional chloride cell. One possibility is that fresh water itself or other hormones are antagonistic to cortisol. Along these lines, a major hormonal modification following seawater transfer of teleosts is a drastic reduction in prolactin secretion (Dharmamba & Nishioka, 1968; Nicoll, Farmer, Nishioka & Bern, 1981) and it may be that the high blood levels of prolactin in freshwater animals block initiation of salt secretion. This idea is supported by a recent series of experiments which demonstrated that although *in vitro* exposure of the opercular membrane to prolactin has no effect within 1 h (Bern *et al.* 1981), several prolactin injections into seawater-adapted tilapia cause substantial parallel inhibitions of chloride secretion and conductance of the opercular membrane (Foskett *et al.* 1982*b*). Similarly, transplantation of the prolactin-cell rich rostral pars distalis into hypophysectomized *Gillichthys* reduces the current and conductance of the skin (Marshall & Bern, 1980).

In an attempt to understand how prolactin inhibits chloride secretion, the tilapia rcular membrane has been modelled as a simple electrical equivalent circuit which

incorporates the effects of a heterogeneous cell population (Foskett *et al.* 1982*b*). This analysis suggested that the normal tissue-to-tissue variability in chloride secretion rates and conductances observed among opercular membranes from different fish was due to variability in the size of the chloride cell population and/or to parallel changes in the active and leak pathway conductances associated with a constant chloride cell population. Variability among tissues in chloride secretion rates has been correlated with the number of chloride cells in the *Gillichthys* skin (Marshall & Nishioka, 1980). Recent vibrating probe studies of the tilapia opercular membrane have demonstrated that variability among chloride cells within a single tissue describes a linear relationship between chloride cell current and conductance, which can best be explained by postulating the existence of a link between the magnitudes of the chloride cell active (cellular) and leak (paracellular?) pathway conductances (J. K. Foskett & T. E. Machen, in preparation). This sort of relationship between cellular and leak pathways may explain the linear relationship between sodium flux and tissue conductance (Degnan & Zadunaisky, 1980; J. K. Foskett, in preparation) and between sodium flux and short-circuit current (J. K. Foskett, in preparation). The nature of this link is unknown, but if the magnitude of the leak pathway is determined by the linear perimeter of the tight junction between the chloride cell and accessory cells (as previously discussed), then increased differentiation of the chloride cell and the chloride cell-accessory cell relation would result in parallel changes in chloride secretion and junctional permeability.

Prolactin appears to inhibit chloride secretion by reducing both the active and leak pathway conductances proportionally, so that the ratio between them is always constant (Foskett *et al.* 1982*b*). These effects are most consistent with the view that prolactin causes a dedifferentiation of the chloride cell population. This might be by reducing the size of the population, possibly by blocking differentiation of new cells to replace those which have worn out or by otherwise 'removing' chloride cells from the tissue (perhaps by preventing exposure to the surface), or by causing dedifferentiation of the existing cells by reducing the magnitudes of both the active and shunt pathways.

This interpretation is consistent with observations that prolactin injections into seawater-adapted fish increase blood ion concentrations (Potts & Fleming, 1971; Utida, Hatai, Hirano & Kamemoto, 1971*a*; Fleming & Ball, 1972; Clarke, 1973; Dharmamba, Mayer-Gostan, Maetz & Bern, 1973; Olivereau & Lemoine, 1973; Johnson *et al.* 1974) and decrease branchial sodium efflux (Potts & Fleming, 1971; Fleming & Ball, 1972; Dharmamba & Maetz, 1976) and branchial Na/K-ATPase levels (Pickford *et al.* 1970*b*; Gallis, Lasserre & Belloc, 1979). Freshwater-adaptation also results in a dedifferentiation of the chloride cell population, reflected by changes in cell density and morphology (Shirai & Utida, 1970; Utida *et al.* 1971*b*) and Na/K-ATPase levels (Utida *et al.* 1971*b*; Kamiya, 1972*b*; Hossler *et al.* 1979; Epstein *et al.* 1980) generally opposite to those induced by seawater adaptation. Similarly, freshwater-adaptation results in a disappearance of accessory cells (Laurent & Dunel, 1980). Thus, the effects of prolactin, as determined from electrophysiological and tracer techniques, are similar to those induced by freshwater adaptation. These data suggest that the enhanced secretion and blood levels of prolactin which accompany transfer to fresh water might be important in shutting down the salt extrusion mechanism in chloride cells, and might explain why the reduction

Table 2. *Effects of environment and hormone treatment on initiation of chloride secretion (= short-circuit current) by chloride cells in the tilapia opercular membrane*

Experiment number	Treatment	Short-circuit current ($\mu A\ cm^{-2}$)	Resistance ($\Omega\ cm^2$)	Transepithelial voltage (mV)	N
1	FW (5D)	2·4 ± 0·3	2036 ± 250	4·9 ± 0·6	5
	1/3 SW (5D)	32·7 ± 7·3	751 ± 175	20·9 ± 3·0	5
2	FW (5D)	1·0 ± 0·1	3067 ± 456	3·0 ± 0·6	5
	300 mM-mannitol (5D)	0·7 ± 0·1	4276 ± 567	3·0 ± 0·9	5
3	FW (5D)	0·9 ± 0·3	2366 ± 566	1·9 ± 0·8	5
	150 mM-NaCl (5D)	5·5 ± 0·9	1245 ± 127	7·2 ± 1·8	5
4	150 mM-NaCl (5D)	7·9 ± 1·4	2063 ± 385	14·5 ± 1·5	5
	1/3 SW (5D)	16·3 ± 6·7	1412 ± 287	16·4 ± 1·9	5
5	150 mM-NaCl + 10 mM-Ca-gluconate (5D)	8·3 ± 3·1	1448 ± 423	8·4 ± 1·4	5
	150 mM-NaCl + 30 mM-mannitol (5D)	9·0 ± 2·6	1548 ± 464	10·6 ± 2·0	5
6	150 mM-NaCl (5D)	18·3 ± 0·8	882 ± 24	16·0 ± 0·9	5
	300 mM-mannitol (5D)	0·7 ± 1·0	2968 ± 68	2·1 ± 0·4	3
	5 mM-Ca-gluconate (5D)	2·1 ± 1·0	1320 ± 259	2·8 ± 0·8	4
7	Cortisol inj (9D), 150 mM-NaCl (5D)	11·9 ± 1·8	1674 ± 107	19·3 ± 2·0	4
	Saline inj (9D), 150 mM-NaCl (5D)	1·4 ± 0·9	6805 ± 852	8·7 ± 2·0	5
8	Cortisol inj (9D), Hypox + 150 mM-NaCl (5D)	20·1 ± 6·4	1104 ± 134	20·0 ± 1·8	5
	Cortisol inj (9D), Sham + 150 mM-NaCl (5D)	24·1 ± 5·2	977 ± 205	19·4 ± 4·0	5
9	Hypox (2-5D)	1·4 ± 0·2	3370 ± 462	4·1 ± 1·5	4
	Hypox + RPD (5D)	1·0 ± 0·3	4100 ± 1170	3·4 ± 1·1	4
10	Cortisol inj (11D), Hypox (4D)	1·2 ± 0·6	4928 ± 993	5·4 ± 1·7	2

Previously freshwater – adapted tilapia were maintained individually in appropriate solutions (D = days of treatment). Cortisol injections (inj) were 5 μg (g body wt)$^{-1}$ day^{-1}. Hypophysectomy (Hypox) was according to Nishioka (1980). RPD is rostral pars distalis re-implantation (Expt 9). N = number of animals.

branchial sodium efflux which normally accompanies freshwater transfer is impaired in hypophysectomized fish (as in Dharmamba *et al.* 1973; Maetz, Sawyer, Pickford & Mayer, 1967; MacFarlane & Maetz, 1974).

Prolactin is required for survival of many euryhaline teleost species, including tilapia, in fresh water (reviewed by Ensor, 1978; Clarke & Bern, 1980; Loretz & Bern, 1982). Death in fresh water in the absence of prolactin appears to be due to salt depletion (see Ensor, 1978 and Clarke & Bern, 1980). In view of the data discussed above, it was possible that in hypophysectomized fish the absence of prolactin causes differentiation of the salt extrusion mechanism, even though the fish is still in fresh water. We have tested this possibility in a series of experiments shown in Table 2. The electrophysiological properties of opercular membranes removed from hypophysectomized, freshwater-adapted tilapia (expt no. 9) are unmodified compared with sham and normal freshwater membranes (see expts nos 1, 2, 3). Thus, hypophysectomy does not induce chloride cell differentiation, and the pituitary does not appear to be required for maintenance of epithelial ion impermeability in fresh water. These data suggest that prolactin has two distinct effects on the branchial epithelium, which depend on the adaptation medium: an effect on chloride cells in sea water and another

effect elsewhere in fresh water which is required for survival. The ability of prolactin to maintain blood ion levels and survival in fresh water may be related to an effect on water permeability since prolactin decreases osmotic water permeability in isolated gills (Lam, 1969; Ogawa, Yagasaki & Yamazaki, 1973; Wendelaar Bonga & Van Der Meij, 1980), and general cell hydration induced by hypophysectomy (Chan, Jones & Mosley, 1968; Loretz, 1979) can be prevented by prolactin (Chan *et al.* 1968).

Other experiments in Table 2 were designed to test the involvement of cortisol in seawater adaptation. A group of tilapia were hypophysectomized to remove prolactin and injected with cortisol, but again chloride secretion was not initiated (expt no. 10). Thus, although cortisol and prolactin appear to be involved in chloride cell differentiation, still other factors, probably associated with the environment, are necessary to initiate salt secretion. The stimulus is probably not increased medium osmotic pressure since transfer to isosmotic sea water stimulates secretion (Foskett *et al.* 1981; Table 2, expt no. 1) but transfer to isosmotic mannitol does not (expts nos 2, 6).

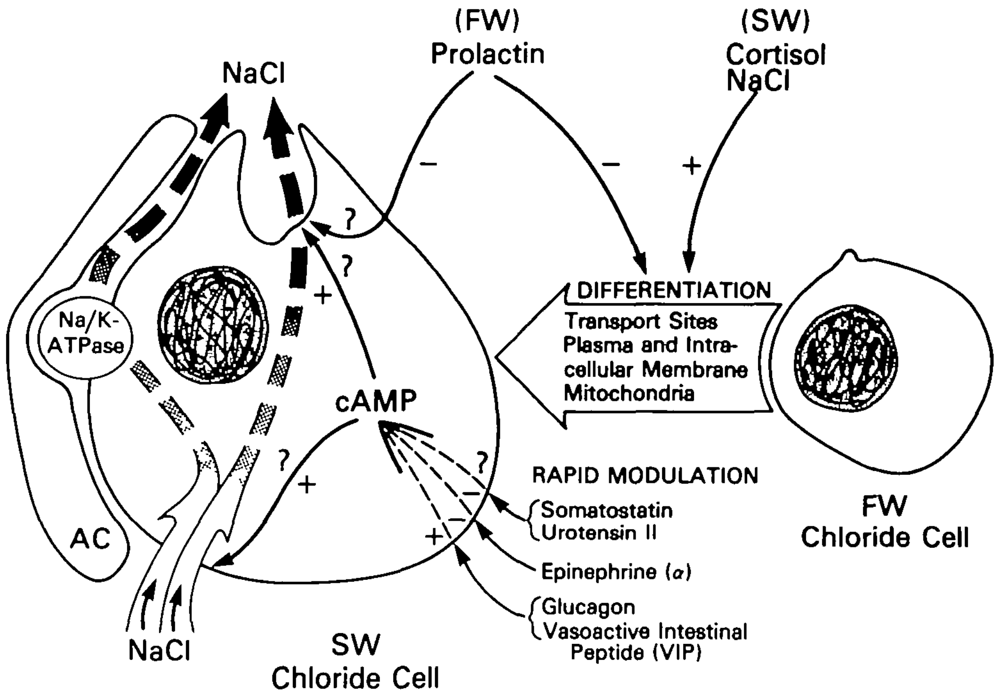

Fig. 5. Summary representation of the regulation of NaCl extrusion by chloride cells. Cortisol and environmental NaCl enhance, and prolactin inhibits, chloride cell differentiation. Differentiation is reflected in membrane and organelle development, the appearance of new transport sites, formation of an apical crypt and development of accessory cells (AC). Ouabain-sensitive Na/K-ATPase on the basolateral membrane maintains low intracellular Na, establishing a gradient which drives uphill movement of Cl into the cell *via* a bumetanide-sensitive NaCl entry mechanism. Chloride exit is down its electrochemical gradient *via* apical membrane conductive pathways; Na transport is driven by the serosa-positive voltage, and probably traverses the chloride cell-accessory cell tight junctional pathway. Several hormones rapidly alter the rate of chloride secretion in differentiated chloride cells: epinephrine (*via* α-receptors), somatostatin and urotension II inhibit, and glucagon and VIP stimulate. Increased intracellular cAMP is associated with enhanced chloride secretion; these hormones may act by modifying these levels. cAMP may ultimately act to modify transport by effects on the basolateral NaCl entry step and the apical Cl exit step.

transfer to 150 mM-NaCl is sufficient to initiate chloride secretion (expts nos 3, 6) but it appears that isosmotic sea water is more effective (expt no. 4), an effect which does not appear to be associated solely with the elevated Ca^{2+} in sea water (expt no. 5). Likewise, external Ca^{2+} does not appear to be a trigger by itself (expt no. 6). Although cortisol injections, either in the presence (Foskett *et al.* 1981) or absence (expt no. 10) of the pituitary, cannot initiate salt secretion in fresh water, cortisol does appear to potentiate the stimulatory effect of 150 mM NaCl (expt no. 7), the magnitude of which appears to be independent of other pituitary influences (expt no. 8). These results suggest that differentiation of chloride cells and chloride secretion probably requires a complex interaction between NaCl and hormones, especially cortisol. Fig. 5 summarizes our knowledge of the control and differentiation of chloride secretion by the chloride cell.

DIFFERENTIATION OF OTHER TELEOST OSMOREGULATORY SURFACES

The effects of prolactin, cortisol and the external environment on branchial salt transport represent a type of regulation of transport which is relatively unstudied and not well understood and involves trophic effects which generally take a few to several days to achieve. In the case of the opercular membrane, these trophic effects involve a combination of hyperplasia, hypertrophy, and differentiation of a single cell type which results in biochemical, morphological and physiological adaptation of the tissue to a chronic environmental and physiological condition. As a result, a particular 'epithelial fabric' is laid down which performs the necessary transport functions within the particular osmoregulatory context. It is on this 'epithelial fabric' that more fast-acting control mechanisms operate acutely to modify transport. Since osmoregulation requires the integrated activities of several effectors, it is likely that those trophic agents responsible for differentiation of branchial transport mechanisms will be the same for the other epithelia involved. There is an abundance of evidence which suggests that this is the case in teleosts, where prolactin and cortisol appear to have differentiative effects in the branchial, gut and renal systems.

Oesophagus

The seawater eel oesophagus is highly permeable to NaCl, but effectively impermeable to water, which allows it to 'de-salt' the ingested sea water before it reaches the intestine. The oesophagus from the freshwater eel has a similarly low water permeability but, in contrast to the seawater condition, also has a low NaCl permeability (Kirsch, Guinier & Meens, 1975; Hirano & Mayer-Gostan, 1976; Kirsch, 1978; Hirano, 1980). Following seawater transfer, the NaCl permeability is enhanced with a time course similar to that described earlier for chloride secretion by the opercular membrane (Kirsch *et al.* 1975; Hirano, 1980). Increased NaCl permeability can be induced in the freshwater oesophagus by a series of cortisol injections, and decreased in sea water by a series of prolactin injections (Hirano, 1980). Seawater adaptation is associated with a profound reorganization of the epithelium: a mucous cell-rich, stratified epithelium in fresh water is replaced by a columnar epithelium free of mucous cells in sea water (Laurent & Kirsch, 1975; Yamamoto & Hirano, 1978).

Thus, it is likely that the changes in NaCl permeability induced by cortisol and prolactin are the result of their effects on cell differentiation, as in the gill.

Intestine

Generally, water and salt absorption rates, osmotic water permeability and Na/K-ATPase activity are higher in intestines from seawater fish compared with freshwater fish, and all increase following seawater transfer of euryhaline fish (reviewed by Hirano, Morisawa, Ando & Utida, 1975). Cortisol injections into freshwater-adapted fish mimic seawater adaptation in all these aspects; prolactin injections have the opposite effect (reviewed by Hirano *et al.* 1975). Seawater adaptation is associated with mucosal hyperplasia as a result of cell proliferation (MacKay & Janicki, 1979) suggesting that prolactin and cortisol might modify transport in the intestine as a result of long-term trophic effects.

Urinary bladder and kidney

In vitro studies of kidney tubular NaCl transport are few and there is no information concerning hormonal regulation. The teleost urinary bladder, unlike those in tetrapods, is of the same embryological origin as the kidney and represents an expansion of the fused mesonephric ducts. Thus, ion and water transport has been examined using the urinary bladder model. It has become clear that the urinary bladder significantly modifies the urine and itself plays an important role in teleost osmoregulation. Generally, bladders from seawater-adapted fish have higher water absorption and osmotic permeability and lower or equal NaCl absorption rates compared with those in fresh water (Hirano, Johnson, Bern & Utida, 1973*b*). Prolactin injections into seawater fish result in decreased water absorption and osmotic permeability and increased (usually) NaCl absorption (reviewed by Clarke & Bern, 1980), mimicking the freshwater situation.

The *Gillichthys* urinary bladder has features permitting the study of hormonal regulation of bladder (= distal tubular?) water and NaCl transport. As in other euryhaline teleosts studied, urinary bladders from 5 %-seawater or freshwater fish have lower water transport and osmotic permeability compared with bladders from seawater *Gillichthys* (Hirano *et al.* 1973*b*; Doneen, 1976). Prolactin injections (Doneen, 1976) or exposure of the bladder in organ culture to prolactin (Doneen & Bern, 1974) reduces water permeability. Cortisol antagonizes this effect in organ culture (Doneen & Bern, 1974). The dorsal, columnar cell region of the bladder is histologically similar to the collecting duct and is responsible for bladder ion transport (Loretz & Bern, 1980, 1983). Freshwater and seawater bladders have the same high rates of neutral NaCl cotransport, but seawater bladders have an additional amiloride-insensitive electrogenic sodium absorption mechanism (Loretz & Bern, 1983). In sea water *Gillichthys*, hypophysectomy reduces the electrogenic component to freshwater levels and cortisol can restore this absorption, and can initiate this mechanism in 5 % seawater fish, where it is normally absent (Loretz & Bern, 1983).

Freshwater-adaptation is associated with enhanced tubular (Wendelaar-Bonga, 1973; Olivereau & Olivereau, 1977*a,b,c*) and bladder (Nagahama, Bern, Doneen & Nishioka, 1975; Loretz & Bern, 1980) cellular development and Na/K-ATPase lev

stein *et al.* 1969; Pickford *et al.* 1970*a*; Utida, Kamiya, Johnson & Bern, 1974), and prolactin injections can enhance both (Olivereau & Olivereau, 1977*a,b,c*; Pickford *et al.* 1970*a*; Utida *et al.* 1974). Prolactin-induced decreased water permeability and increased sodium absorption in the flounder bladder are temporally paralleled by enhanced DNA synthesis (Hirano, Hayashi & Utida, 1973*a*).

CONCLUSION

In the urinary bladder and kidney, as in the gill/opercular membrane, oesophagus and intestine (i.e., in the entire osmoregulatory system of some euryhaline fish) prolactin and cortisol appear to influence epithelial ion and water movements through influences on cell differentiation and proliferation. As a result, these osmoregulatory surfaces become adapted to chronic salt conditions and simultaneously integrated in their functions to achieve whole animal osmoregulation. Other, rapidly-acting hormones can then control transport rates in these differentiated systems to allow 'fine regulation' of ion and water balance in response to rapid changes in the external or internal environment.

It should be emphasized that generalizations for all teleosts in the areas examined here have to be made with caution. In fact, the hormonal regulation of transport has been analysed for very few teleost species. Not all teleosts have opercular membranes capable of chloride transport; oesophageal studies have been restricted largely to the eel; the *Gillichthys* urinary bladder with its seawater electrogenic sodium-transport component is not the same as the flounder bladder, and so forth. Nevertheless, teleost ion and water balance generally is accomplished by a series of osmoregulatory organs whose differentiation is regulated by hormones and whose activity is modulated by hormones.

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