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Review

The mechanism of sodium chloride uptake in hyperregulating aquatic animals

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Summary

The emphasis in this review will be on Na^+ absorption across the skin and gills of vertebrates and the gills of crustaceans. However, some recent studies of Cl^- uptake, especially in crustaceans, will also be described.

Key words: ion transport, Na^+ absorption, crustacean, fish, frog skin model, hyperregulation, Na^+/K^+-ATPase, carbonic anhydrase, H^+ V-ATPase, Cl^- absorption.

Historical prologue

What might be termed the 'modern era' of epithelial ion transport began nearly 70 years ago with the observation that metabolically dependent net Cl^- (presumably NaCl) movement occurred across isolated frog skin bathed on both surfaces by Ringer solutions (Huf, 1936). This was followed shortly after by experiments showing that freshwater (FW) animals, including frogs, could absorb both Na^+ and Cl^- from solutions as dilute as 10^{-5} mol l^{-1} (Krogh, 1937, 1938). But Krogh showed more than this. His work also demonstrated that the uptake of Na^+ and Cl^- were independent; Cl^- was absorbed from salts of impermeant cations and Na^+ was absorbed from salts of non-penetrating anions. Although he made no electrical measurements, he realized the significance of these observations and suggested that the influx of each ion was accompanied by the efflux of an ion of like sign. This was the first step in working out a mechanism for NaCl absorption in FW animals.

During the decade following the end of the Second World War, the basic concepts and experimental approaches to ion movement across epithelia were developed, most of them in the laboratory of Hans Ussing. A rigorous criterion for active transport, the flux ratio equation, was described (Ussing, 1949), voltage clamping allowed relatively easy measurement of ion-induced currents, and isotopes were used to identify the current (Ussing and Zerahn, 1951). The concept of exchange diffusion was introduced (Levi and Ussing, 1948). A more thorough description of Ussing's research career has been published recently (Larsen, 2002), but it is worth emphasizing here that elements of this framework are still used in studying epithelial ion movements. It is also worth noting that, with the exception of exchange diffusion (from a study of muscle), the preparation used in these studies was the isolated frog skin, usually bathed on both sides by Ringer solution. The importance of this model system can't be overemphasized, as will be noted again later.

In addition to these conceptual and methodological tools, Ussing's laboratory also made two observations that became key in providing a definitive model of Na^+ transport across frog skin. First, they showed that transfer of Na^+ from the apical (external) bathing solution into the epithelial cells was passive (Koefoed Johnsen and Ussing, 1958), an observation that led ultimately to the description of the amiloride-sensitive 'epithelial Na channel' (ENaC; cf. Smith and Benos, 1991). In addition, they showed that extrusion of Na^+ from cell to internal bathing medium was mediated by a ouabain-sensitive mechanism, later shown to be the Na^+/K^+-ATPase (Koefoed Johnsen, 1957; Koefoed Johnsen and Ussing, 1958).

The use of a high $[Cl^-]$ medium (Ringer) to bath the apical surface of the skin opened channels permeable to Cl^-. The behavior of these Cl^- channels has been described in detail (Larsen, 1991), but their role in the animal is still not clear; frogs (except for *Rana cancrivora*) are not exposed to bathing media with $[Cl^-]>100$ mmol l^{-1}. In FW, the channels are closed and the skin is nearly impermeable to diffusion of Cl^-. However, during this period (i.e. 1951–1975), the usual procedure was to study the skin with Ringer on both sides. The result was that there was high passive throughput of Cl^-, and in open circuit the active transfer of Na^+ was balanced electrically by Cl^- influx (the Cl^- channel was described by Ussing, 1982); i.e. the two ion fluxes, instead of being independent, were linked. Krogh's experiments were, if not forgotten, not followed up for many years.

The Krogh scenario was finally revisited and the results described in a landmark paper. With improved technology (flame photometry and, later, atomic absorption for measurement of Na^+ and electrometric titration for Cl^-) it was shown that the two fluxes were indeed independent in the intact frog bathed by dilute solutions. Moreover, when the external bathing solution consisted of Na_2SO_4, Na^+ was exchanged for

H^+; the ratio of net fluxes J^{Na}_{net}/J^{H}_{net} was 1:1. In addition, Cl^- was absorbed from several non-penetrating cations in exchange for a base that titrated like HCO_3^- (Garcia Romeu et al., 1969). This was followed later by papers from two laboratories (Garcia Romeu and Ehrenfeld, 1975; Alvarado et al., 1975) in which Cl^- uptake was followed in isolated frog skins bathed on the outside by dilute solutions approximating the ionic concentrations in normal FW. Both groups showed a net uptake of Cl^- that was unaffected by ouabain or amiloride but was inhibited by acetazolamide (AZ; known to inhibit carbonic anhydrase). These papers were followed by an exemplary pair characterizing the membrane potentials and intracellular $[Na^+]$ in the same preparation (Harvey and Kernan, 1984a,b). In summary, the importance of these studies went beyond their data. They showed that the isolated skin could be studied under conditions approximating those encountered by the intact animal; i.e. in open circuit and bathed by FW. Thus, the isolated skin was clearly a tool that could be used to construct models for the two independent influx mechanisms as they operate *in vivo*.

Although the emphasis here is on absorption of Na^+ across the body surface of FW animals, it should be noted that experiments on isolated turtle bladder during the same period were showing the same results: Na^+ absorption was coupled to the active extrusion of H^+ (reviewed by Steinmetz, 1985, 1986).

The model(s)

Using the information described in the previous section, the first phase of modeling Na^+ uptake in the absence of Cl^- was undertaken (Ehrenfeld et al., 1985). Skins were mounted, bathed inside by Ringer solution and outside with dilute, buffered Na_2SO_4, and a microelectrode inserted into an epithelial cell (almost certainly a 'principal cell'). It is relevant here to note that two groups of cells play roles in the movement of ions across frog skin. The numerically dominant group comprises the principal cells (PC). The second group, much fewer in number, comprises mitochondria-rich (MR) cells, about which more will be said later. With a second electrode in the outside bathing medium it was possible to measure the apical membrane potential difference (APD). The transepithelial potential difference (TEP) and resistance were measured between electrodes in the bathing solutions, and a second pair was used to voltage-clamp the preparation. Measurements under open-circuit conditions showed the earlier reported equivalence between the net influx of Na^+ and net efflux of H^+. Moreover, inhibition of either flux was accompanied by an equivalent reduction in the other. Thus, amiloride blockage of the Na^+ channel reduced both J^{Na}_{net} and J^{H}_{net} (Harvey and Ehrenfeld, 1988a), and bafilomycin inhibition of proton efflux caused a similar decrement of both fluxes (Ehrenfeld and Klein, 1997). The APD was usually between –35 mV and – 50 mV (outside medium reference) and, since cell and medium pH were not substantially different (Harvey and Ehrenfeld, 1988b), the extrusion of H^+ was against a gradient of electrochemical potential. Net movements of both ions were sensitive to the APD: hyperpolarizing the apical membrane reduced J^{H}_{net} while augmenting J^{Na}_{net}; depolarizing it had the opposite effects. It had been known that sodium entry into the epithelial cells was passive and electrogenic, but the demonstration that active proton movement was also electrogenic was a key observation. It suggested that the two ion streams were independent and probably, in open circuit, coupled by the APD. This surmise was confirmed by showing that when the APD was clamped near its spontaneous value, amiloride inhibited J^{Na}_{net} but had no effect on J^{H}_{net} (Ehrenfeld et al., 1985). Conversely, when the APD was clamped at its spontaneous value, inhibition of carbonic anhydrase (CA) blocked J^{H}_{out} without effect on intracellular $[Na^+]$, indicating that J^{Na}_{in} was not inhibited (Harvey and Ehrenfeld, 1986).

To these basic observations were added some characteristics of the proton efflux. J^{H}_{net} was shown to be sensitive to the external solution pH, being reduced at lower pH and increased at higher pH. Thus, pumping was subject to both chemical and electrical components of the proton gradient. It was inhibited, as was already known, by inhibitors of CA, showing that the source of H^+ was CO_2. It was also inhibited by dicyclohexylcarbodiimide (DCCD), suggesting that the mechanism underlying H^+ extrusion was a vacuolar-ATPase (an H^+ V-ATPase; Ehrenfeld et al., 1985; hereafter, the terms 'proton pump', 'proton ATPase', 'H^+-ATPase' or 'V-ATPase' refer to this enzyme). This was later confirmed when it was shown to be sensitive to bafilomycin, an inhibitor specific for this enzyme, and the latter was shown by immunohistochemistry to be confined to the apical region of MR cells in frog skin (Ehrenfeld and Klein, 1997). Finally, the action of CA on CO_2 produces an HCO_3^- as well as a proton; it remained to show how the former is handled and to account for the transfer of the absorbed Na^+ across the basolateral membrane. Duranti et al. (1986) demonstrated that the basolateral membrane of the MR cells has a Cl^-/HCO_3^- exchanger sensitive to DIDS (4,4′-diisothiocyano-2,2′-stilbene disulfonic acid) positioned to extrude the bicarbonate into the serosal solution. Extrusion of Na^+ is handled by the Na^+/K^+-ATPase, which provides at least half of the energy for the Na^+/H^+ exchange. The energy stoichiometry of the proton pump appears to be either $2H^+$/ATP (Steinmetz et al., 1981) or $3H^+$/ATP (Dixon and Al Awqati et al., 1980). This, then, is the system that accounts for the net influx of Na^+ in the absence of Cl^-. Because it excretes H^+, it plays a role in acid–base balance as well. The model is shown in Fig. 1.

The MR cell picture is more complex than is suggested in this description. The cell in Fig. 1 is denoted αMR; as noted, it has a proton pump in the apical membrane and a Cl^-/HCO_3^- exchanger in the basal membrane. Most of the Na^+ traffic passes through the principal cells. The system is specialized to absorb Na^+ and excrete protons. A second type of MR cell, described in turtle bladder (Stetson et al., 1985; Stetson and Steinmetz, 1985), has these elements reversed, with the exchanger in the apical membrane and the pump in the basal membrane. It is called βMR and is specialized to excrete base and, in aquatic animals, absorb Cl^- even in Na^+-free solution.

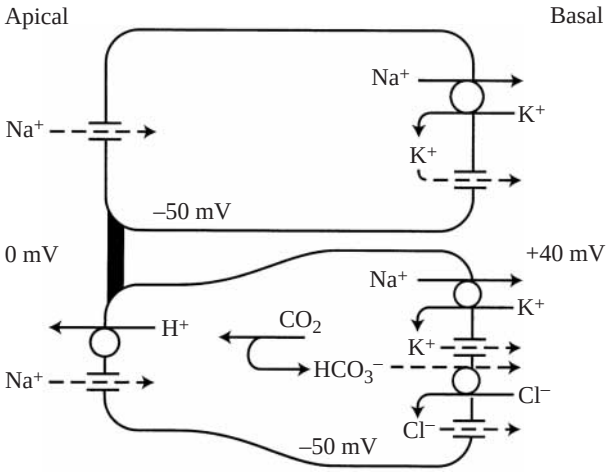

Fig. 1. An αMR cell and associated principal cell. The system will absorb Na^+ and eliminate H^+ even in Cl^--free media. It is unable to absorb Cl^-. Solid lines indicate transport; broken lines indicate diffusion.

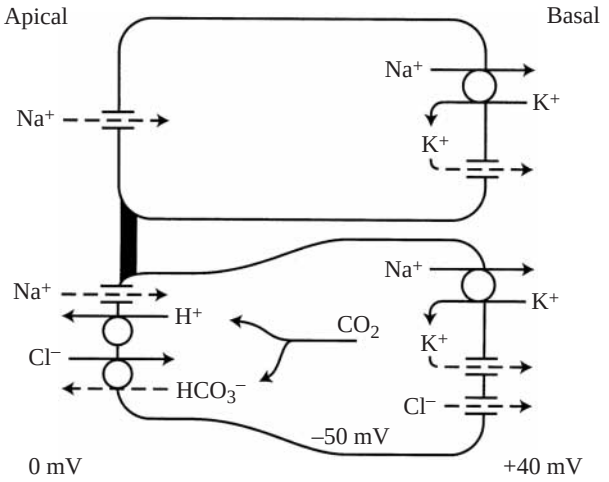

Fig. 2. The γMR cell. With this configuration of transport elements, both Na^+ and Cl^- can be absorbed from ambient NaCl, and Cl^- can be taken up from Na^+-free solution. As pictured, it lacks a basolateral mechanism for eliminating HCO_3^- and hence would be unable to absorb Na^+ from Cl^--free media. Whether this is, in fact, the case remains to be determined. The apical Cl^- channel is not shown, since it would be closed in FW.

A third type, called the γMR cell, has been described in toad skin (Larsen et al., 1992). It has both a proton pump and a Cl^-/HCO_3^- exchanger in the apical membrane, together with the Na channel. It has, in addition, a high conductance, voltage and concentration-activated Cl^- channel in the apical membrane (Voûte and Meier, 1978), although this channel would be closed in normal FW ($[Cl^-]$ <5 mmol l^{-1}). The γMR cell is shown in Fig. 2. As depicted, it would support both Na^+ and Cl^- absorption from NaCl as well as Cl^- uptake from Na^+-free solution. However, it would be unable to take up Na^+ from Cl^--free medium unless there is a still undescribed mechanism on the basal membrane for disposing of HCO_3^-. In addition, it could play no role in maintaining acid–base balance, unless pump and exchanger activities can be independently modulated. Evidence that all three types may be expressed in amphibian skin has been summarized briefly (Jensen et al., 1997). Each of them must contain the CA needed to provide H^+ and/or HCO_3^- required for the exchanges. It is not clear whether the three cell types coexist in an organ such as the amphibian skin or, as would be more economical, that the MR cell can, given the appropriate signal(s), redistribute the key elements between apical and basal membranes. If the latter, the nature of such signals and cell responses would be an interesting problem for investigation.

The rest of this review will be concerned primarily with the uptake of Na^+ in the absence of Cl^- in fully aquatic animals. However, data for the uptake of both Na^+ and Cl^- in hyperregulating crabs will be described.

Modeling in FW fish

Some energetic considerations

The experiments of Krogh and Garcia Romeu were run using animals that had been exposed for some time to distilled water and hence were salt depleted (SD). However, most data are collected on animals adapted to local tap water (TW) or an artificial equivalent ($[NaCl] \approx 1–2$ mmol l^{-1}). It should be noted that some FWs are extremely dilute; e.g. in Vancouver, BC, Canada, $[Na^+]$ is 20–30 μmol l^{-1}, and in Ottawa, ON, Canada, $[Na^+]$ is 145 μmol l^{-1}. It is worth comparing the energetic implications of transport in SD and TW-adapted animals. Krogh estimated that his animals could maintain a sodium steady state with an external $[Na^+]$ of ~10 μmol l^{-1}. This is in reasonable agreement with a recent report that SD crayfish (*Cherax destructor*) maintained a steady state at ~50 μmol l^{-1} (Zare and Greenaway, 1998). The energy barrier for absorption of Na^+ is:

$$\Delta\mu_{Na} = RT \log_e[Na^+]_s/[Na^+]_a + zF\Delta\Psi\,, \qquad (1)$$

where R, the gas constant, is 8.31 J $(°K)^{-1}$ $mole^{-1}$, T is temperature (~287°K), F is the Faraday (96.5 kJ $mole^{-1}$), z is valence (here +1), $\Delta\Psi$ is the TEP (V), and the subscripts s and a denote the serosal (basal) and apical bathing solutions, respectively.

For TW-adapted fish, $\Delta\Psi$ varies substantially and is especially sensitive to environmental $[Ca^{2+}]$ (Eddy, 1975; Potts, 1984). If $[Ca^{2+}]$ is <1 mmol l^{-1}, $\Delta\Psi$ in 1 mmol l^{-1} Na^+ would be approximately –5 mV to –10 mV (outside solution reference). If extracellular fluid (ECF) $[Na^+]$ is ~150 mmol l^{-1}, equation 1 shows that the cost of transport is ~11.3 kJ eq^{-1}. Since three Na^+ are transferred per mole ATP used, the cost of one cycle of the pump is just over 34 kJ. The energy released by ATP hydrolysis in a cycle is ~63 kJ (the data are from frog skin; Civan et al., 1983). Assuming that the free energy yield is similar in fish gill, the Na^+/K^+-ATPase would be operating at <60% efficiency and could handle the energy requirement without calling on another source.

The alternative situation is for a FW animal maintaining a steady state at μmol l^{-1} Na^+ concentrations (i.e. SD animals). The crayfish *C. destructor* maintained a steady state in $[Na^+]\approx 50$ μmol l^{-1}. Hemolymph $[Na^+]$ was ~190 mmol l^{-1}, and $\Delta\Psi$ was 10.3 mV. From equation 1, the energy barrier is 21 kJ eq^{-1}, and hence 63 kJ per ATPase cycle. This equals the free energy released by ATP hydrolysis and, since 100% efficiency is unlikely, a second energy source is required; we might expect this to be the proton ATPase.

Experimental approaches

Krogh had suggested that, since Na^+ was absorbed from salts of impermeant anions, it might be exchanged for NH_4^+. This theme was proposed again when it was shown that net Na^+ uptake was roughly equal to NH_4^+ efflux in crayfish (Shaw, 1960a). It was extended to fish (the goldfish *Carassius auratus*) a few years later when it was shown, in agreement with Krogh, that Na^+ uptake occurred in the absence of anion absorption (Garcia Romeu and Maetz, 1964) and that injection of NH_4^+ into the body fluids stimulated Na^+ influx, while elevating $[NH_4^+]$ in the bathing medium reduced it (Maetz and Garcia Romeu, 1964). Obviously, a Na^+/NH_4^+ exchange at the apical membrane is very different from the events described by the frog skin model. However, the proposal became controversial within a short time. For example, it was shown that when the experimental design engendered changes in J_{net}^{Na}, correlation with $J_{net}^{NH_4}$ was poor, while correlation with the sum of $J_{net}^{NH_4}$ and J_{net}^{H} was much better (Maetz, 1973). The uncertainty was discussed and debated for two decades, and the relevant information was summarized recently (Wilkie, 1997).

The debate appeared to be ended with publication of experiments on a perfused trout (*Salmo gairdneri*) head (Avella and Bornancin, 1989) and intact rainbow trout (*Oncorhynchus mykiss*; Wilson et al., 1994). In the former, it was shown that variations in $J_{net}^{NH_4}$ did not correlate well with J_{in}^{Na} (as had already been shown by Maetz, 1973); moreover, the data indicated that the ammonia efflux was in the form of NH_3. Avella and Bornancin (1989) also showed that J_{in}^{Na} was balanced by an equivalent (net) excretion of protons. This final observation is, in fact, curious, since this would produce excess positive charge moving out; it is J_{net}^{Na}, and not J_{in}^{Na}, that should have balanced proton efflux unless J_{out}^{Na} was zero or there was a large efflux of Cl^-. The first of these suggestions can be ruled out, since they had previously shown (Bornancin et al., 1985) that J_{out}^{Na} was approximately equal to influx (i.e. the net Na^+ flux was about zero) in the perfused head. Chloride fluxes were not reported, so the alternative cannot be assessed. Wilson et al. (1994) measured ammonia fluxes in rainbow trout in which J_{in}^{Na} was altered (low $[Na^+]_{out}$, amiloride), transbranchial gradients of NH_3 and NH_4 were altered or acidification of the gill boundary layer was prevented by buffering. None of these maneuvers produced evidence for a coupled Na^+/NH_4^+ exchange. In agreement with Avella and Bornancin (1989), they concluded that most, if not all, of the ammonia was excreted in the form of NH_3 and that the exchange must be Na^+/H^+. This appears to be the current dominant view and is in accord with the frog skin model. However, one puzzling observation remains. Infusion of NH_4^+, usually in the form of the chloride or sulfate salt, stimulated both J_{in}^{Na} and $J_{out}^{NH_4}$, which was the original support for a Na^+/NH_4^+ exchange. It was later realized that such infusions produce a substantial acidosis, which would provide protons for the recently favored Na^+/H^+ exchange, leaving uncharged NH_3 to diffuse outward. However, in one study, rainbow trout were infused with NH_4HCO_3, which provided a gradient for augmented NH_3 diffusion without producing acidosis. As expected, NH_3 efflux was augmented but, unexpectedly, J_{in}^{Na} was also nearly doubled (Salama et al., 1999). One possible explanation is that NH_4^+ entered the cell *via* the K^+ site on the Na^+/K^+-ATPase. It was shown that NH_4^+ did not stimulate the enzyme in the presence of ambient K^+, but it might well have shared the site with K^+. Such an entry would provide NH_3 for the augmented efflux and a proton for the H^+-ATPase; the latter could account for stimulating J_{in}^{Na}.

Two mechanisms have been proposed to account for an Na^+/H^+ exchange in fish. The first, an exchange protein in the apical membrane of cells in the gill, has a long history, perhaps because the Michaelis–Menten concentration dependence suggested the combination of Na^+ with a membrane protein. However, it was argued that such a neutral exchanger could not take advantage of the membrane potential and that the proton and sodium gradients were insufficient to drive the exchange (Avella and Bornancin, 1989). This is probably true but hard to demonstrate, since intracellular $[Na^+]$ values are uncertain in fish gills. Several measurements have produced wildly disparate values, from approximately 10 mmol l^{-1} to 80 mmol l^{-1} (Li et al., 1997; Morgan et al., 1994; Eddy and Chang, 1993; Wood and LeMoigne, 1991). In frog skin (PCs) bathed with 1 mmol l^{-1} Na on the apical surface, $[Na^+]_{cell}$ was ~4 mmol l^{-1}, and this might serve as a likely benchmark for FW vertebrate transport epithelia. Intracellular pH is also uncertain in fish gill. It has been measured only in rainbow trout, where it was 7.3–7.4 (Wood and LeMoigne, 1991; Goss and Wood, 1991).

Since the striking work on the frog skin model (Ehrenfeld et al., 1985), the proton pump–Na^+ channel has been proposed as the alternative mechanism underlying apical Na^+/H^+ exchange in FW fish gills. It is worth examining current information about the presence, distribution and significance of each of the several components of this system.

The Na^+/K^+-ATPase

This transport enzyme is firmly established as the motor for Na^+ transport in most animal cells, and information regarding its presence in fish gills was summarized some time ago (DeRenzis and Bornancin, 1984). The earliest study of its distribution showed that [^{3}H]ouabain binding in the killifish (*Fundulus heteroclitus*) adapted to 10% seawater (SW) was confined to MR cells in the filament (presumably interlamellar). Although lamellae were not shown, it was stated that respiratory cells [presumably pavement cells (PVCs)] "never exhibited the dense pattern of grains seen over chloride

cells" (Karnaky et al., 1976). Labeling was much denser in SW-adapted animals, corresponding to the well-known marked increase in the enzyme in SW. More recently, the use of antibodies against a subunit of the enzyme has permitted its immunolocalization. In the rainbow trout, labeled cells were found on both the filament and the secondary lamellae but predominantly on the former (Witters et al., 1996). A similar pattern was seen in FW-adapted guppy (*Poecilia reticulata*; Shikano and Fujio, 1998). Subsequently, the method was used in rainbow trout adapted to Vancouver, BC, Canada TW (very low [Na^+]), and the enzyme was found to be widely distributed on both the filament and lamellae. The pattern was similar but less intense for a fish adapted to Ottawa, ON, Canada TW, which also has low ion concentrations. The tilapia (*Oreochromis mossambicus*) also stained for the enzyme, but in this case predominantly on the filament and at the base of the lamellae (Wilson et al., 2000). Labeling occurred on both filament and lamellae in *Oncorhynchus keta* fry (Uchida et al., 1996), while in adults returning from SW (but adapted to FW) labeling was confined to cells on the lamellae (Uchida et al., 1997). In the FW-adapted stingray (*Dasyatis sabina*), the Na^+/K^+-ATPase was found in cells both on the lamellae and in the interlamellar region but was more numerous on the former (Piermarini and Evans, 2000, 2001).

A different approach to enzyme localization has also been described (Galvez et al., 2002). Gill cells from *O. mykiss* were disaggregated enzymatically and separated on a Percoll gradient into three fractions. Two of these were MR cells, as judged by fluorescent dye binding and labeling with an antibody to mitochondrial protein. One of the MR fractions bound peanut lectin agglutinin (PNA^+), while the other did not (PNA^-). It is interesting that the latter has the gross morphology of a PVC. Both MR cells had substantial amounts of the Na^+/K^+-ATPase in a PNA^-:PNA^+ ratio of ~0.3. When the fish was made hypercapnic (1% CO_2), the ratio changed to ~1.3. Unfortunately, because these data were expressed as a ratio, the change might indicate an increase in PNA^- or a decrease in PNA^+. However, either change would point to the PNA^- cell as the pathway for J_{in}^{Na}. The increase during hypercapnia is puzzling, since hypercapnia in these fish is not accompanied by a change in Na^+ fluxes. This is discussed in more detail below. Infusion of HCO_3^- also increased the ratio (to ~2); an interpretation of this change is not obvious.

No consistent distribution pattern is apparent from these studies. Cells containing the enzyme occur on the filament in some, on the lamella in others and, especially in trout exposed to ion-poor water, in both regions. In addition, in most cases, labeled cells are described as 'chloride cells' (without further identification), but in the trout in low ion water both chloride and pavement cells were labeled. This ambiguity is not trivial, since the enzyme should mark the pathway for J_{in}^{Na}, about which there is disagreement, and it may be that the data of Galvez et al. (2002; one class of MR cell has the morphology usually associated with PVCs) will answer this question.

Carbonic anhydrase

Carbonic anhydrase is probably the first non-metabolic enzyme to be associated with epithelial ion transport. Based on reports of such a relationship in kidney and stomach, the effects of sulfonamide inhibitors of CA on Na^+ transport across isolated frog skin were assessed (Fuhrman, 1952). Only modest inhibition was achieved by high inhibitor concentrations. These experiments were run with Ringer solution bathing both sides of the skin and with the preparation short-circuited. Either condition uncouples J_{net}^{Na} from J_{net}^{H}, and the failure of J_{net}^{H} on CA inhibition would not be reflected in the short-circuit current (i.e. J_{net}^{Na}). Experiments with fish proved more illuminating. The enzyme was shown to be present in the gills of *Perca fluviatilis* (freshwater) and *Serranus scriba* (and *S. cabrill*; both marine). In addition, injection of a sulfonamide completely inhibited the enzyme (Maetz, 1953a,b). Maetz then showed that when AZ was injected into the goldfish *Carassius auratus*, J_{in}^{Na} was nearly completely abolished with no effect on J_{out}^{Na} (Maetz, 1956). This was followed by the observation that both J_{in}^{Na} and J_{in}^{Cl} were dependent on CA and that the enzyme's role was to produce the H^+ and HCO_3^- required as exchange partners for the respective fluxes (Maetz and Garcia Romeu, 1964). More recent efforts have been directed toward localizing the enzyme in the gill. In FW rainbow trout, CA was found in the apical region of both MR cells on the filament and PVCs on the lamellae (Conley and Mallatt, 1988; Rahim et al., 1988). The distribution in five other FW species varied. Only *Poecilia velifera* showed enzyme in MR cells (with none in the PVCs). The other species, *Carassius auratus*, *Platydorus costatus*, *Tricogaster leeri and Tilapia shirana*, all stained heavily in the PVC, but MR cells were negative (Conley and Mallatt, 1988). Again, there is the possibility that one group of MRCs was misidentified as PVCs.

In the opercular epithelium of *Fundulus heteroclitus* adapted to FW, the enzyme was found largely in MR cells while PVCs had none (Lacy, 1983). The significance of this observation is not clear, since the isolated preparation, which is very active in SW-adapted fish, does not transport Na^+ in FW (Wood and Marshall, 1994; Marshall et al., 1997).

The proton ATPase

Having ruled out operation of an Na^+/H^+ exchanger, Avella and Bornancin (1989) suggested that the proton pump–Na-channel model provides the mechanism for Na^+/H^+ exchange in fish gill. This was later explored in rainbow trout by noting the effect of putative inhibitors of the H^+-ATPase (vanadate, AZ) as well as amiloride on net proton efflux in rainbow trout. Vanadate and AZ both inhibited proton efflux (~50%). However, vanadate is an inhibitor of P-ATPases (e.g. the Na^+/K^+ enzyme) and does not affect V-ATPases (Forgac, 1989). In addition, proton efflux was unaffected by 0.1 mmol l^{-1} amiloride, which is known to inhibit net Na^+ uptake nearly completely, and even 1 mmol l^{-1} had only a modest effect (Lin and Randall, 1991). Their fig. 9 suggests that the frog skin model functions in fish gill, but vanadate data provide no support for the suggestion, and the apparent

uncoupling of J_{net}^{Na} and J_{net}^{H} (the amiloride data) is simply not consistent with the model. That the trout gill contains the ATPase seems likely, since ATPase activity in gill homogenates was inhibited by several known inhibitors of the enzyme (Lin and Randall, 1993). In fact, since these fish were acclimated to Vancouver, BC TW, the energetic situation (described above) requires an energy source in addition to the Na^+/K^+-ATPase; the proton pump is a likely candidate.

Some recent studies have used immunohistochemistry to localize the enzyme in the gills of several FW fish. In *O. mykiss* gill, an antibody to the H^+-ATPase was found along the lamellar surfaces generally concentrated in the apical regions. The conclusion was that both PVCs and MRCs were labeled (Lin et al., 1994). Another study in the same species found it localized to PVCs (Sullivan et al., 1995). This research group also used probes for the H^+-ATPase mRNA to locate the mRNA and found it in the same cells. Hypercapnic acidosis augmented the mRNA signal as well as antibody staining (Sullivan et al., 1996). These observations suggest an increase in H^+-ATPase during hypercapnia. This is consistent with one study showing that H^+ excretion increased in fish exposed to hypercapnia (Goss and Perry, 1993) but not with another in which it was unchanged (Perry et al., 1987). In addition, J_{in}^{Na} was unchanged during hypercapnia in both of the latter papers. The major adaptive change during hypercapnia was suppression of Cl^-/HCO_3^- exchange with accumulation of bicarbonate in the extracellular fluids. If increased J_{net}^{H} occurs during hypercapnia it appears to be uncoupled from J_{net}^{Na}, and the hypercapnia data provide no support for a frog skin-type Na^+/H^+ exchange.

Additional work examined antibody labeling patterns for several transport proteins in *O. mossambicus* and the rainbow trout. Those relevant to Na^+ uptake were the H^+-ATPase, an Na^+/H^+ exchanger, the Na^+/K^+-ATPase and ENaC. In both species, the H^+-ATPase and Na channel were found together on lamellar PVCs. In the trout, the proteins were also found together but were more widely distributed than in the tilapia (Wilson et al., 2000). In a FW elasmobranch, the stingray *Dasyatis sabina*, the H^+-ATPase and Na^+/K^+-ATPase were found on both interlamellar filaments and on the lamellae but in separate cells (Piermarini and Evans, 2001). Moreover, the H^+-ATPase was on the basolateral membrane and colocalized with a pendrin-based Cl^-/HCO_3^- exchanger on the apical membrane, suggestive of the βMR configuration. The Na^+/K^+-ATPase, in a different cell, was probably part of the system for absorbing Na^+ (Piermarini et al., 2002).

The cell isolation approach described above (Galvez et al., 2002) was also applied to locating the H^+-ATPase. The ratio of PNA^-:PNA^+, for the enzyme in control animals, was ~2 and increased significantly during hypercapnia. This suggests that the Na^+/K^+-ATPase and the H^+-ATPase are located in the same group of cells. Infusion of HCO_3^- had no effect on the ratio.

Finally, bafilomycin (10^{-5} mol l^{-1}) inhibited J_{in}^{Na} in juvenile *O. mossambicus by* ~80%. The K_i was 1.6×10^{-7} mol l^{-1}, and the effect was rapidly reversed on washing out the inhibitor. The inhibitor also reduced J_{in}^{Na} by ~70% in juvenile carp (*Cyprinus carpio*; Fenwick et al., 1999) and by ~60% in trout fingerlings (Bury and Wood, 1999). Bafilomycin also inhibited J_{in}^{Cl} by ~35% in the tilapia; in this case, the inhibition was not reversed on washing but instead became more intense. Interpretation of this effect is not obvious.

The Na channel

The antibody study mentioned above (Wilson et al., 2000) showed that the channel occurred together with the H^+-ATPase in the tilapia and rainbow trout. In a further examination of the PNA-separated MRCs, it was found that Na^+ entry into PNA^- cells was substantial, even in the absence of a proton source. Addition of phenamil (an Na^+ channel blocker) had no effect on Na^+ movement, but 10 nmol l^{-1} bafilomycin inhibited the influx by ~60%. Interpretation of these data is not obvious. However, when a proton source (proprionic acid) was added, the Na^+ influx increased by ~50%, and the increase was inhibited by phenamil and even more by bafilomycin (Reid et al., 2003). Proton fluxes were not measured, but these inhibitor data suggest an Na^+/H^+ exchange mediated by the Na channel–H^+-ATPase couple. These observations support the suggestion that the PNA^- cells are a (the?) route of Na^+ influx across the gill in rainbow trout.

In summary

These data show that key components of the frog skin model exist in the gills of several fish species, and it is reasonable to surmise that the system functions in sodium uptake and proton extrusion in at least some of them. However, the structure of the model and how it functions are still unknown. The path taken by Na^+ is still debated with some not very compelling evidence on both sides. The picture of how the components are distributed is far from uniform in the studies described above; i.e. whether they occupy the same cells or are separated and connected only by a common APD. We have no secure knowledge of key intracellular variables that determine fluxes in the gill (concentrations and membrane potentials) nor can these be easily manipulated in order to test models. And, in most cases, we have only limited ability to control and manipulate the internal environment. What is lacking, of course, is a viable, functional, *in vitro* branchial preparation that allows such uncertainties to be addressed. The frog skin provided such a preparation and was used to generate convincing evidence for the model described earlier. The *Fundulus* opercular epithelium played a significant role in working out a model for NaCl transport in SW-adapted fish. For a time, it was hoped that this preparation, taken from FW-adapted animals, might play such a role for the FW gill. But, while it transports Cl^- weakly, it does not transport Na^+ at all (by the flux ratio criterion; Wood and Marshall, 1994; Marshall et al., 1997).

Since no natural, planar sheet of gill cells has come to light, attempts have been made to disaggregate the cells in a gill and grow them on solid, permeable supports in culture. It is hoped to produce, in this way, a functional transport system that can be addressed experimentally, as in the frog skin. Two preparations have been made. In one of these [single seeded

insert (SSI)], the sheet consists solely of PVCs; in the other [double seeded insert (DSI)], there are ~85% PVCs and ~15% MRCs. A comprehensive review of the behavior of these preparations has appeared (Wood et al., 2002). Briefly, some of the electrical and permeability characteristics resemble those of intact gill. However, neither preparation showed evidence of Na^+ transport. However, a recent study showed that when the cultured preparation was treated with cortisol there was evidence for weak active absorption of both Na^+ and Cl^- (Zhou et al., 2003). The system is not yet a good model, since efflux of the ions greatly exceeds influx; i.e. there was a large net loss of both.

An estuarine fish

Fundulus heteroclitus is estuarine and, while capable of hyperregulation in FW, it presents a picture incompatible with the Na channel–proton pump model. Sodium fluxes in FW are unusually high. J_{in}^{Na} is saturable but appears to be mediated by a low-affinity (half-saturation $[Na^+]_{out}$ nearly 2 mmol l^{-1}), high-capacity system. By contrast, there was virtually no J_{in}^{Cl} at $[Cl^-]_{out}$<7–8 mmol l^{-1}, and J_{out}^{Cl} was also very low (Wood and Marshall, 1994; Patrick et al., 1997). Amiloride (10^{-4} mol l^{-1}) reduced J_{in}^{Na} by ~60% without affecting Na^+ or Cl^- effluxes (Patrick and Wood, 1999), which is similar to its action in other fish. However, compensation for acidosis and alkalosis was completely different. Excretion of an acid load, instead of stimulating J_{in}^{Na}, depressed J_{out}^{Na} and augmented J_{out}^{Cl}. Electrical neutrality, during proton excretion, was maintained by the difference between Na^+ and Cl^- effluxes. However, the data provide no information about molecular mechanisms (i.e. channels, transport proteins). Injection of $NaHCO_3$ did not stimulate J_{in}^{Cl}, nor was the HCO_3 excreted, indicating, in agreement with the negligible control J_{in}^{Cl}, that an apical exchanger is absent (Patrick and Wood, 1999).

In a recent study, the disposition of the H^+-ATPase and the Na^+/K^+-ATPase in *Fundulus* gill was determined by immunohistochemical localization (Katoh et al., 2003). Both enzymes were found on the basolateral membrane of MRCs in the interlamellar filaments. H^+-ATPase concentration was highest in animals adapted to the most dilute medium (0.1 mmol l^{-1} NaCl). A basolateral location suggests the βMR configuration specialized for Cl^- absorption; but, as noted, Cl^- influx in FW is vanishingly small. The authors suggested that both enzymes played a role in Na^+ absorption, perhaps by generating an APD that facilitates Na^+ entry across the apical membrane. However, such a system would engender the fluxes of two cations (Na^+ and H^+) but only one anion (HCO_3^-) across the basolateral membrane. This cannot occur in open circuit, and interpretation of the observations remains unclear.

Finally, the mechanism of hyperregulation by killifish in brackish water is worth more attention in light of the unique system employed by brackish water crabs (described below).

Modeling in FW crustaceans

Crustaceans living in FW present an ion-regulatory picture somewhat different from that of vertebrates. Some, like many fish species, are stenohaline FW animals. Among those large enough for reasonable access to *in vivo* experiments on molecular mechanisms are crayfish, a few crabs and shrimp, but only crayfish have been much exploited in this regard**.** Although the majority of crustacean species are marine**,** some are capable of hyperregulating hemolymph ions in media more dilute than SW. These fall into two groups: weak regulators (*Carcinus maenas, Chasmagnathus granulatus)* can live and hyperregulate in dilute media but their survival in FW is poor; strong regulators include stenohaline FW animals (e.g. crayfish) as well as some crabs (e.g. *Eriocheir sinensis)* that live most of their adult lives in brackish and FW but return to the ocean to reproduce. In contrast to vertebrates that live such a euryhaline pattern with nearly constant extracellular ionic concentrations in both environments (eels, for example), these crabs become essentially isoionic with SW on returning to the ocean.

Another difference between crustacean and vertebrate transport epithelia is the presence, in the former, of a thin, acellular layer, the cuticle, abutting the external (apical) membrane. At least in the FW crayfish this structure is an important element in the uptake of both Na^+ and Cl^-, and, in the weak regulator *C. maenas*, it can influence the interpretation of experimental data. These points will be revisited below.

Crayfish

A striking series of experiments established a picture of Na^+ uptake from FW that is not dissimilar from that in fish but was published before the work on fish appeared. Sodium influx was shown to be concentration dependent, following Michaelis–Menten kinetics. Salt depletion decreased hemolymph $[Na^+]$ and was accompanied by a 3–5-fold increase in J_{in}^{Na} (Shaw, 1959a). J_{in}^{Na} was unchanged when an impermeant anion was used (here SO_4^{2-}), confirming Krogh's observations on FW animals. It was also shown that J_{net}^{Na} was usually about equal and opposite in direction to $J_{net}^{NH_4}$, and an Na^+/NH_4^+ exchange was suggested (Shaw, 1960a). The observation that NH_4^+ in the external solution inhibited Na^+ uptake appeared to confirm this surmise (Shaw, 1960b). Shaw even suggested that an Na^+/H^+ exchange might be involved, since net Na^+ uptake sometimes exceeded NH_4^+ excretion.

Each branchial chamber contains six podobranchs (also 11 arthrobranchs and a pleurobranch, which will not be further discussed). Each podobranch comprises a stem running most of its length and from which projects a series of filaments. At the distal end of the gill the tissue becomes a flattened, V-shaped lamella. The external surface of the single cell layer in both filament and lamella is covered by the cuticle. It was shown some years ago that the lamellar cuticle is permeable to Cl^- and OH^- but nearly impermeable to cations and to other anions. Relative permeabilities were calculated to be: P_{Cl}=1; P_{Na}=0.001; P_{HCO_3}=0.0006 (Avenet and Lignon, 1985). By contrast, the cuticle covering the filaments is cation permeable, with permeabilities in the order $K^+>NH_4^+>Na^+>Li^+\gg Cl^-$ and

SO_4^{2-} (Lignon and Lenoir, 1985). Since ions must cross the cuticle in order to move in either direction, this structure, although acellular, probably determines the possible interactions between ions in the bathing solution and the apical membrane. For example, since $P_{OH} \gg P_{HCO_3}$ in the lamellar cuticle, it was suggested that an apparent Cl^-/HCO_3^- exchange must in fact be Cl^- for OH^- (Avenet and Lignon, 1985). In addition, uptake of Na^+ and Cl^- must be spatially separated, with the former moving through filaments, and the latter through the lamella. Such a separation is supported by the observation that MRCs (presumably the sites of transport) in the lamella had an ultrastructure different from those in the filaments (Dunel-Erb et al., 1997).

In contrast to recent work on fish gills, there has been relatively little research on the putative elements of an Na^+/H^+ (or NH_4^+) exchange, and what there is has relied, for the most part, on the effect(s) of inhibitors added to the apical bathing solution. Thus, amiloride inhibited J_{in}^{Na} with little effect on J_{out}^{Na} and, at least in salt-depleted animals, J_{out}^{H} was equally reduced (Kirschner et al., 1973; Ehrenfeld, 1974). This suggests a role for ENaC in Na^+ uptake and coupling between the fluxes. In addition, AZ inhibited the fluxes, implicating CA, as in fishes (Ehrenfeld, 1974). Direct measurement showed a high concentration of the enzyme in the gills of *Pacifastacus leniusculus* (Wheatly and Henry, 1987). This animal can tolerate a range of environmental salinities up to approximately one-third SW, and it was shown that the CA concentration decreased at high external [NaCl] and rose again when the animal was returned to FW (Henry and Wheatly, 1988).

Na^+/K^+-ATPase activity has been measured in homogenates of the gills of crayfish. It was shown that enzyme concentrations were uniformly high in all gills of *P. leniusculus* and did not show consistent changes on adaptation of the animal to high salinity (in which active ion uptake would be reduced; Wheatly and Henry, 1987). However, enzyme concentration was sensitive to salinity in *Cherax destructor*. It was ~2× higher in SD animals than in those adapted to 25% SW (Mo and Greenaway, 2001). Measurements in *C. destructor* showed constant levels in the gills during intermolt and through premolt, but significantly (~2×) higher amounts during postmolt and in SD animals (Zare and Greenaway, 1998). Net Na^+ fluxes in the last two cases are ~4-fold higher than during intermolt or in TW-adapted animals (Zare and Greeenaway, 1997). The disposition of the enzyme (i.e. whether in lamella and/or filaments and where on the cell membranes) has not been published, but relevant data appeared in a thesis; in *Orchonectes* sp., the Na^+/K^+-ATPase was present in both lamellae and filaments but was higher (~4-fold) in the latter (Putzenlechner, 1994). This would be consistent with the suggestion (above) that Na^+ transport occurs in a population of filaments.

The V-ATPase has been shown to be present in the gills of *C. destructor* during intermolt and premolt, and its concentration increased 2–3-fold when J_{net}^{Na} was high (e.g. during postmolt and in SD animals; Zare and Greenaway, 1998). In *Orchonectes* sp., V-ATPase activity was more than 10× higher in the lamellae than in the 'tubules' (Putzenlechner, 1994). This appears to implicate the enzyme in Cl^- transport. The high concentration in SD animals is probably significant, since a second, ATP-driven system is required in very dilute media (argued above). Molting, like salt depletion, results in a reduced hemolymph [Na^+] (Greenaway and Lawson, 1982), which may be the signal for adjustments leading to augmented J_{in}^{Na}, although the work of Tresguerres et al. (2003; described below) argues for a different signal. However, the presence of an H^+-ATPase in TW-adapted animals need not signal its involvement in Na^+ transport (cf. its presence in *C. maenas*; see below). A possible role for this enzyme was tested in TW-adapted and SD *Procambarus clarkii*. J_{in}^{Na}, J_{net}^{Na} and J_{net}^{H} were measured, and the effects of three inhibitors of the V-ATPase assessed. The inhibitors were DCCD, *N*-ethyl maleimide (NEM) and concanamycin A (CCMA), a relative of bafilomycin. None of them affected either J_{net}^{Na} or J_{net}^{H} in TW-adapted animals. By contrast, all three partially inhibited both net fluxes in the SD animals (Zetino et al., 2001). This suggested that the proton pump–Na channel model functioned in SD crayfish but not in TW-adapted animals. An alternative system worth exploring is a $2Na^+/H^+$ exchanger that has been described in crustacean gut (Ahearn and Clay, 1989) and gill (Shetlar and Towle, 1989). Calculation showed that such an exchanger could maintain net Na^+ uptake in pH neutral media with an [Na^+] of ~1–2 mmol l^{-1} and in more dilute solutions if the pH were alkaline, as are many TWs. An attempt was made to use amiloride (selective for ENaC) and a derivative, ethylisopropyl amiloride (EIPA; reportedly selective for the Na^+/H^+ exchanger), to provide information on the nature of the exchange in crayfish. However, there was no discrimination in either TW-adapted or SD animals. Both compounds inhibited J_{in}^{Na} with half-maximal effects at concentrations of <1 μmol l^{-1} (Kirschner, 2002). Therefore, the test was inconclusive, and questions about the mechanism of Na^+ uptake in TW-adapted crayfish remain unanswered.

Hyperregulating crabs

There are important methodological differences between the research done on crayfish and on euryhaline crabs. The experimental approach to the former has almost exclusively involved work on intact animals. In the ensuing description of research on crabs it will be seen that nearly all of it has involved two *in vitro* preparations. One is a perfused intact gill first described by Koch (Koch et al., 1954; Koch and Evans, 1956), which has a pair of epithelial sheets enclosing a (perfused) hemolymph space. The other is a 'split gill lamella' in which one of the sheets is removed and the other is clamped in a modified Ussing chamber (Schwarz and Graszynski, 1989), so that both sides of the single cellular sheet can be bathed by well-defined media. This preparation can be 'short circuited' and the resulting current (I_{sc}) measured

A second difference involves the location of the transport mechanism(s). In crayfish, all gills appear to transport both Na^+ and Cl^-, although at different sites within the organ. In crabs, both the morphology of the cells (Copeland and Fitzjarrell,

1968; Barra et al., 1983) and the distribution of the Na^+/K^+-ATPase (Towle, 1981; Siebers et al., 1982) indicate that the transport systems are in the posterior three pairs of gills and that the anterior gills are specialized for gas exchange.

Weak hyperregulators

When the shore crab *Carcinus maenas* is in SW its hemolymph is essentially isoionic with the medium. However, it can maintain a steady state down to ~15–20% SW (60–80 mmol l^{-1} NaCl), and it was appreciated early that, although hemolymph concentrations were reduced in dilute media, they were maintained substantially above ambient. The key elements of hyperionic Na^+ regulation were described (Shaw, 1961a), among them the first measurements in crabs of the concentration dependence of both influx and efflux. The non-linear behavior of the former suggested participation of a transport mechanism. A detailed picture of this mechanism has evolved slowly over the past 20 years and in several crab species. An important observation was that Na^+/K^+-ATPase levels in the gills were higher than in any other organ tested and that the concentration in the posterior gills was much higher than in the anterior pairs. Most significantly, the levels in the posterior set (but not the anterior pairs) increased markedly when the animal was transferred from SW to dilute SW (Siebers et al., 1982). Higher Na^+/K^+-ATPase levels in posterior than in anterior gills were also reported in *Uca tangeri*, and the amount nearly doubled when the crab was transferred to a dilute solution (Graszynski and Bigalke, 1986).

An early study of the perfused posterior gill from *C. maenas* showed that when the perfusate [Na^+] was at SW concentration (~500 mmol l^{-1}) there was no evidence for active transport, and a TEP behaved like a diffusion potential. However, when both sides of the gill were bathed by an [Na^+] of ~200 mmol l^{-1}, a small but significant TEP developed (approximately –7 mV). Such a TEP has been reported when the gills of several crabs were perfused with symmetrical solutions more dilute than SW; these are assumed to be a reflection of an active ion transport system. Na^+ flux measurements showed that influx was active (by the flux ratio criterion; Lucu and Siebers, 1986). Similar results were obtained in gills from the crab *Chasmagnathus granulatus*. When the gills were perfused with symmetrical solutions equivalent to hemolymph in SW (1045 mOsm l^{-1}; [Na^+]=468 mmol l^{-1}) the TEP was small, but when solution concentrations were reduced (700 mOsm l^{-1}; [Na^+]=312 mmol l^{-1}) it increased significantly. In the split gill lamella, the I_{sc} doubled when the perfusing solutions were diluted (Tresguerres et al., 2003). In fact, this is what is expected from Shaw's observation that Na^+ transport was controlled by hemolymph (here perfusate) concentration and was activated only when [Na^+] fell below 400 mmol l^{-1}. However, the work on *C. granulatus* showed that activation was due to dilution of the hemolymph; decreased [Na^+] or [Cl^-] at constant osmotic pressure did not activate the transport system. Flux ratio analysis showed that J_{in}^{Na} was also active when posterior gills of *Pachygrapsus marmoratus* (Pierrot et al., 1995a) and *C. granulatus* (Luquet et al., 2002) were perfused with symmetrical dilute solutions.

In the perfused gill of *C. maenas*, ouabain in the perfusate reduced J_{in}^{Na} by 45% with no effect on J_{out}^{Na}. J_{in}^{Cl} was also inhibited with little effect on efflux. This suggested that the two influxes might be linked. When K^+ was omitted from the perfusion fluid, J_{in}^{Cl} was reduced by ~50%, which is consistent with the action of ouabain; i.e. both treatments inhibit the Na^+/K^+-ATPase. In addition, it was also shown that omitting Na^+ from the outer bath reduced J_{in}^{Cl} by 60%, which supported the suggestion that Na^+ and Cl^- fluxes were linked (Lucu and Siebers, 1987). This coupling was later noted in the split gill lamella, where omission of Na^+ from the bathing solutions inhibited the I_{sc} by >90% (Onken and Siebers, 1992). Ouabain also inhibited J_{in}^{Na} in *P. marmoratus* (Pierrot et al., 1995b) and *C. granulatus* (Luquet et al., 2002). Thus, there is substantial evidence that the Na^+/K^+ pump plays a key role in NaCl transport in weakly hyperregulating crabs.

There have been several reports that amiloride applied to the apical side of the perfused gill partly inhibited J_{in}^{Na}. This was examined recently using the split gill lamella of *C. maenas*. The preparation is usually voltage-clamped, and I_{sc} measured. The latter is negative inward and more will be said about it later. Here, it suffices to note that amiloride inhibited both influx and efflux of Na^+ with no effect on Cl^- fluxes and none on I_{sc} (Onken and Riestenpatt, 2002). It is interesting to note that amiloride blocks J_{in}^{Na} without effect on J_{out}^{Na} in crayfish and fishes. Until now it had been assumed that the action of amiloride was on a component (the Na^+ channel) in the apical cell membrane. However, these investigators went on to show that amiloride blocked the current imposed by a 10 mV clamp voltage across isolated cuticle (95% inhibition). In addition, the current fluctuations induced by amiloride were similar in the intact split lamella (cuticle attached) to those in the isolated cuticle. Their conclusion was that the inhibition by amiloride was due to blocking a sodium conductance in the cuticle rather than on a cellular transport component. This may be the general case, at least in these crabs.

Although most flux measurements have been on Na^+, the Cl^- flux data from *C. maenas* (Lucu and Siebers, 1987) indicated that Cl^- influx was also active, and this is supported by the fact that the polarity of the 'transport potential' is hemolymph negative in all cases reported and by the negative inward I_{sc}, which is equal to J_{net}^{Cl} (Riestenpatt et al., 1996). It is therefore germane to inquire about the mechanism transferring Cl^- across the basolateral membrane. It was shown that several Cl^- channel blockers in the perfusate inhibited the TEP and both J_{in}^{Na} and J_{in}^{Cl} across the perfused gill (Siebers et al., 1990). The most potent of these was 5-nitro-2-(3 phenylpropylamino)-benzene (NPPB), which inhibited J_{in}^{Na} by ~50% and J_{in}^{Cl} by ~70%. This was confirmed on the split gill lamella, where NPPB inhibited I_{sc} by ~80% (Riestenpatt et al., 1996). These data show that Cl^- exit from the epithelial cell occurs through a channel; the basolateral membrane potential difference provides the necessary driving force.

The split gill also provides sufficient additional evidence to

suggest a mechanism for NaCl absorption. Application of K^+ channel blockers (Ba^{2+}, Cs^+) to the apical bath virtually abolished I_{sc} and reduced both J_{in}^{Na} and J_{in}^{Cl} in *C. maenas* without affecting effluxes. Cs^+ also inhibited I_{sc} when added to the perfusate (Riestenpatt et al., 1996). These data indicate that K^+ channels exist in both apical and basolateral membranes, and each plays a role in transport. The presence and function of a basal Cl^- channel was noted above, as was evidence for the participation of the Na^+/K^+ pump. Another important observation is that the ratio $J_{net}^{Na}:J_{net}^{Cl}$ is 0.3–0.5 in the voltage-clamped split lamellae ($\Delta\Psi$=0; Onken and Riestenpatt, 2002; Riestenpatt et al., 1996). This suggested that the initial step at the apical membrane might employ the $Na^+/K^+/2Cl^-$ cotransporter. To test this, the basal membrane was made ion-permeable with amphotericin B, thus functionally isolating the apical membrane. Using imposed NaCl and Cl^- gradients to drive ion movement across the membrane, they showed that J_{in}^{Cl} was markedly reduced if either Na^+ or K^+ were omitted from the outside bath. The model shown in Fig. 3 has the cotransporter in the apical membrane, K^+ channels in both membranes to recycle K^+ that enters *via* the cotransporter and the Na pump, and a Cl^- channel to allow transfer of Cl^- into the hemolymph (Riestenpatt et al., 1996). Most of the features of the model were also found in *C. granulatus* (Onken et al., 2003), and it may prove to be generally characteristic of brackish water crabs.

In this model, the H^+-ATPase plays no role, and in support of this are data that appear to show that it does not function in NaCl transport in *Carcinus.* Thus, the amount of the enzyme in the posterior gills of both *C. maenas* and *U. tangeri* is low; only 5–10% of values found in *E. sinensis* and *Orchonectes* sp. (Putzenlechner, 1994). In addition, although mRNA for a subunit of the enzyme was found in the gills, it was more abundant in the anterior than in the posterior gills and did not respond when the crab was transferred to dilute solution. Immunochemical localization showed that the subunit was found distributed throughout the cells rather than targeted to the membrane (Weihrauch et al., 2001). It is worth noting that transcellular Na^+ movement in this model is only half that of Cl^-. To preserve electrical neutrality in open circuit it is necessary that an equal influx of Na^+ take place through paracellular channels, using the TEP as a driving force. This saves half the cost of NaCl absorption; i.e. only half of the total passes through the Na^+/K^+ pump. A leaky paracellular pathway may also explain why amiloride inhibition of J_{in}^{Na} (which occurred at the cuticle) had no effect on Cl^- fluxes; Na^+ could be supplied by diffusing from perfusate to the subcuticular space.

On the other hand, neither furosemide (Riestenpatt et al., 1996) nor bumetanide (Lucu, 1989) in the external bath had much effect on fluxes, TEP or I_{sc}. These compounds are known to be potent inhibitors of the $Na^+/K^+/2Cl^-$ cotransporter, and their lack of action provides no support for the model proposed. It remains to be determined whether the cuticle prevents them from approaching the apical membrane (as is apparently the case with amiloride) or whether a different model is required.

Finally, the participation of carbonic anhydrase in NaCl transport has given rise to some controversy. It was reported (Böttcher et al., 1991) that AZ had no effect on the (presumably transport-related) TEP in perfused gill of *C. maenas*, although it inhibited HCO_3^- extrusion into the bathing medium. The apparent lack of effect on ion fluxes was confirmed, and a role for the enzyme in cell pH regulation was suggested (Siebers et al., 1994). The inhibitor also had no effect on I_{sc} in the split gill lamella (Onken and Siebers, 1992). It was reported to have a modest inhibitory action on J_{in}^{Cl} and the TEP (~25%) in *C maenas*, with less effect on J_{in}^{Na}, but the concentration (10 mmol l^{-1}) was 100× higher than is usually used (Lucu, 1989). It also had a modest effect (~20%) on J_{in}^{Na} in *C. granulatus* (Onken et al., 2003). Neither of these effects is impressive. However, it was later argued (Henry, 2001) that Böttcher's lack of results in *Carcinus* were due to the low permeability of membranes to AZ and insufficient time allowed for it to act. One notes here that it did have a pronounced effect on both H^+ transfer into the perfusate and HCO_3^- excretion into the bath, so it probably entered the cell. If the model proposed by Onken and Riestenpatt (1998) is correct, neither H^+ nor HCO_3^- plays a significant role in NaCl absorption in weak regulators, although CA might be important in acid–base regulation.

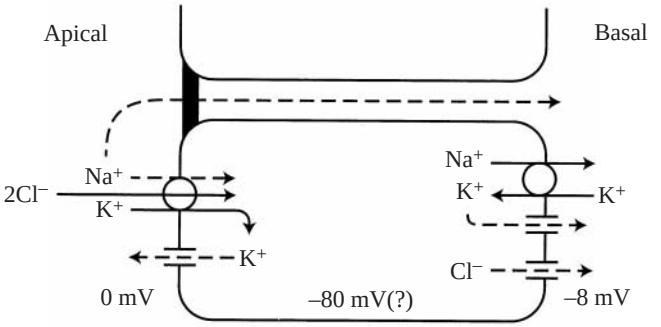

Fig. 3. A tentative model for Na^+ and Cl^- cotransport by *Carcinus* gill. Note that transcellular Na^+ influx is only half that of Cl^-. To maintain electrical neutrality, paracellular Na^+ influx must equal transcellular Na^+ influx. The model requires higher ambient Na^+ and K^+ than is usually found in freshwater in order to operate the cotransporter, but it would be effective in brackish water. The apical membrane potential difference (–80 mV) is an estimate, supposing that it is a K^+ diffusion potential with $[K^+]\approx 2$ mmol l^{-1} in 20% seawater. The transepithelial potential difference (–8 mV) is based on many published values between –5 mV and –10 mV.

Strong hyperregulators

After Krogh's initial observations on *E. sinesis*, Na^+ uptake was examined in more detail in the intact animal (Shaw, 1961b) and in *Potomon niloticus* (Shaw, 1959b). Amiloride was shown to inhibit unidirectional Na^+ fluxes in *Callinectes sapidus* with no effect on Cl^- fluxes. J_{in}^{Na} was also markedly inhibited in *Callinectes* by injected AZ (Cameron, 1979). Nearly all subsequent studies have employed either the perfused gill leaflet or the split gill lamella, and most of them have been on *E. sinensis.*

When the isolated gill of *Eriocheir* was perfused on both sides with a saline approximating hemolymph concentrations in FW, there was a TEP of 5–50 mV negative on the basal side. It was observed that when Na^+ was omitted from the external solution (replaced by tris or choline), the TEP increased markedly, as it also did when amiloride was added to the external bath. Conversely, replacing Cl^- with gluconate or SO_4^{2-} resulted in a TEP of opposite sign (positive inside), and this was abolished by amiloride (Pequeux and Gilles, 1988; Onken and Graszynski, 1989). Both research groups suggested that the data reflected the independent, electrogenic influxes of Na^+ and Cl^-.

Fluxes of Na^+ and Cl^- were measured under isoionic conditions in the perfused gill. J_{in}^{Na} appeared to show saturation kinetics, while J_{out}^{Na} was undetectable. This supported the surmise, based on TEP measurements, that Na^+ uptake was active (Pequeux and Gilles, 1981). One curious observation in this paper was that ouabain had no effect on Na^+ influx when applied in the perfusate but that it was inhibitory when applied to the apical side. An apical location of the Na^+/K^+-ATPase would be unusual in a transport epithelium, but the observation has not been confirmed (see below). There was a measurable J_{out}^{Cl}, but J_{in}^{Cl} was higher at all concentrations above 1 mmol l^{-1}. Net uptake took place in the absence of a concentration gradient and against a voltage gradient and hence was active (Gocha et al., 1987).

The voltage-clamped, split lamella was employed to investigate the mechanisms involved in Na^+ and Cl^- movements. The exposed area of this preparation (~1–2 mm^2) is too small to make isotope experiments generally useful, and most of the data involved clamp currents. However, the preparation allowed insertion of a microelectrode to measure the apical and basal membrane potential differences. The APD was about –100 mV (cell negative) while the basal membrane potential difference was about –80 mV (cell negative). In the absence of Na^+ in the outer solution, the current was negative inward, and using ^{36}Cl to measure J_{in}^{Cl}, it was shown that, under these conditions, $I_{sc}=J_{net}^{Cl}$ (noted I_{Cl}; Onken and Riestenpatt, 1998). Apical addition of blockers of Cl^-/HCO_3^- exchange (SITS, SCN^-) inhibited I_{Cl}, indicating that the exchange was an important step in Cl^- uptake. In agreement with this, the current was inhibited by AZ in the perfusate. Ouabain had no effect on the current. Elevating $[K^+]$ in the perfusate resulted in depolarization of the basolateral membrane, which would reduce the driving force for Cl^- exit from the cell, and partially inhibited I_{Cl}. The Cl^- channel blocker diphenylamine carboxylate (DPC) applied inside also markedly inhibited I_{Cl}. Thus, exit across the basolateral membrane involves diffusion, driven by the membrane potential, through Cl^- channels (Onken et al., 1991). The proton ATPase was also implicated in the mechanism. First, it was shown that the enzyme was present in gill homogenates and did not copurify with the Na^+/K^+-ATPase or with acid phosphatase (an intracellular organelle marker) on centrifugation, and hence was probably on the apical membrane. Further, vesicles made from a fraction containing the V-ATPase generated an ATP-driven H^+ transport. And finally, I_{Cl} across the split lamella was inhibited by apical addition of bafilomycin (Onken and Putzenlechner, 1995). Immunolocalization showed that the V-ATPase was confined to the 'Pillar' cells (Putzenlechner et al., 1992). Whether the exchanger and CA are also confined within these cells or whether the relationship is less direct is unknown.

In the absence of Cl^- outside, the current was positive inward and was completely abolished by amiloride applied outside or by substituting tris for Na^+ (Schwarz and Graszynski, 1990; Zeiske et al., 1992). It was also abolished by ouabain applied inside (Schwartz and Graszynski, 1990), in disagreement with the report of Pequeux and Gilles (1981). Thus, I_{sc} in the absence of Cl^- (noted I_{Na}) was a measure of J_{net}^{Na} . It was shown that amiloride-sensitive Na^+ entry was *via* apical membrane channels (Zeiske et al., 1992). The APD (–100 mV) would provide the force necessary for diffusion through these channels. It is likely, but not demonstrated, that the V-ATPase is responsible for the APD, and hence is also involved in Na^+ uptake. A model for transport of both ions, which must be considered tentative at present, is essentially the same as for the frog skin γMR cell, with the same uncertainty about how the gill transports Na^+ in the absence of Cl^-.

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