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ION TRANSPORT IN THE FRESHWATER BIVALVE,
CORBICULA FLUMINEA (MÜLLER)

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Zoology and Physiology

by

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ABSTRACT

Corbicula fluminea (Müller) maintains hydromineral balance in dilute media by active transepithelial transport of Na and Cl. Cl uptake depended on external [Cl] and displayed saturable kinetics. The transport capacity in pondwater(PW)-acclimated animals was $7.00 \pm 0.51 \mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$ and the transport affinity $0.21 \pm 0.08 \text{ mM}$. Prolonged salt depletion increased Cl transport capacity without changing the affinity.

In PW-acclimated *C. fluminea*, Na and Cl transport were independent, and were stimulated by serotonin. In salt depleted (SD) animals, Na transport was partially Cl-dependent but Cl transport was Na-independent. Acetazolamide increased Na and Cl efflux. Both serotonin and acetazolamide promoted the loss of titratable base.

N, N'-dicyclohexylcarbodiimide (DCCD) inhibited Na and Cl transport. The inhibition of Na transport by DCCD was Cl-dependent, but the inhibition of Cl transport was Na-independent. DCCD increased the loss of titratable base in Cl-free PW medium. 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid decreased Cl influx and Na net flux. Furosemide inhibited Na and Cl transport in PW-acclimated animals.

Exposure of *C. fluminea* to hyperosmotic nonelectrolytes resulted in an elevation of blood solutes due to dehydration followed by a precipitous decrease in blood Na and the accumulation of nonelectrolytes. Lanthanum was rarely observed to penetrate the paracellular spaces without hyperosmotic stress, but was observed in the paracellular junctional complexes between gill epithelial cells under hyperosmotic conditions. Longer exposure resulted in greater amounts of lanthanum precipitation in more locations compared to shorter exposure.

Carunculina texasensis was able to maintain normal blood ion concentration for 8 h with minimal dehydration under hyperosmotic condition. Longer exposure caused a precipitous decrease in most blood solutes and an extensive accumulation of nonelectrolytes. More lanthanum was observed in the paracellular spaces of *C. texasensis* compared to *C. fluminea* for identical treatments.

This study suggests that Na and Cl transport in *C. fluminea* are energized by a proton-pump. The entrance of Na is likely *via* an apical Na-conductive channel and Cl *via* an apical Cl/HCO₃ exchange pathway. It also suggests that under normal conditions the epithelium of *C. fluminea* is relatively “tight” and the paracellular pathway for solutes is insignificant.

CHAPTER ONE INTRODUCTION

Freshwater bivalves have the same osmoregulatory problems as do other freshwater organisms. They must accumulate salts from dilute environment to compensate for the loss of solutes by diffusion and excretion. This can be accomplished by transepithelial ion uptake in the ion transporting epithelia. There are two pathways available for ion movement across epithelia; 1) a transcellular pathway whereby ions traverse at least two cell membranes arranged in series; and, 2) a paracellular or shunt pathway which by-passes the transcellular route (Gerencser, 1982). In high-resistance epithelia where the tight junctions constitute a tight seal restricting diffusion, the principal pathways of transepithelial ion transport are confined to the transcellular route (Larsen, 1991). Most of the ion absorbing epithelia in vertebrates, such as amphibian skin, urinary bladders and kidney collecting ducts are high-resistance epithelia.

Limited information is available for the ion absorbing epithelia of invertebrates. The gill epithelia of freshwater bivalves that have been studied have septate junction whose function is similar to vertebrate zonula occludens (Green *et al*, 1980). In recent studies *Dreissena polymorpha* was shown to have low resistant epithelia with “leaky” junctional complexes. This allows significant paracellular solute movement under hyperosmotic conditions (Dietz *et al*, 1995). In addition, *D. polymorpha* also has a substantial diffusive uptake of Rb (Wilcox and Dietz, 1995). However, there have been no studies on *C. fluminea* where paracellular/transcellular ion movement has been

partitioned. The majority of the ions transported into the blood must move across the epithelial cells *via* specific ion transporting pathways located on the cell membranes.

The movement of the ions against their electrochemical gradients can be accomplished by polarized epithelial cells in which the apical and basal surface membranes contain different transport systems.

Several models have been proposed for the transcellular transport of Na and Cl. In most cases Na and Cl are inextricably linked to acid/base balance by direct or indirect exchanges with acid and base equivalent. Na and Cl uptake mechanisms are traditionally thought to involve electroneutral exchange with H (and or NH₄) and HCO₃, respectively (Krogh, 1939). The concept of Cl/HCO₃ exchange for Cl uptake has generally been accepted. Transcellular Cl uptake *via* a Cl/HCO₃ exchanger on the apical cell membrane of the transporting epithelium is supported by a various studies of numerous epithelia including the gill or operculum of freshwater fishes (Marshall, 1995; Heisler, 1993; Perry and Laurent, 1993; Perry *et al.*, 1981; Perry and Randall, 1981), amphibian skin and the epithelia of vertebrate renal distal tissues (urinary bladder and kidney collecting duct) and intestine (Knickelbein, et al, 1985; Larsen, 1991; Larsen *et al.*, 1992). A Cl/HCO₃ exchanger for Cl uptake has also been demonstrated in invertebrates such as the low-salinity-adapted crabs (Lee and Pritchard, 1985), and freshwater bivalves (Dietz, 1979, 1985; Dietz and Branton, 1979; Dietz and Findley, 1980; Dietz and Hagar, 1990). In ion transporting epithelia of vertebrates that have been studied, the Cl/HCO₃ exchanger is energized by an electrogenic proton-pump

located on the same membrane as the Cl/HCO_3 exchanger (amphibian skin) or on the opposite membrane (renal distal epithelia). Cellular substrates for the anion exchanger and proton-pump are provided by the hydration of CO_2 facilitated by carbonic anhydrase. CO_2 may be supplied by the oxidative metabolism of a transporting epithelial cell itself or/and may be derived from the serosal fluid by diffusion into the cell across the basolateral membrane (Larsen, 1991, Larsen *et al.*, 1992). Few studies have identified the location of the Cl/HCO_3 exchanger or how this anion exchanger is energized and regulated in invertebrates.

Transcellular Na uptake, on the other hand, is more controversial. In amphibian skin, Na uptake is accomplished through amiloride-sensitive conductive Na channels located apically and energized by basolateral located Na-K-ATPase pumping Na out and K in (Larsen, 1991). This model also is applied to the distal renal epithelia. Apical Na uptake *via* a Na/H (NH_4) exchange was considered to be the major pathway in transepithelial Na transport and H excretion in freshwater fishes (Wright and Wood, 1985), but has recently been challenged (Lin and Randall, 1995). In the new model, Na uptake is accomplished through a conductive apical Na channel energized by an electrogenic proton-pump located on the apical membrane of the gill epithelial cells. The protons are primarily supplied by the hydration of CO_2 as in Cl transport.

The transepithelial Na uptake mechanisms and their regulation in invertebrate systems have been less frequently studied. In the freshwater unionid mussels that have been studied, the entry of Na is thought to be through an apical Na/H exchange

pathway based on the amiloride-sensitive inhibition. It is energized, presumably, by the basolateral Na-K-pump as in amphibian skin (Dietz, 1978, 1979, 1985; Dietz and Findley, 1980). In other freshwater bivalves such as *C. fluminea* and the zebra mussel *Dreissena polymorpha* (Horohov *et al.*, 1992; McCorkle and Dietz, 1980) Na uptake is not sensitive to amiloride, suggesting multiple Na uptake pathways may occur in freshwater bivalves.

Most of the information concerning ion transport and regulation of freshwater bivalves is derived from the unionids (Dietz, 1978, 1979, 1985; Dietz and Branton, 1979; Dietz and Findley, 1980; Dietz and Hagar, 1990; Scheide and Dietz, 1986). The members of the unionid family have a long history in freshwater (Keen and Casey, 1969). Ion transport studies have recently been completed on the zebra mussel, *Dreissena polymorpha*, which is the most recent freshwater bivalve introduced into the United State from Europe (Dietz *et al.*, 1994; Horohov *et al.*, 1992; Mackie *et al.*, 1989; Wilcox and Dietz, 1995). *Corbicula fluminea* (Müller) is a freshwater bivalve commonly found in shallow lentic and lotic habitats throughout the United States (McMahon, 1983). It belongs to the subclass Heterodonta; order Veneroida; superfamily Corbiculacea and family Corbiculidae (McMahon and Williams, 1984). *C. fluminea* is a relatively recent invader of freshwater (Keen and Casey, 1969) and has a different blood ion profile compared to the unionid mussels and the zebra mussel (Dietz, 1979; McCorkle and Dietz, 1980; Dietz *et al.*, 1994; Byrne, *et al.*, 1989; Horohov *et al.*, 1992; Wilcox and Dietz, 1995). Previous studies of Na transport in *C.*

fluminea have demonstrated that the transport rate is 5-10 times faster than that of unionid mussels but 2-3 times slower than that of *Dreissena polymorpha*. (Horohov *et al.*, 1992; McCorkle and Dietz, 1980). Cl transport in *C. fluminea* has not been studied previously.

The present study was undertaken to elucidate the mechanisms of Na and Cl transport and their regulation in *C. fluminea*. This study has four components. The first (Chapter Two) investigates the Cl transport kinetics of pondwater-acclimated and salt-depleted *C. fluminea*. This study together with the second has been submitted to Biological Bulletin (H. Zheng and T. H. Dietz, in review). The second study (Chapter Three) examines the interrelationship of Cl and Na transport under normal, stimulated, and drug-inhibited conditions in pondwater- acclimated or salt-depleted *C. fluminea*. This study was presented at the annual meeting of American Society of Zoologists in 1995. The third study (Chapter Four) emphasizes the function of Cl/HCO₃ exchange and the proton-pump on the Cl and Na transport mechanisms of *C. fluminea*. The fourth study (Chapter Five) explores the importance of paracellular solute uptake of *C. fluminea* in comparison to *Carunculina texasensis* (a unionid mussel). The final chapter summarizes and integrates the general results and conclusions, and proposes a working model for transepithelial Cl and Na transport in *C. fluminea*.

CHAPTER TWO CL TRANSPORT KINETICS IN THE FRESHWATER BIVALVE, *CORBICULA FLUMINEA*

INTRODUCTION

Freshwater bivalves must accumulate salts from the dilute environment to compensate for the loss of solutes by diffusion and excretion. The rate of ion uptake depends on the corresponding external ion concentration, and the transport processes exhibit saturable kinetics in the freshwater bivalves that have been studied (Dietz, 1978; Dietz and Branton, 1979; Dietz and Byrne, 1990; Dietz and Hagar, 1990; Krogh, 1939; McCorkle and Dietz, 1980; Wilcox and Dietz, 1995). In unionid clams, the transport capacity for Na, Cl or K is about $1\text{--}2 \mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$ with a transport affinity about 0.1 to 0.2 mM (Dietz, 1978; Dietz and Branton, 1979; Dietz and Byrne, 1990; Dietz and Hagar, 1990; Krogh, 1939; McCorkle and Dietz, 1980). *Corbicula fluminea* (Müller), a member of the Corbiculidae, has higher blood osmolality than the unionids (Dietz, 1979; McCorkle and Dietz, 1980; Byrne, *et al.*, 1989), and therefore, may take up ions faster than the unionids. Studies of Na transport have shown that the transport capacity of *C. fluminea* is about $12.90 \pm 3.01 \mu\text{eq Na}(\text{g dry tissue} \cdot \text{h})^{-1}$, which is about ten times the rate found in the unionid *Carunculina texasensis* (Dietz, 1978; McCorkle and Dietz, 1980). K transport capacity of *C. fluminea* is about twice that of the unionids (Dietz and Byrne, 1990).

The stimulation of ion uptake rate by salt depletion has been demonstrated in several studies of freshwater bivalves (Krogh, 1939; Murphy and Dietz, 1976; Scheide

and Dietz, 1986). Elevation of the transport capacity with little or no change in transport affinity after appropriate salt depletion has been shown in the studies of Na, Cl and K uptake by unionids, K uptake by the zebra mussel *Dreissena polymorpha* and Na and K uptake by *C. fluminea* (Dietz, 1978, 1985; Dietz and Branton, 1979; Dietz and Byrne, 1990; McCorkle and Dietz, 1980; Wilcox and Dietz, 1995).

Cl is the most abundant anion in the blood of *C. fluminea* and the uptake of Cl is very important for the ionic balance in this species. However, little work has been done on Cl transport in Corbiculidae. In this study we reported that Cl uptake displays higher transport capacity than that found in other freshwater mussels.

MATERIALS AND METHODS

Animals

C. fluminea were collected under permit from the Tangipahoa River in Mississippi. The animals were acclimated to aerated artificial pondwater (PW) containing 0.5 NaCl, 0.4 CaCl₂, 0.2 NaHCO₃, 0.05 KCl mM at 22-25°C for at least one week before use. Salt-depleted (SD) animals were stored deionized water for at least 14 days before use. The deionized water was changed daily for the first two weeks and every other day for extended periods of the salt depletion.

Ion transport

The animals were rinsed with deionized water (DW) for about 30 min, and transferred to individual beakers with 20 to 30 ml of appropriate bathing medium containing the radioactive isotope ³⁶Cl. Bath samples were collected at intervals and the radioactivity of ³⁶Cl was analyzed by a liquid scintillation counter using a cocktail

based on Triton X-114/xylene (Wiegman *et al.*, 1975). The unidirectional Cl influx (J_i^{Cl}) used in this chapter was determined by the modification of a conventional methods (see Dietz and Branton, 1975). In the conventional method, unidirectional flux is calculated, in part, based on the measurement of Cl net flux. Because of the lack of sensitivity of the Cl titrator, Cl net flux can not be accurately measured if the Cl concentration is too low. This problem can be overcome by using a modified method, with Cl influx being determined from the ratio of the change of ^{36}Cl (^{36}Cl specific activity was about 2,000 CPM/ μM Cl) and the average ^{36}Cl CPM in the bathing solution. To verify the validity of the method, the values of Cl influx from the modified methods was compared to those using the conventional method (Fig 2.1). Although the influx values from the modified method tended to overestimate the Cl flux, by assuming that there was no change of Cl concentration in the bathing solution during the experiment, there was no significant difference between these two data sets. With this modified method, J_i^{Cl} was calculated as follows:

$$J_i^{Cl} = ((C_{CPM}/A_{CPM}) * V_T * [Cl]) / (W * H)$$

$$C_{CPM} = \text{change of } ^{36}Cl \text{ cpm in bathing solution} = (\text{initial cpm} - \text{final cpm})$$

$$A_{CPM} = \text{average cpm of bathing solution} = (\text{initial cpm} + \text{final cpm})/2$$

$$V_T = \text{total bathing volume in ml}$$

$$[Cl] = \text{Cl concentration in mM}$$

$$W = \text{dry tissue weight in g}$$

$$H = \text{interval in hours}$$

Figure 2.1. Comparisons of Cl influx from two different calculation methods. (A): J_i^{Cl} was calculated with the modified method which was determined from the ratio of the change of CPM in ^{36}Cl and the average CPM in the bath. (B): J_i^{Cl} was calculated with the conventional method based on values of Cl net flux. There was no significant difference between the values from these two determination methods for the Cl concentrations compared ($P > 0.25$).

Statistical analyses

Data were expressed as mean and standard error. Differences were tested with a Student's T-test and considered to be significant if the corresponding P-value was equal or less than 0.05. A rectangular hyperbolic function was chosen to fit the data. The significance of the curve fitting and the variables in the function ($J_{\max}$ and K_m) were calculated by a statistical program (Inplot 4, Graph Pad Software Inc.).

RESULTS

The rate of unidirectional Cl influx was dependent on the external Cl concentration for both PW-acclimated and SD *C. fluminea* (Fig. 2.2). When exposed to a range of NaCl concentrations, both PW-acclimated and SD animals displayed saturation kinetics. Animals stored in DW for two weeks were not sufficiently SD and did not differ from PW-acclimated animals ($P>0.25$). The Cl influxes of these “inadequately SD” (ISD) animals overlapped with the influx of PW-acclimated animals. Transport capacity ($J_{\max}$) for PW-acclimated and ISD animals was $7.46 \pm 0.94 \mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$ and $6.63 \pm 0.59 \mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$, respectively, and were not significantly different. Likewise, the transport affinity (K_m) of PW-acclimated animals was not statistically different from that of ISD animals ($0.22 \pm 0.15 \text{ mM}$ and $0.18 \pm 0.10 \text{ mM}$ for PW-acclimated and ISD animals, respectively). Since the rate of Cl influx of ISD animals was in the range of that of the PW-acclimated animals, we used the combined data set to represent the Cl uptake kinetics for PW-acclimated animals. This combined transport capacity and the corresponding transport affinity were calculated to be $7.00 \pm 0.51 \mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$ and $0.21 \pm 0.08 \text{ mM}$, respectively.

Figure 2.2. Effects of NaCl concentration on the average unidirectional Cl influx in PW-acclimated and SD *C. fluminea*. Each point represents a mean and a standard error (only minus standard error bar is shown) of 5 animals. Down triangles: PW-acclimated animals, $J_{\max} = 7.46 \pm 0.94 \mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$, $K_m = 0.22 \pm 0.15 \text{ mM}$; Squares (SD.A): salt depletion for two weeks, $J_{\max} = 6.63 \pm 0.59 \mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$, $K_m = 0.18 \pm 0.10 \text{ mM}$; Circles (SD. B): SD for four weeks, $J_{\max} = 10.85 \pm 0.91 \mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$, $K_m = 0.20 \pm 0.17 \text{ mM}$; Up triangles (SD. C): salt depletion for 8 weeks, $J_{\max} = 13.49 \pm 1.06 \mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$, $K_m = 0.10 \pm 0.12 \text{ mM}$.

The rate of unidirectional Cl uptake was dependent on the degree of salt depletion of the animals. Salt depletion of four weeks or longer resulted in the elevation of unidirectional Cl uptake. The Cl influx of the animals subjected salt depletion for four weeks or longer was consistently higher than that of the PW-acclimated animals under all Cl concentrations tested. Compared to the combined Cl transport capacity from PW-acclimated and ISD animals, there was about a 50% increase in maximal Cl influx ($J_{\max} = 10.85 \pm 0.91 \mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$) estimated for four week SD animals, and about an 85% increase for eight week SD animals ($J_{\max} = 13.49 \pm 0.16 \mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$). Animals subjected to eight weeks of salt depletion exhibited higher net Cl uptake rate than that of the four week SD group. It appeared that the longer the duration of salt depletion, the higher the rate of Cl uptake. While the increase of the transport capacity in SD animals was substantial, the change of its affinity for Cl was not significant. The estimated values of the transport affinity for SD animals were not significant different than that of the PW-acclimated animals.

DISCUSSION

Unidirectional ion uptake depends on the corresponding external ion concentration and it displays saturation kinetics in the freshwater bivalves studied to date (Dietz, 1978; Dietz and Branton, 1979; Dietz and Byrne, 1990; Dietz and Hagar, 1990; McCorkle and Dietz, 1980; Wilcox and Dietz, 1995). The Cl influx kinetics observed in *C. fluminea* do not differ from that of other freshwater clams in this regard. However, a high transport capacity ($J_{\max}$) seems to be a distinctive characteristic in *C. fluminea*. The Cl transport capacity is about seven times higher in *C. fluminea* than

that of the unionids *C. texasensis* and *Ligumia subrostrata* (Dietz and Branton, 1979).

The transport capacity for Na in *C. fluminea* is ten times that of the unionid *C. texasensis* (Dietz, 1978; McCorkle and Dietz, 1980). About twice as much K transport capacity was in *C. fluminea* compared to that of the unionid *C. texasensis* and the zebra mussel *D. polymorpha* (Dietz and Byrne, 1990; Wilcox and Dietz, 1995).

Salt depletion causes ion transport capacity to be stimulated while transport affinity remains nearly unchanged in most of the freshwater bivalves studied (Dietz, 1978, 1985; Dietz and Branton, 1979; Dietz and Byrne, 1990; Dietz and Hagar, 1990; McCorkle and Dietz, 1980; Wilcox and Dietz, 1995). In this study, Cl transport capacity increase 50% and 85% after salt depletion for four and eight weeks, respectively. The increase in transport capacity after an appropriate period of salt depletion was found to be from 100% to 150% for Na in *C. fluminea* and *C. texasensis*, about 100% for Cl in *C. texasensis* or *L. subrostrata*, and 57% for K in *D. polymorpha*. Although two week salt depletion is sufficient to stimulate the rate of ion uptake in most unionid clams and in the zebra mussels, it is not adequate to elevate Cl uptake or its transport capacity in *C. fluminea*. It appears that the degree of salt depletion of the animals depends on the duration of their exposure to the DW. The magnitude of elevation of Cl influx, or transport capacity, corresponds directly with the degree of salt depletion in *C. fluminea*. The fact that *C. fluminea* needed more time for sufficient salt depletion suggests that there is a low epithelial permeability in this species.

With regard to transport affinity, the published values for Na, Cl or K K_m values in PW-acclimated or SD freshwater clams are between 0.05 and 0.3 mM. The transport affinity for Cl in this study is 0.21 ± 0.08 mM Cl for PW-acclimated *C. fluminea*. The estimated transport affinity for *C. fluminea* subjected to four week or longer salt depletion did not deviate from that of the PW animals (Fig. 2.2). This elevation in transport capacity with no change in transport affinity suggests more epithelial transport sites are activated during salt depletion treatment.

The passive diffusion components of ion uptake may play a relatively important role at high ion concentrations. Studies of K transport kinetics using radioactive Rb as a substitute tracer for K in the zebra mussel *D. polymorpha* have revealed a greater proportion of diffusive uptake at higher external Rb concentrations (Wilcox and Dietz, 1995). The ion concentrations used for most of the Cl and Na transport kinetic studies of freshwater bivalves was usually less than 3 mM. In this Cl transport study, a much greater range of Cl concentrations was applied in an attempt to partition the importance of passive diffusion to the uptake of Cl. Over a range of Cl concentration up to 12 mM, a Michaelis-Menten rectangular hyperbolic function fits very well with the Cl influx data ($R > 0.95$) of both PW-acclimated and SD *C. fluminea*. Attempts using a higher range of Cl concentrations (up to 20 mM) were not successful because the animals did not open their valves when exposed to Cl concentration higher than 12 mM. Nevertheless, no evidence of an elevation in Cl influx was found in any of these high Cl concentrations (data not show) in either PW-acclimated or SD animals, suggesting that passive diffusion of Cl uptake is not an important component in *C. fluminea*.

The epithelium of *C. fluminea* is much “tighter” than that of unionids or the zebra mussels. This tight or low permeable characteristic of the epithelia in this species is reflected by the longer time required for salt depletion to be effective, and the lack of evidence for a passive diffusion component in Cl uptake. *C. fluminea* inhabit brackish water up to 5‰ salinity and has higher blood osmolality than unionid clams and the zebra mussels (Filice, 1958; Hayashi, 1956; Dietz, 1979; McCorkle and Dietz, 1980; Keen and Casey, 1969; Byrne, *et al.*, 1989; Horohov *et al.*, 1992; Dietz *et al.* 1994). In addition, *C. fluminea* is very tolerant to salt depletion and can be kept in DW for several months with little mortality (unpublished observations). Thus, *C. fluminea* can tolerate much a broader range of ambient osmolality than unionid clams and zebra mussels. The characteristic of “tight” epithelia combined with the high uptake rate and transport capacity for Cl in this study corresponds to the relatively “euryhaline” characteristics of *C. fluminea*.

CHAPTER THREE
INTERRELATIONSHIP BETWEEN CHLORIDE AND SODIUM TRANSPORT
IN THE FRESHWATER BIVALVE,
CORBICULA FLUMINEA

INTRODUCTION

Freshwater animals must take up ions from the environment to maintain internal salt balance. Active Na and Cl uptake have been reported in many freshwater mussels (Dietz, 1978; Dietz, 1979; Krogh, 1939). The independence of Cl and Na uptake has been shown in many freshwater mussels (Dietz, 1978; Dietz and Branton, 1979; Dietz and Hagar, 1990), and other invertebrate and vertebrate species (Alvarado *et al.*, 1975; Kristensen, 1978; Kerstetter and Kirschner, 1972; Romeu *et al.*, 1969; Stobbart, 1971).

Cl/HCO₃ exchange is considered to be the major pathway for transepithelial Cl transport. The Na-independence Cl uptake has been shown in many invertebrate and vertebrate species (Alvarado *et al.*, 1975; Kerstetter and Kirschner, 1972; Lee and Pritchard, 1985; Romeu *et al.*, 1969; Stobbart, 1971). Cl uptake *via* Cl/HCO₃ exchange has been demonstrated in variety of animals (Alvarado *et al.*, 1975; Kerstetter and Kirschner, 1972; Lee and Pritchard, 1985; Madshus and Olsnes, 1987; Romeu *et al.*, 1975; Olsnes *et al.*, 1987; Tønnessen *et al.*, 1987; Tønnessen *et al.*, 1989; Smith, 1988; Stobbart, 1971). In freshwater mussels that have been studied, Na and Cl transport are independent and Cl uptake is suggested to be *via* a Cl/HCO₃ exchange pathway (Dietz, 1978, 1985; Dietz and Branton, 1979; Dietz and Findley, 1980; Henry and Saintsing, 1983). The Cl transport mechanism of *C. fluminea*, however, has not been studied.

Serotonin (5HT, 5-hydroxytryptamine) has been shown to be an effective stimulator of many processes in freshwater mussels. It dilates gill ostia openings, stimulates active Na uptake, gill cAMP production, lateral ciliary of the gill, gill muscle relaxation, foot movement, spawning and some other reproductive activities (Dietz, 1985; Fong *et al.*, 1993, 1994; Gardiner *et al.*, 1991; Horohov *et al.*, 1992; Scheide and Dietz, 1986). Yet the effect of 5HT on Cl transport has not been explored.

Acetazolamide (AZ), an inhibitor of carbonic anhydrase (CA), has been demonstrated to decrease Na influx, increase Cl and Na efflux, decrease blood Na and Cl, and increase blood bicarbonate concentrations in freshwater mussels (Dietz, 1978; Dietz and Branton, 1979; Henry and Saintsing, 1983). The action of AZ is thought to inhibit CA function in the gills and thereby reduce the amount of H and HCO₃ counterions available for the uptake of Na and Cl. However, previous studies involving AZ focused on unionid clams with little comparative information available for corbiculid clams.

MATERIALS AND METHODS

Animals

Corbicula fluminea (Müller) were collected under permit from the Tangipahoa River in Mississippi. The animals were acclimated to aerated artificial pondwater (PW) containing, in mM, 0.5 NaCl, 0.4 CaCl₂, 0.2 NaHCO₃, 0.05 KCl at 22-25°C for at least one week before use. Salt-depleted (SD) animals were stored the animals in deionized water for at least 14 days. The deionized water was changed daily for the first two weeks and every other day for extended salt depletion period.

Ion analysis

The animals were rinsed for about 30 min in deionized water and transferred to individual beakers with 20 to 30 ml of appropriate bathing medium containing radioactive isotope ^{36}Cl or ^{22}Na . In the experiments where the net flux of titratable base was measured, tris (tris(hydroxymethyl)-aminomethane) was added into the bathing medium at a final concentration of 0.1 mM, and the pH was adjusted to 7.3 with tris-HCl for either PW or Na-free PW, and with tris- H_2SO_4 for Cl-free solutions. Choline was used to replace Na in Na-free bathing medium and sulfate was used to replace Cl in Cl-free bathing medium. When the clams began siphoning (10 to 15 min), samples were taken from the bath at intervals of 2 h. The Cl concentration in the bath samples was determined by electrometric titration and the Na concentration was assayed by flame photometry. To measure the net loss of titratable base (J_{a}^{B}), samples were sonicated to remove respiratory CO_2 . The samples were titrated to pH 4.5 using standardized 5 mM HCl. The difference between initial and final buffer capacity was used to calculate net base production.

Ion transport

Unidirectional influx (J_{i}) was calculated from the disappearance of isotope (specific activity for ^{22}Na or ^{36}Cl was about 2000 to 3000 CPM/ μM) from the bathing medium, as previously described (Dietz, 1978; Graves and Dietz, 1982). Radioactivity was assayed with a liquid scintillation counter using a cocktail based on Triton X-114/xylene (Wiegman *et al.*, 1975). Net flux (J_{n}) was estimated from the changes in bath ion concentration. The bath volume was kept small (30 ml) to allow rapid changes

in the radioactivity of the bath with limited accumulation of isotope in the body fluid.

These conditions minimized the back flux of isotope that would cause an underestimation of J_i (Dietz, 1978).

Administration of drugs

5HT was added directly into the bathing medium during the experiment at a final concentration of 0.1 mM, and pH was adjusted to 7.3. AZ was dissolved in the deionized water and the animals were immersed in the solution with the final concentration of AZ of 0.5 mM for 12 h before the experiments. No AZ was added in the bathing medium during the flux measurement.

Statistics

A two level factorial experimental design, using specific ion as one factor and drug treatment as the other, was employed to correlate Cl or Na transport and loss of base under the condition of presence or absence of specific ions, and the presence or absence of specific drugs, simultaneously. Data were expressed as mean $\pm$ sem, and were analyzed with SAS by a linear regression model (REG). Differences were considered significant if $P < 0.05$.

RESULTS

The relationship between Cl and Na transport and the effects of 5HT on PW-acclimated animals

Cl transport was not affected by the absence of external Na, but was significantly stimulated by 5HT in PW-acclimated *C. fluminea* (Table 3.1). With 5HT in the bath, animals displayed active siphoning, valve gaping and extension of the foot.

Table 3.1. The effects of 5HT and external Na on Cl transport and net flux of titratable base in PW-acclimated *C. fluminea*. 5HT was added to the bathing solution with a final concentration of 0.1 mM. J_n , net flux; J_i , influx; J_o , efflux. Data were means and standard errors of 10 animals and expressed as $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$. * $P < 0.05$, ** $P < 0.01$. Na/5HT: interaction between Na and 5HT.

Flux	+Na		-Na		F-Test	
	-5HT	+5HT	-5HT	+5HT	$\pm\text{Na}$	$\pm\text{5HT Na/5HT}$
J_n^{Cl}	-1.70 ± 0.63	1.45 ± 0.59	-2.44 ± 0.48	0.70 ± 0.93		**
J_i^{Cl}	2.07 ± 0.30	4.07 ± 0.35	1.91 ± 0.37	4.23 ± 0.55		**
J_o^{Cl}	3.77 ± 0.81	2.62 ± 0.82	4.35 ± 0.69	3.53 ± 0.87		
J_n^{B}	-1.78 ± 0.39	-4.19 ± 0.46	-2.51 ± 0.51	-5.73 ± 0.52	*	**

The stimulation of Cl transport by 5HT was Na-independent. With or without Na, 5HT significantly increased influx and net flux of Cl with little change in efflux. Cl net flux increased about 3.2 and 3.1 $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$ and influx about 2.0 and 2.3 $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$ in PW and Na-free PW bathing medium, respectively. No statistical interactions (Na/5HT) were found between external Na and 5HT. The lack of effect of external Na on Cl transport and the Na-independence of 5HT stimulation of Cl uptake indicate that Cl uptake in PW-acclimated *C. fluminea* is a 5HT stimutable and Na-independent process.

To determine the relationship between Cl and Na transport, Na transport was also examined. The effect of 5HT on Na transport was similar to that on Cl transport (Table 3.2). With or without Cl, 5HT increased Na net flux 2.7 and 2.4 $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$, and influx 2.3 and 1.6 $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$ in PW and Cl-free PW, respectively. Na transport was not affected by the absence of external Cl. There were no statistical interactions between external Cl and 5HT (Cl/5HT), indicating the effect of 5HT on Na transport was not dependent on external Cl. Therefore, Na uptake of PW-acclimated *C. fluminea* is a Cl-independent process which can also be elevated by 5HT.

The loss of titratable base was significantly elevated by 5HT. This effect was independent of the external Na or Cl concentration, as seen in the lack of interactions between external Cl or Na and 5HT (Table 3.1 and 3.2). 5HT increased the net flux of base by about 1.5 $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$ and 1.6 $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$ in the presence

Table 3.2. The effects of 5HT and external Cl on Na transport and net flux of titratable base in PW-acclimated *C. fluminea*. 5HT was added to the bathing solution with a final concentration of 0.1 mM. J_n , net flux; J_i , influx; J_o , efflux. Data were means and standard errors of 10 animals and expressed as $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$. * $P < 0.05$, ** $P < 0.01$. Cl/5HT: interaction between Cl and 5HT.

Flux	+Cl		-Cl		F-Test		
	-5HT	+5HT	-5HT	+5HT	$\pm\text{Cl}$	$\pm 5\text{HT}$	Cl/5HT
J_n^{Na}	1.31 $\pm$ 0.53	4.05 $\pm$ 0.33	1.43 $\pm$ 0.69	3.79 $\pm$ 0.51		**	
J_i^{Na}	5.88 $\pm$ 0.68	8.21 $\pm$ 0.29	5.95 $\pm$ 0.47	7.57 $\pm$ 0.86		**	
J_o^{Na}	4.57 $\pm$ 0.34	4.16 $\pm$ 0.17	4.52 $\pm$ 0.30	3.78 $\pm$ 0.44			
J_n^{B}	-2.72 $\pm$ 0.51	-4.18 $\pm$ 0.54	-2.23 $\pm$ 0.55	-3.82 $\pm$ 0.68		**	

and absence of external Cl, respectively. The absence of Na caused a small but significant increase in the net flux of base. A greater net flux of base was consistently observed in the absence of external Na. In contrast, a small decrease of the base net flux was consistently, though not significantly, observed in the absence of external Cl.

Although Cl and Na uptake were not directly dependent upon each other in PW-acclimated *C. fluminea*, as indicated in Tables 3.1 and 3.2, they were correlated in an indirect way. A regression of individual net flux between Cl and Na revealed a significant linear relationship (Fig 3.1), in which a higher Cl net flux correlated with a higher Na net flux ($R=0.84$; $P<0.05$).

The relationship between Cl and Na transport and the effects of AZ on SD animals

To study the effects of AZ on Cl and Na transport, SD *C. fluminea* were used to increase the basal rate of Cl and Na uptake. Studies with PW-acclimated animals were variable and it was common to observe no effect of AZ on ion transport (data not shown). As shown in Table 3.3, AZ was an effective inhibitor of Cl transport in SD *C. fluminea*. AZ pre-treatment resulted in significant reduction of Cl net flux by increasing Cl efflux. The increase of Cl efflux induced by AZ was $3.1 \mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$ in the presence of Na and $6.0 \mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$ in the absence of Na. AZ exerted greater effects on Cl efflux when there was no Na in the bathing medium, as indicated by the significant interaction between external Na and AZ in Cl efflux (Table 3.3).

Na efflux was also significantly elevated by AZ (Table 3.4). Na efflux was elevated about $1.8 \mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$ in the presence of external Cl and $3.6 \mu\text{eq}(\text{g}$

Figure 3.1. Relationship between net flux of Cl and Na of PW-acclimated *C. fluminea*. J_n , net flux; $R=0.85$, $N=35$, $P<0.01$.

Table 3.3. The effects of AZ and external Na on Cl transport and net flux of titratable base in SD *C. fluminea*. The animals were pre-treated for 12 h before the experiments with AZ added to the bathing solution with a final concentration of 0.5 mM. No AZ was present in the bathing solution during the experiments. J_n , net flux; J_i , influx; J_o , efflux. Data were means and standard errors of 10 animals and expressed as $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$. * $P < 0.05$, ** $P < 0.01$. Na/AZ: interaction between Na and AZ.

Flux	+Na		-Na		F-test		
	-AZ	+AZ	-AZ	+AZ	$\pm\text{Na}$	$\pm\text{AZ}$	Na/AZ
J_n^{Cl}	3.89 $\pm$ 1.18	2.17 $\pm$ 0.88	4.46 $\pm$ 0.77	1.73 $\pm$ 0.59		*	
J_i^{Cl}	6.41 $\pm$ 1.07	7.77 $\pm$ 0.68	5.27 $\pm$ 0.57	8.55 $\pm$ 0.60		**	
J_o^{Cl}	2.52 $\pm$ 0.62	5.60 $\pm$ 0.89	0.81 $\pm$ 0.51	6.82 $\pm$ 0.73		**	*
J_n^{B}	-6.95 $\pm$ 1.37	-10.83 $\pm$ 1.08	-5.65 $\pm$ 0.69	-8.83 $\pm$ 0.48		**	

Table 3.4. The effects of AZ and external Cl on Na transport and net flux of titratable base in SD *C. fluminea*. The animals were pre-treated for 12 h before the experiments with AZ added to the bathing solution with a final concentration of 0.5 mM. No AZ was present in the bathing solution during the experiments. J_n , net flux; J_i , influx; J_o , efflux. Data were means and standard errors of 10 animals and expressed as $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$. * $P < 0.05$, ** $P < 0.01$. Cl/AZ: interaction between Cl and AZ.

Flux	+Cl		-Cl		F-Test		
	-AZ	+AZ	-AZ	+AZ	$\pm\text{Cl}$	$\pm\text{AZ}$	Cl/AZ
J_n^{Na}	7.75 $\pm$ 0.92	7.18 $\pm$ 0.72	5.38 $\pm$ 0.90	3.81 $\pm$ 0.83	*		
J_i^{Na}	12.33 $\pm$ 1.34	13.55 $\pm$ 1.12	8.45 $\pm$ 1.64	10.49 $\pm$ 0.87	*		
J_o^{Na}	4.58 $\pm$ 0.50	6.37 $\pm$ 0.65	3.07 $\pm$ 0.79	6.69 $\pm$ 0.82		**	
J_n^{B}	-1.47 $\pm$ 0.97	-5.74 $\pm$ 0.70	-2.46 $\pm$ 1.02	-4.33 $\pm$ 0.53		**	

dry tissue $\cdot$ h)⁻¹ in the absence of external Cl. Net flux was not changed due to the small but significant increase of Na influx by AZ.

Unlike PW-acclimated *C. fluminea* in which Cl and Na transport were independent, as described earlier, the Cl and Na transport in SD animals appeared to be more related (Table 3.3 and 3.4). Although Cl uptake was Na-independent in SD *C. fluminea*, as indicated by the lack of alteration of Cl influx in the absence of external Na, Na uptake was significantly reduced by the absence of external Cl. With or without exposure to AZ, there was about a 3 to 4 $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$ reduction in Na influx in the absence of external Cl, resulting in a significant decrease in the Na net flux. This suggests that the component of Na uptake stimulated by salt depletion is at least in part dependent on the presence of external Cl. The dependence of Na transport of SD animals on external Cl will be further explored in Chapter Four.

As shown in Table 3.3 and 3.4, the effect of AZ on base net flux was consistent in the two experiments with SD *C. fluminea*. AZ significantly increased the net loss of titratable base. The increase of net base flux was about 4 $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$ in regular PW medium, 3.2 $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$ in Na-free bathing medium, and 1.9 $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$ in Cl-free bathing medium. Interestingly, the net flux of base was little affected by the absence of external Cl or Na. No interaction between external Cl or Na and AZ was observed.

In both PW-acclimated and SD *C. fluminea*, the net flux of titratable base was not affected by the absence of external Cl. However, a regression between Cl influx and

net flux of base from individual animals demonstrated a significant linear relationship (Fig. 3.2, $R=0.78$ and 0.82 for SD and PW-acclimated animals, respectively, $P<0.01$). In all experiments, a greater Cl influx was positively correlated with a greater base net flux. The slope of the regression for PW-acclimated animals was not statistically different from that of SD animals, suggesting that the relationship between Cl influx and base net flux is stoichiometrically similar in both acclimation groups.

DISCUSSION

The relationship between Cl and Na transport in *C. fluminea*

Na transport in *C. fluminea* can be Cl-independent or Cl-dependent depending on the acclimation condition of the animals. Although Na transport and the component of Na active uptake stimulated by 5HT are Cl independent in PW-acclimated animals, a substantial portion of Na uptake in SD *C. fluminea* depends on the presence of external Cl. This suggests that multiple Na uptake pathways may exist in *C. fluminea*, and that the pathway stimulated by salt depletion is different from that stimulated by 5HT.

Although the Na/H exchange mechanism has been considered to be a major pathway for Na uptake in freshwater bivalves including *C. fluminea* (Dietz, 1978, 1985; Henry and Saintsing, 1983; McCorkle and Dietz, 1980), other Na uptake pathways, such as indirect coupling to the activity of proton-ATPase (proton-pump) may also exist.

Recent studies have demonstrated that Na uptake in the gills of freshwater rainbow trout is energized by the electrogenic proton-ATPase located on the apical membrane of the gill (Lin and Randall, 1995).

Figure 3.2. Relationship between Cl influx and net loss of titratable base in PW-acclimated and SD *C. fluminea*. A: Open circle, SD animals, $R=0.77$, $N=37$, $P<0.01$. B: Solid square, PW-acclimated animals, $R=0.82$, $N=38$, $P<0.01$. J_n , net flux. The slopes of two lines were not significantly different ($A=0.65\pm0.07$; $B=0.56\pm0.08$).

Our recent studies have also shown that Na influx of *C. fluminea* was inhibited by N, N'-dicyclohexylcarbodiimide (DCCD), an effective H-pump inhibitor, and that this inhibition was Cl-dependent in SD animals (see Chapter 4). Perhaps there are two independent Na entrance processes in *C. fluminea* and the partition of Na entrance through different pathways may depend on the condition of the animals or regulation differences depending on environmental stress. The Na/H exchange pathway may be substantial in PW-acclimated animals. This process may be more sensitive to 5HT and independent of external Cl. The Na uptake pathway and its coupling to the proton-pump may be more sensitive to salt depletion, and more dependent on the external Cl. Chronic adaptation of freshwater rainbow trout to water containing low Na increased the amount of H-ATPase recruited onto the gill epithelium and the enzymatic activity of H-ATPase in the gill membrane fraction (Lin and Randall, 1995). Exocytic insertion of H pump-bearing vesicles into the plasma membrane has been reported (Schwartz and Al-Awqati, 1986). If the functional significance of H-ATPase in the gills of *C. fluminea* is to energize active Na uptake from dilute medium as in the pavement cells of freshwater fish (Lin and Randall, 1995), then the increase in the amount and activity of H-ATPase after salt depletion would be expected.

The effect of 5HT on Cl and Na transport in *C. fluminea*

5HT stimulated Cl influx by activating the active transport component, presumably the Cl/HCO₃ exchange pathway. The Na-independent stimulation of Cl uptake by 5HT suggests that the active uptake of Cl in PW-acclimated *C. fluminea* is a Na-independent Cl/HCO₃ exchange. 5HT may stimulate the active Cl uptake pathway

similar to that stimulated by salt depletion. Salt depletion was reported to stimulate the activity of CA in the gills of *C. fluminea* (Henry and Saintsing, 1983). The increased amount of HCO_3^- due to the stimulation of CA activity may promote the $\text{Cl}^-/\text{HCO}_3^-$ exchange pathway directly or indirectly by providing more HCO_3^- .

$\text{Cl}^-/\text{HCO}_3^-$ exchange is an important mechanism for Cl^- uptake in the freshwater bivalves that have been studied. Cl^- - and HCO_3^- -stimulated ATPase activity has been demonstrated in the microsomal membranes of the gills of the unionid mussel *Carunculina texasensis* (Dietz and Findley, 1980). The highly significant correlation between Cl^- influx and the net flux of titratable base in both PW-acclimated and SD *C. fluminea* in this study also suggests the importance of this exchange mechanism in Cl^- uptake. In PW-acclimated *C. fluminea* both the active uptake component *via* a $\text{Cl}^-/\text{HCO}_3^-$ exchange and the exchange diffusion through Cl^-/Cl^- turnover maybe important for total Cl^- isotope turnover. Exchange diffusion was demonstrated to be important for the total Na inward movement in *C. fluminea* (McCorkle and Dietz, 1980). The exchange diffusion could be significant when there is insufficient exchangeable HCO_3^- , or when there is an elevation of intracellular Cl^- due to the blockage of the Cl^- exit pathway on the basolateral membrane. An increase of exchange diffusion, however, would result in the elevation of both Cl^- influx and efflux simultaneously. The observed significant increase of Cl^- influx without an elevation of Cl^- efflux by 5HT in this study suggests that there is stimulation of the active Cl^- uptake by exchange with HCO_3^- . This is further supported by the small but consistent decrease of net base flux in the absence of external Cl^- (Table 3.2).

Gills are a major site of Na and Cl transport in freshwater mussels (Dietz and Findley 1980; Dietz and Hagar, 1990), and the epithelial cells of gills are abundantly innervated by serotonergic neurons (Dietz, 1985; Dietz *et al.*, 1992). 5HT has been reported to elevate active Na transport by increasing cAMP levels in gill tissue of freshwater mussels *Carunculina texasensis* and *Ligumia subrostrata* (Scheide and Dietz, 1986). The presence of a 5HT-stimulated adenylate cyclase has been found in gill homogenate, and the stimulation of adenylate cyclase-catalyzed cAMP synthesis was reported in several freshwater mussels (Dietz, 1985). The stimulation of Na uptake by 5HT in *C. fluminea* is probably through a similar cAMP pathway as found in *C. texasensis* and *L. subrostrata*.

The stimulation of net flux of titratable base by 5HT has not been reported in freshwater bivalves. The amount of net flux of titratable base in this study was determined by the amount of exogenous acid required to titrate the base in the bathing solution. Although the primary source of the titratable base in *C. fluminea* is HCO_3^- , the amount of exogenous titrating acid required can be affected by the amount of endogenous H released into the bathing solution. Therefore, the net change of titratable base reflects the combined results of the activities of the Cl/HCO_3^- exchanger, Na/H antiporter, proton-pump and other possible pathways that generate acid or base equivalents. The stimulation of Na uptake may extrude more H^+ , presumably through a Na/H antiporter and/or proton-pump, which would autotitrate the base in the bathing solution. The activation of Cl uptake, on the other hand, elevates the amount of base in the medium *via* a Cl/HCO_3^- exchanger.

Although the increase of Na and Cl uptake by 5HT accounts for some fraction of the alteration of net base flux, a substantial proportion of the net base flux that was stimulated by 5HT is independent of the uptake of Na and Cl. This is indicated by the observation that in the absence of external Na or Cl there was a persistent loss of base that was elevated by 5HT (Table 3.1 and 3.2). The mechanism for elevation of the loss of base by 5HT is not clear. It could be an indirect result of the increased amount of HCO_3 filtered by the renal tissue. 5HT appears to be a “universal” stimulator of freshwater bivalves including *C. fluminea*. 5HT has been reported to increase gill ciliary activities, dilate gill ostia openings, increase foot movement, and stimulate gill muscle relaxation, spawning and other reproductive activities in freshwater mussels (Gardiner *et al.*, 1991; Fong *et al.*, 1993, 1994). The stimulation of all these activities may increase metabolic CO_2 production resulting in the elevation of blood HCO_3 due to the hydration of CO_2 . Alternatively, serotonin may directly stimulate HCO_3 excretion by the renal tissue.

The effects of AZ on the Cl and Na transport of *C. fluminea*

While 5HT acts on the Cl and Na uptake process, AZ works predominately on the Cl and Na exit pathway in SD *C. fluminea*. In SD *C. fluminea* AZ depressed Cl transport by exerting a greater influence on the Cl efflux than influx. The significant increase of Cl efflux by AZ in this study agrees with previous studies of the freshwater mussels *C. texasensis* and *L. subrostrata* (Dietz and Branton, 1979; Henry and Saintsing, 1983). The increase of Cl influx by AZ has not been reported previously. Few studies have been performed examining the effect of AZ on the Na transport in

freshwater bivalves. AZ has been shown to inhibit Na transport by reducing influx in *L. subrostrata* (Henry and Saintsing, 1983). In SD *C. fluminea*, AZ exerted its effect on the Na efflux, and no evidence of a reduction in influx was observed. This disagreement is possibly due to species differences in response to AZ and differences in the Na uptake process.

The effects of AZ on the change of titratable base has not been reported in freshwater bivalves. In SD *C. fluminea*, AZ significantly increased the net loss of base into the bathing solution. Although gill epithelia are important sites for HCO_3 and H exit by the exchange activities, the filtration and excretion in the renal tissue also could be a significant process in the regulation of acid and base exit. An increase of blood HCO_3 by AZ has been demonstrated in *C. fluminea* and other freshwater mussels (Henry and Saintsing, 1983). The blockage of the HCO_3 exit pathway in the gill epithelia would result in the accumulation of HCO_3 in the blood, and increase the amount of HCO_3 being subjected to filtration or excretion by the renal tissue.

CHAPTER FOUR

CHLORIDE AND SODIUM TRANSPORT MECHANISMS IN THE FRESHWATER BIVALVE, *CORBICULA FLUMINEA*

INTRODUCTION

Na and Cl uptake mechanisms are traditionally thought to involve electroneutral exchange with H (and or NH_4) and HCO_3 , respectively (Krogh, 1939). The concept of Cl/HCO_3 exchange for Cl uptake has generally been accepted. Transcellular Cl uptake *via* an apical Cl/HCO_3 exchanger has been found in a various studies in epithelia of many vertebrates including gill and operculum of freshwater fishes (Marshall, 1995; Heisler, 1993; Perry and Laurent, 1993; Perry *et al.*, 1981; Perry and Randall, 1981), amphibian skins and vertebrate renal distal tubule and intestine (Knickelbein *et al.*, 1985; Larsen, 1991; Larsen *et al.*, 1992). In ion transporting epithelia of vertebrates such as amphibian skins and renal distal tubules, the Cl/HCO_3 exchanger is energized by an electrogenic proton-pump. The cellular substrates for the anion exchanger and the proton-pump are provided by the hydration of CO_2 catalyzed by carbonic anhydrase (Larsen, 1991). Limited information is available for the invertebrates. Cl/HCO_3 exchange has been shown to be important in the Cl uptake of the low-salinity-adapted crabs (Lee and Pritchard, 1985) and freshwater bivalves (Dietz, 1979, 1985; Dietz and Branton, 1979; Dietz and Findley, 1980; Dietz and Hagar, 1990). Proton-pumps responsible for the acid secretion have been demonstrated in the shell-facing membrane of mantle epithelium in the freshwater clam, *Unio complanatus* (Hudson, 1993). The

involvement of a proton-pump in the Cl/HCO_3 exchanger, however, has not been reported in invertebrates.

Transcellular Na uptake through amiloride-sensitive apical conductive Na channels, energized by the basolateral Na-K-pump, has been demonstrated in amphibian skins and vertebrate renal distal epithelia (Larsen, 1991). Apical Na uptake *via* Na/H (NH_4) exchange was also considered to be the major pathway in Na transport and H excretion in freshwater fishes (Wright and Wood, 1985), but this concept has recently been challenged (Lin and Randall, 1995). In a new model of freshwater fishes, Na uptake is accomplished through conductive apical Na channels energized by electrogenic proton-pumps. The protons are primarily supplied by the hydration of CO_2 as would occur in Cl transport. The transepithelial Na uptake mechanism(s) and their regulation in invertebrate systems have been less well studied. In the freshwater unionid mussels that have been studied, the entry of Na is considered, based on the amiloride sensitivity, to be through an apical Na/H exchange pathway. The basolateral Na-K-pump is the likely energy source as in amphibian skin (Dietz, 1978, 1979, 1985; Dietz and Findley, 1980). In other freshwater bivalves such as *C. fluminea* and the zebra mussel *Dreissena polymorpha* (Horohov *et al.*, 1992; McCorkle and Dietz, 1980) Na uptake is not sensitive to amiloride, suggesting multiple Na uptake pathways may occur among freshwater bivalves.

N, N'-dicyclohexylcarbodiimide (DCCD), an effective proton-pump inhibitor, has been useful in localizing and characterizing the proton-pump (Forgac, 1989; Sbia *et al.*, 1992). 4,4'-diisothiocyano stilbene-2,2'-disulfonic acid (DIDS), an inhibitor of

anion exchange, has been used in the studies of Cl/HCO_3 or Cl/Cl self-exchange systems in cells and tissues (Janas *et al.*, 1989; Remjeesingh *et al.*, 1981; Restrepo *et al.*, 1991).

MATERIALS AND METHODS

Animals

Corbicula fluminea (Müller) were collected under permit from the Tangipahoa River in Mississippi. The animals were acclimated to aerated artificial PW (PW) containing (in mM) 0.5 NaCl, 0.4 CaCl_2 , 0.2 NaHCO_3 , 0.05 KCl at 22-25°C for at least one week before use. Salt depleted (SD) animals were obtained by storing the animals in deionized water for at least 14 days. The deionized water was changed daily for the first two weeks and every other day for salt depletion of extended duration.

Ion transport

Unidirectional influx (J_i) was calculated from the disappearance of isotope (specific activity for ^{22}Na or ^{36}Cl was about 2000 to 3000 CPM/ μM) from the bathing medium, as previously described (Dietz, 1978; Graves and Dietz, 1982). Radioactivity was assayed with a liquid scintillation counter using a cocktail based on Triton X-114/xylene (Wiegman *et al.*, 1975). Net flux (J_n) was estimated from the changes in bath ion concentration. The bath volume was small (30 ml) and allowed rapid changes in the radioactivity of the bath with limited accumulation of isotope in the body fluid. These conditions minimized the back flux of isotope that would cause an underestimation of J_i (Dietz, 1978). The Cl concentration in the bath samples was determined by electrometric titration and the Na concentration was assayed by flame

photometry. Choline was used to replace Na in Na-free bathing medium and sulfate was used to replace Cl in Cl-free bathing medium.

In the experiments where the net flux of titratable base was measured, tris (tris(hydroxymethyl)aminomethane) was added into the bathing medium at a final concentration of 0.1 mM, and the pH was adjusted to 7.3 with tris-HCl for either PW or Na-free PW, and with tris-H₂SO₄ for Cl-free solutions. To measure the net loss of titratable base (J_n^B), samples were sonicated to remove respiratory CO₂. The samples were titrated to pH 4.5 using standardized 5 mM HCl and the difference between initial and final buffer capacity was used to calculate net base production.

The administration of pharmacological agents

The animals were rinsed for about 30 min in deionized water before the administration of pharmacological agents. Atrial natriuretic peptide (ANP) and Phenylalanyl-methionyl-argininyl-phenylalanyl-amide (FMRFamide) were dissolved in distilled water. All other chemicals were dissolved in 100% ethanol. Most chemicals were carefully injected into the foot muscle of the animals. 3-[Aminosulfonyl]-5-[butylaminol]-4-phenoxy-benzoic acid (bumetanide, 0.5 mM) was added directly to the incubating solution with 2.0 mM dimethylsulfoxide (DMSO). 4,4'-diisothiocyano stilbene-2,2'-disulfonic acid (DIDS) was dissolved in PW (0.5 mM) and the pH adjusted to 7.3 with Tris. After 1 h equilibration in deionized water following the injection, animals were transferred to individual beakers with appropriate bathing medium containing radioactive isotope ³⁶Cl or ²²Na. Control animals were sham-injected with a similar amount of deionized water or of 100% ethanol accordingly.

When clams began siphoning (10 to 15 min), samples were taken from the bath at intervals of 2 h.

Statistics

Data were expressed as mean and standard error. The differences were examined with one-way ANOVA and considered to be significant if P-value was equal or less than 0.05.

RESULTS

The effects of N, N'-dicyclohexylcarbodiimide (DCCD) on ion transport

In preliminary studies, the effects of DCCD on net Na and Cl flux was observed in PW-acclimated animals. Both Cl and Na net flux were significantly decreased by DCCD (Table 4.1). To examine the maximal effects of DCCD on ion transport, the animals were salt depleted. The effects of DCCD on the Cl and Na fluxes of SD *C. fluminea* are illustrated in Table 4.2. Injection of DCCD significantly decreased Cl uptake from PW and Na-free PW media, suggesting the direct action of DCCD on Cl transport. The reduction of net Cl uptake from Na-free PW medium was due to the inhibition of Cl influx in which 66% of the Cl influx was inhibited. Cl efflux was little affected by DCCD. Na transport was significantly affected by DCCD in PW medium, but was not affected in Cl-free medium. This suggests that the inhibition of DCCD on Na transport is Cl dependent. In PW medium, about 70% of the Na net flux and 55% of the influx was inhibited by DCCD injection.

The net loss of titratable base was significantly increased by the treatment with DCCD only when animals were bathed in the Cl-free medium during the experiment

Table 4.1. The effects of N, N'-dicyclohexylcarbodiimide (DCCD) injection (0.5 μmol) on the Na and Cl net fluxes in PW-acclimated *C. fluminea*. DCCD was dissolved in 100% ethanol. Control animals were sham injected with equal amounts of ethanol. PW was used as a bathing solution during the experiment. J_n , net flux expressed as $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$. Data are expressed as a mean and a standard error with the number of animals in the parenthesis. * is significantly different from the control value in the same row ($P < 0.05$).

J_n	control	DCCD-treated
Cl	$0.88 \pm 0.37(9)$	$-2.0 \pm 0.76(10)^*$
Na	$2.62 \pm 0.44(9)$	$-2.54 \pm 1.24(10)^*$

Table 4.2. The effects of N, N'-dicyclohexylcarbodiimide (DCCD) injection (0.25 μmol) on the Na and Cl fluxes in SD *C. fluminea* in different bathing solutions. DCCD was dissolved in 100% ethanol. Control animals were sham injected with equal amounts of ethanol. PW, PW; Na-F PW, Na-free PW; Cl-F PW, Cl-free PW. J_n , net flux; J_i , influx; J_o , efflux. Flux is expressed as $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$. Data are expressed as a mean and a standard error with the number of animals in the parenthesis. * is significantly different from the control values in the same row ($P < 0.05$).

flux	exp. medium	control	DCCD-treated
J_n^{Cl}	PW	$3.79 \pm 0.74(8)$	$-0.18 \pm 0.489(10)^*$
J_n^{Cl}	Na-F PW	$7.69 \pm 0.52(16)$	$1.66 \pm 0.63(12)^*$
J_i^{Cl}	Na-F PW	$10.22 \pm 0.47(16)$	$3.50 \pm 0.83(12)^*$
J_o^{Cl}	Na-F PW	$2.47 \pm 0.74(16)$	$1.88 \pm 0.38(12)$
J_n^{Na}	PW	$6.63 \pm 0.81(8)$	$1.99 \pm 0.33(10)^*$
J_i^{Na}	PW	$10.20 \pm 1.15(8)$	$4.56 \pm 0.56(10)^*$
J_o^{Na}	PW	$3.57 \pm 0.39(8)$	$2.58 \pm 0.37(10)$
J_n^{Na}	Cl-F PW	$5.88 \pm 0.22(14)$	$6.16 \pm 0.49(9)$
J_i^{Na}	Cl-F PW	$8.66 \pm 0.31(14)$	$9.24 \pm 0.65(9)$
J_o^{Na}	Cl-F PW	$2.78 \pm 0.16(14)$	$3.06 \pm 0.23(9)$

(Table 4.3). In PW or Na-free PW media, however, titratable base were not affected by the treatment with DCCD. The net flux of titratable base was also affected by the different bathing media used in the experiment. The greatest net base flux was recorded in Na-free media. This was likely because much less endogenous protons can be released by the proton-pump and/or the Na/H exchanger. Conversely, the loss of titratable base was the smallest in Cl-free PW medium probably because no external Cl was available for the exchange with HCO_3 and proton transport would autotitrate the base. In PW media where Na/H antiporter, Cl/ HCO_3 exchanger, and proton-pump were functioning. The net flux of titratable base in PW was intermediate to that of the other two media as would be expected.

The effects of 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) on ion transport

Table 4.4 demonstrates the effects of DIDS on the Cl flux and the net flux of Na and titratable base in PW-acclimated and in SD *C. fluminea*. In both acclamations, Cl influx was significantly decreased by the DIDS, suggesting the involvement of a Cl/ HCO_3 exchange mechanism in these animals. The reduction of Cl influx by DIDS treatment in PW-acclimated animals was more than 60% and accounted for the total decrease of Cl net flux, suggesting that the inhibition of DIDS is mostly on the Cl/ HCO_3 exchange rather than on the Cl/Cl self exchange.

Although the absolute reduction of Cl influx by DIDS treatment in SD animals ($4.8 \mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$) was more than that of PW-acclimated clams ($3.3 \mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$), the proportional reduction relative to the corresponding control value was much less in SD animals (less than 30%). This suggests that the proportion of Cl

Table 4.3: The effects of N, N'-dicyclohexylcarbodiimide (DCCD) injection (0.25 μmol) on the net flux of titratable base (J_n^B) of SD *C. fluminea*. DCCD was dissolved in 100% ethanol. Control animals were sham injected with equal amount of ethanol. Animals were bathed in different media during the experiments. PW, PW; Na-F PW, Na-free PW; Cl-F PW, Cl-free PW. Data are expressed as a mean in the units of $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1} \pm$ standard error with the number of animals in the parenthesis. * is significantly different from the control values in the same row ($P < 0.05$).

exp. medium	control	DCCD-treated
PW	$-3.39 \pm 0.93(8)$	$-3.22 \pm 0.47(10)$
Na-F PW	$-6.40 \pm 1.74(7)$	$-5.46 \pm 0.12(6)$
Cl-F PW	$0.50 \pm 0.46(13)$	$-1.90 \pm 0.65(9)^*$

Table 4.4: The effects of 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS, 0.5 mM) in the bath on the ion fluxes of *C. fluminea*. PW with ^{36}Cl and 0.1 mM of Tris was used as the bathing medium. The DIDS used in the experiment was a Na salt, and the concentration of Na in the control medium was adjusted with Na_2SO_4 to the same Na level as in DIDS treated animals to eliminate the possible effects due to the concentration difference of Na between the two solutions. PW, PW-acclimated animals; SD, salt depleted animals. J_n , net flux; J_i , influx; J_o , efflux. Flux was expressed as $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$. *Mean is significantly different from its controlled value in the same row ($P < 0.05$).

flux	acclimation	control	DIDS-treated
J_n^{Cl}	PW	$1.48 \pm 0.59(10)$	$-1.76 \pm 0.90(10)^*$
	SD	$14.44 \pm 2.01(10)$	$11.37 \pm 1.07(10)$
J_i^{Cl}	PW	$5.38 \pm 0.24(10)$	$2.09 \pm 0.29(10)^*$
	SD	$16.17 \pm 1.94(10)$	$11.41 \pm 1.06(10)^*$
J_o^{Cl}	PW	$3.91 \pm 0.51(10)$	$3.85 \pm 0.99(10)$
	SD	$1.72 \pm 0.41(10)$	$0.04 \pm 0.04(10)^*$
J_n^{Na}	PW	$2.08 \pm 0.66(10)$	$0.35 \pm 0.71(10)^*$
	SD	$15.92 \pm 2.30(10)$	$14.78 \pm 0.94(10)$
J_n^{B}	PW	$-4.97 \pm 0.46(10)$	$-3.27 \pm 0.46(10)^*$
	SD	$-4.69 \pm 0.67(10)$	$-0.19 \pm 0.67(10)^*$

uptake through DIDS-sensitive Cl/HCO_3 exchange to the total Cl uptake was decreased after salt depletion. The net change of Cl flux elicited by DIDS treatment for SD animals was due to the combined effects on both efflux and influx with the later being much more important. The reduction of efflux was small but significant.

The net flux of Na was significantly affected by DIDS in PW-acclimated *C. fluminea*, in which 83% of the Na net flux was inhibited. The magnitude of reduction in Na net flux was less than that of Cl, suggesting that only a portion of the Na uptake was linked to the DIDS sensitive Cl/HCO_3 exchange system. Interestingly, Na net flux of SD animals was not affected by DIDS treatment. This suggests that the Na uptake pathway stimulated by SD is not DIDS-sensitive.

Net loss of titratable base was significantly reduced by DIDS treatment in both PW-acclimated and SD *C. fluminea*. The magnitude of reduction in net loss of titratable base was greater in SD animals than in PW-acclimated animals; the opposite of the effect seen on Na and Cl transport. While the decrease was only about 34% in PW-acclimated animals, there was a 96% reduction of J_n^B in SD animals.

The effects of DIDS on the blood Cl and Na concentration were in agreement with the flux data (Table 4.5). The concentrations of blood Cl and Na were significantly reduced by the treatment of DIDS resulting in a reduction of total blood osmolarity in SD *C. fluminea*. The change was greater in Cl than Na in the blood in both PW-acclimated and SD animals (PW, 6.0% reduction in Na and 9.1% in Cl; SD, 6.7% reduction in Na and 10% in Cl). Therefore, it appears that about two thirds of the Na transport is related to a DIDS sensitive process regardless of the acclimation condition

Table 4.5. The effects of 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) on the blood ion concentrations and blood osmolality of *C. fluminea*. In the treated group, DIDS was added into the PW bathing medium to a final concentration of 0.5 mM. Since DIDS used in the experiment was a Na salt, the concentration of Na in the control medium was adjusted with Na₂SO₄ to the same Na level as in DIDS-treated animals to eliminate the possible effects due to the concentration difference of Na between the two solutions. PW, PW-acclimated animals; SD, salt depleted animals. TBS, total blood solutes in mOsm. * Mean is significantly different from its controlled value in the same row with P<0.05.

solute	acclimation	control	DIDS-treated
TBS	PW	54.72±1.06(10)	52.11±0.76(10)
	SD	47.91±0.90(10)	44.18±0.91(10)*
Na	PW	26.73±0.34(10)	25.13±0.35(10)*
	SD	22.62±0.27(10)	21.10±0.49(10)*
Cl	PW	22.62±0.51(10)	20.56±0.31(10)*
	SD	16.43±0.35(10)	14.77±0.40(10)*

of the animals. The consistent degree of inhibition in SD and non-SD animals indicates that the activation of Cl and Na uptake by salt depletion is mostly through DIDS-insensitive pathways.

The effects of furosemide on ion transport

The effect of furosemide inhibition on Cl and Na transport of PW-acclimated *C. fluminea* is illustrated in Table 4.6. Furosemide was dissolved in distilled water in experiment A. In experiment B furosemide was dissolved in distilled water with 5.0 mM serotonin to enhance the ion flux. In both experiment A and B, furosemide significantly decreased the net Cl flux by reducing the influx with little influence on the efflux, suggesting that the action of furosemide was on the uptake process of Cl transport. About 43% of the Cl influx was inhibited by the injection of furosemide in DW without serotonin. With serotonin in the injection solution, more than 60% of the Cl flux was reduced. Although this greater inhibition could be due to the higher injection dosage used in the experiment B (Table 4.6), it is possible that the serotonin stimulated portion of Cl influx was sensitive to furosemide. This inference is supported by the observation that the furosemide-insensitive portion of Cl influx was consistent regardless of the injection dosage.

Although to a lesser extent, Na transport was also significantly inhibited by furosemide. As with Cl, the inhibition of Na was due to the reduction of the influx without alteration of the efflux. With serotonin in the injection solution, 22% of the Na influx was inhibited by furosemide.

Table 4.6. Effects of furosemide on Cl and Na flux in PW-acclimated *C. fluminea*. Treated animals were injected with 10 μ l DW containing 1.0 μ mol of furosemide in DW (A), or with 10 μ l containing 3.0 μ mol of furosemide in DW with 5.0 mM of serotonin (B). Control animals were injected with equal volume of DW (A) or DW with 5.0 mM serotonin (B). J_n , net flux; J_i , influx; J_o , efflux in μ eq(g dry tissue $\cdot$ h)⁻¹. Data are expressed as mean and standard error with the number of animals in the parenthesis. * significantly different from the control value in the same row (P<0.05).

flux	exp.	control	treated
J_n^{Na}	B	4.04 $\pm$ 1.08(10)	1.67 $\pm$ 1.03(9)*
J_i^{Na}	B	10.37 $\pm$ 0.94(10)	8.13 $\pm$ 0.94(9)*
J_o^{Na}	B	6.33 $\pm$ 1.29(10)	6.46 $\pm$ 1.55(9)
J_n^{Cl}	A	0.68 $\pm$ 0.80(10)	-3.16 $\pm$ 0.73(10)*
J_n^{Cl}	B	1.12 $\pm$ 0.60(18)	-5.32 $\pm$ 0.80(19)*
J_i^{Cl}	A	6.91 $\pm$ 0.98(10)	3.93 $\pm$ 0.49(10)*
J_i^{Cl}	B	8.42 $\pm$ 0.75(18)	3.17 $\pm$ 0.37(19)*
J_o^{Cl}	A	6.22 $\pm$ 0.95(10)	7.09 $\pm$ 1.0(10)
J_o^{Cl}	B	7.30 $\pm$ 0.85(18)	8.49 $\pm$ 1.10(19)

The effects of other pharmacological agents on ion transport

The effects of other pharmacological agents on Na or Cl transport are illustrated on Table 4.7. With the exception of indomethacin which appeared to increase the Na influx, all other chemicals tested had no effects on the Na or Cl transport.

DISCUSSION

In PW-acclimated or SD *C. fluminea*, DCCD significantly decreased net Na and Cl uptake from PW or Na-free PW medium by reducing the influx with little change in the efflux. These data indicate an involvement of proton-pump activity in the process of Na and Cl entrance. The observation that inhibition of Cl uptake by DCCD was independent of external Na suggests the action of DCCD is directly on the Cl uptake pathway rather than by an indirect inhibition of Na uptake. The pumping of protons out of the epithelial cells by a DCCD-sensitive proton-pump may generate an important force driving Na entry into the epithelial cells, probably through conductive apical Na channels (proton-pump Na channel). Na uptake in freshwater rainbow trout is accomplished through conductive apical Na channels in the gill epithelial cells (Lin and Randall, 1995). The driving force for Cl could come from the increased intracellular HCO_3^- which could exchange with external Cl through an apically located Cl/HCO_3^- exchange pathway. The uptake of Cl through a Cl/HCO_3^- exchanger energized by an electrogenic proton-pump has been reported in amphibian skins and vertebrate renal distal tubules (Larsen *et al.*, 1992). The co-regulation of the active transport of

Table 4.7. Effects of other pharmacological agents on the net flux of Na and Cl in *C. fluminea*. Animals were PW-acclimated unless indicated. ANP and FMRFamide were dissolved in distilled water and all other chemicals except bumetanide were dissolved in 100% ethanol. Chemicals were injected into the foot muscle of the animals except bumetanide was added directly into the incubating solution at a final concentration of 2.0 mM DMSO (dimethylsulfoxide). ANP, atrial natriuretic peptide (0.03 μM); BUM, Bumetanide (3-[Aminosulfonyl]-5-[butylaminol]-4-phenoxy-benzoic acid (0.5 mM); DES, diethylstilbestrol (0.5 mM); DPC, diphenylamide-2-carboxylic acid (0.5 μM); FMRF, Phenylalanyl-methionyl-argininyl-phenylalanyl-amide (A, 0.01 μmol , PW animals; B, 0.08 μmol , SD animals); INDO: indomethacin (0.15 μmol); PGE₂, Prostaglandin E₂ (0.2 μmol). Jn: net flux in $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$.

chemicals	J _n Cl		J _n Na	
	control	treated	control	treated
ANP	-2.11 $\pm$ 2.08(5)	0.19 $\pm$ 1.94(5)	-0.31 $\pm$ 1.64(5)	1.03 $\pm$ 1.34(5)
BUM	-0.03 $\pm$ 0.92(5)	-0.06 $\pm$ 0.49(5)	1.66 $\pm$ 0.66(5)	0.04 $\pm$ 0.33(5)
DES	-0.10 $\pm$ 0.52(15)	-0.33 $\pm$ 0.53(15)	1.67 $\pm$ 0.72(15)	2.28 $\pm$ 0.37(15)
DPC	-0.37 $\pm$ 0.70(9)	-0.11 $\pm$ 0.10(7)	2.67 $\pm$ 0.48(9)	2.91 $\pm$ 0.80(7)
FMRF, A	0.53 $\pm$ 0.78(7)	2.04 $\pm$ 0.35(10)	4.21 $\pm$ 0.82(9)	4.86 $\pm$ 0.42(10)
FMRF, B	6.85 $\pm$ 0.77(5)	6.92 $\pm$ 1.12(5)	13.48 $\pm$ 2.58(5)	16.01 $\pm$ 0.85(5)
INDO			-0.08 $\pm$ 0.46(30)	1.38 $\pm$ 0.42(33)*
PGE ₂			-0.08 $\pm$ 0.46(30)	-0.35 $\pm$ 0.50(10)

Cl and Na by intracellular H could be of great advantage to the animals, i.e., hydromineral and acid-base balance would all be regulated simultaneously.

Cellular substrates of the proton-pump could originate from CO₂. Gills are the primary site of gas exchange in aquatic organisms and normally encounter high concentrations of metabolic CO₂. The oxidative metabolic activities in the epithelial cell itself can be another source of CO₂. Rather than simply diffusing across the gill epithelial cells in the process of gas exchange, CO₂ can be hydrated into H and HCO₃ accelerated by carbonic anhydrase. In this way, the metabolic waste CO₂ can be utilized as an important source of counter ions for the active uptake of Na and Cl. It has been reported that the cellular substrates for the active Cl influx *via* a Cl/HCO₃ exchange transport system in mitochondria-rich cells of toad skin epithelium depends on carbonic anhydrase-dependent hydration of CO₂ (Larsen *et al.*, 1992). Carbonic anhydrase also was localized on the apical surface of mitochondria rich cells in unionid gills (Kays *et al.*, 1990).

While the effect of DCCD on the Cl transport was independent of external Na, the action of DCCD on Na transport was dependent on the presence of external Cl in SD *C. fluminea* (Table 4.2). The absence of Cl in the bathing medium abolished the effect of DCCD on the Na uptake, indicating the function of the DCCD-sensitive proton-pump is, at least in part, Cl dependent. The dependence of Na uptake of SD *C. fluminea* on the external Cl was already demonstrated in the previous chapter (Table 3.4). In *C. fluminea*, as much as 40% of blood Cl can be lost after about 20 days of salt depletion (data not shown). Therefore, the external Cl could become critically

important in maintaining the level of intracellular Cl for the normal functioning proton-pump. The dependence of the proton-pump upon Cl ions has been found in various regions of mammalian renal tissues and tissues of other (Hilden *et al.*, 1988; Kaunitz *et al.*, 1985; Moriyama and Nelson, 1987; Ruiz and Arruda, 1992). In rabbit renal tissues, the activity of a DCCD-sensitive pumps and H ion translocation are critically dependent upon the presence of Cl. In this case Cl influences H ion translocation by dissipating the H ion gradient and acting at the catalytic site of the pump (Ruiz and Arruda, 1992). ATP driven proton-pumping that is DCCD-sensitive is absolutely dependent on the presence of external Cl in endosomal fractions isolated from the rabbit renal cortex (Hilden *et al.*, 1988). The biological and physiological importance of a Cl-dependence for the proton-pump function is not yet fully understood. It could be very important in maintaining the homeostasis of intracellular acid-base balance by adjusting the amount of intracellular H and HCO_3 .

Salt depletion enhances the mechanism(s) responsible for Cl and Na uptake of *C. fluminea* that are insensitive to DIDS but sensitive to DCCD. The most likely candidate stimulated by salt depletion is the proton-pump in the cells of gill epithelia. The proton-pump energizes the active uptake of both Cl and Na in *C. fluminea*. Although salt depletion was suggested to stimulate the active Na uptake through a Na/H exchange in unionid mussels, based on the inhibition of amiloride, the Na transport in PW-acclimated *C. fluminea* was not sensitive to amiloride (Dietz, 1978; Dietz and Branton, 1979; McCorkle and Dietz, 1980). This suggests that different Na transport mechanisms might be utilized by *C. fluminea*, especially in SD animals. Since

salt depletion increases Cl and Na transport capacity with little influence in the transport affinity in *C. fluminea* (see Chapter 2; McCorkle and Dietz, 1980), it is reasonable to assume that salt depletion increases the number of proton-pumps in cell membranes or the number of H-pump-containing cells in the gill epithelia. Chronic adaptation of freshwater rainbow trout to low Na water increases the number of proton-pumps recruited into the gill epithelium (Lin and Randall, 1995). Exocytic insertion of the proton-pump into the plasma membranes of mitochondria-rich cells of vertebrate urinary epithelia upon stimulation has been reported (Schwartz and Al-Awqati, 1986).

While Cl efflux was not affected by DIDS treatment in PW-acclimated animals, it was decreased significantly by DIDS in SD animals. The most likely sites for ion loss are gill and kidney epithelia. Because DIDS was added directly to the bathing solution, its pharmacological effects may be primarily on the apical surface of the gill epithelia rather than kidney. The lack of effect of DIDS on the Cl efflux of PW-acclimated animals suggests that the loss of Cl from the Cl/HCO₃ exchange pathway (which might operate in a Cl/Cl self-exchange fashion) in the gill epithelia is insignificant. Therefore, the loss of Cl from gill epithelia, if any, would be mostly through passive diffusion *via* transcellular or paracellular pathways. The magnitude of Cl efflux in SD animals is smaller than that of PW-acclimated animals for both control and DIDS treated groups. This decrease is probably for two reasons: 1) a decrease of membrane or paracellular permeability to Cl to minimize the passive loss of Cl in the gill epithelia; 2) an increase of Cl reabsorption in the renal tissue. The inhibition of a Cl/HCO₃ exchanger in the gill

epithelia by DIDS would increase the HCO_3^- concentration in the blood. The circulating HCO_3^- would bathe the kidney tubule (which is less affected by DIDS) and may be exchanged with Cl resulting in the decrease of Cl efflux. Therefore, the greatest Cl retention can be achieved by activating the Cl/ HCO_3^- exchange in the gill epithelia to take up more Cl, and activating Cl/ HCO_3^- exchange system in the renal tissue to prevent the loss of Cl. This elevation of ion influx and reduction of ion efflux by salt depletion, commonly seen in freshwater bivalves (Dietz, 1978; Dietz and Branton, 1979; McCorkle and Dietz, 1980), ensures the maximum ion retention when animals are subjected to the challenge of salt depletion.

The decrease of Na transport in PW-acclimated *C. fluminea* by DIDS suggests a linkage between Na transport and the Cl/ HCO_3^- exchange system in these animals. Although this decrease was not statistically significant in SD animals probably due to high variability, the reduction was apparent. This is further supported by the significant reduction of blood Na ion concentration by DIDS treatment. However, the magnitude of reduction in Na net flux was much less than that of Cl, suggesting this linkage is not a one-to-one stoichiometric relationship. This inference is supported by the differential degree of reduction in the concentration of blood Na and Cl. Based on the magnitude of DIDS inhibition, about one half to two thirds of the Na uptake may be associated with a Cl/ HCO_3^- exchange component of Cl uptake in PW-acclimated *C. fluminea*. The most likely linkage between Na transport and Cl/ HCO_3^- exchange is through the coupling to H exit out of the gill epithelium. The coupling of Na and H transport could

be either directly through a Na/H antiporter as demonstrated by previous studies of unionids (Dietz, 1978, 1979, 1985), or through a proton-pump Na channel.

The significant reduction of Na and Cl uptake by furosemide in this study conflicts with previous studies in which furosemide failed to inhibit Na or Cl transport of intact animals or isolated gills of freshwater unionid mussels (Dietz, 1978; Dietz and Branton, 1979; Dietz and Hagar, 1990). This disagreement could be explained by the different administration of the drug, or by a different species specific response to this drug. In previous studies, furosemide was applied externally by adding it directly into the bathing solution where it would be expected to exert its effect on the apical surface of the transporting epithelium. In this study, furosemide was injected into the animals. Therefore, it would be carried by the blood to act on the basolateral side of the epithelium. The inhibitory mechanism of furosemide on Na and Cl transport of freshwater bivalves is not clear. Furosemide has traditionally been used as a Na-K-2Cl cotransport inhibitor. However, the basolateral exit of Na and Cl *via* a Na-K-2Cl cotransport system has not been reported in bivalves. It is more likely that furosemide suppressed the activity of proton-pump. The inhibition of the H-pump that is Cl-dependent and DCCD-sensitive by furosemide has been reported in the endosomal fraction isolated from the rabbit renal cortex (Hilden *et al.*, 1988).

In PW and Na-free PW media, DCCD treatment did not affect the net flux of titratable base in SD *C. fluminea* even though the uptake of Cl and/or Na was significantly reduced. This is probably because the reduction of HCO₃ by the inhibition of DCCD equals the reduction of H autotitration. This inference can be supported by

the evidence that the net reduction of Na transport by DCCD was very close to the change of Cl transport in the PW media. Furthermore, DCCD caused a small but significant increase in the net loss of titratable base when Cl-free PW was used as a medium. In Cl-free PW medium, H becomes the primary factor influencing the acidity of the bathing solution because the Cl/HCO_3 exchange is not functioning under these conditions. The small and varying amount of HCO_3 can all be autotitrated by H released, as supported by the positive value of net base flux in the control animals in Cl-free PW. The inhibition of a proton-pump by DCCD would reduce the H being released into the bathing solution resulting an increase in the net loss of titratable base.

Other pharmacological agents reported to have effects on membrane ions permeability in other animals were examined. Of the agents tested, only indomethacin stimulated the net flux of Na. The other chemicals had no effect on Cl or Na net flux. Indomethacin is an inhibitor of prostaglandin synthesis which has been shown to stimulate Na uptake in unionids (Graves and Dietz, 1979).

CHAPTER FIVE
PARACELLULAR SOLUTE UPTAKE IN THE FRESHWATER
BIVALVES, *CORBICULA FLUMINEA* AND
CARUNCULINA TEXASENSIS

INTRODUCTION

The paracellular pathway is known to be the site responsible for the high electrical conductance of “leaky” epithelia in vertebrates (Frömter and Diamond, 1972). This particular pathway is usually limited by tight junctions, but can operate as a simple “gate” in several epithelia (Diamond, 1977). Paracellular movement of solute has been demonstrated in the epithelia of vertebrate gallbladders, small intestine, and proximal renal tubules (Boulpaep, 1971; Barry *et al.*, 1971; Frömter and Diamond 1972). In mollusks and some other invertebrates, the septate junction performs a similar function as the vertebrate tight junction (Lord and DiBona, 1976). Dehydrated terrestrial slugs *Lehmanna valentiana* display rapid water uptake, and accumulate nonelectrolytes (mannitol and inulin) through opened paracellular pathways in their foot epithelium (Uglen *et al.*, 1985). In the intestine of *Aplysia juliana*, the paracellular pathway for both Na and Cl constitutes the major conductive route for these ions (Gerencser, 1982).

Recent studies of *Dreissena polymorpha* (zebra mussels) have demonstrated unusually “leaky” characteristics in their gill epithelia (Dietz *et al.*, 1995). When exposed to solutions that are hyperosmotic to their blood, zebra mussels display substantial solute movement through paracellular pathways (Dietz *et al.*, 1995). Following exposure to hyperosmotic but low ionic strength mannitol, glucose or

sucrose solution (100 mM), zebra mussels lost 80-85 % of blood Na and Cl within 12 h with concomitant accumulation of the nonelectrolytes from the bathing medium through the paracellular pathway.

Corbicula fluminea (Müller) belong to the family Corbiculidae and are recent invaders of freshwater (Keen and Casey, 1969). *C. fluminea* have been reported to inhabit brackish water up to 5‰ salinity although they are considered to be freshwater species (Filice, 1958; Hayashi, 1956). *C. fluminea* have higher blood osmolality, higher ion transport rate, and a different blood ionic composition compared to unionid mussels. In contrast to the corbiculids, the unionid bivalves invaded fresh water millions of years ago (Keen and Casey, 1969). The unionid *C. texasensis* and the corbiculid *C. fluminea* may have evolved different epithelial characteristics and, thus were chosen as bivalve models for a comparative study of paracellular permeability.

MATERIALS AND METHODS

Animals

C. fluminea were collected under permit from the Tangipahoa river in Mississippi. *Carunculina texasensis* were obtained under permit from ponds in Louisiana. The animals were acclimated to an artificial pondwater (PW) containing (in mM) 0.5 NaCl, 0.4 CaCl₂, 0.2 NaHCO₃, 0.05 KCl at 22-25°C for at least one week before use.

Blood Analyses

Blood was collected anaerobically by cardiac puncture (Fyhn and Costlow, 1975) and centrifuged 1 min at 8,000 X g. The supernatant was used for solute

determinations. Total solute was measured by freezing point depression on undiluted samples. Na and potassium were measured by flame photometry and Cl by electrometric titration. Calcium and magnesium concentration were determined from samples diluted with LaO_3/HCl with an atomic absorption spectrophotometer. The phenol/sulfuric acid method for total sugars was used to determine glucose concentration (Montgomery, 1957). Mannitol was determined by HPLC separation using a normal phase APS-hypersil column and a refractive index detector (Gäde, 1991; Dietz *et al.*, 1995). A 5 μl sample of blood in acetonitrile was injected onto the column. Mannitol concentrations calculated from the HPLC chromatography peak area had less than 5% error. Glucose was measured using a specific glucose kinase procedure (Sigma Chemical) for some samples.

Ion transport

Net ion flux (J_n) was calculated from the change in bath ion concentration. (Dietz, 1978). The animals were rinsed with deionized water (DW) for about 30 min, and transferred to individual beakers with appropriate bathing medium as outlined extensively in chapter 3. Bath samples were collected at intervals and J_n calculated and expressed as $\mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$.

Hyperosmotic conditions and paracellular permeability

PW containing 100 mM mannitol or glucose was used to establish the hyperosmotic conditions without the elevation of bath ion concentrations. In other experiments, sucrose was used as an additional osmotic stressor and lanthanum as an electron dense diffusion tracer. Animals were exposed to CO_2 -free PW containing 0.1

mM LaCl_3 and 100 mM sucrose for 2h or 12h. Lanthanum carbonate forms an insoluble precipitate at the site of La contact with endogenous HCO_3^- . Blood samples were collected from control and treated animals and total solute determined. Gills were excised and fixed for 2 h in 2% glutaraldehyde prepared with phosphate buffer that was isosmotic to the blood at pH 7.3 (Gardiner *et al.*, 1991). Tissues were rinsed with buffer, exposed to 1% osmium tetroxide for 1 h, and dehydrated in an ethanol series. The tissues were infiltrated with 50% LR White resin in ethanol overnight, transferred to 100% LR White resin (2-3 changes), and polymerized at 59 °C overnight. Tissues were thin sectioned with an ultramicrotome (50-70 nm), and examined with a JOEL CX-100 transmission electron microscope operating at 40 or 80 keV without staining.

Statistical analyses

Data were expressed as the Mean $\pm$ SEM with the number of animals in parentheses. Statistical significance was determined by one way ANOVA or Student's T-test. For some data sets where larger variances were associated with the relatively higher mean values, the data were transformed by the natural log (Ln) for the statistical analysis.

RESULTS

The responses of *C. fluminea* to hyperosmotic stress

Mannitol

Analysis of the blood of animals exposed to 100 mM mannitol demonstrated that hyperosmotic stress induced an elevation in blood ion concentrations and thus total blood solute concentration (Table 5.1). After 12 h exposure to 100 mM mannitol in

Table 5.1. Comparisons of blood ion concentrations, total blood solute and the accumulation of mannitol in *C. fluminea* over different exposure times to 100 mM mannitol in PW. Means, expressed as mM or mOsm, in the same row having different letters are significantly different with $P < 0.05$.

solute	control	100 mM mannitol	
		12h	24 h
Na	28.1±0.2(23) ^a	38.0±0.7(18) ^b	43.2±1.0(15) ^c
Cl	23.3±0.5(24) ^a	30.7±0.6(18) ^b	31.1±1.2(15) ^b
Ca	9.0±0.5(24) ^a	14.6±1.5(18) ^b	16.9±1.4(15) ^b
K	0.8±0.0(24) ^a	0.9±0.0(18) ^a	0.8±0.0(15) ^a
mannitol		1.6±1.1(18) ^a	13.9±3.3(15) ^b
mOsm	61.5±0.6(24) ^a	85.8±2.2(18) ^b	97.1±2.0(15) ^c

pondwater, blood osmolality and all blood ion concentrations of the animals were significantly elevated, with the exception of blood potassium.

A small amount of mannitol was detected in the blood after 12 h of exposure to 100 mM mannitol (1.6 ± 1.1 mM), but after 24 h exposure mannitol a significant ($P < 0.05$) amount of mannitol accumulated in the blood (13.9 ± 3.3 mM), suggesting an apparent change in permeability of the epithelium after prolonged exposure to the hyperosmotic 100 mM mannitol solution. Although blood Na concentration was further elevated from 12 to 24 h exposure to hyperosmotic mannitol, there was no difference between the 12 and 24 h treated groups for the other blood ions. These data are consistent with a dehydration-induced elevation of blood ion concentration. The penetration of mannitol into the body fluids is likely *via* paracellular routes.

Glucose

Glucose was selected as an alternative nonelectrolyte to induce a hyperosmotic challenge that allowed more rapid chemical analysis. The changes of blood ion concentration induced within 24 h by hyperosmotic glucose were similar to that of mannitol in most cases (Table 5.2). At 12 h of exposure to 100 mM glucose, blood osmolality and all blood ion concentrations except blood potassium were significantly elevated. With the exception of blood calcium that continued to rise during the 24 h of exposure, the other blood ion concentrations were not further altered until 48 h at which time a critical change in membrane permeability occurred. Small amounts of glucose entered the blood after 12 h of exposure and remained relatively constant up to

Table 5.2. Comparisons of blood ion concentrations, total blood solute and the accumulation of glucose in *C. fluminea* over different exposure times to 100 mM glucose in PW. Means, expressed as mM or mOsm, in the same row having different letters are significantly different with $P < 0.05$, ($n=10$).

solute	control	100 mM glucose			
		12h	24h	36h	48 h
Na	29.7±0.5 ^b	44.2±0.9 ^c	45.4±1.0 ^c	43.5±0.8 ^c	13.2±1.5 ^a
Cl	27.6±0.4 ^a	35.1±0.5 ^b	32.5±1.0 ^b	32.1±1.3 ^b	26.3±3.0 ^a
Ca	8.9±0.7 ^a	13.6±1.0 ^b	19.8±1.8 ^c	14.60±1.0 ^b	12.0±1.3 ^b
Mg	2.8±0.3 ^a	5.2±0.4 ^b	5.0±0.4 ^b	4.5±0.3 ^b	3.3±0.4 ^a
K	0.6±0.0 ^b	0.6±0.0 ^b	0.1±0.0 ^a	0.1±0.0 ^a	0.0±0.0 ^a
glucose	0.7±0.1 ^a	4.4±0.8 ^b	10.1±2.3 ^b	5.5±0.5 ^b	44.0±8.0 ^c
mOsm	64.7±1.0 ^a	86.6±0.9 ^b	96.6±0.9 ^c	94.7±1.4 ^c	95.0±5.1 ^c

36 h of exposure. These data are consistent with an initial dehydration induced elevation of blood ion concentration over 12 to 36 h.

Although all of the animals lived and appeared to be in normal condition after prolonged hyperosmotic stress, there were acute decreases in major blood ion concentrations concomitant with a rapid rise in glucose concentration in the blood during 36 to 48 h of exposure. During this period, 70% of blood Na and 18 to 26% of blood Cl, calcium and magnesium were lost. Concomitantly, the amount of glucose in the blood increased about 8 times. The concentration of blood potassium after 48 h of exposure was so low that it was below the detection limit (0.05 mM) of the equipment. These data suggest an acute change in permeability of the epithelial tissue following prolonged exposure to hyperosmotic stress.

Due to the elevation of blood ion concentrations and a rather small amount of glucose in the blood, blood osmolality became isosmotic with the bathing medium within 24 h of exposure. Although most of the blood ion concentrations decreased during 36 to 48 h of exposure, blood osmolality remained isosmotic to the bathing solution because of the simultaneous accumulation of glucose in the blood.

In PW-acclimated animals, blood osmolality of *C. fluminea* (about 60-65 mOsm) is higher than that of *C. texasensis* (about 40-50 mOsm). 100 mM glucose in the bathing medium is about double the blood osmolality of *C. texasensis*. In order to compare the responses to hyperosmotic stress between these two species, 140 mM glucose was added to PW to challenge *C. fluminea* with double the normal

osmolality. After 12h exposure to glucose in PW, both the 100 mM and 140 mM glucose treatments had significantly higher blood osmolality and blood ion concentrations than PW controls (Table 5.3). However, with the exception of blood calcium, major blood ions and blood osmolality in 140 mM treatments were not statistically different from the 100 mM treatments. Likewise, the higher osmotic gradient did not significantly increase the amount of glucose entering the blood. The responses of *C. texasensis* to hyperosmotic stress

Glucose

Table 5.4 shows the time course of change in *C. texasensis* blood solute concentration, total blood solute and the accumulation of glucose over 24 h exposure to 100 mM glucose. During the 4 to 8 h exposure to 100 mM glucose in PW, *C. texasensis* were able to maintain their blood ion concentrations in normal range. During this period, there was a small but significant amount of glucose entering the blood. Total blood solute was significantly increased due to the combined results of all individual ions and the accumulated glucose. After 12h or longer exposure to hyperosmotic glucose, there was a precipitous decrease in blood ion concentrations with concomitant accumulation of a large amount of glucose in the blood. The concentration of glucose in the blood was about 70 mM after 12 h exposure to 100 mM glucose accounting for 80 to 90 percent of blood osmolality. "Other" solute concentration was relatively unaffected by hyperosmotic challenge although the variability was large. "Other" is mostly HCO_3^- in *C. texasensis*, and is the difference between the blood osmolality and the measured ions. The measured blood ion morality

Table 5.3. Comparisons of blood ion concentrations, total blood solute and the accumulation of glucose in *C. fluminea* after 12 h exposure to 100 mM and 140 mM glucose in pondwater. Means, expressed as mM or mOsm, in the same row having different letters are significantly different with $P < 0.05$, ($n=5$).

solute	control	glucose	
		100 mM	140 mM
Na	35.0±1.2 ^a	47.3±0.4 ^b	48.4±1.5 ^b
Cl	25.6±0.9 ^a	34.9±0.5 ^b	35.7±1.9 ^b
Ca	12.2±0.6 ^a	16.4±2.1 ^b	25.0±1.2 ^c
Mg	1.7±0.2 ^a	1.8±0.2 ^a	3.4±0.6 ^b
K	0.9±0.0 ^a	0.8±0.0 ^a	0.8±0.1 ^a
glucose		3.6±1.8 ^a	3.9±0.5 ^a
mOsm	67.7±1.5 ^a	90.8±0.8 ^b	95.8±3.0 ^b

Table 5.4. Comparisons of blood ion concentrations, total blood solute and the accumulation of glucose in *C. texasensis* over different exposure times to 100 mM glucose in PW. Means, expressed as mM or mOsm, in the same row having different letters are significantly different with $P < 0.05$, ($n = 5-10$).

solute.	control	100 mM glucose			
		4 h	8h	12 h	24 h
Na	17.2±0.8 ^b	19.0±2.2 ^b	20.1±1.0 ^b	1.6±0.8 ^a	0.0±0.0 ^a
Cl	10.4±0.5 ^c	11.3±1.4 ^c	11.5±0.5 ^c	2.4±0.4 ^b	0.8±0.2 ^a
Ca	3.6±0.5 ^b	5.6±0.9 ^b	3.9±0.3 ^b	1.1±0.1 ^a	1.4±0.1 ^a
Mg	0.3±0.1 ^b	0.4±0.0 ^b	0.2±0.0 ^b	0.0±0.0 ^a	0.2±0.0 ^b
K	0.5±0.0 ^b	0.6±0.0 ^b	0.5±0.1 ^b	0.0±0.0 ^a	0.0±0.0 ^a
other	5.1±0.8 ^a	5.6±0.8 ^a	17.4±1.5 ^b	4.0±1.6 ^a	14.9±7.4 ^b
glucose	3.5±0.4 ^a	10.4±5.5 ^b	10.5±3.2 ^b	73.2±7.6 ^c	66.6±8.8 ^c
mOsm	40.6±1.8 ^a	53.0±4.9 ^b	64.0±3.4 ^b	80.3±6.0 ^c	83.4±9.4 ^c

(mM) was used as an estimated molality in the calculation of “other”. Because the blood ion concentrations in *C. texasensis* can be considered to be very dilute (less than 20 mM).

Mannitol

The changes in blood ion concentrations following the treatment with hyperosmotic 100 mM mannitol were similar to those after a glucose challenge for most of the blood ions in *C. texasensis* (Table 5.5). After 24 h exposure to 100 mM mannitol in PW, all blood ion concentrations were significantly decreased with the exception of “other” solute (mostly HCO_3) which was variable but not statistically different. During the 24 h exposure, a large amount of mannitol accumulated in the blood and reached 61.5 ± 11.0 mM after 24 h exposure, accounting for about 71 percent of the total blood osmolality.

Localization of electron-dense lanthanum carbonate precipitate

In order to exclude the existence of the possible, but unlikely, epithelial glucose transport system in the gills of these animals, we used sucrose instead of glucose as the hyperosmotic osmolyte in lanthanum studies. Preliminary studies had demonstrated that both *C. fluminea* and *C. texasensis* behaved the same in sucrose induced hyperosmotic stress as in glucose treatment (data not shown).

C. fluminea exposed to lanthanum

Exposure of *C. fluminea* to 0.1 mM lanthanum in HCO_3 -free PW (control animals) for 2 or 12 h resulted in either no or only trace amount of electron-dense

Table 5.5. Comparisons of blood ion concentrations, total blood solute, and the accumulation of mannitol in *C. texasensis* following 24 h exposure to 100 mM mannitol in PW. Means, expressed as mM or mOsm, in the same row having different letters are significantly different with $P < 0.05$.

solute	control	mannitol
Na	16.9±1.0(9) ^b	2.4±1.2(6) ^a
Cl	9.9±0.8(9) ^b	2.0±0.6(6) ^a
Ca	3.1±0.3(9) ^b	1.8±0.5(6) ^a
K	0.6±0.0(9) ^b	0.2±0.0(6) ^a
mannitol		61.5±11.0(6)
other	9.6±0.9(9) ^a	19.0±10.4(6) ^b
mOsm	40.3±2.6(9) ^a	86.8±10.1(6) ^b

lanthanum carbonate deposits in a few locations in the junctional complexes of the gill epithelial cells (Fig. 5.1, A and C). Only one out of eight control animals was found to contain lanthanum precipitate in either 2 or 12 h exposure. In contrast, exposure of four *C. fluminea* to 0.1 mM lanthanum in HCO₃-free PW containing 100 mM sucrose for 2 or 12 h resulted in lanthanum precipitates appearing in the intercellular spaces between many epithelial cells in all the gill sections examined (Fig. 5.1, B and D). More interestingly, some lanthanum deposits were found to be inside the vesicles or “blister-like” structures inside the epithelial cells of the gill filaments or in the intercellular spaces between the cells (Fig. 5.1, E). The lanthanum-containing “blisters” were more prominent in the 12 h hyperosmotic treatment than 2 h treatments. These “blisters” were probably formed by the deformation of cells or the disruption of the lateral membranes due to the hyperosmotic-induced dehydration. This inference was supported by the evidence of the tissue condensation in the gill filament (Fig 5.1, E), and the dehydration-induced elevation of blood solute concentrations under hyperosmotic glucose or mannitol challenge. (Table 5.1, 5.2). Longer term hyperosmotic stress (12 h) also resulted in more extensive lanthanum deposits in the paracellular spaces of gill epithelial cells than the shorter-term treatments (2 h) indicating a time dependence of the hyperosmotic induction of paracellular leakage.

C. texasensis exposed to lanthanum

Animals after both 2 h and 12 h exposure to HCO₃-free PW containing 0.1 mM lanthanum showed only a few locations in one of six controls containing a limited

Fig. 5.1. Transmission electron micrographs of gill of *C. fluminea*. All sections are unstained. Apical surface is at the top of each micrograph. A: Gill epithelium after animals were exposed to 0.1 mM lanthanum in pond water for 2 h (control animals). There were no electron-dense precipitates of lanthanum in the lateral spaces between the epithelial cells. Bar = 0.51 μm . B: Gill epithelium after animals were exposed to 100 mM sucrose in pond water with 0.1 mM lanthanum for 2 h. Small amounts of lanthanum deposits were noticeable in the lateral membranes between cells (arrowheads). Bar = 0.19 μm . C: Gill epithelium after animals were exposed to 0.1 mM lanthanum in pondwater for 12 h (control animals). Note presence of a small deposits of electron-dense lanthanum precipitate in the lateral space between cells in the center of the micrograph (arrowhead). Bar = 0.33 μm . D: Gill epithelium after animals were exposed to 100 mM sucrose in pondwater with 0.1 mM lanthanum for 12 h. Extensive electron-dense precipitates of lanthanum were found to penetrate deeply into the lateral spaces between cells (arrowheads). Bar = 0.19 μm . E: Gill epithelium after animals were exposed to 100 mM sucrose in pondwater with 0.1 mM lanthanum for 12 h. Note the condensation of the tissue, and a series of lanthanum-containing “blisters” in the lateral membrane region between the epithelial cells (arrowheads). These “blisters” may appear as vesicles that are incorporating portions of lateral membranes. Bar = 0.67 μm .

amount of lanthanum deposit in the junctional complexes of the gill epithelial cells (Fig. 5.2 A and C). When animals were exposed to 0.1 mM lanthanum in HCO₃-free PW containing 100 mM sucrose for 2 or 12 h, lanthanum was found to penetrate intercellular spaces between many epithelial cells in all the gill sections examined (Fig. 5.2, B and D). Longer exposure to hyperosmotic conditions always resulted in a larger quantity of lanthanum precipitate than shorter exposure. In some sections from the 12 h treated animals, lanthanum precipitate were found inside of the “blisters” as observed in *C. fluminea*.

C. texasensis was more sensitive to hyperosmotic stress than *C. fluminea*. Both the quantity of lanthanum in each location and the number of locations of lanthanum precipitate in the intercellular spaces of *C. texasensis* were qualitatively greater than that of *C. fluminea* after the similar treatment time. These data suggest that the paracellular pathways between cells of epithelia of *C. texasensis* were more sensitive to hyperosmotic stress than that of *C. fluminea*.

DISCUSSION

The epithelia of *C. fluminea* was relatively “tight” and resistant to the penetration of nonelectrolytes under short term hyperosmotic challenge compared to other freshwater bivalves studied (Dietz *et al.*, 1995). The initial response of *C. fluminea* to 100 mM hyperosmotic stress was to increase blood ion concentrations by dehydration. During the initial period, *C. fluminea* tolerated a 20 to 50% increase of major blood ion concentrations and total blood osmolality without a significant change in the epithelial permeability to the nonelectrolytes. However, small but significant

Fig. 5.2. Transmission electron micrographs of gill of *C. texasensis*. All sections are unstained. Apical surface is at the top of each micrograph. A: Gill epithelium after animals were exposed to 0.1 mM lanthanum in pondwater for 2 h (control animals). Close to the apical surface in the lateral spaces between the epithelial cells, electron-dense precipitates of lanthanum were noticeable (arrowhead). Bar = 0.67 μ m. B: Gill epithelium after animals were exposed to 100 mM sucrose in pondwater with 0.1 mM lanthanum for 2 h. Electron-dense lanthanum deposits were found to penetrate into the lateral space between cells (arrowheads). Bar = 0.17 μ m. C: Gill epithelium after animals were exposed to 0.1 mM lanthanum in pond water for 12 h (control animals). Close to the bottom of the micrograph, there were noticeable amounts of lanthanum deposits in the lateral membranes between cells (arrowheads). Bar = 0.17 μ m. D: Gill epithelium after animals were exposed to 100 mM sucrose in pondwater with 0.1 mM lanthanum for 12 h. Extensive electron-dense precipitates of lanthanum were found to spread around the lateral spaces between cells (arrowheads). Note the formation of lanthanum-containing “blisters” on the right side of the micrograph (arrows). Bar = 0.17 μ m.

amounts of nonelectrolytes were able to diffuse into the blood probably through paracellular leakiness in gill epithelia. The paracellular permeability was demonstrated by the increase of lanthanum carbonate precipitates between the cell junctions of gill filaments after 12 h exposure to hyperosmotic stress.

A distinct change of epithelial permeability, however, can be induced by prolonged exposure to hyperosmotic condition. This is suggested by the acute decrease of blood ion concentrations and the concomitant rise in glucose during 36 to 48 h exposure of *C. fluminea* to hyperosmotic glucose. This distinct increase of epithelial permeability was most likely due to the increase of paracellular permeability through which large amounts of glucose or mannitol diffuse into the body fluids. It is very interesting that the decrease of blood ions is more selective to Na than to any other ion. As much as 70 percent of Na ions but only 18 to 26 percent of other ions (Cl, calcium or magnesium) were lost from the blood during 36 to 48 h of exposure to hyperosmotic conditions. The reason for this selectivity was not clear. Perhaps the paracellular pathway was more permeable to monovalent cations and constitutes some proportion of diffusive loss of Na in *C. fluminea* even under normal conditions. Nevertheless, even after the significant increase of paracellular leakage, the animals were still able to stabilize their blood ion concentrations at the control levels except for Na and potassium (Table 5.2).

The most distinct difference between *C. texasensis* and *C. fluminea* is the former's much lower resistance to long-term hyperosmotic challenge probably due to the relative leakiness of their gill epithelia. The distinct change of epithelial permeability

of *C. texasensis* occurred after only 8 h of exposure to hyperosmotic glucose at which time acute loss of large amounts of blood ions occurred concomitant with the rapid rise of glucose concentration in the blood. As in *C. fluminea*, this increased epithelial permeability is mostly paracellular as suggested by the increased lanthanum precipitates between the intercellular spaces after hyperosmotic treatments. Unlike *C. fluminea*, *C. texasensis* were unable to retain their blood ion concentrations after this distinct change of epithelial permeability. After 12 h of exposure to the hyperosmotic glucose, more than 75% of the ionic solutes were lost from the blood. Furthermore, the loss of the solutes is relatively non-selective.

Another significant difference was that instead of experiencing an elevation of blood ion concentrations by dehydration, *C. texasensis* maintained a nearly normal blood ion concentration for a short period of time (up to 8 h) after exposure to a hyperosmotic challenge of glucose. The maintenance of blood ion concentration was coupled with the low glucose accumulation in the blood, suggesting a relatively low paracellular permeability, initially. The slightly increased total blood osmolality was attributed to the small amount of accumulated glucose in the blood. No statistical evidence of an elevation in individual blood ion concentrations suggested minimal dehydration occurred in *C. texasensis*.

The physiological responses of *C. texasensis* to hyperosmotic stress are more similar to the zebra mussel *Dreissena polymorpha* (Dietz *et al*, 1995) than to *C. fluminea*. In *Dreissena polymorpha* approximately 80-85% of Na and Cl in the blood were lost after 12 h of exposure to 100 mM mannitol or glucose in PW. Blood total

solute was elevated within 4 h of exposure to hyperosmotic conditions due to the gain of glucose or mannitol in the blood and not to the osmotic loss of water. The responses of *C. texasensis* to hyperosmotic challenge are similar to that of *D. polymorpha* in, at least, two respects. There is no significant elevation of blood ion concentrations by dehydration in either species for short term hyperosmotic exposure. In addition, within 12 h for both species, a hyperosmotic challenge induced a substantial increase in paracellular permeability in the epithelium allowing both the blood solutes to diffuse out and bath glucose to diffuse in.

The tolerance to osmotic challenge of freshwater bivalves might reflect their habitats and possibly to their origin. Corbiculidae are recent invaders of freshwater (Keen and Casey, 1969), and *C. fluminea* has been reported to inhabit brackish water up to 5‰ salinity although it is considered to be a freshwater species (Filice, 1958; Hayashi, 1956). Corbiculids have higher blood osmolality compared to unionid clams (Dietz, 1979, McCorkle and Dietz, 1980). The characteristic of relatively lower paracellular permeability in epithelia of *C. fluminea* than the unionid clam *C. texasensis* allows these animals to maintain a higher blood osmolality. Thus, *C. fluminea* are less sensitive to osmotic stress and more resistant to the environmental osmotic challenges than *C. texasensis*.

CHAPTER SIX CONCLUSION, INTERPRETATION AND FUTURE DIRECTIONS

Corbicula fluminea (Müller) maintains hydromineral balance in dilute media by active transepithelial transport of Cl and Na into the blood primarily through transcellular pathways. Cl uptake of *C. fluminea* depends on the external Cl concentrations and displays similar saturable kinetics to that reported for Na uptake (McCorkle and Dietz, 1980). The transport capacity in PW acclimated animals is $7.00 \pm 0.51 \mu\text{eq}(\text{g dry tissue} \cdot \text{h})^{-1}$ with the transport affinity of $0.21 \pm 0.08 \text{ mM}$. Prolonged salt depletion increases Cl transport capacity with little influence on the transport affinity. This is probably due to increased amounts of numbers of proton-pumps in the gill epithelial cells.

The possible transcellular Cl and Na uptake mechanisms in *C. fluminea* are summarized in Fig 6.1. Based on this model, Cl and Na transport are through independent pathways but correlate with each other by the activity of the proton-pump and the hydration of CO_2 . The apical entrance of Na into the gill epithelial cells is likely through a proton-pump Na channel. This process may be energized by a DCCD-sensitive and Cl-dependent proton-pump located on the basolateral membrane of the gill epithelial cell. Operation of the ATP-consuming proton pump drives the formation of H and HCO_3 from CO_2 and H_2O with the latter reaction catalyzed by carbonic anhydrase. The active pumping of proton out of the epithelial cell generates an electrochemical driving force for the entrance of Na. A Na/K-pump also is located on

Fig 6.1. Model of transcellular Cl and Na transport in the gill epithelial cells of *C. fluminea*. ATP-driven pumps are denoted by solid circles and ion exchangers by open circles. Downward arrows on the membranes represent ion conductive channels. AM, apical membrane; BM, basolateral membrane; CA, carbonic anhydrase; GEC, gill epithelial cells.

the basolateral membrane and is responsible for the basolateral Na exit. The activity of the Na/K-pump may also create a transmembrane Na gradient facilitating the apical entrance of Na.

In addition to the proton-pump Na channel, another Na entrance pathways may also exist. Although Na transport in *C. fluminea* was not inhibited by amiloride (McCorkle and Dietz, 1980), the presence of a Na/H antiporter cannot be completely excluded. The amiloride-insensitive Na/H antiporter has been reported in mammalian proximal tubule cell, functioning in transepithelial transport (see Alpern *et al.*, 1995). Although a Na/H antiporter in this model is depicted in the same cell as a proton-pump Na channel, it could be located in different cells. The partition of Na entrance *via* different pathways may depend on the acclimated conditions or the regulatory activities of the animal.

The apical entrance of Cl is likely *via* a Cl/HCO₃ exchange pathway driven by the transmembrane HCO₃ gradient. In the process of proton-pumping, the proton-pump also raises the intracellular activity of HCO₃ to levels above that of Cl in the external bath, providing the condition for uphill transport of Cl⁻ across the apical membrane in exchange for cellular HCO₃. The exit of Cl in the basolateral membrane could be *via* a passive Cl conductive channel.

The cellular substrates for the proton-pump can originate from the hydration CO₂. Gills are the major respiratory sites of gas exchange for aquatic animals and the gills would normally be exposed to large amounts of metabolic CO₂. Furthermore, the oxidative activities inside the epithelial cell itself may be another source of CO₂. Rather

than simply diffusing across the gill epithelial cells in the process of gas exchange, CO_2 can be hydrated into H and HCO_3^- catalyzed by carbonic anhydrase. Thus, the metabolic waste CO_2 can be re-utilized as an important source of counterions for the active uptake of Na and Cl. The regulation of Cl and Na entrance through H and HCO_3^- could be of great advantage to animals because hydromineral and acid-base balance could be regulated simultaneously.

Salt depletion increases Cl and Na transport capacity without changing transport affinity and increases the Cl dependency of Na transport in *C. fluminea*. Furthermore, salt depletion is a relatively slow process in *C. fluminea*. It is reasonable to assume that salt depletion increases the number of proton-pumps in epithelial cells, or the numbers of proton-pump containing cells in the epithelia. Serotonin independently stimulates Cl and Na transport without increasing the Cl-dependence of Na uptake. The stimulation by serotonin is a rapid event. Apparently, serotonin action is different from salt depletion in activating Cl and Na transport in *C. fluminea*.

The epithelia of *C. fluminea* is relatively "tight" compared to the unionid *C. texasensis* or to the zebra mussel *Dreissena polymorpha* (Dietz *et al*, 1995). Paracellular solute permeability in *C. fluminea* is insignificant under normal pondwater conditions, but can be increased to a certain extent by prolonged hyperosmotic challenge especially for the Na ion. Compared to Cl and/or other blood ions, Na is a relatively penetrable ion through the paracellular pathway. This paracellular "leakage" for Na may constitute a substantial part of passive Na loss under certain conditions. It

may also account for some of the high Na/Na exchange diffusion reported for *C. fluminea* (McCorkle and Dietz, 1980).

The gill epithelia of *C. fluminea* can serve as a useful model for the study of linkage between hydromineral balance and acid/base regulation in the freshwater invertebrates. Continued definitive localization, characterization and quantification of the proton-pump, Cl/HCO₃ exchanger, Na/H antiporter and carbonic anhydrase are necessary. The use of the advanced cellular localization techniques, such as immunocytochemistry and autoradiography, will benefit our understanding of the mechanism of osmoregulation and acid/base balance in freshwater bivalves.

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Title of Dissertation: Ion Transport in the Freshwater Bivalve, Corbicula fluminea (Muller)

Approved:

Major Professor and Chairman

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EXAMINING COMMITTEE:

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