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Electrogenic 2 Na^+/1 H^+ Exchange in Crustaceans

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Summary. Hepatopancreatic brush border membrane vesicles of the freshwater prawn, *Macrobrachium rosenbergii* and the marine lobster, *Homarus americanus* exhibited ^{22}Na uptake which was Cl-independent, amiloride sensitive, and stimulated by a transmembrane H gradient ($H_i > H_o$). Sodium influx by vesicles of both species were sigmoidal functions of $[Na]_o$, yielding Hill coefficients that were not significantly different ($P > 0.5$) than 2.0. Estimations of half-saturation constants (K_{Na}) were 82.2 mM (prawn) and 280.1 mM (lobster), suggesting a possible adaptation of this transporter to environmental salinity. *Trans*-stimulation and *cis*-inhibition experiments involving variable [H] suggested that the exchangers in both species possessed single internal cation binding sites (pK 6.5–6.7) and two external cation binding sites (prawn, pK 4.0 and 5.7; lobster pK 3.5 and 6.1). Similar *cis* inhibition studies using amiloride as a competitive inhibitor of Na uptake supported the occurrence of dual external sites (prawn, K_i 50 and 1520 μM; lobster K_i 9 and 340 μM). Electrogenic Na/H exchange by vesicles from both crustaceans was demonstrated using equilibrium shift experiments where a transmembrane potential was used as the only driving force for the transport event. Transport stoichiometries of the antiporters were determined using Static Head analysis where driving forces for cation transfer were balanced using a 10 : 1 Na gradient, a 100 : 1 H gradient, and a stoichiometry of 2.0. These electrogenic 2 Na/1 H exchangers appear thermodynamically capable of generating sufficient gastric acidification for organismic digestive activities.

Key Words Na/H exchange · antiport · countertransport · brush border membrane vesicles · electrogenic · stoichiometry · hepatopancreas · crustaceans

Introduction

A wide variety of epithelial and nonepithelial vertebrate cell types possess a Na/H exchange protein in their plasma membranes that catalyzes the net uptake of extracellular sodium for the net extrusion of cytoplasmic protons. The reported biological functions of this antiport mechanism include the regulation of intracellular pH (Moolenaar et al., 1981; Weinman & Reuss, 1982; Piwnica-Worms & Lieberman, 1983); cell volume (Cala, 1980, 1983; Ericson & Spring, 1982; Parker, 1983), and transcellular transport of Na and HCO_3 (Murer, Hopfer & Kinne, 1976; Boron & Boulpaep, 1983; Knickelbein et al., 1983). In the last few years several reviews have been written about the properties of this transporter, describing its stoichiometry, specificity, kinetics, and interaction with a variety of extra- and intracellular cation species (Krulwich, 1983; Aronson, 1985; Aronson & Igarashi, 1986; Grinstein & Rothstein, 1986; Sacktor & Kinsella, 1986). These reviews suggest that, in vertebrates, this protein is highly conservative in its physiological properties across a range of cell types universally displaying an electroneutral 1 Na/1 H exchange stoichiometry, which is energized by the cation illustrating the predominant transmembrane chemical driving force.

Recently, three reports have been published concerning Na/H exchange by crustacean gill (Towle et al., 1988) and hepatopancreatic (Ahearn & Clay, 1989; Ahearn & Franco, 1989) epithelial cells which suggest that in these eukaryotic cell types electrogenic exchange mechanisms, exhibiting transport stoichiometries of 2 Na/1 H, exist with physiological properties that depart significantly from those of the vertebrate paradigm. The present investigation is a detailed kinetic comparison of electrogenic 2 Na/1 H exchange in the hepatopancreas of freshwater and marine crustaceans and a discussion of the potential relevance of this process to gastric digestion in these animals.

Materials and Methods

Animals and Vesicle Preparation

Live intermolt freshwater prawns (*Macrobrachium rosenbergii*) and Atlantic lobsters (*Homarus americanus*) were purchased from commercial dealers in Hawaii and maintained unfed for up to 1 week at room temperature (24°C) in aquaria containing running freshwater (prawns) or at 15°C in a refrigerated aquarium containing filtered seawater (lobsters).

Hepatopancreatic brush border membrane vesicles (BBMV) were prepared from fresh organs of individual 0.5-kg

lobsters or from pooled tissue of 8–10 prawns using a magnesium precipitation technique developed previously (Ahearn, Grover & Dunn, 1985). Purity of BBMV prepared by this method was assessed by comparing the activities of membrane-bound enzymes in the final membrane pellet with those of the original tissue homogenate. Results of these studies suggested that brush border membrane preparations from both crustacean species were highly enriched in apical membranes and contained minimal contamination from basolateral and organelle sources (Ahearn et al., 1985; Ahearn & Clay, 1989).

Transport Measurements

Transport studies using BBMV were conducted at 22°C (prawn) or 15°C (lobster) using the Millipore filtration technique developed by Hopfer et al. (1973). Both long-term and short-term incubations of membrane vesicles from both species with ^{22}Na (New England Nuclear) were conducted in this study using radioisotopic and liquid scintillation methods previously described (Ahearn et al., 1985; Ahearn & Clay, 1989).

^{22}Na uptake values are expressed as pmol or nmol (using specific activity of the isotope in the medium) per mg protein (Bio Rad protein assay) per filter. Each experiment was repeated two or three times using membranes prepared from different animals. Within a given experiment 3–5 replicates were used, and data are presented as mean values and their standard errors.

Chemicals

Valinomycin, amiloride, tetramethylammonium chloride (TMA-Cl), tetramethylammonium hydroxide, D-gluconic acid lactone, and other reagent grade chemicals, were obtained from Sigma Chemical.

Results

Demonstration of Na/H Exchange in Crustacean Hepatopancreas

Short-circuited brush border vesicles of hepatopancreatic epithelial cells of both lobster and prawn were examined for the presence of Na/H exchange by loading with 200 mM mannitol, 50 mM K-gluconate, and 50 μM valinomycin at pH 5.5 (MES-Tris) and incubating in media containing either 0.1 (prawn) or 1.0 (lobster) mM ^{22}Na, 100 mM TMA-gluconate, and 50 mM K-gluconate at pH 5.5 (MES-Tris) or 8.5 (HEPES-Tris). One external medium at pH 8.5 had 2.0 mM amiloride, while the second at this pH lacked the drug.

Figure 1 shows the time course of ^{22}Na uptake by hepatopancreatic vesicles from both crustacean species under the above media conditions and indicates that Na transfer was strongly stimulated in vesicles possessing an outwardly directed, transmembrane proton gradient (pH_i 5.5; pH_o 8.5). In the prawn intravesicular ^{22}Na content at 15 sec of incubation was transiently more than double the value at the 60-min equilibrium point, while the 1-min uptake value in the lobster exceeded its respective equilibrium by almost a factor of 7. These results suggest that in both species a transmembrane proton gradient led to Na transport against considerable concentration gradients. The other two medium conditions (equal pH values on both membrane surfaces, pH gradient plus amiloride) resulted in slow, nonconcentrative Na uptake time courses, suggesting the occurrence of carrier-mediated, amiloride-sensitive Na/H exchange mechanisms in the epithelial brush borders of both crustaceans.

Initial Rates of Na/H Exchange

In order to establish an exposure interval that approximated initial uptake rates of ^{22}Na by Na/H exchange, the time course of sodium uptake by short-circuited BBMV from prawn and lobster was examined at several $[^{22}Na]_o$ (prawn: 5, 35, 50, 75 mM; lobster: 25, 100, 200, 300 mM) for very short time intervals (1 to 5 sec), using a rapid uptake apparatus that automatically controlled incubation period to 1 sec. Vesicles were loaded with either 200 mM mannitol (prawn) or 300 mM TMA-gluconate (lobster), 50 mM K-gluconate, and 50 μM valinomycin at pH 6.0 (MES-Tris) and were incubated in media at pH 8.0 (HEPES-Tris) containing the above ^{22}Na concentrations as Na-gluconate, 50 mM K-gluconate, and TMA-gluconate where needed for osmotic balance.

For vesicles prepared from the prawn, uptake of sodium was a linear function of time from 1 to 5 sec for 5 and 35 mM concentrations, while accumulation of the radiolabeled cation was linear for only the first 3.5 sec at 50 and 75 mM Na (Fig. 2, left panels; data for uptake at 35 and 75 mM are not shown). Uptake of ^{22}Na by BBMV from the lobster was linearly related to time over 5 sec at 25 mM Na, over 4 sec at 100 and 200 mM Na, and only over $3\frac{1}{2}$ sec at 300 mM Na (Fig. 2, right panels; data for uptake at 100 and 300 mM are not shown). Based on these findings, subsequent estimations of Na influx in both species over a wide range of $[Na]_o$ used either $2\frac{1}{2}$ or 3 sec incubations.

Slopes of linear regression lines through the data in Fig. 2 provided estimates of unidirectional ^{22}Na entry rates, and vertical intercepts yielded information about the magnitude of nonspecific isotope binding to the membranes at each concentration. ^{22}Na binding values, determined from extrapolated vertical intercepts at each external sodium concentration, were not significantly different ($P > 0.05$) from those obtained using vesicles injected into ice-cold (0°C) uptake medium at time

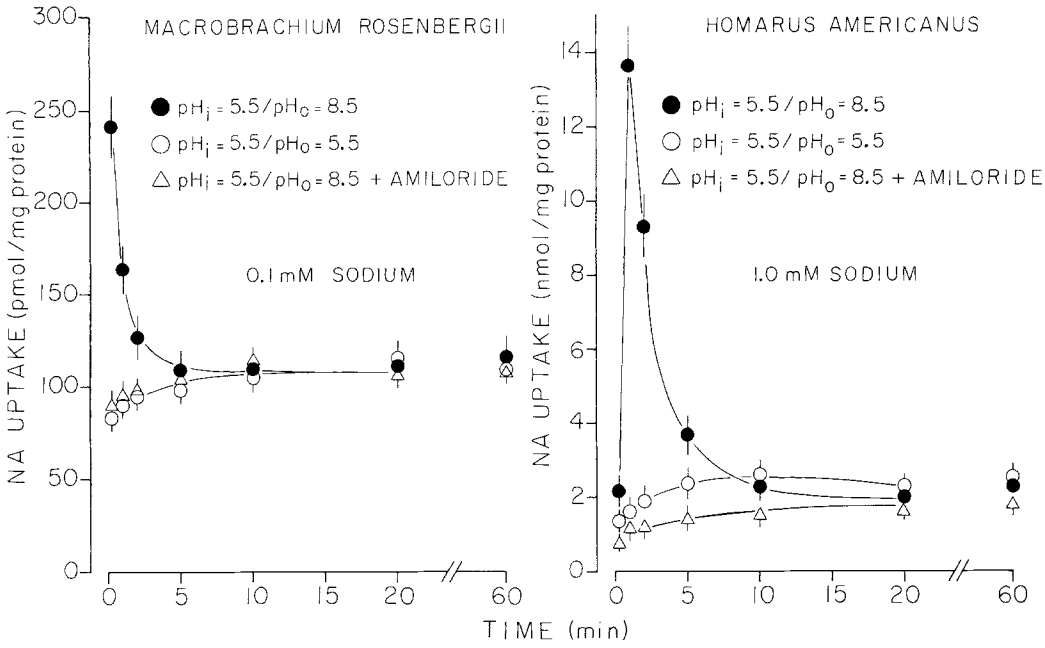

Fig. 1. Stimulation of ^{22}Na uptake by outwardly directed proton gradients in short-circuited hepatopancreatic brush border membrane vesicles (BBMV). Vesicles from both species were loaded with 200 mM mannitol, 50 mM K-gluconate, and 50 μM valinomycin, and 20 mM MES-Tris at pH 5.5 and were incubated in media containing 0.1 mM (prawn) or 1.0 mM (lobster) ^{22}Na-gluconate, 100 mM TMA-gluconate, and 50 mM K-gluconate at pH 5.5 (20 mM MES-Tris) or pH 8.5 (20 mM HEPES-Tris). One external medium at pH 8.5 had 2.0 mM amiloride

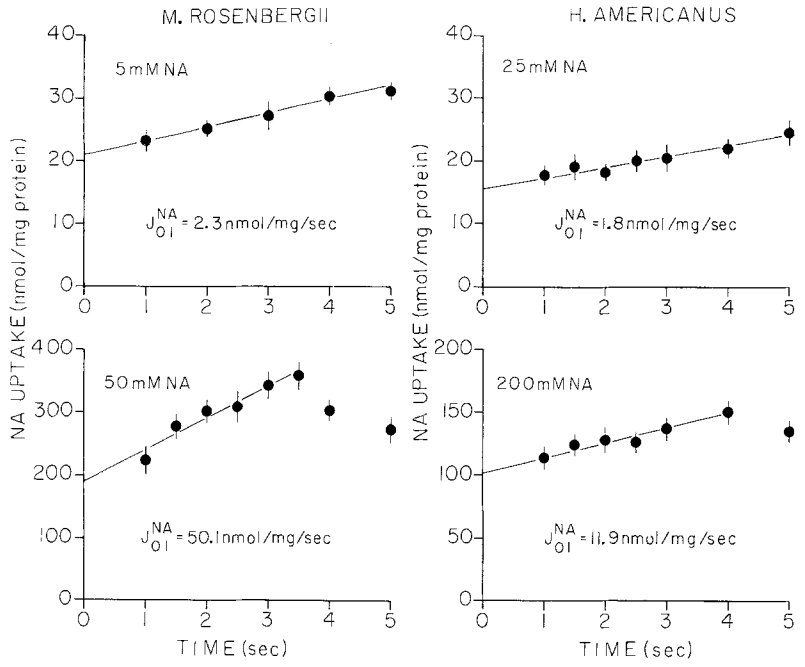

Fig. 2. Determination of exposure interval for estimation of ^{22}Na influx. Vesicles were loaded with either 200 mM mannitol (prawn) or 300 mM TMA-gluconate (lobster), 50 mM K-gluconate, and 50 μM valinomycin at pH 6.0 (20 mM MES-Tris) and were incubated in media at pH 8.0 (20 mM HEPES-Tris) containing the above ^{22}Na concentrations as Na-gluconate, 50 mM K-gluconate, and additional TMA-gluconate for osmotic balance

zero and then rapidly filtered ("blank uptake values"). Sodium influxes at 5 and 50 mM (prawn), as shown on the left of Fig. 2, were 2.3 ± 0.4 and 50.1 ± 12.4 nmol/mg protein/sec, respectively, while those at 25 and 200 mM (lobster), illustrated to the right on Fig. 2, were 1.8 ± 0.2 and 11.9 ± 2.2 nmol/mg protein/sec, respectively.

For vesicles from the prawn, nonspecific sodium binding to filters and membranes, determined from the vertical intercepts of uptake regression

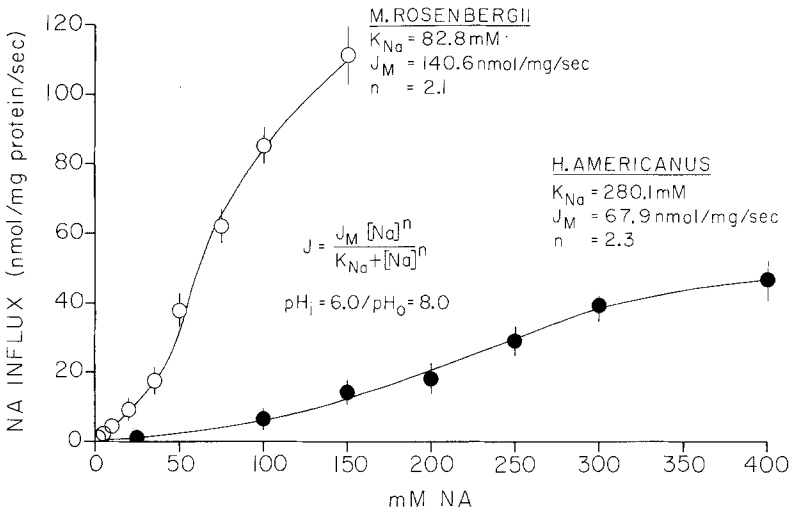

Fig. 3. Effect of $[Na]_o$ on 2.5 sec (lobster) and 3.0 sec (prawn) ^{22}Na influx in short-circuited hepatopancreatic BBMV in the presence of an outwardly directed H gradient. Vesicles were loaded with 300 mM mannitol, 50 mM K-gluconate, 50 μM valinomycin, and TMA-gluconate for osmotic balance at pH 6.0 (20 mM MES-Tris) and were incubated in media containing 50 mM K-gluconate and variable concentrations of ^{22}Na-gluconate and TMA-gluconate at pH 8.0 (20 mM HEPES-Tris). Lines drawn through data and resulting values for kinetic constants were calculated from Hill Equation on Figure

lines for 5, 35, 50, and 75 mM $[Na]_o$ as described above, were 77.8, 74.2, 56.5, and 55.6% of respective 3-sec uptakes. Therefore, subsequent 3-sec estimations of ^{22}Na influx at $[Na]_o$ from zero to 35 mM were corrected for an average 76 ± 2% nonspecific component, while an average correction of 56 ± 1% was used for sodium concentrations of 50 mM or greater. Similar corrections for nonspecific ^{22}Na binding to lobster vesicles were determined from the vertical intercepts of uptake regression lines for 25, 100, 200, and 300 mM $[Na]_o$. In this instance the nonspecific components amounted to 79.5, 85.1, 77.8, and 75.4% of the respective 2.5-sec uptakes. Because these values were much closer to one another than were those for the prawn, an overall average nonspecific binding component of 79.5 ± 2.1% was used for 2.5-sec influx estimations for vesicles from the lobster at all $[Na]_o$. Control experiments with vesicles from both crustacean species were conducted which showed that variation in $[H]_i$, $[H]_o$, or $[amiloride]_o$ had no significant influence on the quantitative values of nonspecific ^{22}Na binding components.

Kinetics of Na/H Exchange

The kinetics of 2.5-sec (lobster) or 3-sec (prawn) ^{22}Na influx as a function of $[Na]_o$ in short-circuited BBMV from both crustacean species is illustrated in Fig. 3. Vesicles were loaded at pH 6.0 (MES-Tris) with 300 mM mannitol, 50 mM K-gluconate, 50 μM valinomycin and TMA-gluconate for osmotic balance and were incubated in media containing 50 mM K-gluconate and variable ^{22}Na-gluconate and TMA-gluconate concentrations at pH 8.0. Influx values at each $[Na]_o$ were corrected for the percentage of total activity associated with the vesicles and filters that was due to nonspecific binding as described above, or from "blank values" where vesicles and radiolabeled incubation medium were simultaneously injected into a stop solution without prior mixing.

In the presence of a fixed outwardly-directed proton gradient, Na influx (J_{Na}) in both the prawn and the lobster was a sigmoidal function of $[Na]_o$ and followed the Hill equation (Fig. 3; Ahearn & Clay, 1989), where J is maximal Na influx, K_{Na} is an apparent affinity constant modified to accommodate multisite interactions (interaction coefficient), and the Hill Coefficient, n, is an estimate of the number of reactive Na binding sites. Quantitative values for these transport constants were estimated using a Marquardt nonlinear iterative computer program providing the best-fit curve through the experimental points in Fig. 3. The coefficient of determination (r^2) for the best-fit lines drawn through the data for both curves using these quantitative values were both greater than 0.99, suggesting highly satisfactory fits to the experimental results.

The sigmoidal nature of the results presented in Fig. 3, and the occurrence of Hill Coefficients of approximately 2, indicate that Na/H exchange by BBMV prepared from both prawn and lobster occurs by carrier processes with at least two external cation sites that exhibit binding cooperativity. In addition, the apparent affinity constant (K_{Na}) for Na binding was threefold greater in the marine animal, *Homarus*, than in the freshwater species, *Macrobrachium*, suggesting a possible adaptation of the carrier mechanism to habitat salinity and substrate availability.

Effects of Intravesicular [H] on Na Influx

The effects of intravesicular proton concentration ($[H]_i$) on the kinetics of 3-sec influx of 50 mM ^{22}Na

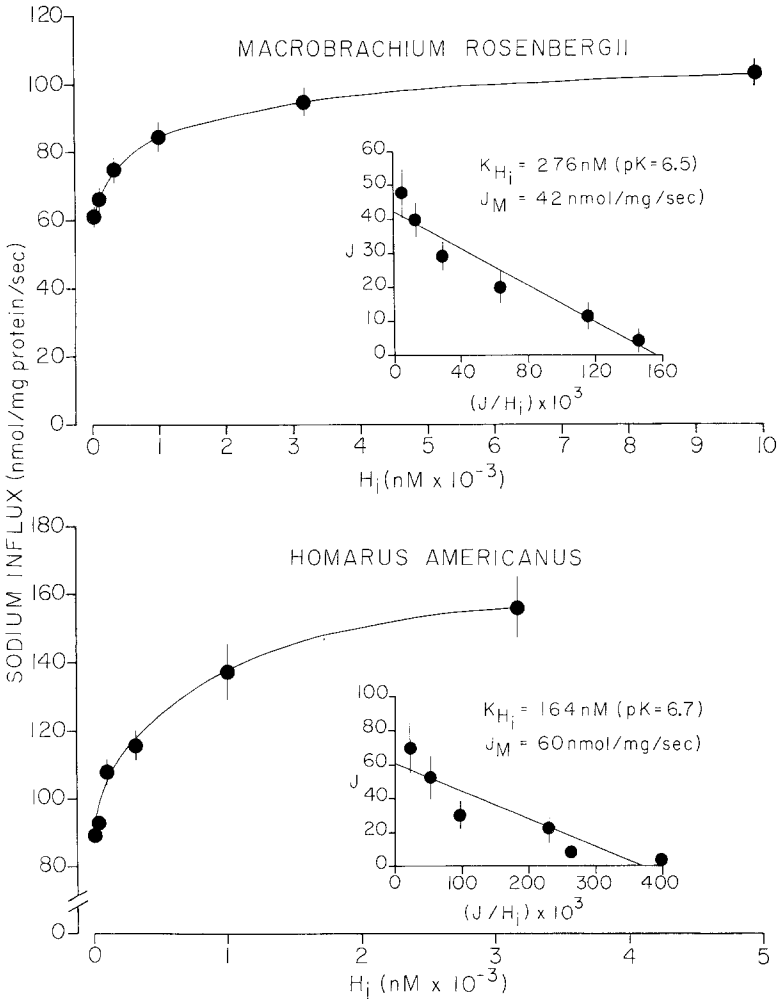

Fig. 4. Effect of variable internal [H] on 50 and 100 mM ^{22}Na influx in short-circuited prawn and lobster hepatopancreatic BBMV, respectively. Vesicles were preloaded for 2 hr at 22°C with 50 mM K-gluconate, 50 μM valinomycin, and appropriate concentrations of TMA-gluconate at pH values from 7.5 to 5.0 (50 mM MES-Tris or HEPES-Tris) and were then incubated in media containing 50 mM K-gluconate at pH 7.5 (50 mM HEPES-Tris) and either 50 mM ^{22}Na-gluconate (prawn) or 100 mM ^{22}Na-gluconate (lobster). Insets are Eadie-Hofstee plots of data after subtraction of estimated vertical intercept (nonspecific binding) from values in the main body of figure. Lines in Eadie-Hofstee plots were calculated by regression analysis

(prawn) or 2.5-sec influx of 100 mM ^{22}Na (lobster) in short-circuited BBMV are displayed in Fig. 4. Vesicles were preloaded for 2 hr at room temperature (22°C) with 50 mM K-gluconate, 50 μM valinomycin, and appropriate concentrations of TMA-gluconate at pH values from 7.5 to 5.0 (MES-Tris and HEPES-Tris). They were then incubated in media containing 50 mM K-gluconate, at pH 7.5 (HEPES-Tris) and either 50 mM ^{22}Na-gluconate (prawn) or 100 mM ^{22}Na-gluconate (lobster).

For both species of crustaceans ^{22}Na influx was a hyperbolic function of $[H]_i$ over the pH range used approaching an asymptote at the highest proton concentrations and illustrating a significant extrapolated vertical axis intercept representing nonspecific binding of isotope to vesicles and filters. After subtracting this nonspecific binding from each influx determination, the resulting data were graphed in Eadie-Hofstee plots (insets, Fig. 4). This transformation of the data yielded values for K_H, internal [H] resulting in $\frac{1}{2}$ maximal ^{22}Na influx (prawn: 276 ± 38 nM H; lobster: 164 ± 36 nM H), and J_M, maximal ^{22}Na influx (prawn: 42.8 ± 3.1; lobster: 60 ± 8.1 nmol/mg protein/sec). The hyperbolic nature of the effect of $[H]_i$ on Na influx in both animals and the magnitude of the resulting K_H values suggests that a single internal proton binding site was used during cation exchange with apparent pK values between 6.5 and 6.7. Because transmembrane gradients of both Na and protons were used in this experiment, the combined driving forces of both gradients were likely responsible for observed isotope accumulation within vesicles.

Effects of Extravesicular [H] on Na Influx

Because crustacean hepatopancreatic and stomach luminal pH values in vivo range from pH 4.0 to 6.0 (Gibson & Barker, 1979), an experiment was conducted with both *Macrobrachium* and *Homarus* to ascertain the extent to which external protons and Na ions interact with each other during Na/H exchange in BBMV. Vesicles were loaded with 100 mM mannitol, 50 mM K-gluconate, and 50 μM valinomycin at pH 7.0 (HEPES-Tris) and were incubated for 3 sec in media at pH 5.0 to 8.0 containing

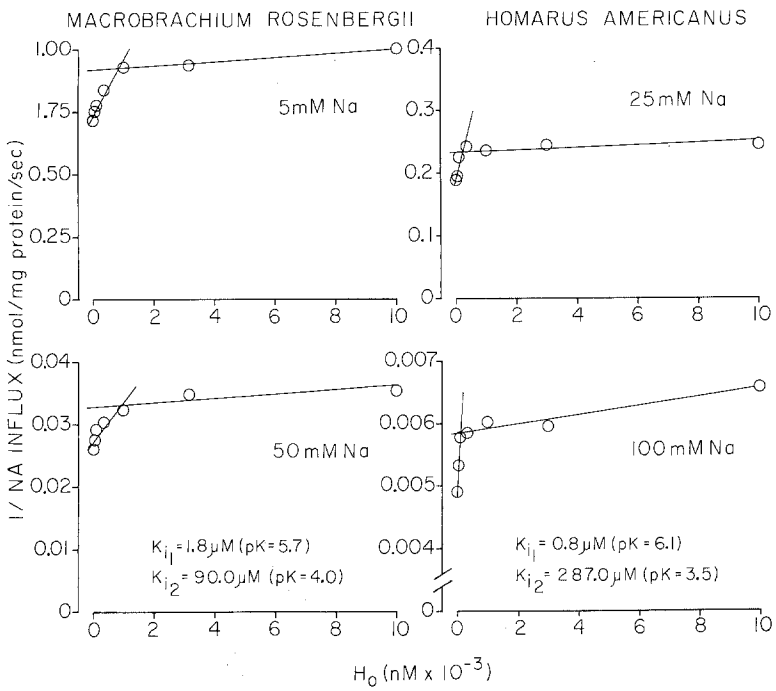

Fig. 5. Effect of variable $[H]_o$ on ^{22}Na influxes at two $[Na]_o$ in short-circuited hepatopancreatic BBMV. Vesicles were loaded with 100 mM mannitol, 50 mM K-gluconate, and 50 μM valinomycin at pH 7.0 (50 mM HEPES-Tris) and were incubated in media at pH 5.0 to 8.0 containing either 5 or 50 mM ^{22}Na-gluconate and TMA-gluconate for osmotic balance (prawn), or were incubated in media over the same pH range containing 25 or 100 mM ^{22}Na-gluconate and appropriate balancing mannitol (lobster). All influx values were corrected for nonspecific binding

either 5 or 50 mM ^{22}Na-gluconate and TMA-gluconate for osmotic balance (prawn), or were incubated for 2.5 sec in media over the same pH range containing 25 or 100 mM ^{22}Na-gluconate and appropriate balancing mannitol (lobster). All influx values were corrected for nonspecific binding as previously discussed.

Figure 5 indicates that at all external [Na] for both crustacean species, reducing pH_o from 8.0 to 5.0 had small, but significant, inhibitory effects on Na influx. In all cases biphasic Dixon plots (1/Na influx *vs.* $[H]_o$) were obtained from the interactions between external Na ions and protons, suggesting that these cations interact at two external binding sites having different apparent cation binding affinities. Inhibitory constants (K_i) for H_o competing with Na_o at both high and low affinity binding sites were estimated from this figure by extrapolating each curve to the left of the vertical axis. A vertical line, drawn from the respective intersections of each pair of lines to the *X* axis, provided values for these constants. The inhibitory constants for proton inhibition of Na influx at the high and low affinity sites in *Macrobrachium* were 1.8 μM (apparent pK = 5.7) and 90.0 μM (apparent pK = 4.0) and in *Homarus* were 0.8 μM (apparent pK = 6.1) and 287.0 μM (apparent pK = 3.5). These results suggest that the two external cation sites in these animals exhibited a 50-fold (prawn) and a 350-fold (lobster) difference in apparent binding affinities for protons. Furthermore, these data also indicate that a marked difference in apparent proton binding affinity occurred between the inner and outer membrane face of the exchange proteins (cytoplasmic pK = 6.5 to 6.7; Fig. 4) with the luminal surface illustrating a far lower affinity in both species. While transmembrane proton gradients were used in this experiment, the inhibitory effects of variable external concentrations of this cation on ^{22}Na influx appear to relate strictly to competitive interactions at shared sites rather than being a function of variable proton driving forces.

Effect of $[Amiloride]_o$ on Na Influx

Figure 1 and other previous work (Ahearn & Clay, 1989) suggested that 2.0 mM external amiloride abolished ^{22}Na uptake by hepatopancreatic BBMV. In order to establish the inhibitory properties of this drug on Na/H exchange by BBMV in both crustacean species, an experiment was conducted where vesicles were loaded with 200 mM mannitol, 50 mM K-gluconate, and 50 μM valinomycin at pH 5.5 (MES-Tris), and were incubated for 15 sec in media containing 0.1 or 0.5 mM ^{22}Na-gluconate (prawn), or 1.0 or 5.0 ^{22}Na-gluconate (lobster), 100 mM TMA-gluconate, 50 mM K-gluconate at pH 8.5, and one of the following concentrations of amiloride (in μM): 0, 5, 10, 50, 100, 500, 1000, or 5000.

A control experiment was conducted using these internal and external media to determine if variable amiloride concentrations had differential effects of nonspecific ^{22}Na binding to vesicles. Nonspecific binding of radiolabeled sodium to these in-

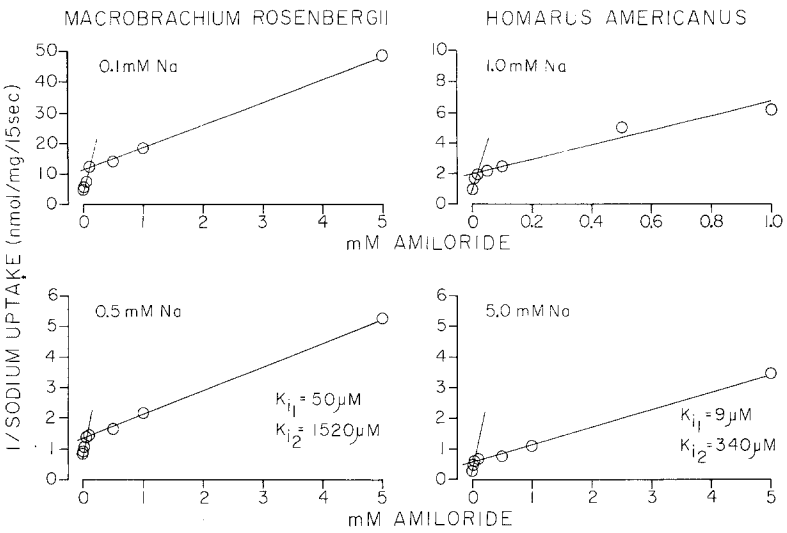

Fig. 6. Effect of variable $[amiloride]_o$ on Na influx in hepatopancreatic BBMV. Vesicles were loaded with 200 mM mannitol, 50 mM K-gluconate, and 50 μM valinomycin at pH 5.5 (50 mM MES-Tris) and were incubated in media containing 0.1 or 0.5 mM ^{22}Na-gluconate (prawn), or 1.0 or 5.0 mM ^{22}Na-gluconate (lobster), 100 mM TMA-gluconate, 50 mM K-gluconate at pH 8.5 (50 mM HEPES-Tris) and concentrations of amiloride from 0 to 10 mM. Inhibition constant (K_i) values calculated from intersections to left of vertical axis of respective extrapolations of high and low affinity components were as described in text

creased as external [^{22}Na] was elevated, but there was no significant difference ($P > 0.05$) in the quantitative magnitude of these binding values at any given $[Na]_o$ for a range of $[amiloride]_o$ (Ahearn & Clay, 1989).

Figure 6 presents Dixon plots of 15 sec ^{22}Na uptake by short-circuited BBMV of prawns and lobsters at a variety of external [amiloride] after correcting for nonspecific ^{22}Na binding as discussed above. All four graphs exhibited Dixon plots with two slopes over the range of [amiloride] used similar to the results obtained when ^{22}Na uptake was measured using variable $[H]_o$ (Fig. 5). These responses suggest that both H_o and external amiloride inhibit Na transport by hepatopancreatic vesicles at two independent binding sites having markedly dissimilar apparent binding affinities. Amiloride inhibitory constants (K_i) were determined from the graphs in Fig. 6 as described above for proton inhibition (Fig. 5). For BBMV from the prawn, calculated K_i values were 50 and 1520 μM, while those for vesicles from the lobster were 9 and 340 μM. In both crustacean species an approximately 30-fold difference in apparent affinity between the two binding sites were disclosed. Because one binding site saturates with amiloride at a far lower external drug concentration than the other, an implication of these results is that Na may still be transferred across the vesicle membrane by one binding site while the other is simultaneously occupied by amiloride.

Effect of Transmembrane Electrical Potential Difference on Na/H Exchange

The effects of an imposed membrane potential on Na/H exchange in both *Macrobrachium* and *Homarus* BBMV were evaluated in experiments where vesicles were first equilibrated with ^{22}Na, K-gluconate and valinomycin at pH 5.5 and were then incubated in an external medium at the same pH containing isotope at an identical specific activity, but which lacked K, a condition leading to the imposition of a transmembrane potassium diffusion potential. Under these conditions, the membrane potential (inside negative) served as the only driving force for net ^{22}Na transport. In other conditions, following the pre-incubation interval, some vesicles were incubated in external medium containing K at the same concentration as in the internal medium (short-circuited conditions). The final groups of vesicles were incubated in external media containing 2.0 mM amiloride alone or 2.0 mM amiloride plus 1.0 mM D-glucose.

Figure 7 indicates that a significant ($P < 0.05$) net uptake of ^{22}Na occurred in vesicles from both crustacean species in the presence of an inside negative membrane potential. This net uptake was abolished by the addition of external amiloride or by short-circuiting the membranes, indicating that the net transfer of Na in the presence of the membrane potential was largely by an electrogenic, amiloride-sensitive carrier process rather than by membrane potential-stimulated Na diffusion. In *Macrobrachium* net Na uptake was also stimulated when both amiloride and D-glucose were present simultaneously in the external medium, suggesting that when Na/H exchange was blocked, Na-dependent D-glucose transport alone led to net uptake of the cotransported cation. This experiment also shows that the presence of amiloride does not, by itself, influence the membrane potential, since electrogenic Na-D-glucose cotransport was able to proceed under these conditions. After 180 min of incubation

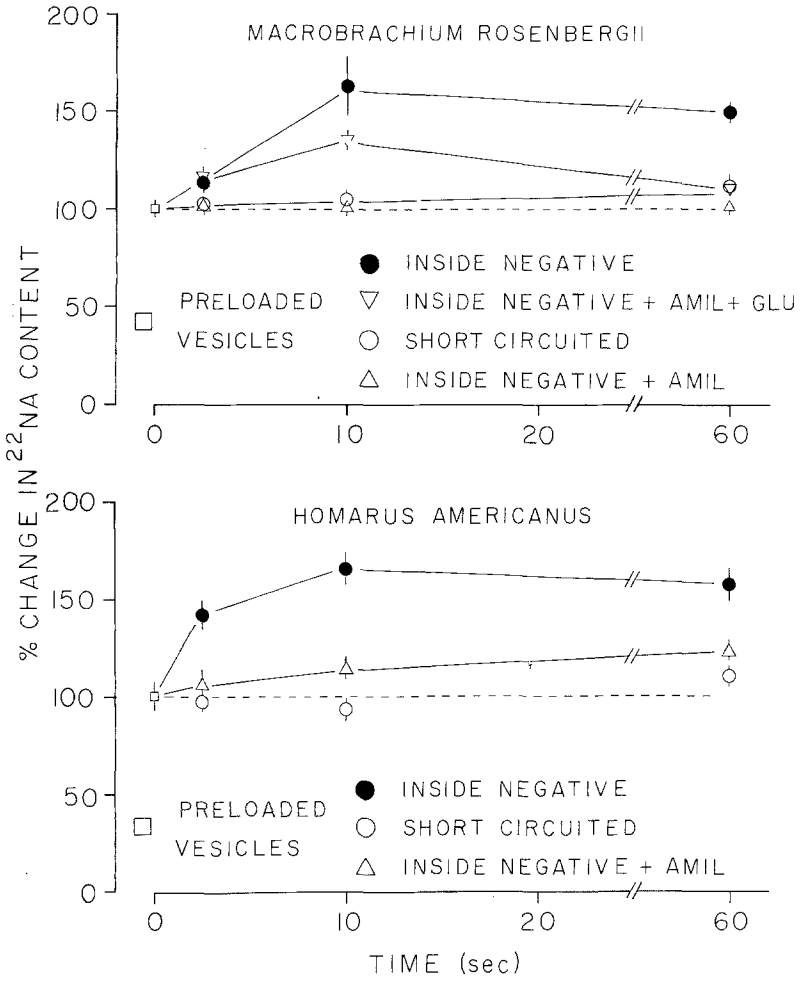

Fig. 7. Effect of a transmembrane electrical potential difference on Na-H exchange in hepatopancreatic BBMV. Vesicles were preloaded for 30 min at 22°C with 50 or 200 mM ^{22}Na-gluconate for prawn and lobster, respectively, 100 mM K-gluconate, and 50 μM valinomycin at pH 5.5 (40 mM MES-Tris). Following this preloading period, vesicles from the prawn were incubated for 2.5, 10, and 60 sec in one of the following media (in mM) at the same pH: (i) 50 ^{22}Na-gluconate, 100 TMA-gluconate; (ii) 50 ^{22}Na-gluconate, 100 K-gluconate; (iii) 50 ^{22}Na-gluconate, 100 TMA-gluconate, 2.0 amiloride; (iv) 50 ^{22}Na-gluconate, 100 TMA-gluconate, 2.0 amiloride, 1.0 D-glucose. Preloaded vesicles from the lobster were incubated for the same time intervals in one of the following media (in mM) at the same pH: (i) 200 ^{22}Na-gluconate, 100 K-gluconate; (ii) 200 ^{22}Na-gluconate, 100 TMA-gluconate; (iii) 200 ^{22}Na-gluconate, 100 TMA-gluconate, 2.0 amiloride. Dotted lines represent unchanged ^{22}Na content after the preloading period

the ^{22}Na content of all vesicles for both species were not significantly different ($P < 0.05$) from that of the respective preloaded vesicles, indicating that isotopic equilibration had occurred by this period of time (*data not shown*).

Results from all treatments with both prawn and lobster BBMV presented in this figure provide strong support for the occurrence of an electrogenic Na/H exchange mechanism capable of transferring net positive charge to the vesicle interior. In addition, at least in the prawn, these data corroborate earlier studies with lobster hepatopancreas showing the presence of a second electrogenic transport system in this membrane which is responsible for Na-glucose cotransport (Ahearn et al., 1985).

Static Head Demonstration of Transport Stoichiometry

Figure 3 indicated that Na influx as a function of $[Na]_o$ in BBMV from both the prawn and lobster followed sigmoidal kinetics yielding Hill coefficients of approximately 2.0, suggesting the possible involvement of more than one Na binding site during Na/H exchange. Figure 8 shows that the antiport processes in both crustaceans was an electrogenic process involving the net movement of positive charge into vesicles. Both figures, therefore, suggest that the transport stoichiometry of Na/H exchange in crustacean BBMV may significantly depart from the established electroneutral 1 Na/1 H antiport system characterized for vertebrate cells (Aronson, 1985).

In order to clarify the stoichiometric relationship between the simultaneous fluxes of Na ions and protons in these vesicle preparations, the Static Head method of transport analysis proposed by Turner and Moran (1982) was applied to Na/H exchange in hepatopancreatic BBMV. In this procedure a pH gradient was established across the vesicle wall (pH_i 5.0; pH_o 7.0) which served as a fixed driving force for ^{22}Na/H exchange and various Na gradients were tested as opposing driving forces to balance this proton gradient. When the driving

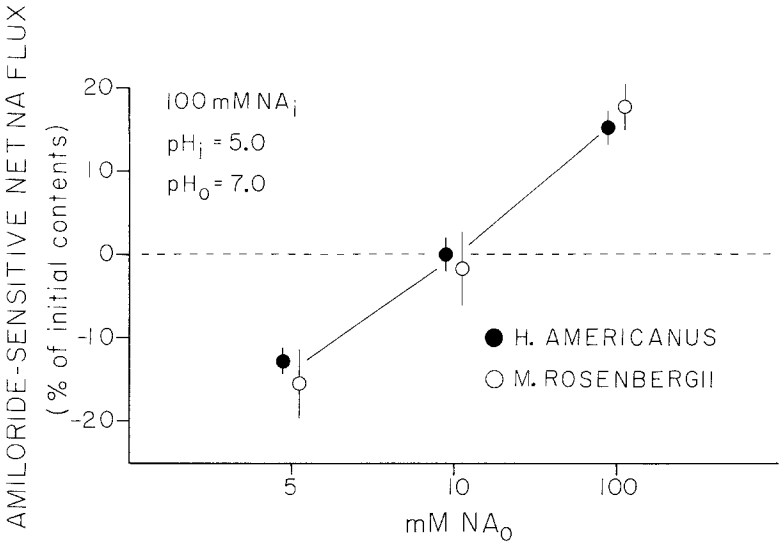

Fig. 8. Demonstration of Na/H transport stoichiometry using Static Head analysis. Two groups of vesicles from both species were preloaded for 30 min at 22–24°C with 100 mM ^{22}Na-gluconate, 50 mM K-gluconate, and 50 μM valinomycin at pH 5.0 (50 MES-Tris). Following loading, one group of vesicles was incubated for 2.5 sec in external media at pH 7.0 (50 mM HEPES-Tris) containing 5, 10, or 100 mM ^{22}Na-gluconate, 50 mM K-gluconate, and mannitol for osmotic balance. The other group was exposed to identical media with 2.0 mM amiloride added. Values displayed represent the % change in vesicular ^{22}Na content over 2.5 sec resulting from amiloride-sensitive net influx or efflux of labeled sodium in response to variable driving forces

forces from proton and Na gradients were balanced, no net flux of ^{22}Na across the membrane in exchange for protons was observed. The thermodynamic equation that describes the condition of no net flux of either H or Na by way of an Na/H antiporter when driving forces of both cations are balanced is shown below:

$$\ln[H_i/H_o] = n(\ln[Na_i/Na_o] + F\Delta\psi/RT) \quad (1)$$

where $\Delta\psi$ is the transmembrane potential, n is the number of Na ions simultaneously transported for each proton (e.g. transport stoichiometry), and F, R, and T have their usual meanings. In the following experiment, BBMV were short circuited with K/valinomycin so that the resulting relationship defining static head conditions in these vesicles is reduced to

$$\ln[H_i/H_o] = n(\ln[Na_i/Na_o]. \quad (2)$$

Two groups of brush border vesicles from both crustacean species were preloaded for 30 min at room temperature (24°C) with 100 mM ^{22}Na-gluconate, 50 mM K-gluconate, and 50 μM valinomycin at pH 5.0 (MES-Tris). One group of preloaded vesicles was then incubated for 2.5 sec in external media at pH 7.0 (HEPES-Tris) containing 5, 10, or 100 mM ^{22}Na-gluconate (same specific activity as internal medium), 50 mM K-gluconate, and mannitol for osmotic balance. The other group was exposed to identical external media except that 2.0 mM amiloride was added to each medium. Preloaded vesicle samples were taken and analyzed for ^{22}Na content at the end of the preloading period for comparison with vesicle isotopic content following exposure to each external medium. Amiloride-sensitive net Na flux for each external medium represents the difference between the 2.5-sec ^{22}Na uptake in the presence and absence of the drug.

Figure 8 shows the relationship between amiloride-sensitive net Na flux (expressed as a % of the preloaded vesicle isotope contents) and external [Na]. Static head conditions were attained for both crustacean species at 100 mM Na_i and 10 mM Na_o. At either $[Na_o]$ = 5 or 100 mM, significant net flow of ^{22}Na across the vesicle wall was observed, directionality being determined by the predominant cation driving force. Because a 10-fold transmembrane gradient of Na balanced a 100-fold transmembrane gradient of protons and led to static head conditions, the transport stoichiometry of Na/H exchange in crustacean hepatopancreatic BBMV is likely to approximate 2.0.

Discussion

The results of this study indicate that hepatopancreatic epithelial brush border membranes of both freshwater and marine crustaceans possess an electrogenic Na/H exchange mechanism that may bring about the net uptake of luminal Na and the net secretion of cytoplasmic protons. Electrogenic net positive charge transfer across these isolated membranes, and presumably across hepatopancreatic apical membranes in living animals, appears to be attained by a membrane carrier protein exhibiting a 2 Na/1 H transport stoichiometry which, in vivo, is likely powered by the combination of a transapical Na gradient and membrane potential. These results depart markedly from the vertebrate Na/H ex-

Table. Kinetic constants for Na/H exchange

	K_{Na} (mM)	J_{max} (nmol/mg/sec)	n	K_H (nM)	K_i^{amil} (μM)	K_i^H (μM)
Crustacean hepatopancreatic cells						
M. rosenbergii	82.8	140.6	2.1	276 (pK 6.5)	50	1.8 (pK 5.7)
(FW prawn)					1520	90.0 (pK 4.0)
H. americanus	280.1	67.9	2.3	164 (pK 6.7)	9	0.8 (pK 6.1)
(SW lobster)					340	287.0 (pK 3.5)
Mammalian kidney cells						
Rabbit renal cortex[a]	13.0	18.0	1.0	—	7	0.035 (pK 7.5)
LLC-PK$_1$ cells[b]	19.9	0.3	1.0	—	30	0.066 (pK 7.2)
Mammalian intestinal cells						
Rabbit ileum[c]	16.2	2.2	1.0	—	—	—
Human jejunum[d]	29.0	3.2	1.0	—	99	—

[a] Aronson et al., 1983 (data collected at pH 7.5).
[b] Moran, 1987.
[c] Knickelbein et al., 1983 (data collected at pH 7.5).
[d] Kleinman et al., 1988.

change paradigm recently summarized by Aronson (Aronson, 1985; Aronson & Igarashi, 1986), where electroneutral 1 Na/1 H exchange takes place in a variety of cell types and is powered by the predominant chemical driving force independent of membrane potential.

The Table illustrates a comparison of kinetic constants obtained from ^{22}Na/H exchange experiments performed in this study with prawn and lobster hepatopancreatic brush border membrane vesicles and those for similar antiport systems in vesicles from mammalian epithelia. Markedly different quantitative values for all Na influx kinetic parameters (K_{Na}, J_{max}, n) are apparent between the crustacean tissues and those of mammals, while more numerical similarities occur for tissues within the same animal group. While the half-saturation constants for Na binding (K_{Na}) are uniformly low in the four representative vertebrate cell types, both crustacean values are considerably greater, a finding which may be related to differences in blood or environmental sodium levels. Within the two species of crustaceans this relationship appears to hold as prawn hemolymph contains approximately 220 mM Na (Ahearn et al., 1977) while lobster blood has 470 mM Na (Prosser, 1973). Mammal plasma possesses Na concentrations significantly less than either of these values. Therefore, the half-saturation constant for Na/H exchange across animal phyla may illustrate a degree of adaptation to substrate availability.

The second major difference between crustacean and mammalian kinetic parameters concerns the respective Hill coefficients (n), an index of the approximate number of external cation binding sites. In all mammalian cell types examined to date, four of which are included in this table, Hill coefficients of 1.0 characterize Na/H exchange, indicating that single cations bind to each side of the carrier prior to transmembrane translocation. In contrast, both crustacean hepatopancreatic BBMV illustrate Hill coefficients of approximately 2, reflecting an asymmetric number of cation binding sites on the two carrier faces. The asymmetric nature of cation binding to the crustacean antiporters is supported in the present study by several lines of evidence including: (i) sigmoidal Na influx *vs.* $[Na]_o$ curves (Fig. 3), (ii) proton and amiloride inhibition of Na influx (Figs. 5, 6), (iii) electrogenic cation exchange (Fig. 7), and (iv) static head conditions attained at 100 H/10 Na gradient driving forces (Fig. 8).

Further evidence supporting an asymmetry of cation binding sites for Na/H exchange in crustacean hepatopancreatic epithelial brush border membranes is shown in Fig. 4 where hyperbolic relationships were disclosed for Na influx as a function of intravesicular [H]. The hyperbolic nature of these curves for both crustacean species suggests that single proton binding sites occur on the cytoplasmic side of the membrane. Similarity of the pK values for this binding site for the two animal species (prawn, pK = 6.5; lobster pK = 6.7) suggests that intracellular pH conditions are likely similar even though the crustaceans experience markedly different blood and environment ionic conditions. Intracellular pK values also indicate that striking apparent affinity differences of the invertebrate

exchanger toward protons exist on the two membrane faces with that of the cytoplasmic face being significantly greater and similar to pK values illustrated by proton binding to the external face of the Na/H exchanger in mammal renal vesicles (Table).

The last major point of comparison between the kinetics of Na/H exchange in crustaceans and mammals in the Table relates to the inhibitory effects of external protons and amiloride on ^{22}Na influx. In mammalian cells both protons and amiloride inhibit Na transport, by competitive inhibition, at single external binding sites (Aronson, Suhm & Nee, 1983; Aronson & Igarashi, 1986; Moran, 1987; Kleinman et al., 1988). In crustacean BBMV this inhibition is also of a competitive nature, but occurs at two distinct external sites with markedly dissimilar apparent binding affinities for the inhibitors (Figs. 5 and 6).

Inhibitory constants for proton binding to external exchanger sites (K_i) in mammals range between 0.035 and 0.066 μM (pK 7.2 to 7.5), suggesting a relatively high apparent binding affinity of the site for the cation (Aronson & Igarashi, 1986; Nord et al., 1986; Moran, 1987). In contrast, crustacean hepatopancreatic Na/H exchange illustrated two K_i values for external proton binding to the antiport process that were both considerably greater than those reported for the mammalian cells (Fig. 5; Table). Such results imply that external cation binding sites in the crustacean cells possess much lower apparent affinities for protons than do those of the vertebrates. This difference in apparent binding affinities between the two animal groups may relate to relative physiological proton concentrations in the respective tissues. In mammals, the pH of the renal tubular lumen is close to neutrality, whereas in the lumen of crustacean hepatopancreatic tubules the pH has been reported to be as low as pH 4.7 (Gibson & Barker, 1979).

In a wide variety of mammalian cells, amiloride interacts with the Na/H exchanger at a single external site with K_i values reported between 7 and 99 μM (Kinsella & Aronson, 1981; Aronson et al., 1983; Mahnensmith & Aronson, 1985; Grinstein & Furuya, 1986; Moran, 1987; Kleinman et al., 1988). This relatively narrow range of K_i values suggests the likelihood of a highly conservative conformational structure of the external binding site among vertebrate cells. In crustacean BBMV amiloride acted as a competitive inhibitor to Na binding at two external carrier sites having markedly dissimilar apparent affinities for the drug (Fig. 6; Table). One of these sites illustrated a high apparent binding affinity for the drug which was within the range of values reported for mammalian cells (9 μM, *Homarus*; 50 μM, *Macrobrachium*) and may share similar structural and/or chemical properties with this portion of the vertebrate antiporter. The other crustacean amiloride site exhibited a much lower apparent binding affinity for the drug (340 μM, *Homarus;* 1520 μM *Macrobrachium*), which was considerably outside of the mammalian range of values. The electrogenic nature of this invertebrate exchange system, as well as other distinguishing properties of the carrier process, may be a function of the occurrence of this lower affinity amiloride binding site.

Epithelial cells of the crustacean hepatopancreas perform a variety of functions. Some of the cells synthesize an array of digestive enzymes and secrete them into tubular lumens where they flow to the stomach via paired hepatopancreatic ducts and mix with food undergoing trituration (Vonk, 1960; van Weel, 1974; Gibson & Barker, 1979). Other cells are believed absorptive, transferring organic solutes from lumen to cytosol by several Na-dependent and Na-independent apical carrier processes (Ahearn, 1987, 1988; Ahearn & Clay, 1988). Crustacean stomach contents of both freshwater and marine species during digestion have been recorded between pH 4.7 and 7.6 (Gibson & Barker, 1979), while hemolymph pH values generally range between 7.0 and 8.0. Prior to this study and our previous work (Ahearn & Clay, 1989), the source of protons leading to this gastric acidification was unknown. The present investigation provides evidence for a discrete hepatopancreatic brush border mechanism in both freshwater and marine crustaceans that generates sufficient luminal protons to account for recorded values of gastric pH during digestion.

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References

Ahearn, G.A. 1987. Nutrient transport by the crustacean gastrointestinal tract: Recent advances with vesicle techniques. *Biol. Rev.* **62:**45–63

Ahearn, G.A. 1988. Nutrient absorption by the invertebrate gut. *In:* Advances in Environmental and Comparative Physiology. R. Gilles, editor. Vol. 2, Chap. 3, pp. 91–129. Springer-Verlag, Berlin

Ahearn, G.A., Clay, L.P. 1988. Sodium-coupled sugar and amino acid transport in an acidic microenvironment. *Comp. Biochem. Physiol.* **90A:**627–634

Ahearn, G.A., Clay, L.P. 1989. Kinetic analysis of electrogenic 2 Na/1 H antiport in crustacean hepatopancreas. *Am. J. Physiol.* **257:**R484–R493

Ahearn, G.A., Franco, P.L. 1989. Hepatopancreatic electrogenic 2 Na/1 H antiporters occur in both freshwater and marine crustaceans. *FASEB J.* **3(3):**A563

Ahearn, G.A., Grover, M.L., Dunn, R.E. 1985. Glucose transport by lobster hepatopancreatic brush border membrane vesicles. *Am. J. Physiol.* **248:**R133–R141

Ahearn, G.A., Maginniss, L.S., Song, Y.K., Tornquist, A. 1977. Intestinal water and ion transport in freshwater malacostracan prawns (*Crustacea*), *In:* Water Relations in Membrane Transport in Plants and Animals. A.J. Jungreis, T. Hodges, A. Kleinzeller, and S.G. Schultz, editors. pp. 129–142. Academic, New York

Aronson, P.S. 1985. Kinetic properties of the plasma membrane Na-H exchanger. *Annu. Rev. Physiol.* **47:**545–560

Aronson, P.S., Igarashi, P. 1986. Molecular properties and physiological roles of the renal Na-H exchanger. *In:* Current Topics in Membranes and Transport. P.S. Aronson and W.F. Bacon, editor. Vol. 26, pp. 57–75. Academic, New York

Aronson, P.S., Suhm, M.A., Nee, J. 1983. Interaction of external H with the Na-H exchanger in renal microvillus membrane vesicles. *J. Biol. Chem.* **258:**6767–6771

Boron, W.F., Boulpaep, E.L. 1983. Intracellular pH regulation in the renal proximal tubule of the salamander. Na-H exchange. *J. Gen. Physiol.* **81:**29–52

Cala, P.M. 1980. Volume regulation by *Amphiuma* red blood cells. The membrane potential and its implications regarding the nature of the ion-flux pathways. *J. Gen. Physiol.* **76:**683–708

Cala, P.M. 1983. Cell volume regulation by *Amphiuma* red blood cells. The role of Ca^{2+} as a modulator of alkali metal/H^+ exchange. *J. Gen. Physiol.* **82:**761–784

Ericson, A.C., Spring, K.R. 1982. Volume regulation by *Necturus* gallbladder: Apical Na-H and Cl-HCO_3 exchange. *Am. J. Physiol.* **243:**C146–C150

Gibson, R., Barker, P.L. 1979. The decapod hepatopancreas. *Oceanog. Mar. Biol. Annu. Rev.* **17:**285–346

Grinstein, S., Furuya, W. 1986. Characterization of the amiloride-sensitive Na-H antiport of human neutrophils. *Am. J. Physiol.* **250:**C283–C291

Grinstein, S., Rothstein, A. 1986. Mechanism of regulation of the Na/H exchanger. *J. Membrane Biol.* **90:**1–12

Hopfer, U., Nelson, K., Perrotto, J., Isselbacher, K.J. 1973. Glucose transport in isolated brush border membrane from rat intestine. *J. Biol. Chem.* **248:**25–32

Kinsella, J.L., Aronson, P.S. 1981. Amiloride inhibition of the Na-H exchanger in renal microvillus membrane vesicles. *Am. J. Physiol.* **241:**F374–F379

Kleinman, J.G., Harig, J.M., Barry, J.A., Ramaswamy, K. 1988. Na^+ and H^+ transport in human jejunal brush-border membrane vesicles. *Am. J. Physiol.* **255:**G206–G211

Knickelbein, R., Aronson, P.S., Atherton, W., Dobbins, J.W. 1983. Sodium and chloride transport across rabbit ileal brush border: I. Evidence for Na-H exchange. *Am. J. Physiol.* **245:**G504–G510

Krulwich, T.A. 1983. Na/H antiporters. *Biochim. Biophys. Acta* **726:**245–264

Mahnensmith, R.L., Aronson, P.S. 1985. Interrelationships among quinidine, amiloride, and lithium as inhibitors of the renal Na-H exchanger. *J. Biol. Chem.* **260:**12586–12592

Moolenaar, W.H., Boonstra, J., Van der Saag, P.T., De Laat, S.W. 1981. Sodium/proton exchange in mouse neuroblastoma cells. *J. Biol. Chem.* **256:**12883–12887

Moran, A. 1987. Sodium-hydrogen exchange system in LLC-PK_1 epithelium. *Am. J. Physiol.* **252:**C63–C67

Murer, H., Hopfer, U., Kinne, R. 1976. Sodium/proton antiport in brush-border membrane vesicles isolated from rat small intestine and kidney. *Biochem. J.* **154:**597–604

Nord, E.P., Goldfarb, D., Mikhail, N., Moradeshagi, P., Hafezi, A., Vaystub, S., Cragoe, E.J., Jr., Fine, L.G. 1986. Characteristics of the Na-H antiporter in the intact renal proximal tubular cell. *Am. J. Physiol.* **250:**F539–F550

Piwnica-Worms, D., Lieberman, M. 1983. Microfluorometric monitoring of pH_i in cultured heart cells: Na-H exchange. *Am. J. Physiol.* **244:**C422–C428

Parker, J.C. 1983. Volume-responsive sodium movements in dog red blood cells. *Am. J. Physiol.* **244:**C324–C330

Prosser, C.L. 1973. Comparative Animal Physiology. W.B. Saunders, Philadelphia

Sacktor, B., Kinsella, J.L. 1986. Hormonal regulation of renal Na-H exchange activity. *Curr. Top. Membr. Transp.* **26:**223–244

Towle, D.W., Hunter, K.C., Maiolo, N.J. Jr., Wrestler, J.C., Shetlar, R.E., Holleland, T. 1988. Stoichiometry of sodium/proton exchange in membrane vesicles from gills of osmoregulating and osmoconforming crabs. *Am. Zool.* **28(4):**18A

Turner, R.J., Moran, A. 1982. Stoichiometric studies of the renal outer cortical brush border membrane D-glucose transporter. *J. Membrane Biol.* **67:**73–80

van Weel, P.B. 1974. "Hepatopancreas?" *Comp. Biochem. Physiol.* **47A:**1–9

Vonk, H.J. 1960. Digestion and metabolism. *In:* Physiology of Crustacea. T.H. Waterman, editor, Vol. 1, pp. 291–316. Academic, New York

Weinman, S.A., Reuss, L. 1982. Na/H exchange at the apical membrane of *Necturus* gallbladder. *J. Gen. Physiol.* **80:**299–321

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