RICHARD J. NEVES

Dietz
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1979

Reprinted from PHYSIOLOGICAL ZOOLOGY
Vol. 52, No. 4, October 1979

CHLORIDE TRANSPORT IN FRESHWATER MUSSELS[1]

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(Accepted 3/5/79)

Chloride transport in unionid bivalves is cation independent and is apparently an anion exchange system. The influx of Cl is highly correlated with the appearance of titratable base in the bathing medium. There is a substantial exchange diffusion component of Cl fluxes in these mussels. The influx of Cl displays saturation kinetics with a maximum velocity of 1 μEq (g dry tissue·h)$^{-1}$ and an affinity of 0.1 mM Cl/liter. Salt depletion doubles the rate of Cl influx by increasing the active transport component, with no change in the affinity. Chloride uptake is inhibited about 80% by 2 mM/liter thiocyanate but is not affected by acetazolamide or furosemide.

INTRODUCTION

Freshwater animals must take up ions from the environment to maintain salt balance. Krogh (1939) demonstrated the presence of well-developed salt transport systems in a variety of aquatic animals, including bivalves. Recent studies have shown that many epithelial salt transport systems employ ion exchange mechanisms: Na/H or NH_4 and Cl/HCO_3 or OH (Maetz and Garcia Romeu 1964; Garcia Romeu, Salibian, and Pezanni-Hernandez 1969; Stobbart 1971; Kerstetter and Kirschner 1972; Dietz 1974; Alvarado, Poole, and Mullen 1975; Garcia Romeu and Ehrenfeld 1975). Although Na transport has been studied extensively, until recently there was limited information on the mechanism of Cl transport.

Mussels are unusual in maintaining low blood solute concentrations (Dietz 1977, 1979). The principal anions in unionid bivalve blood are Cl and HCO_3, in nearly equal concentrations. Furthermore, when bivalves are subjected to salt depletion they tolerate a loss of more than 70% of their body fluid Cl with no mortality (Murphy and Dietz 1976). Although freshwater mussels tolerate large changes in blood Cl concentrations, they can actively transport Cl from the bathing medium (Dietz and Branton 1975; Murphy and Dietz 1976).

This paper characterizes Cl transport in two freshwater bivalves. Chloride transport is by Cl/base exchange and is stimulated by salt depletion. Chloride transport is inhibited by thiocyanate but not by furosemide or acetazolamide.

MATERIAL AND METHODS

Animals.—The unionid mussels *Ligumia subrostrata* and *Carunculina texasensis* were obtained from ponds near Baton Rouge. The animals were acclimated to an artificial pond water (0.5 NaCl, 0.4 $CaCl_2$, 0.2 $NaHCO_3$, 0.05 KCl, in mM/liter) for at least 1 wk (22–25 C) before use. Salt-depleted mussels were obtained by storing the animals in deionized water, which was replaced daily, for 14 days or more.

Ion analyses.—Sodium was determined

[1] This work was supported by National Science Foundation grants PCM75-05483 A01 and PCM77-08818. We thank C. Booker and P. McMurtry for technical assistance and Ms. B. Fabregas for typing the manuscript. Hoeschst-Roussel provided furosemide for this study.

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by flame photometry and Cl by electrometric titration. To measure the net loss of titratable base (J_n^{base}), the bathing solutions were buffered with 1 mM tris(tris[hydroxymethyl]aminomethane)-H_2SO_4 to pH 7.3. Bath samples were collected at the beginning and end of an experiment and sonicated to remove respiratory CO_2. The bath samples were titrated to pH 4.5 using standardized 5 m*N* HCl and the difference between initial and final buffer capacity was used to calculate base production.

Ion transport.—Unidirectional influx (J_i) was determined by the disappearance of isotope (2,000 cpm/μM Cl) from the bathing solution (see Dietz and Branton 1975). Samples of the bathing medium were collected at 1–3-h intervals and radioactivity in each sample was determined by a Beckman LS-8000 liquid scintillation counter using a triton-X100, toluene, p-terphenyl counting fluid. Net flux (J_n) was estimated from the changes in bath ion concentration. Efflux (J_o) was calculated from: $J_o = J_i - J_n$. The bath volume was small (30 ml) to allow rapid changes in the radioactivity of the bath with small changes in the body fluids. This restriction is important because of the low blood Cl concentration of <12 mM/liter. If the Cl isotope uptake exceeds about 20% of the initial radioactivity in the bath, the body fluid–specific activity could approach 10% of the bath-specific activity. Isotope back flux would be significant causing an underestimation of J_i.

Polyethylene glycol clearance.—Carbon-14 labeled polyethylene glycol was injected into the blood sinus in the foot of the animals (0.03 μCi in 25 μl) and they were left overnight in pond water. After rinsing, each animal was placed in pond water and bath samples were collected initially and after 30 min. Blood was collected by cardiac puncture immediately after the last bath sample Clearance was calculated from the increase in bath radioactivity divided by the blood-specific activity (see Murphy and Dietz 1976).

Statistical analyses.—Data are expressed as mean ± SEM (Standard Error of the Mean). Differences between group means were tested using the Student's *t*-test and were considered significant if $P < .05$. Linear regression coefficients were calculated by the method of least squares.

RESULTS

When acclimated to pond water both *Ligumia subrostrata* and *Carunculina texasensis* are able to maintain Cl and Na ion balance (table 1). Rates of exchange of Cl and Na are similar in the two species.

In previous studies, inulin clearance from the body fluids of *L. subrostrata* was about 115% of the soft tissue weight per day (Murphy and Dietz 1976). Clearance rates of ^{14}C-polyethylene glycol (PEG) in *C. texasensis* gave similar results (127 ± 28% of soft tissue weight per day, no. = 8). These clearance data

TABLE 1

UNIDIRECTIONAL Na AND Cl FLUXES IN POND-WATER-ACCLIMATED MUSSELS

SPECIES	No.	J_i^{Cl}	J_o^{Cl}	No.	J_i^{Na}	J_o^{Na}
		μEq (g Dry Tissue·h)$^{-1}$				
Ligumia subrostrata	17	.96 ± .09[a]	.87 ± .13	8	1.13 ± .16	1.17 ± .23
Carunculina texasensis	15	1.20 ± .16	1.35 ± .20	13	1.31 ± .09	1.12 ± .11

[a] Mean ± SEM.

suggest the osmotic water uptake may be rather high in bivalves. However, because the nephropore is in the suprabranchial chamber and cannot be blocked, we do not have a direct measurement of osmotic water movement. Most of the PEG loss is from the kidney rather than extrarenal since the clearance of PEG is dependent upon the osmotic uptake of water. When the bath is made isosmotic with the blood by adding mannitol, the PEG clearance is reduced to 28 ± 7% (no. = 6) of the control rate.

If water uptake is substantial, there could be a solvent drag or convective component of Cl influx. To determine the solvent drag on J_i^{Cl}, we measured Cl influx in pond water, then sufficient mannitol was added to make the bath isosmotic with the mussel body fluids (table 2). The data are listed as the group averages. On a paired basis the mannitol treated J_i^{Cl} was 75 ± 10% of the control. Therefore abolishing the osmotic uptake of water did not significantly reduce J_i^{Cl} and convective Cl uptake is minimal.

Unidirectional Cl influx for pond-water-acclimated *L. subrostrata* exposed to a range of NaCl concentrations displays saturation kinetics (fig. 1). The transport capacity (V_{max}) is 0.95 μEq (g dry tissue·h)$^{-1}$. The transport affinity (K_s) for Cl is about 0.11 mM/liter. These coefficients do not change when pond-water-acclimated mussels are exposed to a range of choline chloride concentrations (fig. 2). The V_{max} is 1.0 μEq (g dry tissue·h)$^{-1}$, and K_s is 0.13 mM/liter. However, salt depletion (2 wk or more) elevates V_{max} (2 μEq [g dry tissue·h]$^{-1}$) without affecting K_s (0.14 mM/liter). The response of *C. texasensis* is essentially the same as for *L. subrostrata*.

Chloride is taken up from choline chloride solutions at the same rate as

TABLE 2

EFFECT OF ISOSMOTIC MANNITOL ON UNIDIRECTIONAL Cl FLUXES IN "CARUNCULINA TEXASENSIS" ACCLIMATED TO POND WATER

	μEq (g Dry Tissue·h)$^{-1}$	
BATH	J_i^{Cl}	J_o^{Cl}
Pond water.....	1.36 ± .21[a]	1.77 ± .19
Pond water+ 45 mM/l mannitol	1.05 ± .19	1.52 ± .19

[a] Mean ± SEM, no. = 10.

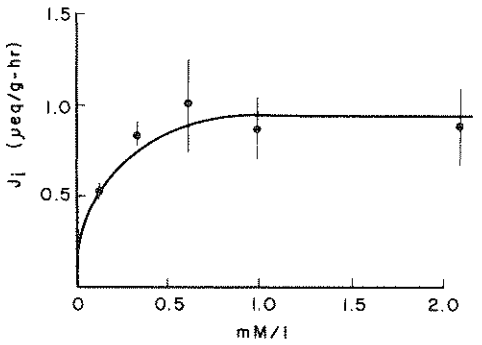

FIG. 1.—Effect of NaCl concentration in the bathing medium on the unidirectional influx of Cl in pond-water-acclimated *Ligumia subrostrata*. Each point represents the mean ± SE of 5–8 animals.

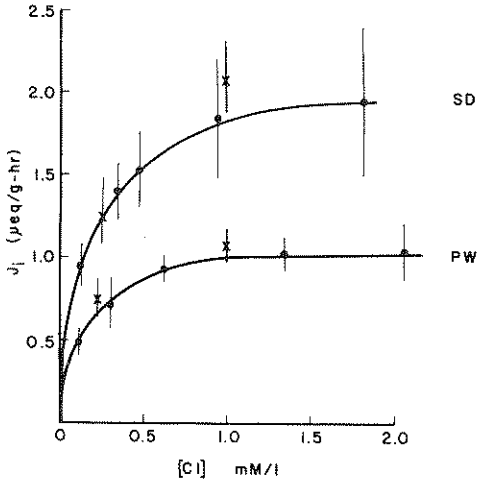

FIG. 2.—Effect of choline chloride concentration in the bathing fluid on the unidirectional influx of Cl in *Ligumia subrostrata* (●) and *Carunculina texasensis* (X). Each point represents the mean ± SE of 5–10 animals. *SD* refers to salt-depleted animals and *PW* refers to pond-water-acclimated mussels.

from pond water. Choline, however, is a nonpenetrating cation. The measured rate of choline influx by pond-water-acclimated *L. subrostrata* in 1 mM/liter ^{14}C-choline is very low (0.08 ± 0.02 μEq [g dry tissue·h]$^{-1}$, no. = 5).

Since Cl uptake occurs without the concomitant uptake of a cation, Cl must be exchanged for an endogenous anion. Table 3 shows the Cl exchanges and net base production (J_n^{base}) by pond-water-acclimated and salt-depleted *C. texasensis* in choline chloride. Salt depletion stimulates J_i^{Cl} and the animals experience a net uptake of Cl. Chloride efflux is significantly lower in salt-depleted mussels than in nondepleted animals. There is a substantial production of titratable base, possibly HCO_3, in both groups of animals. Although the average production of base does not differ between groups, this is due to the variability within each group. Paired comparisons of J_i^{Cl} with J_n^{base} revealed a highly significant correlation for both nondepleted and salt-depleted animals (r = .90 and .72, respectively; $P < .001$). In addition, salt-depleted mussels display a significant correlation between J_n^{Cl} and J_n^{base} ($r = .89$, $P < .001$).

The relationship between the appearance of titratable base in the bathing medium and Cl influx for both nondepleted and salt-depleted animals is shown in figure 3. The relationship is highly significant ($r = .79$, $P < .01$). The slope of the line is 0.72 ± 0.04 μEq base/μEq Cl, which is not significantly different from 1, indicating good stoichiometry between Cl and base exchange. The baseline in the figure represents the appearance of base in the bath (0.46 ± 0.14 μEq [g dry tissue·h]$^{-1}$) when the animals are in Cl-free media where $J_i^{Cl} = 0$. Presumably, this baseline represents an excretory component of base loss or diffusion out of the animal. Tris (1 mM/liter) was used to adjust the initial bathing medium pH to 7.3, but Tris has no effect on J_i^{Cl} (control, 0.69 ± 0.14; Tris, 0.80 ± 0.17 μEq [g dry tissue·h]$^{-1}$).

The total unidirectional fluxes can be partitioned into several components. The efflux is composed of an excretory/diffusive loss, which cannot be separated in the bivalves, and exchange diffusion. The influx can be separated into diffusion, the same exchange diffusion component as in the efflux and active transport.

The Cl fluxes from table 3 are partitioned in table 4. The sum of diffusion and excretion was obtained by transferring bivalves into deionized water. There is no significant change in electrical potential (Dietz and Branton 1975), and we are assuming no change in the epithelial permeability to Cl. When pond-water-acclimated and salt-depleted *C. texasensis* are in deionized water, the initial loss of Cl is 0.37 ± 0.08 and 0.03 ± 0.01 μEq (g dry tissue·h)$^{-1}$, respectively. Subtracting the diffusion/excretory component from total efflux gives the exchange diffusion. The inward diffusion of Cl is negligible, as calculated from the flux ratio equation (Ussing 1949). Blood Cl is about 12 mM/liter in pond-water-acclimated mussels and 2–3 mM/liter in salt-depleted mussels. The body fluids are about -11 to -15 mV, and the voltage is independent of bath Cl (Dietz and Branton 1975). Subtracting the diffusion and exchange diffusion components from total influx gives the active Cl transport.

Salt depletion results in a decreased Cl efflux due to a reduction of all of the components. A substantial exchange diffusion is evident in these bivalves. The influx of Cl is stimulated in salt-depleted animals because of an increase in the active transport of Cl. The active transport component in nondepleted animals

is low, and this group of animals experienced a net loss of Cl. Active Cl transport in pond-water-acclimated animals, in a steady state, is probably about 0.4 μEq (g dry tissue·h)$^{-1}$, or about 40% of the total unidirectional influx.

Attempts to alter J_i^{Cl} with HCO_3 were ineffective. Elevating the bath HCO_3 to 10 mM/liter $NaHCO_3$ (and 1 mM Cl/liter) had no effect on J_i^{Cl} in pond-water-acclimated animals (Control, 0.71 ± 0.16; HCO_3, 0.61 ± 0.19 μEq [g dry tissue·h]$^{-1}$, no. = 4). Injections of $NaHCO_3$ to raise blood HCO_3 5–7 mM/liter in pond-water-acclimated animals did not change J_i^{Cl}. Control animals' (injected with 100 μl 0.5 M/liter Na_2SO_4) J_i^{Cl} was 0.80 ± 0.29 μEq (g dry tissue·h)$^{-1}$ (no. = 4), and HCO_3-injected (100 μl of 1 M/liter $NaHCO_3$) mussels' J_i^{Cl} was 0.60 ± 0.10 μEq (g dry tissue·h)$^{-1}$ (no. = 5). The injections caused an elevation in J_n^{base} in both groups (control, −1.52 ± 0.62; HCO_3, −2.82 ± 1.83 μEq [g dry tissue·h]$^{-1}$). Compared with the data in table 3, the HCO_3-injected animals are significantly higher in J_n^{base} ($P < .05$). However, in the HCO_3-injected animals the base production does not correlate with J_i^{Cl} ($r = .28$, $P > .1$); but it does in the control animals ($r = .95$, $P < .05$). The HCO_3 injections may cause an increased renal output of base or outward

TABLE 3

EFFECTS OF SALT DEPLETION ON ION EXCHANGES IN "CARUNCULINA TEXASENSIS" IN 1 mM/LITER CHOLINE CHLORIDE

		μEq (g Dry Tissue·h)$^{-1}$			
TREATMENT	No.	J_i^{Cl}	J_o^{Cl}	J_n^{Cl}	J_n^{base}
Nondepleted.....	23	1.02 ± .06[a]	1.27 ± .15	−.25 ± .18	−.95 ± .13
Salt depleted.....	11	1.47 ± .19**	.65 ± .16*	.82 ± .21**	−1.22 ± .12

[a] Mean ± SEM.
* Significantly different from nondepleted animals, $P < .05$.
** Significantly different from nondepleted animals, $P < .01$.

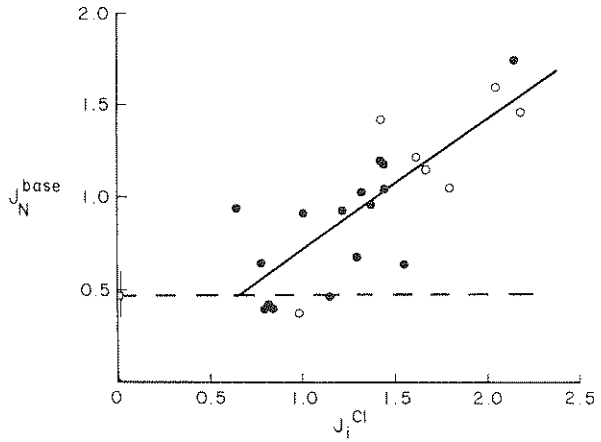

FIG. 3.—Relationship between the influx of Cl and the net loss of titratable base in *Carunculina texasensis* in 1 mM choline chloride solutions. Closed circles are pond-water-acclimated animals, open circles are salt-depleted mussels. The open square represents base production in Cl-free solutions. The units are μEq (g dry tissue·h)$^{-1}$.

TABLE 4

PARTITIONING OF Cl FLUXES IN POND-WATER-ACCLIMATED AND SALT-DEPLETED "CARUNCULINA TEXASENSIS"

	μEq (g Dry Tissue·h)$^{-1}$							
		Efflux			Influx			
TREATMENT	Net Flux	Total	Diffusion and Excretion	Exchange Diffusion	Total	Diffusion	Exchange Diffusion	Active Transport
Nondepleted....	−.25	1.27[a]	.37[b]	.90	1.02[a]	<.02[c]	.90	.10
Salt depleted....	.82	.65[d]	.03	.62	1.47[d]	<.01	.62	.84

[a] In pond water.
[b] Loss to deionized water.
[c] Flux ratio equation.
[d] In 1 mM/liter choline chloride.

diffusion of HCO_3 across the body surface, independent of Cl transport.

A number of drugs inhibit J_i^{Cl} in some freshwater animals. However, in mussels, thiocyanate is the only effective inhibitor of several drugs we tested (table 5). A relatively high concentration of thiocyanate (SCN) (2 mM/liter) was required to inhibit J_i^{Cl} by 84%. Acetazolamide, injected to have a blood concentration of about 0.1 mM/liter, had no effect on J_i^{Cl} but significantly increased J_o^{Cl}. Furosemide (1 mM/liter) in the bath had no significant effect on J_i^{Cl}.

DISCUSSION

Chloride transport in freshwater bivalves is by an active transport process with a substantial Cl/Cl exchange diffusion component. Salt depletion stimulates the active transport system and causes a reduction in the exchange diffusion component. In salt-depleted bivalves, the active transport component is about 60% of total J_i^{Cl}. Pond-water-acclimated animals have an active transport component of less than 40% of total J_i^{Cl}. When pond-water mussels are in a negative Cl balance, the loss is due to a decrease in active transport and an apparent increase in Cl/Cl exchange diffusion. A high exchange diffusion component of J_i^{Cl} has been noted in other animals (Alvarado and Dietz 1970; Alvarado et al. 1975; Alvarado, Dietz, and Mullen 1975; Dietz 1974).

The chloride transport system in mussels is saturable. The affinity, K_s, is

TABLE 5

EFFECTS OF PHARMACOLOGICAL AGENTS ON UNIDIRECTIONAL Cl FLUXES IN "CARUNCULINA TEXASENSIS" IN 1 mM/LITER CHOLINE CHLORIDE SOLUTIONS

				μEq (g Dry Tissue·h)$^{-1}$			
				Control		Treated	
DRUG	DOSE	LOCATION	No.	J_i	J_o	J_i	J_o
Thiocyanate....	2 mM/liter	Bath	5	.87±.12[a]	.88±.14	.23±.07**	.61±.17
Acetazolamide..	100 μg/g dry	Inject	5	.64±.09	.55±.06	.70±.06	1.51±.34*
Furosemide.....	1 mM/liter	Bath	11	.61±.11	.83±.12	.54±.13	1.16±.18

[a] Mean ± SEM.
* Significantly different from control fluxes, $P < .05$.
** Significantly different from control fluxes, $P < .01$.

about 0.1 mM Cl/liter and is similar to that reported in other freshwater animals (Shaw 1960; Kerstetter and Kirschner 1972; DeRenzis and Maetz 1973; Dietz 1974; Dietz and Alvarado 1974). The capacity (V_{max}) for Cl transport in bivalves is about 1 μEq (g dry tissue·h)$^{-1}$ (1.3 μEq [10 g soft tissue·h]$^{-1}$) which is comparable with values for earthworms (Dietz 1974) and amphibians (Alvarado and Dietz 1970; Dietz and Alvarado 1974) but lower than the V_{max} in freshwater fish (Kerstetter and Kirschner 1972; DeRenzis and Maetz 1973).

Chloride uptake in salt-depleted mussels is stimulated when they are returned to solutions containing Cl. The transport capacity is at least double the V_{max} of pond-water-acclimated unionids, with no change in K_s. Similar responses have been seen in other salt-depleted animals (Alvarado and Dietz 1970; Dietz 1974). Bivalves apparently have control mechanisms to regulate Cl balance. However, the regulation of Cl balance is rather loose since bivalves will survive for months in deionized water, which results in blood Cl concentrations decreasing to about 2 mM/liter (Murphy and Dietz 1976). Some of the Cl lost from the blood during salt depletion is replaced by HCO_3.

The blood HCO_3 may serve as the substrate for a Cl/base exchange mechanism. Subtracting the "excretory" component of base production (0.5 μEq [g dry tissue·h]$^{-1}$) from the total base output (table 3) leaves 0.45 μEq base (g dry tissue·h)$^{-1}$ available for a Cl/base exchange in pond-water-acclimated mussels. This is about equal to the active transport component of J_i^{Cl} if the mussels are in a steady state. The quantity of base available for anion exchange in salt-depleted animals is 0.7 μEq base (g dry tissue·h)$^{-1}$ (1.2–0.5) which is about equivalent to the active transport component of J_i^{Cl} in salt-depleted mussels.

The highly significant correlation ($P < .001$) between J_i^{Cl} and J_n^{base} and the correlation between J_n^{Cl} and J_n^{base} in salt-depleted mussels suggests a Cl/base exchange system. The uptake of Cl from choline chloride solutions requires an anion for anion exchange. The presence of an anion exchange system was proposed by Krogh (1939), and recent studies have confirmed their presence as a general characteristic of aquatic vertebrates and invertebrates (Jorgensen, Levi, and Zerahn 1954; Shaw 1960; Maetz and Garcia Romeu 1964; Garcia Romeu et al. 1969; Stobbart 1971; Kerstetter and Kirschner 1972; Dietz 1974; Dietz and Alvarado 1974; Alvarado, Poole, and Mullen 1975; Garcia Romeu and Ehrenfeld 1975).

It is interesting that HCO_3 in the bath or injected into the blood does not influence Cl transport in pond-water-acclimated bivalves. The lack of effect of bath HCO_3 on J_i^{Cl} may reflect the high affinity for Cl and a low affinity for HCO_3. The lack of effect of injected HCO_3 on J_i^{Cl} may be due to the Cl transport system being saturated at the 1-mM bath Cl concentrations (see figs. 1 and 2). The increased J_n^{base} observed in HCO_3-injected animals could be due to elevated renal output and Cl-independent outward diffusion of HCO_3 across permeable epithelia. Alternatively, it is possible that the endogenous base used in Cl exchange is not HCO_3 (see Bentley and Yorio 1978).

The site of Cl transport in freshwater mussels is unknown but may be in the epithelial cells of the gills or mantle. Pond-water-acclimated mussels' body fluid is electrically negative by about 10 mV (Dietz and Branton 1975), and the cytoplasm of mantle cells is electrically negative to the bathing medium (Istin and Kirschner 1968). The Cl con-

centration in the blood (12 mM/liter) and the cytoplasm (2 mM/liter) is higher than the bath (Murphy and Dietz 1976). Therefore, Cl transport from the bath into the epithelial cells occurs against the electrochemical gradient (see Kristensen 1972; Alvarado, Poole, and Mullen 1975).

The similar rates of Cl transport by mussels in pond water, NaCl or choline chloride solutions indicates Cl movement is independent of cation transport. Others have noted a partial coupling of Na and Cl transport in fish and amphibians (Garcia Romeu et al. 1969; DeRenzis and Maetz 1973; Alvarado, Dietz, and Mullen 1975). In an earlier report (Dietz and Branton 1975) we had indicated a partial coupling between Na and Cl transport. However, this may have been due to differential levels of salt depletion in the two groups of mussels.

Although Cl transport can be stimulated, the system is effectively inhibited by thiocyanate in the bath (2 mM/liter). Chloride uptake in other animals is inhibited by SCN (Kristensen 1972; Alvarado, Dietz, and Mullen 1975; Epstein, Maetz, and DeRenzis 1973). Of interest is the recent observation of a Cl/HCO_3 stimulated–SCN-inhibited ATPase in the gills of goldfish (DeRenzis and Bornancin 1977). This enzyme is located in the cell membrane fraction of gill tissue and is stimulated by Cl and HCO_3 concentrations within the range expected in freshwater and cytoplasm, respectively. We have detected the presence of a HCO_3 stimulated–SCN inhibited ATPase in bivalve gills (unpublished observations).

The freshwater mussels are similar to some fish in the insensitivity of Cl transport toward acetazolamide inhibition (Kerstetter and Kirschner 1972; Degnan, Karnaky, and Zudunaisky 1977). However, in other animals, Cl transport is significantly depressed by acetazolamide (Dietz 1974; Garcia Romeu and Ehrenfeld 1975; Alvarado, Dietz, and Mullen 1975; Alvarado, Poole, and Mullen 1975). If HCO_3 is the endogenous anion used for Cl exchange, some animals may require HCO_3 generated by carbonic anhydrase at or near the site of Cl/HCO_3 exchange. It is noteworthy that J_o^{Cl} in bivalves is significantly elevated following acetazolamide injection. The route of Cl loss is partially renal, suggesting that the mechanism of Cl reabsorption by kidney tissue differs from that in the epithelial tissues involved in ion uptake from the environment.

Furosemide is a potent diuretic that has been shown to inhibit J_i^{Cl} in a number of animals (Burg et al. 1973; Prusch and Otter 1977; Degnan et al. 1977). However, furosemide (1 mM/liter) is ineffective in blocking J_i^{Cl} in bivalves. Apparently there are basic functional similarities in Cl/base exchange systems among animals but there are significant differences. Some animals (bivalves and trout) have a Cl transport system which is not inhibited by acetazolamide or furosemide. Other animals (earthworms, amphibians, goldfish), having a Cl transport system which is inhibited by acetazolamide, also display a sensitivity toward furosemide. The bases behind this dichotomy are, as yet, unresolved.

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