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INVITED PERSPECTIVES IN PHYSIOLOGICAL AND BIOCHEMICAL ZOOLOGY

Ion Transport Processes of Crustacean Epithelial Cells

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ABSTRACT

Epithelial cells of the gut, antennal glands, integument, and gills of crustaceans regulate the movements of ions into and across these structures and thereby influence the concentrations of ions in the hemolymph. Specific transport proteins serving cations and anions are found on apical and basolateral cell membranes of epithelia in these tissues. In recent years, a considerable research effort has been directed at elucidating their physiological and molecular properties and relating these characteristics to the overall biology of the organisms. Efforts to describe ion transport in crustaceans have focused on the membrane transfer properties of Na^+/H^+ exchange, calcium uptake as it relates to the molt cycle, heavy metal sequestration and detoxification, and anion movements into and across epithelial cells. In addition to defining the properties and mechanisms of cation movements across specific cell borders, work over the past 5 yr has also centered on defining the molecular nature of certain transport proteins such as the Na^+/H^+ exchanger in gill and gut tissues. Monovalent anion transport proteins of the gills and gut have received attention as they relate to osmotic and ionic balance in euryhaline species. Divalent anion secretion events of the gut have been defined relative to potential roles they may have in hyporegulation of the blood and in hepatopancreatic detoxification events involving complexation with cationic metals.

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Introduction

Crustaceans are a very diverse group of organisms, living in a wide variety of habitats, including freshwater, marine, and terrestrial. The large numbers of species found in these very different environments are largely a function of the physiological plasticity of the group as a whole. Functional challenges to organisms inhabiting these markedly dissimilar environments often involve specialized adaptations of epithelial cell layers found in the gills, integument, gut, and antennal glands, which allow the animals to regulate the passage of molecules, such as ions, between the external environment and the hemolymph. These adaptations can be at the molecular, cellular, or tissue level of anatomical complexity, and these tissue-specific specializations may be modified by systemic hormonal control processes. Over the past 20 yr a large number of excellent publications have appeared in print that describe the organismic abilities of a significant number of crustaceans to inhabit different places on earth, but few have addressed the specific cellular, membrane, or molecular processes by which each tissue is able to contribute to the overall physiological adaptation of the animal. The goals of this review are to present experimental data and theoretical models, largely published over the past 5 yr, which describe the roles of crustacean epithelial cells in regulating cellular and hemolymph ion content of animals living in a wide variety of environments. Ion transport mechanisms are compared between species, and convergent mechanisms for dealing with similar regulatory problems in dissimilar habitats are elucidated. It is also hoped that this review will provide the reader with insight into some of the more challenging physiological problems facing experimentalists in crustacean biology today and will therefore potentially lead to new ways of addressing some of the more outstanding questions that still remain to be elucidated about the biology of a highly successful group of organisms.

Na^+/H^+ Exchange in Invertebrate Gut, Renal, and Gill Epithelia

Many of the larger invertebrate representatives of the phyla echinodermata, mollusca, and arthropoda possess extensive gastrointestinal diverticula that are composed of hundreds of blind-ended tubules that exchange their luminal contents with those of either the stomach or the intestine during normal digestive and absorptive activities. Recently, the crustacean hepatopancreas, a bilobed stomach diverticulum, has come under intense investigation, using membrane vesicle techniques

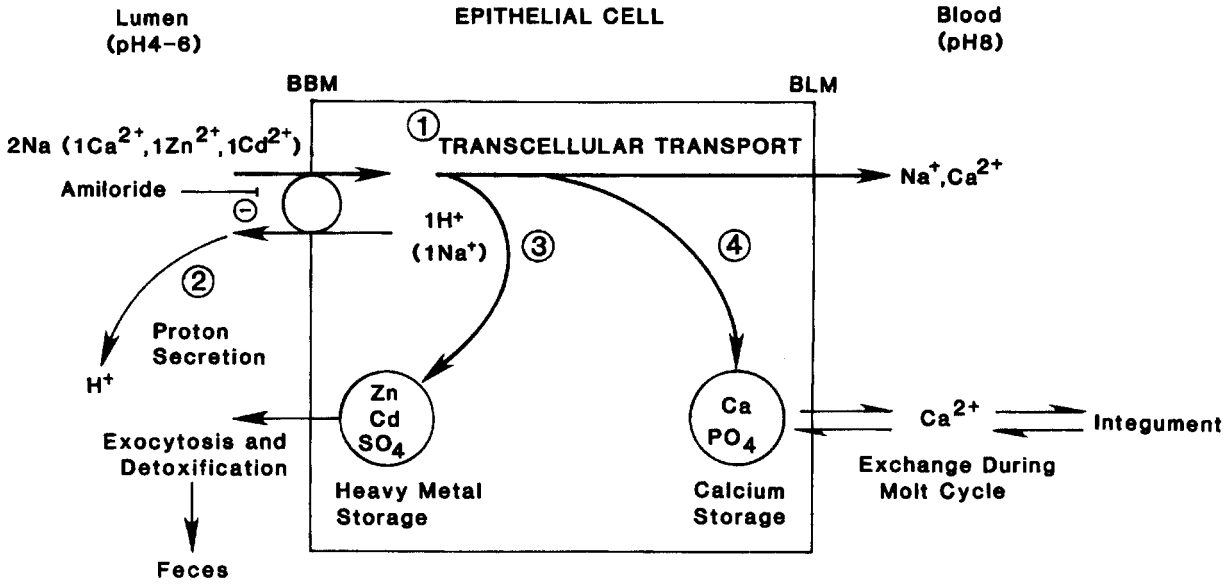

Figure 1. Polyfunctional role of invertebrate electrogenic $2Na^+/1H^+$ antiporter for absorption, secretion, and sequestration of monovalent and divalent cations in crustacean hepatopancreatic epithelium. Involvement of apical membrane (*BBM*) exchanger with the four physiological activities of this tissue shown is described in the text. Sodium is transferred across the basolateral membrane (*BLM*) by an electroneutral $1Na^+/1H^+$ exchanger (Duerr and Ahearn 1996) and an electrogenic $3Na^+/2K^+$ sodium/potassium ATPase, while basolateral calcium transfer takes place by the carrier and channel proteins described later in this review. Some or all of these processes are common to a wide variety of invertebrate epithelial cells. (From Ahearn 1996.)

originally developed to study transport phenomena in vertebrate epithelial cells. Reports over the past 12 yr with this methodology showed the epithelial brush border membrane (e.g., apical membrane) of this organ to possess a wide variety of transport proteins for the absorption of sugars (Ahearn et al. 1985), amino acids (Ahearn et al. 1986; Ahearn and Clay 1987*a*, 1987*b*, 1988; Balon and Ahearn 1991), vitamins (Siu and Ahearn 1988), and peptides (Thamotharan and Ahearn 1996). The physiological role of the hepatopancreas as a nutrient absorptive organ has been suggested for at least 75 yr (Yonge 1924; van Weel 1955), and these new data lend strong support for this contention (Ahearn 1987, 1988; Ahearn and Clay 1988; Ahearn et al. 1992).

More recently, membrane vesicle methodology has been applied to the study of ion transport processes of the hepatopancreas. Because of its ubiquitous nature among the vertebrates and its well-characterized transport properties, the plasma membrane Na^+/H^+ antiporter was first examined in the apical membrane of the hepatopancreatic epithelium (Ahearn and Clay 1989; Ahearn et al. 1990). These studies provided evidence that the antiporter found in this location displayed transport properties quite different from those universally reported for vertebrate cells. Sodium/proton exchange in hepatopancreatic apical membrane was amiloride sensitive and electrogenic and had a transport stoichiometry of $2Na^+/1H^+$. These physiological properties of the $2Na^+/1H^+$ exchanger also hold for crustacean antennal glands (Ahearn and Franco 1990) and starfish pyloric ceca (Ahearn and Franco 1991). The divalent cation, calcium, was shown to be a transported substrate by this carrier process in place of two monovalent sodium ions (Ahearn and Franco 1990, 1993), and as a result, a model was proposed for the epithelium of the crustacean hepatopancreas linking this transport process to such biological activities as gastric acidification, transcellular ion absorption, and calcium regulation during the molt cycle (Fig. 1; Ahearn et al. 1994; Ahearn 1996).

A similar electrogenic $2Na^+/1H^+$ transport system was described in crab gill (*Carcinus maenas*) epithelium using acridine orange as a technique for quantifying the rates of exchange across vesicular membranes in this animal (Shetlar and Towle 1989). As with apical membranes from crustacean hepatopancreas and antennal glands, the gill electrogenic antiporter was amiloride sensitive. A recent model describing ion transport by crab gill epithelium proposes an apical location (facing the external environment) of this Na^+/H^+ exchanger together with Cl^-/HCO_3^- and $Ca^{2+}/1H^+$ antiporters (Towle 1993).

Na^+/H^+ exchange also has been characterized for the hepatopancreatic basolateral membrane in lobsters, and the properties of this carrier system on the blood side of the epithelium vary considerably from those facing the lumen (Duerr and Ahearn 1996). In contrast to the brush border antiporter, the exchanger on the basolateral membrane was electroneutral, ex-

hibited a transport stoichiometry of $1Na^+/1H^+$, and was insensitive to divalent cations. In fact, the hepatopancreatic basolateral exchanger very closely resembled the basolateral antiporter described for vertebrate epithelial cells of the intestine and kidney known as NHE1 (Aronson 1985; Sardet et al. 1989). These results support the paradigm established for vertebrates that epithelial cells exhibit a specialized brush border Na^+/H^+ exchanger that is physiologically attuned to the specific cell specialization, while the basolateral transporter is more conserved in function, and largely plays housekeeping roles of cell volume regulation, pH control, and maintenance of intracellular monovalent ion activities.

Very recently, using a combination of acridine orange fluorometric methods and $^{22}Na^+$ radioisotopic tracer procedures in the same study, an electroneutral apical membrane Na^+/H^+ antiporter was described for the hepatopancreatic epithelium of the marine shrimp *Penaeus japonicus* (Vilella et al. 1998). The characteristics of this transport system compared favorably with those described for the basolateral $1Na^+/1H^+$ antiporter described in the lobster (Duerr and Ahearn 1996), but control experiments from the laboratory of Vilella et al. showed that phlorizin-sensitive ^{3}H-D-glucose transport occurred on the vesicle preparation displaying electroneutral cation exchange, providing clear evidence of an apical location of the described antiporter. Additional control studies using BBMV (apical membrane vesicles) from lobster (*Homarus americanus*) hepatopancreas from the same research team disclosed electrogenic $2Na^+/1H^+$ antiport for this tissue, confirming published accounts of the lobster exchanger. Lobster hepatopancreatic R-cells were reported to be the cell type responsible for the expression of electrogenic $2Na^+/1H^+$ antiport in this animal, using immunolocalization methodology (Kimura et al. 1994). Histological examination of *P. japonicus* hepatopancreatic epithelium revealed a paucity of R-cells, relative to F- and B-cell types (Vilella et al. 1998), and the authors proposed that the electroneutral Na^+/H^+ exchange characterized from this animal may have been due to expression of an exchange process displayed in the brush borders of the remaining epithelial cell types.

Regulation of Na^+/H^+ Exchange in Crustacean Hepatopancreas

Physiological control mechanisms regulating invertebrate plasma membrane transport proteins have not been investigated to any significant extent. Control of housekeeping operations by basolaterally localized NHE1 in vertebrate epithelial cells has been studied in a variety of cell types. In these animals, Na^+/H^+ exchange is allosterically regulated by intracellular proton concentration in such a way that the antiporter can respond immediately and autonomously to an acidification of the cytoplasm (Aronson et al. 1982). This allosteric regulation is brought about by the presence, on the cytoplasmic surface of the transporter, of a H^+-binding modifier site that is distinct from the H^+ and Na^+ transport sites and activates cation exchange when occupied by intracellular protons. The exchanger can also be activated by phosphorylation by protein kinase C, which in turn is activated by diacylglycerol or by tumor-promoting phorbol esters (Grinstein et al. 1985).

More recent studies with regulation of mammalian NHE1 have shown in Na^+/H^+-exchanger-deficient fibroblast cells transfected with NHE1 that increases in cytosolic calcium concentration stimulate EIPA-sensitive $^{22}Na^+$ uptake by a Ca^{2+}-calmodulin-dependent kinase (Wakabayashi et al. 1994). Furthermore, ATP depletion in A431 carcinoma cells caused by metabolic inhibitors dramatically reduces the exchange rate of the transporter, and the ATP sensitivity appears to be determined by several cytoplasmic regions of the NHE1 (Cassel et al. 1986; Goss et al. 1994). Other studies have indicated that novel types of heterotrimeric plasma membrane G proteins in mammalian epithelial cells ($G_{\alpha 12}$ and $G_{\alpha 13}$) may possibly be directly coupled to the Na^+/H^+ exchanger influencing ion transport activity (Kitamura et al. 1995). Finally, cell spreading recently has been shown to activate the Na^+/H^+ exchange process in endothelial cells through an integrin-mediated process (Ingber et al. 1990), and growth factor activation of NHE1 appears to depend greatly upon the degree of cell adhesion to the substratum, strongly suggesting an interaction of the exchanger with the cell cytoskeleton (Schwartz et al. 1992). All these recent studies concerning the vertebrate electroneutral exchanger point to an increasing intracellular complexity of regulatory events that control the movements of these monovalent ions across the plasma membranes of the respective cells. Perhaps in the near future, the complex regulatory cascade for an NHE1 isoform will be known in totality for at least a single cell type and can be used as a primary model system to test the conservation of these processes across cell types.

Cellular mechanisms of hormonally mediated regulation of plasma membrane–bound transport proteins in crustaceans are little known, but the evidence available suggests that protein kinases may be involved with such processes as crustacean hyperglycemic hormone (CHH) regulation of blood sugar in some species (Sedlmeier 1985) and transepithelial sodium transport in Chinese crab (Asselbourg et al. 1991). To assess the nature of intracellular regulatory mechanisms controlling Na^+/H^+ antiport in lobster hepatopancreatic epithelial cells, a centrifugal elutriation method was applied to dissociated heterogeneous cell suspensions, initially composed of four different epithelial cell types, producing a >95% pure preparation (assessed visually) of undifferentiated mitotic E-cells (Duerr and Ahearn 1998). These cells are located at the distal tips of hepatopancreatic tubules and differentiate into two cell lines, R-cells (absorptive in nature) and F-cells (secretory in nature), as they migrate proximally down the tubule during maturation (Al-Mohanna and Nott 1989). E-cells were selected as an initial model for investigation of transport regulation because it was

thought that an undifferentiated cell likely would only possess the housekeeping antiporter and later would express the apical specialized exchanger as it matured.

The fluorescent pH-indicator dye 2′,7′-bis(carboxyethyl)-5,6-carboxy-fluorescein (BCECF) at 2.5 μM was loaded into purified suspensions of hepatopancreatic E-cells by incubation for 20 min at room temperature in a lobster physiological saline. The dependence on extracellular Na^+ by E-cell Na^+/H^+ exchange was quantitatively described by varying extracellular Na^+ from 1 mM to 200 mM in a choline medium. E-cells were suspended in choline medium and acidified with 30 mM K^+-propionate and pH_i monitored (Duerr and Ahearn 1998). Na^+-dependent proton efflux was measured over the first minute of incubation and could be described by a saturable carrier system exhibiting Michaelis-Menten kinetics according to Equation (1):

$$J_H = J_{max} \times (Na^+)/[K_m + (Na^+)], \quad (1)$$

where J_H is the outward H^+ flux in μmol/s × L cell water, J_{max} is the maximal carrier-mediated H^+ flux in μmol/s × L cell water, K_m is the concentration of Na^+ resulting in 1/2 J_{max}, and (Na^+) is the concentration of Na^+ in mM. The hyperbolic curve in Figure 2 represents carrier-mediated Na^+/H^+ antiport by a transport protein with an apparent 1 : 1 exchange stoichiometry such as occurs on the basolateral membrane of hepatopancreatic epithelial cells (Duerr and Ahearn 1996). Kinetic constants were calculated by iterative curve-fitting software (KaleidaGraph 3.0) using the Michaelis-Menten function, providing a K_m of 41.5 ± 10.8 mM and a J_{max} of 68.8 ± 7.0 μmol/s × L cell water. The hyperbolic nature of this relationship suggests that E-cell plasma membranes do not express the electrogenic $2Na^+/1H^+$ antiporter. Previous studies with apical vesicles produced from a mixture of all cell types resulted in a sigmoidal Na^+/H^+ exchange relationship with a 2 : 1 transport stoichiometry as a result of the transport contribution from mature R-cell types (Ahearn and Clay 1989; Ahearn et al. 1990; Kimura et al. 1994).

The resting pH_i of E-cells is 7.19 ± 0.008 ($n = 12$) in standard lobster physiological saline (Duerr and Ahearn 1998). Two chemical agents capable of stimulating either protein kinase A or protein kinase C pathways were applied to BCECF-stained E-cell suspensions. Addition of 20 nM PMA induced a considerable alkalinization over a 10-min period, which became apparent within 3 min (Fig. 3). In contrast, 2 μM 8-Br-cAMP produced no such stimulation of Na^+/H^+ exchange over the entire 10-min interval. The final pH_i attained after the addition of 20 nM PMA for three separate experiments was 7.45 ± 0.02, an average pH_i increase of 0.21. This alkalinization was inhibited by dimethyl amiloride (Duerr and Ahearn 1998), strongly suggesting that the increase in pH_i in the presence of

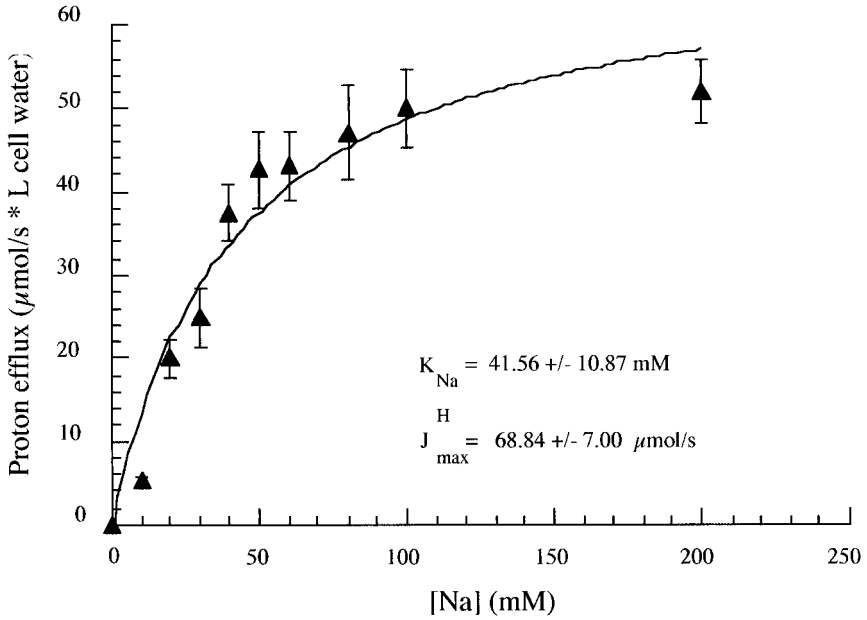

Figure 2. Na^+/H^+ exchange kinetics of hepatopancreatic E-cells. External Na^+ concentrations ranged from 0 to 200 μM. Cells were acidified by exposing them to 30 μM K^+-propionate, and the rate of H^+ efflux was calculated over the first minute. Data plotted represent means ± SEM of five calculations. Hyperbolic curve and kinetic constants were calculated using iterative curve-fitting software (KaleidaGraph 3.0). (From Duerr and Ahearn 1998.)

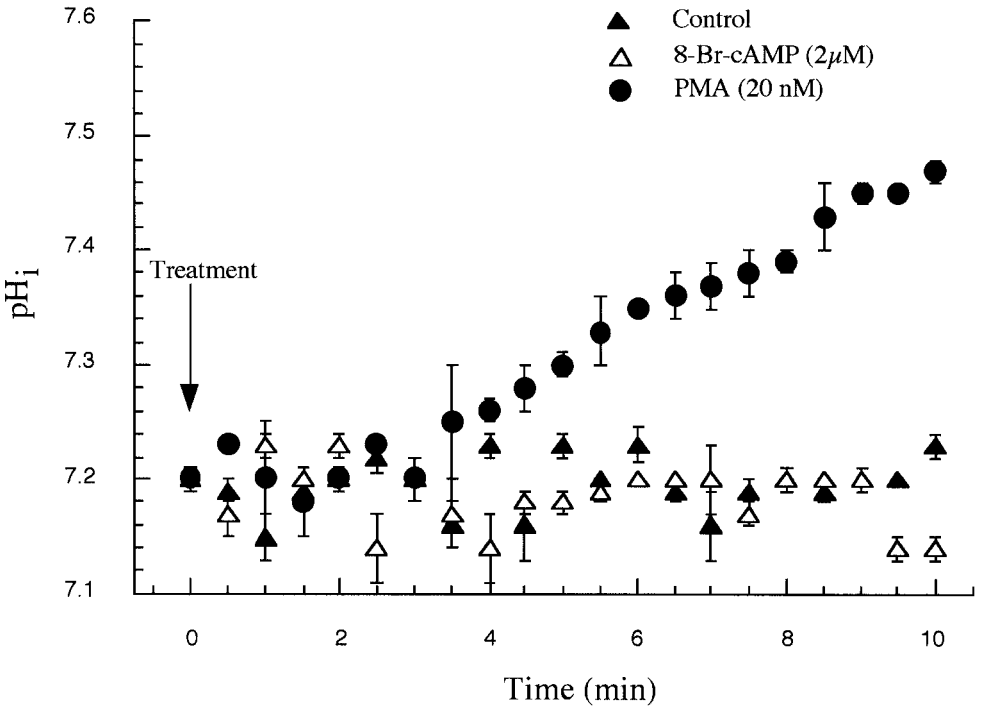

Figure 3. Effects of putative activating agents of crustacean hepatopancreatic E-cell Na^+/H^+ exchange. E-cell suspensions in a standard lobster physiological saline were exposed to 20 nM PMA (phorbol 12-myristate, 13-acetate) and 1 μM 8-Br-cAMP (8-bromo-cyclic adenosine monophosphate) in order to assess any activating effect. Over a period of 10 min, the presence of 20 nM PMA caused an increase of 0.28 pH units, whereas 8-Br-cAMP was without effect relative to control conditions. Data are means $\pm$ SEM of three separate preparations. (From Duerr and Ahearn 1998.)

PMA was due to an increase in Na^+/H^+ exchange. The activation of Na^+/H^+ exchange by PMA, and the lack of effect on this carrier system by cAMP, suggests that regulation of this exchange system in lobster hepatopancreatic E-cells more strongly resembles the regulatory cascade for NHE1 of mammal basolateral membranes, which also are stimulated by phorbol esters through phosphorylation of protein kinase C and are unaffected by protein kinase A or cAMP (Grinstein et al. 1985; Sardet et al. 1990; Borgese et al. 1992) than the regulatory cascade of the NHE1 homologue, NHEβ, found in teleost red blood cells, which is activated by cAMP (Borgese et al. 1994).

Molecular Biology of Na^+/H^+ Exchange in Crustacean Epithelia

During the past 5 yr investigators have applied molecular biology techniques to sequence and clone cDNAs coding for Na^+/H^+ antiporters in crustacean epithelial cells of the gills, gut, and antennal gland so that nucleotide and amino acid comparisons can be made between invertebrate and published vertebrate systems. cDNA sequences, coding for vertebrate electroneutral $1Na^+/1H^+$ antiporters, have been cloned in several mammals, including humans (Sardet et al. 1989), pigs (Reilly et al. 1991), rats (Orlowski et al. 1992), and rabbits (Tse et al. 1991), and designated as NHE1. Also, three additional isoforms recently have been described, primarily in mammalian intestine and kidney, and have been designated as NHE2, NHE3, and NHE4 (Orlowski et al. 1992; Tse et al. 1993; Wang et al. 1993). In fish red blood cells a related isoform, named NHEβ, was described (Borgese et al. 1994) that exhibits a 75% homology to mammalian NHE1. Most recently, a cDNA from crab gill coding for a Na^+/H^+ exchanger in this location was cloned and expressed in *Xenopus* oocytes (Towle et al. 1997). Injection of *Xenopus laevis* oocytes with cRNA transcribed from the cloned crab sequence substantially enhanced Na^+-dependent H^+ efflux from the oocytes, as determined using a miniature pH microelectrode placed in the surrounding buffer. On the basis of these results, Towle et al. (1997) proposed a hypothetical secondary Na^+/H^+ antiporter protein structure within a lipid bilayer that exhibits 10 transmembrane-spanning α-helices and a long intracellular loop terminating in a -COOH that likely participates in regulation of the antiporter. It is unclear whether the Na^+/H^+ antiporter cDNA sequence reported for crab gill is functionally an electroneutral or electrogenic exchanger, but the study does indicate that the crab gill clone may be related

NHE Amino Acid Sequence Alignment

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          1                                                          60
Crab      AVYFMPNRLFFDNLFTILVFAVIGTIWNALTIGITMYAISLTGLFG-LDIPMLHMFLFSS
Lobster   AVYFMPNRLFFDNLFTILVFAVIGTIWNALTIGITMYAISLTGLFG-ESIPMLHMFLFSS
Human     AGYFLPLRQFTENLGTILIFAVVGTLWNAFFLGGLMYAVCLVGGEQINNIGLLDNLLFGS

          61                                                        120
Crab      LISAVDPVAVLAVFEEMQVEEVLFILVFGESLLNDGVTVVLYHLFEGFSELGE-ANIMAV
Lobster   LISAVDPVAVLAVFEEMHVEEVLFILVFGESLLNDGVTVVLYHLFEGFSELGE-ANIMLV
Human     IISAVDPVAVLAVFEEIHINELLHILVFGESLLNDAVTVVLYHLFEEFAN---YEHVGIV

          121                                                       180
Crab      DIASGVASFLLVALGGTAIGIIWGFLTAFVTRLTSGVRVIEPVFVFVMAYLAYLNAEIFH
Lobster   DIASGVASFLLVALGGTVIGIIWGSLTAFVTKFTSGVRVIEPVFVFVMAYLAYLNAEIFH
Human     DIFLGFLSFFVVALGGVLVGVVYGVIAAFTSRFTSHIRVIEPLFVFLYSYMAYLSAELFH

          181                                                       240
Crab      LSGILSITFCGITMKNYVEQNISAKSHTTIKYAMKMLASSSETIIFMFLGVSTIQSDHQW
Lobster   LSGILRITFCGITMKNYVEQNISTKSHTTIKYAMKMLASSSETVIFMFLGVSTVQSKHQW
Human     LSGIMALIASGVVMRPYVEANISHKSHTTIKYFLKMWSSVSETLIFIFLGVSTVAGSHHW

          241                                                       300
Crab      NTWFVILTILFCSIYRILGVLIFSAVCNRFRVKKIGFVDKFVMSYGGLRGAVAFALVITI
Lobster   NTWFVIFTIFFCSIYRILGVWIFSTMCNRFRVKKIAFVDKFITSYGGLRGAVAFALVLTI
Human     NWTFVISTLLFCLIARVLGVLGLTWFINKFRIVKLTPKDQFIIAYGGLRGAIAFSLGYLL

          301                                 330
Crab      NPIHIPLQPMFLTATIAMVYFTVFVQGITI
Lobster   NSLHIPLQPMFLTANIAMVYFTVFIQGITI
Human     DKKHFPMCDLFLTAIITVIFFTVFVQGMTS
```

Figure 4. Amino acid sequence alignment of the 1-kb fragment of the lobster NHE with the corresponding sequences of the crab and human NHE1 gene. Amino acids differing from lobster are shown boxed. Lobster NHE shows an 82% similarity with crab at the nucleotide level, which translates into a 92% amino acid similarity.

to NHE1 in the vertebrates because the membrane-spanning region of the crab sequence shows a 72% amino acid homology to the same region of the rat basolateral, electroneutral isoform (Towle et al. 1997).

Because Na^+/H^+ antiport experiments with lobster hepatopancreatic epithelial basolateral membranes suggested the occurrence of an NHE-like transport system in this location that functionally resembled members of the mammalian NHE family (Duerr and Ahearn 1996), work was initiated recently to determine the molecular structure of this invertebrate protein for comparison with that of the mammalian system. RT-PCR (reverse transcription polymerase chain reaction) was used to amplify defined segments of cDNA. Degenerate primers for low-stringency PCR were made from sequence alignment of the NHEs that have been cloned, including that from crab gill (Towle et al. 1997), and were used to produce a 1,000-base-pair (bp) cDNA clone whose nucleotide sequence very closely resembled a similar length segment from the crab gill sequence (Killebrew et al. 1998).

Nucleic acid alignment and homology estimates were generated using the Lasergene biocomputing software system for the Macintosh (DNASTAR Inc., Madison, Wis.). Crab and human NHE sequences were obtained using their respective accession numbers from the NCBI Genbank World Wide Web sequence retrieval page (http://www.ncbi.nlm.nih.gov/).

The cloned 1,000-bp lobster fragment was then sequenced by automated fluorescent sequencing techniques; its deduced amino acid sequence is presented in Figure 4, aligned with the final seven transmembrane-spanning homologous regions of the crab and human NHE1 isoforms. This region of the total protein presumably occurs just prior to the long intracellular -COOH terminal region that likely participates in protein regulation. This putative lobster cDNA fragment exhibits a 92% amino acid identity and an 82% nucleic acid identity with the crab NHE-like isoform, a 59% nucleic acid identity with human NHE1, and between 57% and 77% nucleic acid identity with the remaining NHE isoforms cloned from vertebrate tissues. The crab gill NHE-like cDNA clone is approximately 2,500 bp in length, and if the full-size lobster clone retains its high degree of similarity with that of the crab, the partial clone identified in this study therefore likely represents about 40% of its full length. Efforts are currently under way to complete the partial sequence reported here.

Similar cloning results for the Na^+/H^+ antiporter of mosquito Malpighian tubules have been reported recently (Petzel et al. 1998). In this instance, RT-PCR methods were used to

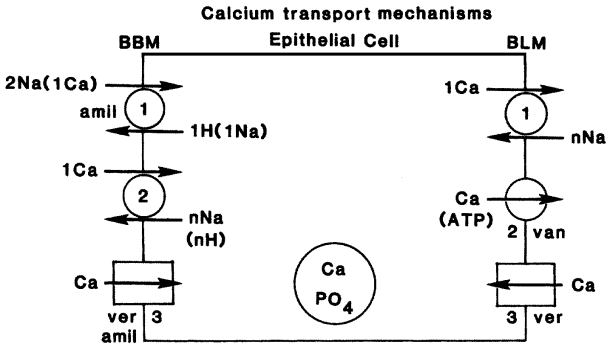


Figure 5. Proposed apical and basolateral calcium transport processes of the lobster hepatopancreatic epithelium. The luminal membrane (*BBM*) transporters include (1) an amiloride-sensitive electrogenic $2Na^+/1H^+$ antiporter, which can accommodate both external monovalent and divalent cations in exchange for either intracellular protons or sodium ions, (2) an amiloride-insensitive Ca^{2+}/nNa^+ exchanger, which probably exhibits a 1 : 1 stoichiometry and can accommodate intracellular protons or sodium ions, and (3) a verapamil-sensitive calcium channel. The basolateral transporters (*BLM*) include (1) a Ca^{2+}-ATPase, (2) an electrogenic ($3Na^+/1Ca^{2+}$) cation exchanger, and (3) a verapamil-sensitive calcium channel. Calcium phosphate vacuoles occur in specific cell types and represent a discrete intracellular calcium pool that undergoes cyclic formation and dissolution during the molt cycle of the lobster. These processes are likely linked to the plasma membrane calcium transporters through undefined regulatory mechanisms. (From Ahearn and Zhuang 1996; Zhuang and Ahearn 1998.)

amplify NHE-specific cDNA, which was subsequently cloned and sequenced. A prominent RT-PCR 510-bp band from the Malpighian tubules was isolated that was greater than 60% and 77% identical, respectively, to the nucleotide and amino acid sequences of specific transmembrane domains of the green crab NHE, rabbit NHE3, human NHE3, and rat NHE3, suggesting that this arthropod has an antiporter sequence that is highly homologous to published NHE systems of other species.

As suggested above, published work in progress in lobster and mosquito tissues suggests that these two arthropods likely exhibit cDNAs that are very similar to the sequence reported for crab gill NHE and indicate that similar "housekeeping" transport systems are probably present in epithelia of all three species and control cation movements by electroneutral exchange. It is not known at present whether the brush border electrogenic $2Na^+/1H^+$ antiporter disclosed for all three crustacean epithelia is related to NHE or represents a member of a completely novel gene family.

Calcium Transport Processes of Crustacean Epithelial Cells

There are four epithelial-lined organs that control the concentration of calcium in the blood of a crustacean: the gut, the kidneys (e.g., antennal glands), the gills, and the integumentary epidermis. At any stage of the feeding or molting cycles, these four cell layers are hormonally linked and transfer the cation across the respective cell layers following physiological dictates of the overall organism. Over the past 15 yr, a number of excellent reviews of the physiology of calcium balance in crustaceans inhabiting a variety of habitats (marine, freshwater, terrestrial) have been published (Greenaway 1985, 1993; Wheatly and Gannon 1995; Wheatly 1996, 1997, 1998). This review focuses on defining the characteristics of specific calcium transport systems described within the past 5 yr that occur in epithelial layers of the gut and gills, in order to better define the individual roles played by each tissue in overall organismic calcium regulation.

Epithelial cells of the lobster hepatopancreas have a significant role in animal calcium balance during the molt cycle. In order for lobsters and other crustaceans to grow, the old exoskeleton must be shed and replaced with a newer, larger model that accommodates increased animal mass. During molting, much of the calcium that is in the old exoskeleton is solubilized, transferred to the blood, stored in hepatopancreatic epithelial cells as concretions with SO_4 and PO_4, or transferred through these cells to the stomach lumen where it is stored as solid material in sacs called gastroliths. Following the molt, the new exoskeleton is soft and must be rapidly calcified for protection. At this time the calcium storage processes reverse, and calcium is released from gastroliths and hepatopancreatic concretions and dumped into the blood to join calcium entering the animal from the external seawater, where it eventually is incorporated into the new shell and hardens. Hepatopancreatic epithelial cells must be adapted to transport and store considerable quantities of this cation during these cyclic periods of massive calcium movements, and an understanding of plasma membrane calcium transport systems is important to be able to evaluate the overall role of this organ in organismic calcium balance.

Recently we characterized the calcium transport mechanisms of lobster hepatopancreatic and antennal gland apical membranes and found similar transfer processes in both tissue locations (Ahearn and Franco 1990, 1993; Ahearn and Zhuang 1996; Zhuang and Ahearn 1996). These studies showed that calcium influx across the apical membranes of both organs occurred by the combination of three transport processes: (1) an amiloride-sensitive carrier system; (2) an amiloride-insensitive carrier system; and (3) a verapamil-inhibited, PD-dependent, ion channel (Fig. 5). As suggested in Figure 1, the amiloride-sensitive carrier process for calcium uptake in hepatopancreatic apical membrane is likely the same protein accommodating the electrogenic exchange of $2Na^+/1H^+$ across the same membrane (Ahearn 1996). During postmolt, when calcium must be solubilized rapidly from stomach and hepatopancreatic storage sites and transferred through hepatopancreatic epithelial cells to the blood in massive quantities, basolateral transport mechanisms must be present that have the capability of shifting from low-transport-rate "housekeeping" activities to high-volume transfer processes, delivering most of

Table 1: Kinetic constants for epithelial basolateral Ca^{2+} transport

	Ca^{2+}-ATPase		Ca^{2+}/Na^{+} Exchanger		
Species and Tissue	K_t (nmol 1^{-1})	J_{max} (pmol μg^{-1} protein $8s^{-1}$)	K_t (nmol 1^{-1})	J_{max} (pmol μg^{-1} protein $8s^{-1}$)	Reference
Lobster hepatopancreas (*Homarus americanus*)[a]	65	1.07	14,570	2.72	Zhuang and Ahearn 1998
Seawater-adapted fish gill (*Oreochromis mossambicus*)	495	.91	1,880	2.08	Verbost et al. 1994
Crab gill (*Carcinus maenas*)	149	.23	1,780	1.32	Flik et al. 1994
Fish intestine (*O. mossambicus*)	27	.08	181	.96	Flik et al. 1990
Rat kidney cortex	110	10.80	200	.43	Van Heeswijk et al. 1984

Source. Zhuang and Ahearn 1998.

[a] Lobster hepatopancreas data collected at 25°C; all others at 37°C.

the calcium needed to harden the new exoskeleton in as short a period of time as possible. Recent studies have shown that in addition to a verapamil-sensitive ion channel, at least two calcium-transporting proteins occur in hepatopancreatic basolateral membranes: (1) an ATP-dependent Ca^{2+}-ATPase and (2) an electrogenic $1Ca^{2+}/3Na^{+}$ antiporter (Fig. 5; Zhuang and Ahearn 1998).

In order to obtain accurate assessments of the kinetic constants of the basolateral calcium transport mechanisms in lobster hepatopancreatic epithelium and therefore gain an appreciation of their respective roles in transferring this divalent cation from cytosol to hemolymph during the molt cycle, a calcium-chelating buffer system developed in Holland and applied to the investigation of calcium fluxes in fish intestine (Flik et al. 1990) and crab gills (Flik et al. 1994) was employed with lobster basolateral membrane vesicles. The basis of this technique is the use of the calcium chelators ethylene glycol-bis(β-aminoethylether)N,N,N′N′-tetraacetic acid (EGTA), N-hydroxy ethyl-ethylenediamine-triacetic acid (HEEDTA), nitrilotriacetic acid (NTA), and ATP in a buffer system that controls for the calcium activity in solution. The computer software program Chelator can be used in combination with these buffers to obtain a precise measure of the calcium available for transport across a specific membrane (Schoenmakers and Flik 1992).

Using the methods described above, the kinetic constants of basolateral calcium transport by way of the Ca^{2+}-ATPase localized to this membrane were disclosed. Over the selected calcium activity range, ATP-dependent Ca^{2+} influx was a hyperbolic function of external calcium activity, suggesting the presence of a saturable carrier system, which can be described by the Michaelis-Menten equation (Eq. [1]). Apparent kinetic parameters for the ATP-dependent carrier process calculated in this manner were $K_t = 65.28 \pm 14.39$ nM and $J_{max} = 1.07 \pm 0.06$ pmol/μg protein × 8 s (Table 1; Zhuang and Ahearn 1998).

The chelator-buffering system was also used to obtain an estimate of ATP-independent Na^{+}/Ca^{2+} exchange in hepatopancreatic basolateral membranes. $^{45}Ca^{2+}$ influxes into vesicles preloaded with either intravesicular Na^{+} or Li^{+} were both hyperbolic functions of external calcium activity, possessing rates that could be described by the Michaelis-Menten equation (Eq. [1]). Iterative, nonlinear, computer curve fitting of the resulting data yielded the following kinetic constants for calcium influx in vesicles preloaded with Na^{+}: $K_t = 14{,}570 \pm 5{,}020$ nM and $J_{max} = 2.72 \pm 0.23$ pmol/μg protein × 8 s (Table 1). Similar values for these parameters were obtained in vesicles preloaded with Li^{+}: $K_t = 20{,}170 \pm 6{,}990$ nM and $J_{max} = 1.03 \pm 0.10$ pmol/μg protein × 8 s (Zhuang and Ahearn 1998). These results suggest that both Na^{+} and Li^{+} could support cation exchange, but that the maximal transport velocity was about three times greater with Na^{+} than with Li^{+}.

To estimate the stoichiometry of the exchange process, $^{45}Ca^{2+}$ influx (at a fixed external calcium activity; 5 μM) was measured as a function of intravesicular sodium concentration (0, 5, 10, 20, 35, and 75 mM). Results indicated that a sigmoidal function existed between the variables where calcium influx followed the Hill equation:

$$J = J_{max}(Na^{+})^{n}/[(K_t)^{n} + (Na^{+})^{n}], \quad (2)$$

where J_{max} is maximal $^{45}Ca^{2+}$ influx, $(K_t)^{n}$ is an affinity constant modified to accommodate multisite interactions (interaction coefficient), and the Hill coefficient n is an estimate of the number of reactive Na^{+}-binding sites on the internal vesicular surface. A curve-fitting program was used to obtain estimates for the three kinetic parameters using Equation (2) above. The best-fit curve provided the following values for these constants: $J_{max} = 1.40 \pm 0.02$ pmol/μg protein × 8 s; $(K_t)^{n} = 10.34 \pm 0.29$ mM; and $n = 2.91 \pm 0.22$ (Zhuang and Ahearn 1998).

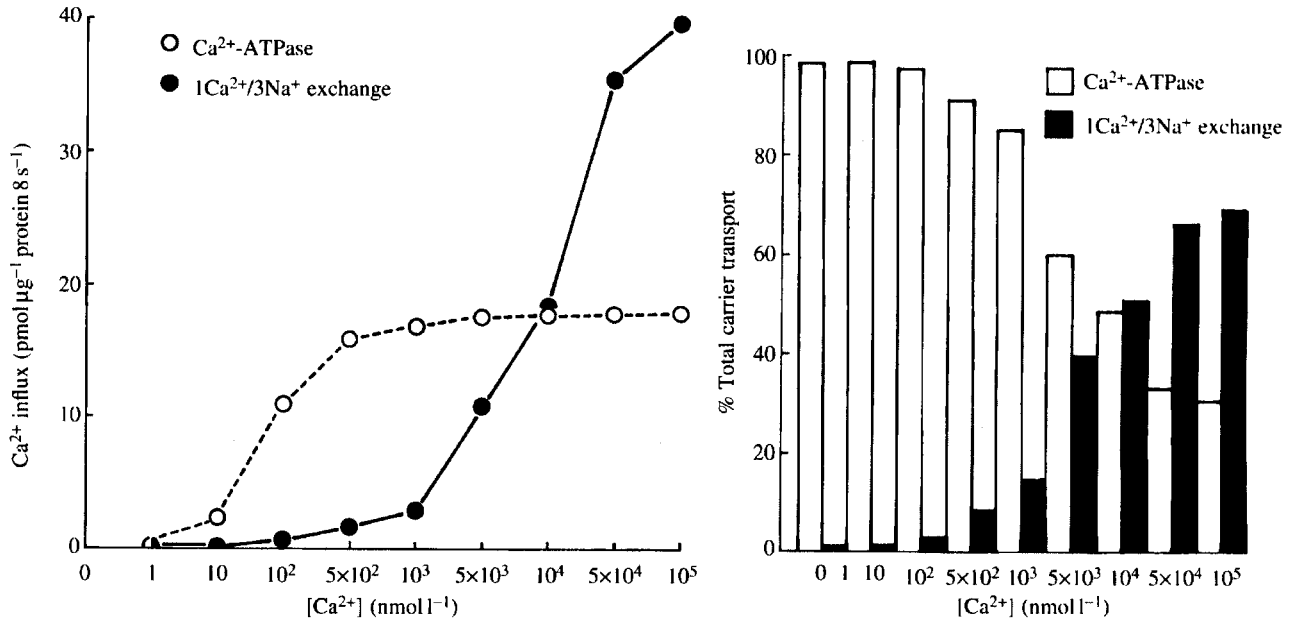


Figure 6. Relative roles of the Ca^{2+}-ATPase and the electrogenic $1Ca^{2+}/3Na^{+}$ antiporter in Ca^{2+} transport across lobster hepatopancreatic basolateral membranes over a wide intracellular Ca^{2+} activity range, such as might occur during the intermolt, premolt, and postmolt stages of the molt cycle. Values in both panels were derived using the kinetic constants derived in our study for each carrier system and the percentage protein associated with only the inside-out sealed basolateral membrane vesicles (6%). Under these conditions, J_{max} values for calcium influx become 17.83 pmol/mg protein × 8 s for the Ca^{2+}-ATPase and 45.33 pmol/mg e s for the $1Ca^{2+}/3Na^{+}$ exchanger. (From Zhuang and Ahearn 1998.)

These results suggest that this cation antiporter has an apparent exchange stoichiometry of approximately $3Na^{+}/1Ca^{2+}$.

Figure 6 is a comparison of the transporting roles of the lobster hepatopancreatic basolateral Ca^{2+}-ATPase and electrogenic $1Ca^{2+}/3Na^{+}$ antiporter at intracellular calcium activities between 1 nM and 100,000 nM, calculated using the apparent kinetic constants shown in Table 1. This figure suggests that at intracellular calcium activities as might occur in typical epithelial cells (e.g., 100–500 nM), greater than 90% of calcium efflux takes place by way of the Ca^{2+}-ATPase. From 1,000 nM to 10,000 nM, intracellular activities that might occur during temporary calcium storage or transcellular calcium movements associated with certain times in the molt cycle, the electrogenic $1Ca^{2+}/3Na^{+}$ exchanger assumes a greater role in moving the divalent cation out of the cell. In this figure the crossover point (where efflux by both processes is about equal) is 10,000 nM (a value near the apparent K_t for the exchanger). Flik et al. (1994) described calcium efflux by these two transporters in crab gill epithelium and found a crossover point at 500 nM, considerably below that of the hepatopancreas. These results suggest that this crossover point may have biological relevance to the roles the different cell types play in calcium balance in the two animals where markedly dissimilar quantitative fluxes of this cation would likely occur through the two epithelia.

Both ATP-dependent and ATP-independent calcium transporters occur on basolateral membranes of epithelial cells of gut, kidney, and gill tissues from both vertebrates and invertebrates (Table 1), and each displays a role in maintenance of appropriate intracellular calcium activities. Among the crustaceans, animals from marine, dilute seawater, and freshwater habitats uniformly display high-affinity Ca^{2+}-ATPases (K_t between 65 and 300 nM), while exhibiting lower-affinity Ca^{2+}/Na^{+} exchangers (K_t between 150 and 14,570 nM) (Table 1; Flik et al. 1994; G. Flik and C. Haond, unpublished data; M. G. Wheatly, unpublished data). The K_t values for ATP-dependent calcium transport in crustacean gills are quantitatively similar to those discussed above for lobster hepatopancreatic basolateral membranes, while those for the ATP-independent system are approximately five times higher in affinity than analogous hepatopancreatic proteins. These values support the hypothesis that higher intracellular calcium activities may be reached in the hepatopancreas during the molt cycle than in the gills, and a lower-affinity, higher-capacity transfer system in this gut organ may be needed to facilitate the transfer of this cation during these periods.

In general, data from crustacean epithelial cells of lobster, crab, and crayfish from gut and gill tissues studied over the past 5 yr suggest a common theme across crustacean species that seems to be relatively independent of environmental or hemolymph ionic composition and is supported by available

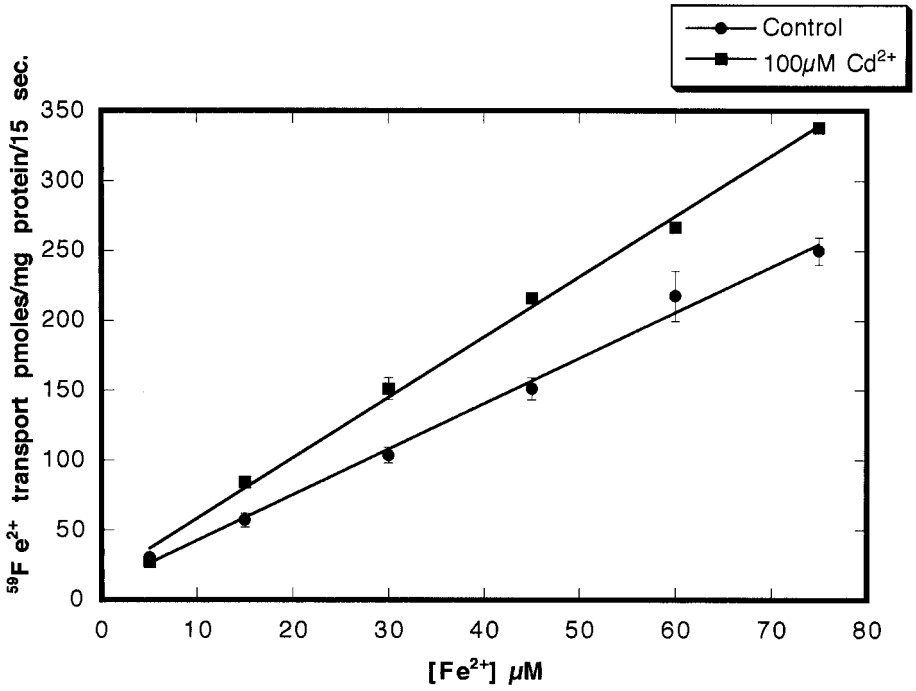


Figure 7. The effect of external Cd^{2+} on influx kinetics of $^{59}Fe^{2+}$ uptake in hepatopancreatic BBMV. Loading medium was composed of 100 μM mannitol, 50 μM KCl, 50 μM K^+-gluconate, 25 μM HEPES, 1 μM ascorbate, 50 μM valinomycin, and 1 μM citrate. External medium was the same as loading medium, except the former had $^{59}Fe^{2+}$ activities of 5, 15, 30, 45, 60, and 75 μM. In the condition with Cd^{2+}, cadmium was added so that its activity was 100 μM. The uptake duration was 15 s. Values were corrected for nonspecific $^{59}Fe^{2+}$ binding and are presented as means $\pm$ SEM ($n = 3$).

evidence from vertebrate cells as well. Basolateral calcium transport proteins appear to have developed across tissues and phyla to regulate intracellular calcium activities within a relatively narrow range regardless of other biological functions occurring in the whole animal, such as molting, feeding, or osmoregulation. The combination of high- and low-affinity transporters in the same membrane ensures that intracellular calcium activities will be sufficiently controlled to tissue-specific values, whether small or large changes in calcium activities occur in the environment or hemolymph. It will be interesting to see whether this initial trend observed in only three species of crustaceans holds for other animals from a variety of aquatic and terrestrial habitats.

Heavy Metal Transport by Crustacean Hepatopancreas

Invertebrate gastrointestinal epithelial cells are known to sequester and detoxify environmental heavy metals (Viarengo and Nott 1993). This detoxification process occurs in three steps: (1) entry of the metal into the epithelial cytoplasm across the plasma membrane; (2) transport into endosomal compartments and complexation with sulfur or phosphorus; and (3) exocytosis across the luminal membrane of the gut cell and loss from the animal by excretion. Although the general aspects of this process have been known for a number of years from electron microscopic studies, the physiological details of metal transport across plasma membranes or organelle membranes remains to be elucidated.

Recently, as part of an investigation to characterize the mechanisms for calcium transport across the luminal hepatopancreatic membrane of lobsters, the heavy metals zinc and cadmium were found to be competitive inhibitors of calcium uptake and were capable of trans-stimulating the efflux of intracellular calcium by way of one or more shared apical carrier process(es) (Ahearn et al. 1994; Zhuang and Ahearn 1996). Because calcium enters these cells by both an amiloride-sensitive, electrogenic $1Ca^{2+}/1H^+$ antiporter and an amiloride-insensitive, electroneutral $2Na^+/1\ Ca^{2+}$ exchanger, the precise target for heavy metals is unclear at present. The following experiments were designed to shed some light on the pathway(s) by which some metals gain access to hepatopancreatic epithelial cells.

The uptake of iron is well characterized in yeast, where ferric iron (Fe^{3+}) is reduced to ferrous iron (Fe^{2+}) by ferric reductase enzymes (FR1 and FR2) on the plasma membrane prior to membrane transfer (Anderson et al. 1994). The ferrous iron is then taken up by either a low-affinity iron transporter (FET4)

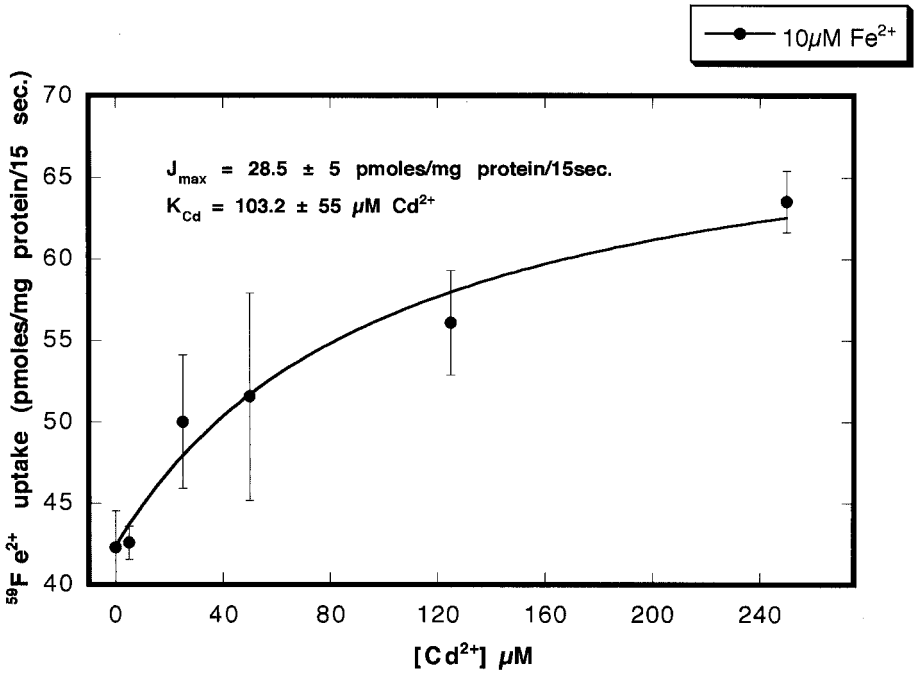


Figure 8. The influx of 10 μM $^{59}Fe^{2+}$ in the presence of varying activities of Cd^{2+}. Loading medium was the same as in Fig. 10. External medium was also the same except that $^{59}Fe^{2+}$ activity was 10 μM and Cd^{2+} activities were varied from 0 to 250 μM. The uptake duration was 15 s. Values were corrected for nonspecific $^{59}Fe^{2+}$ binding and are presented as means $\pm$ SEM ($n = 3$).

or a high-affinity transporter complex composed of two proteins (FET1 and FET3) (Dix et al. 1994; Eide 1997). This two-step process of reduction of ferric iron and uptake of ferrous iron also appears to be important for iron uptake in some plants (Fox et al. 1996). This recurring theme may also occur in mammals. Furthermore, there is some indication of a brush border ferric reductase in the duodenum, the prominent site of iron uptake (Riedel et al. 1995). Recently, a transporter (DCT1), expressed in duodenal enterocytes, that transports ferrous iron has been cloned from the rat (Gunshin et al. 1997). This transporter appears to take up ferrous iron coupled with a proton (symport). It also transports a variety of other divalent ions, thereby exhibiting a fairly broad specificity range (Gunshin et al. 1997; Hediger 1997).

Vesicles of lobster hepatopancreatic BBMV were prepared as described previously (Ahearn et al. 1985) and were used in initial uptake rate experiments (15-s uptakes) of $^{59}Fe^{2+}$ (Aslamkhan et al. 1998). Because metals bind to ligands in media, it was necessary to control their activities with metal buffers (metal chelates), just as it was necessary to control proton activities (e.g., pH) with proton buffers (Bers et al. 1994; Fox et al. 1996). Therefore, a citrate-based metal-chelate buffer system was used to control the activity of free ferrous iron. The activity of free ferrous iron was modeled on WinMax v1.70 (a Windows 3.1–based version of Max Chelator [Bers et al. 1994]) with carefully selected constants from the six-volume series titled *Critical Stability Constants* (Martel and Smith 1974–1989).

Figure 7 illustrates the effects of external cadmium on the influx kinetics of $^{59}Fe^{2+}$. Over the activity range selected, the influx of $^{59}Fe^{2+}$ was a linear function of external iron activity showing no indication of saturation at the higher metal activities. Iron activities greater than 75 μM were difficult to buffer accurately and were not attempted. Addition of 100 μM external Cd^{2+} significantly increased $^{59}Fe^{2+}$ influx at each iron activity, and, once again, iron influx kinetics under these conditions showed no indication of saturation. These results suggest that cadmium acted as an activator of iron influx, contrasting markedly with previous reports of cadmium functioning as an inhibitor of the low-affinity iron transporter found in yeast (FET4) (Dix et al. 1994).

To assess the nature of the cadium-stimulating effect on $^{59}Fe^{2+}$ influx in hepatopancreatic BBMV, an experiment was conducted in which iron influx (10 μM) was measured as a function of variable external activities of cadmium; the results of this experiment are displayed in Figure 8. Increasing Cd^{2+} activity stimulated $^{59}Fe^{2+}$ influx hyperbolically, following Michaelis-Menten kinetics, suggesting saturation of a finite number of cadmium-binding sites. These results suggest that Cd^{2+} stimulation of $^{59}Fe^{2+}$ uptake may be by way of trans-stimulation

following passage of the unlabeled metal across the membrane and binding to an intravesicular translocation site on a transporter shared with iron.

The countertransport experiment illustrated in Figure 9 was designed to show whether enhanced carrier protein activity in the presence of cadmium could account for the stimulatory effect of this metal on $^{59}Fe^{2+}$ influx. In comparison to the control, both the cold ferrous iron and the cadmium significantly increased the efflux of internal $^{59}Fe^{2+}$, suggesting that both iron and cadmium were able to exchange with radiolabeled iron by way of an antiport carrier process in these membranes. These results suggest that the enhancing effect observed by cadmium on $^{59}Fe^{2+}$ influx was likely due to the entry of Cd^{2+} by a membrane channel or other process and the subsequent transstimulation of low-affinity iron uptake by antiport (Aslamkhan et al. 1998). These findings are in agreement with recent observations on the mammalian iron transporter (DCT1), where both cadmium and iron were shown to share a common low-affinity metal carrier process in intestinal epithelium (Gunshin et al. 1997). Moreover, the presence of a cadmium channel in the brush border of a gastrointestinal epithelium was also disclosed in teleosts (Verbost et al. 1987). This study with lobster hepatopancreatic epithelium is the first to propose the coupling of a metal-transferring channel with a shared metal antiport process on the same membrane. Future studies will define the details of this proposed coupling of metal-translocating mechanisms.

Anion Transport by Crustacean Gill and Gut Epithelial Cells

Anion transport properties of epithelia of the antennal glands, gill, and gut of crustaceans have been studied extensively over the past 15 yr. Excellent descriptions of organic anion and cation transport by crustacean antennal gland urinary bladder and comparisons with analogous properties of mammalian renal epithelia were published in the past decade (Holliday and Miller 1980, 1982, 1984*a*, 1984*b*; Miller and Holliday 1982, 1987; Miller et al. 1989; Smith et al. 1990). Similarly, inorganic anion transport mechanisms for these epithelia and their potential roles in organismic biology have been described from the 1980s and early 1990s and should be consulted for experimental details and comparative information (Mantel and Farmer 1983; Lee and Pritchard 1985; Lucu 1990, 1993; Wheatly and Henry 1992; Towle 1993; Wheatly 1993).

Within the past 5 yr, new methodology involving the use of

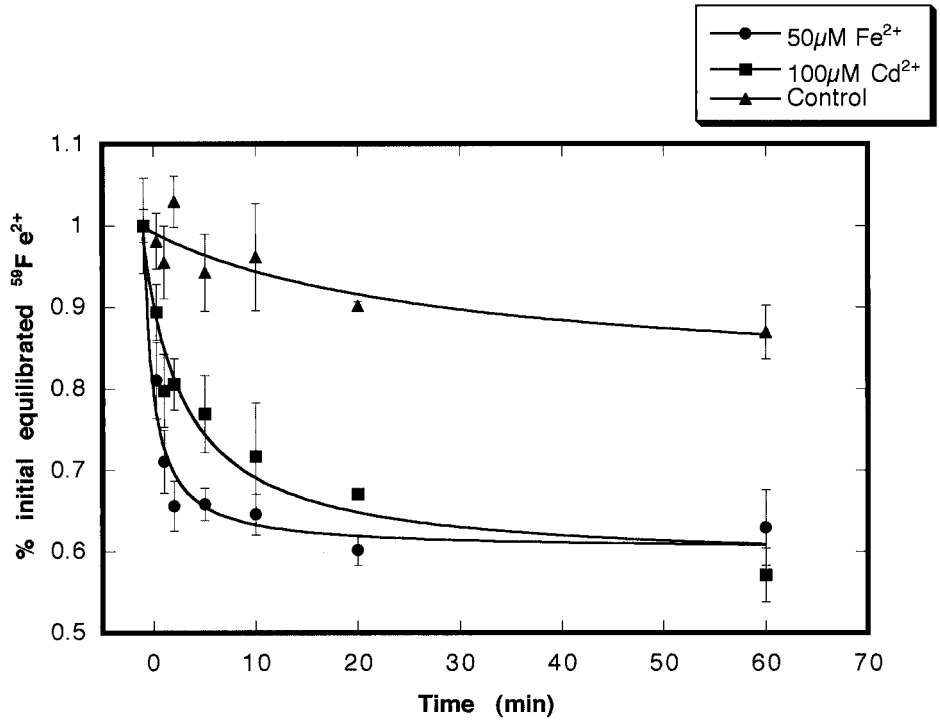
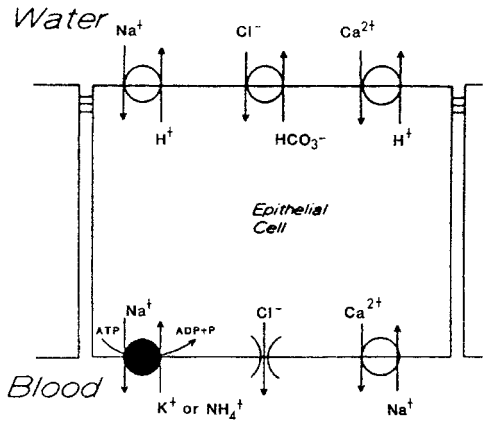
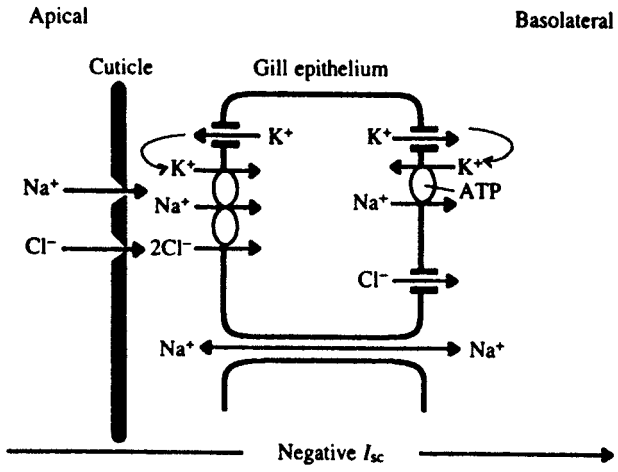


Figure 9. The effect of adding 100 μM Cd^{2+}, 50 μM Fe^{2+}, or mannitol (adjusted to same osmolarity) to BBMV equilibrated for 1 h in outside medium with the same composition as in Fig. 10, except that $^{59}Fe^{2+}$ activity was 5 μM. Samples were taken prior to adding countertransport substrates (ca. 1 min) and at time points following addition of the external substrates (15 s and 1, 2, 5, 10, 20, and 60 min). Values were corrected for nonspecific $^{59}Fe^{2+}$ binding and are presented as means $\pm$ SEM ($n = 3$).



(A)



(B)

Figure 10. *A*, Model of NaCl transport mechanisms in epithelial cells of crab gill, based on studies with perfused gills and isolated membrane vesicles. In this model the apical cell membrane is thought to possess parallel Na^+/H^+ and Cl^-/HCO_3^- antiporters, while the basolateral membrane demonstrates a chloride channel in combination with a Ca^{2+}/Na^+ antiporter. The driving force for transcellular NaCl movement is brought about by the ATP-dependent, basolateral Na^+/K^+-ATPase (from Towle 1993). *B*, Model of NaCl transport mechanisms in crab gill epithelial cells based on electrophysiological studies. In this model a negative short-circuit current (I_{sc}) is carried via apical K^+ channels and basolateral Cl^- channels. Transapical NaCl transport proceeds via $Na^+/K^+/2Cl^-$ symport. This cotransporter is secondarily energized by the activity of the basolateral Na^+/K^+-ATPase, which generates a Na^+ gradient directed into the cell. Whereas K^+ leaves the cell via the conductive pathways in the apical and basolateral membranes, basolateral NaCl exit is effected via the Cl^- channels and the Na^+/K^+-ATPase. (From Riestenpatt et al. 1996.)

isolated and purified membrane vesicles from epithelial apical and basolateral membranes and split gill lamellae have extended the results of these early observations and have provided a more detailed look at anion plasma membrane transport systems than was previously available. Gill epithelia of hyperosmoregulating crabs regulate the absorption of both sodium and chloride and, therefore, the concentrations of these ions in the hemolymph. Figure 10 compares two recent models for monovalent anion transfer across this epithelium of euryhaline crabs. Figure 10*A* is the paired antiport model for NaCl absorption by posterior gills in *Carcinus maenas*, the green shore crab (Towle 1993). In this model NaCl absorption across the gill apical membrane is facilitated by the combination of Na^+/H^+ and Cl^-/HCO_3^- exchangers. Chloride exits the epithelial cell by way of a chloride channel in the basolateral membrane (Onken et al. 1991). Support for the presence of an apical, amiloride-sensitive Na^+/H^+ exchanger in this cell layer comes from vesicle studies described previously (Shetlar and Towle 1989), while the presence of a functioning Cl^-/HCO_3^- exchanger in crab gill was suggested by reduction in Cl^- uptake into these cells in the presence of acetazolamide (carbonic anhydrase inhibitor) (Lucu 1989) and by stimulation of $^{36}Cl^-$ uptake into gill plasma membrane vesicles by preloading with HCO_3^- (Lee and Pritchard 1985). Both Lucu (1989) and Shetlar and Towle (1989) also showed that addition of SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid), a known blocker of Cl^-/HCO_3^- exchange, substantially inhibited uptake of chloride in both perfused gill preparations as well as in gill vesicles.

Figure 10*B* describes an alternative model for NaCl absorption by the same epithelium from the shore crab, based on electrophysiological evidence from single split gill lamellae containing both epithelium and cuticle (Riestenpatt and Siebers 1994; Riestenpatt et al. 1996). In this model, Na^+ and Cl^- enter the gill epithelium across the apical membrane by cotransport on a $Na^+/2Cl^-/K^+$ symporter in parallel with a K^+ channel. This model also suggests that a Cl^- channel in the epithelial basolateral membrane is responsible for anion efflux to the blood. This apical uptake model was generated as a result of the dependence of Cl^- influx upon the presence of both apical Na^+ and K^+, a finding that would appear to be at variance with the paired antiport model presented above. Therefore, while details of the epithelial apical transfer of Na^+ and Cl^- in hyperegulating crab gills are still unsettled, the exit process for the anion, by way of a basolateral channel, seems fairly strongly established.

In recent years monovalent and divalent anion transport by the crustacean hepatopancreatic epithelium has been investigated as part of a research program examining the secretory nature of this organ as well as its role in heavy metal sequestration and detoxification (Cattey et al. 1992, 1994; Gerencser et al. 1995, 1996). Hemolymph sulfate is regulated below its concentration in seawater in marine crustaceans, but the physiological nature of this regulation is unclear. In order to estab-

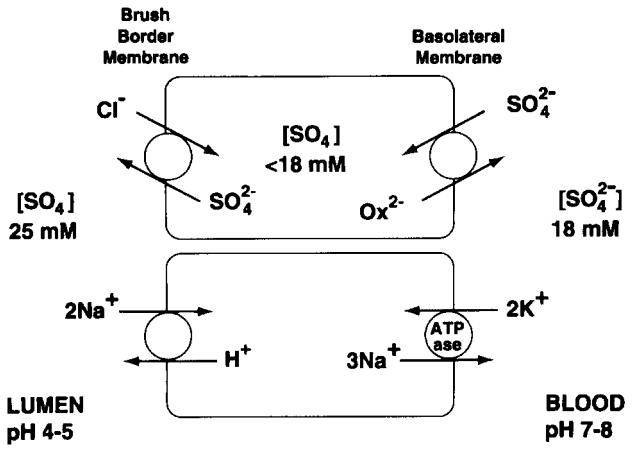


Figure 11. Schematic diagram of hepatopancreatic epithelium illustrating a proposed model of sulfate secretion. The approximate concentrations of sulfate in the hemolymph and tubular lumen are indicated, and the intracellular sulfate concentration is assumed to be less than that of the blood and lumen. In the upper cell, sulfate initially enters the cytoplasm by moving down a concentration gradient in electroneutral exchange for oxalate. Sulfate then leaves the cell via electrogenic exchange with chloride. The secretion of sulfate against its concentration gradient is driven by chloride moving down its concentration gradient. The net movement of a negative charge out of the epithelial cell facilitates the action of the transporter. The lower cell indicates the brush border sodium-proton exchanger, which is driven by the transmembrane sodium gradient and membrane potential. During digestion and absorption, the lumen is acidified by the protons, which in turn stimulate the chloride-sulfate antiporter by an external regulatory site. (From Gerencser et al. 1996.)

lish whether the epithelium of the hepatopancreas played a role in sulfate regulation, a series of investigations were undertaken to characterize the mechanisms of sulfate transfer across isolated and purified brush border and basolateral membranes of this tissue in the Atlantic lobster, *Homarus americanus*. Figure 11 summarizes the results of these studies and indicates that sulfate transfer across the basolateral membrane of this cell layer, down a concentration gradient, occurs by way of a highly specific electroneutral sulfate/oxalate exchanger (Gerencser et al. 1995). In contrast, transfer of the divalent anion across the apical membrane took place by way of an electrogenic, $1SO_4^{2-}/1Cl^-$ exchanger employing both the transmembrane chloride concentration gradient and membrane potential to power the antiport (Cattey et al. 1992, 1994). The apical mechanism was sensitive to an imposed membrane potential, was modulated by luminal proton concentration, and was strongly inhibited by the stilbenes SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid) and DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acid). Working in series, these two transport systems were seen capable of bringing about a net secretion of sulfate from hemolymph to gut lumen and could be, at least partially, responsible for the hemolymph hyporegulation of this anion. Roles of both gills and antennal glands in sulfate regulation remain to be established.

In addition to facilitating the net secretion of sulfate across the hepatopancreatic epithelial cell layer, these divalent anion transport systems may also have a function in heavy metal sequestration and detoxification in this tissue. It is well known that many invertebrate epithelial cells possess sequestration mechanisms in membrane-bound endosome compartments that complex potentially toxic divalent cations such as copper, iron, zinc, and lead with divalent anions such as sulfate or phosphate, forming insoluble precipitates in the process (Al-Mohanna and Nott 1986; Hopkin 1989; Viarengo 1989; Viarengo and Nott 1993). These concretion-containing endosomes eventually are transferred across the epithelial apical membrane for excretion with the feces, thereby ridding the organism of potentially harmful heavy metals. The hepatopancreatic sulfate transport mechanisms described in Figure 11 may be under regulatory control so that the rate of transepithelial sulfate secretion is tightly associated with the cellular need for a heavy metal anionic complexing agent so that at any particular time the processes of anion secretion and heavy metal detoxification exist in an equilibrium state.

Conclusions

Epithelial cells of the gills, gut, antennal glands, and integument in crustaceans control the movements of cations and anions between the hemolymph and environment and, in so doing, regulate such biological activities as ionic and osmotic regulation, gastric acidification, molting, and heavy metal detoxification. Because recent methods to produce purified apical and basolateral plasma membrane domains of these epithelial cells have been developed, we are slowly gaining an appreciation for the roles that each cell pole may play in ionic cellular physiology. Recent research with crustacean gill and hepatopancreatic epithelial Na^+/H^+ exchange has disclosed the occurrence of two distinctly different antiporters on apical and basolateral membranes of the same cell, one likely performing a housekeeping role of intracellular pH and ionic regulation (basolateral isoform), the other serving as a significant divalent cation uptake center (apical isoform), therefore having a major role in the calcification processes that occur during the molt cycle. Studies of the molecular biology of crustacean Na^+/H^+ antiporters have disclosed a high degree of structural similarity to vertebrate isoforms of these transport systems, suggesting a remarkable degree of molecular conservation between animals and the likely important role these transport systems have played throughout evolutionary time. Specific epithelial cells of the gut have a role in heavy metal detoxification by employing cellular mechanisms for trapping (sequestering) and complexing a variety of these cationic substances with divalent anions prior to elimination across the apical border and excretion with the feces. Therefore, an increasing number of organismic bi-

ological processes are becoming known that are influenced by cellular functions performed by epithelial cell layers separating the animal from its environment. In coming years, the powerful combination of cellular physiology and molecular biology will increasingly be brought to bear on clarifying the nature of regulatory events controlled by these epithelial cells and on defining the effects these regulatory events have on the overall biology of the animals under study.

Among the more exciting challenges facing crustacean epithelial experimentalists in the coming years will be the elucidation of the molecular and physiological association between the apical and basolateral Na^+/H^+ exchangers in such tissues as the hepatopancreas. Are these transporters structurally similar to one another and perhaps part of the same gene family or completely structurally dissimilar and regulated independently of one another? Another functional enigma facing crustacean biologists is whether the calcium transport mechanisms of the apical, basolateral, and organellar (e.g., mitochondria, lysosomal, etc.) membranes change their kinetic properties during different phases of the molt cycle. Are the apparent affinity constants or maximal velocity values described in this review for the basolateral Ca^{2+}/ATPase and Ca^{2+}/Na^+ exchanger altered as massive movements of calcium through epithelial cells of the hepatopancreas take place during premolt and postmolt, or are other undiscovered mechanisms taking place during these times that are not apparent during intermolt? What is the intracellular mechanism of heavy metal sequestration and detoxification at the organellar level of tissue complexity? Are metals directly transported into endosomal compartments by a Ca^{2+}-ATPase or perhaps as antiported substrates exchanging with intravesicular protons transferred into the organellar compartment by a V-ATPase? What is the mechanism of concretion formation within these cellular compartments that involves the chemical interaction between the cationic metal and multivalent anions? Does intravesicular pH regulate this process, and if so, how? These and many other questions about the roles of crustacean epithelial cell layers in ion balance and regulation remain to be elucidated in the future. It is hoped that this review will serve as a catalyst to find the answers to some of them.

Acknowledgments

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