J. Membrane Biol. 118, 193–214 (1990)

The Journal of
Membrane Biology

Interactions of External and Internal H^+ and Na^+ with Na^+/Na^+ and Na^+/H^+ Exchange of Rabbit Red Cells: Evidence for a Common Pathway

Kevin Morgan and Mitzy Canessa

Endocrine-Hypertension Division, Brigham and Women's Hospital, and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02215

Summary. We have studied the kinetic properties of rabbit red cell (RRBC) Na^+/Na^+ and Na^+/H^+ exchanges (EXC) in order to define whether or not both transport functions are conducted by the same molecule. The strategy has been to determine the interactions of Na^+ and H^+ at the internal ($_i$) and external ($_o$) sites for both exchanges modes. RRBC containing varying Na_i and H_i were prepared by nystatin and DIDS treatment of acid-loaded cells. Na^+/Na^+ EXC was measured as Na_o-stimulated Na^+ efflux and Na^+/H^+ EXC as Na_o-stimulated H^+ efflux and ΔpH_o-stimulated Na^+ influx into acid-loaded cells.

The activation of Na^+/Na^+ EXC by Na_o at pH_i 7.4 did not follow simple hyperbolic kinetics. Testing of different kinetic models to obtain the best fit for the experimental data indicated the presence of high (K_m 2.2 mM) and low affinity (K_m 108 mM) sites for a single- or two-carrier system. The activation of Na^+/H^+ EXC by Na_o (pH_i 6.6, $Na_i < 1$ mM) also showed high (K_m 11 mM) and low (K_m 248 mM) affinity sites. External H^+ competitively inhibited Na^+/Na^+ EXC at the low affinity Na_o site (K_H 52 nM) while internally H^+ were competitive inhibitors (pK 6.7) at low Na_i and allosteric activators (pK 7.0) at high Na_i.

Na^+/H^+ EXC was also inhibited by acid pH_o and allosterically activated by H_i (pK 6.4). We also established the presence of a Na_i regulatory site which activates Na^+/H^+ and Na^+/Na^+ EXC modifying the affinity for Na_o of both pathways. At low Na_i, Na^+/Na^+ EXC was inhibited by acid pH_i and Na^+/H^+ stimulated but at high Na_i, Na^+/Na^+ EXC was stimulated and Na^+/H^+ inhibited being the sum of both pathways kept constant. Both exchange modes were activated by two classes of Na_o sites, *cis*-inhibited by external H_o, allosterically modified by the binding of H^+ to a H_i regulatory site and regulated by Na_i. These findings are consistent with Na^+/Na^+ EXC being a mode of operation of the Na^+/H^+ exchanger.

Na^+/H^+ EXC was partially inhibited (80–100%) by dimethyl-amiloride (DMA) but basal or pH_i-stimulated Na^+/Na^+ EXC (pH_i 6.5, Na_i 80 mM) was completely insensitive indicating that Na^+/Na^+ EXC is an amiloride-insensitive component of Na^+/H^+ EXC. However, Na^+ and H^+ efflux into Na-free media were stimulated by cell acidification and also partially (10 to 40%) inhibited by DMA; this also indicates that the Na^+/H^+ EXC might operate in reverse or uncoupled modes in the absence of Na^+/Na^+ EXC.

In summary, the observed kinetic properties can be explained by a model of Na^+/H^+ EXC with several conformational states, H_i and Na_i regulatory sites and loaded/unloaded internal and external transport sites at which Na^+ and H^+ can compete. The occupancy of the H^+ regulatory site induces a conformational change and the occupancy of the Na_i regulatory site modulates the flow through both pathways so that it will conduct Na^+/H^+ and/or Na^+/Na^+ EXC depending on the ratio of internal $Na^+ : H^+$.

Key Words rabbit red cells · Na/Na exchange · Na/H exchange · H^+ and Na^+ interactions

I. Introduction

Rabbit red blood cells are similar to human erythrocytes with respect to the mechanisms present within their cell membranes for the translocation of Na^+. Both species exhibit a ouabain-sensitive Na^+ pump (Villamil & Kleeman, 1959; Glynn & Karlish, 1975) together with ouabain-insensitive Na^+/Na^+ EXC which is also capable of transporting Li^+ (Haas, Schooler & Tosteson, 1975; Duhm & Becker, 1979) and can be inhibited by phloretin (Pandey et al., 1978). The maximal velocity (V_{max}) of Na^+/Li^+ countertransport in human red cells is of low magnitude (Haas et al., 1975; Canessa et al., 1980) and it has been assumed that in-vivo it operates an obligatory one-for-one Na^+/Na^+ EXC. In rabbit red cells, the V_{max} of this system is at least 10-fold higher than in human erythrocytes but only partially blocked by phloretin (Duhm & Becker, 1979).

Several studies have confirmed that the activity of the Li^+/Na^+ EXC is elevated in red cells of patients with essential hypertension and it is genetically determined (Canessa et al., 1980; for reviews, Canessa, 1984; Canessa, Brugnara & Escobales, 1987; Canessa, Morgan & Semplicini, 1988). Aronson (1982), Sachs, Faler and Rabson (1982) and Funder et al. (1984) have suggested that Na^+/Na^+, Na^+/Li^+ EXC might operate as a Na^+/H^+ EXC, a transport system involved in proximal tubular reabsorption (Murer, Hopfer & Kinne, 1976; Aronson &

Igarashi, 1986) and regulation of cell pH in vascular smooth muscle cells (Berk et al., 1986) and other cell types.

Recently, evidence has been presented which confirms the presence of a Na^+/H^+ EXC system in human (Escobales & Canessa, 1986) and rabbit erythrocytes (Escobales & Rivera, 1987) which is capable of performing net Na^+ transport using the H^+ gradient as the driving force. The activity of this and other Na^+ transport systems are less in RBC than in the kidney, vascular smooth muscle or lymphocytes; as in the case of Na^+ pump kinetic studies, this drawback can be compensated for by using a larger number of cells and longer times for flux measurements. Furthermore, RBC is uniquely suited to kinetic studies because of its low passive permeability and the ease with which it is possible to manipulate intra and extracellular conditions. For instance, no study to date has measured the activity of Na^+/H^+ EXC in Na_i-depleted cells.

Our strategy to investigate the relationship between Na^+/Na^+ and Na^+/H^+ EXC has been to study the kinetics of the interactions of internal and external H^+ and Na^+ with both exchangers. Before any potential H^+ interactions with Na^+/Na^+ EXC could be determined, we needed to study the kinetic parameters of the Na^+/Na^+ EXC under physiological conditions (i.e., $pH_i = pH_o = 7.4$). Subsequently, we determined the kinetic effects of H_i and H_o on Na^+/Na^+ EXC and the effects of H_i and Na_i on Na^+/H^+ EXC. Finally, in order to gain an understanding of the regulation of both Na^+/Na^+ and Na^+/H^+ EXC modes, we investigated the *trans*-effect of Na_i on the K_m for Na_o of each process.

The results provide evidence that H_i and H_o interact asymmetrically with Na^+/H^+ as well as with Na^+/Na^+ EXC suggesting that both processes are coupled. Similarly, Na_i appears to regulate the flow through both pathways depending on the Na_i: H_i ratio; thus, Na^+/H^+ EXC operated as Na^+/Na^+ EXC at physiological pH_i and at acid pH_i when Na_i is very high. To account for the experimental results, we propose a transport model of Na^+/H^+ EXC with two or more conformational states controlled by H_i and Na_i regulatory sites and several modes of operation determined by the relative ratio of substrate concentrations at both membrane sites.

II. Materials and Methods

A. Chemicals

NaCl, $MgCl_2$ and glucose were obtained from Fisher Scientific (Fairlawn, NJ). Ouabain, Tris, MES, MOPS, DIDS, nystatin and albumin (bovine fraction V) were purchased from Sigma Chemical (St. Louis, MO). KCl was purchased from Mallinckrodt (St. Louis, MO). ^{22}Na was purchased from Amersham (Arlington Heights, IL). Phloretin was from Nutritional Biochemicals (Cleveland, OH) and neptazane was from Lederle Laboratories Division (Pearl River, NY).

B. Preparation of Red Blood Cells

Blood was obtained by ear venipuncture from New Zealand White male rabbits. The sample was centrifuged (2000 × *g*, 4 min, 4°C) and the plasma and buffy coat removed by aspiration. The cells were then washed three times with potassium wash solution (KWS) containing (in mM): 140 KCl, 0.15 $MgCl_2$, 10 Tris-MOPS (pH 7.4 at 4°C) and resuspended with KWS to 50% hematocrit. Aliquots of this suspension were used for determinations of hemoglobin (optical density at 540 nM), hematocrit, and Na^+ concentration by using appropriate dilutions in double-distilled water containing 0.02% Acationox® detergent of cell lysates (American Scientific Products, Boston, MA)

C. Cation and Isotope Loading of Red Cells

Na^+ content was varied using the ionophore nystatin (Canessa et al., 1986). Washed packed cells (prepared as above) were incubated at 15% hematocrit in cold nystatin loading solution (NLS) containing 40 μg/ml nystatin for 20 min at 4°C in the dark. The nystatin was dissolved in dimethyl sulfoxide (5 mg in 1.3 ml). NLS contained (in mM): 140 (NaCl + KCl), 50 sucrose. To vary the cellular Na^+, the NLS contained different ratios of NaCl : KCl according to the desired Na^+ content. To prepare cells with low internal Na^+ (depleted or less than 10 mmol/liter cells) the supernatant was removed and the cells were incubated for a further 20 min in NLS without nystatin.

To load the cells with ^{22}Na, the isotope was added after the first incubation with NLS at 4°C in order to do it at very high specific activity; after nystatin was incorporated, the supernatant was removed and the cells were suspended at 50% hematocrit in the same loading solution, without nystatin, containing 5 μCi ^{22}Na per ml packed cells. Under these conditions (low temperature) nystatin is still in the membrane and isotope equilibration can be rapidly achieved. After 20-min incubation at 4°C, with adequate shaking, the suspension was warmed to 37°C for 5 min and the red cells were washed four times with warm (37°C) nystatin wash solution (NWS). Removal of nystatin while keeping a high specific activity yielded red cells loaded with very high specific activity. The NWS had the same composition as the NLS with the pH adjusted to 7.4 with 1 mM $KHPO_4$ buffer and 10 mM glucose and 0.1% albumin added. After the final wash the supernatant was removed and the cell pH adjusted as outlined below.

D. Internal pH Manipulation

Red cells with different pH_i were obtained using a modification of a previously described procedure for human red cells (Canessa, 1989). Cells with the desired intracellular Na^+ were incubated at 37°C for 10 min at 5% hematocrit in media which contained (in mM): 170 KCl, 0.15 $MgCl_2$, 0.1 ouabain, 10 glucose and 20 Tris-MES (for pH 6 to 7 at 37°C) or Tris-MOPS (for pH > 7 to 8 at 37°C) to obtain varying pH_i (from 6 to 8). To avoid cell swelling, 40 mM sucrose was added to the media with $pH_i < 7$ (final osmolarity, 360 mOsm) and the KCl reduced to 130 mM (final osmolarity, 260 mOsm) for media with $pH_i > 7.6$. All other solutions and media had osmolar concentrations between 300 and 315 mOsm.

In order to clamp pH_i, DIDS and neptazane were added to final concentrations of 125 and 400 μM, respectively, following the 10-min preincubation with different acid-loading solutions. After incubation for 30 min at 37°C, the cells were centrifuged and washed five times with the corresponding ice-cold pH washing solution (pHWS) to remove external cations. The composition of the unbuffered pHWS was (in mM): 170 KCl, 0.15 $MgCl_2$ which was osmotically adjusted as described for the incubation media. After the final wash the cells were centrifuged (2000 × *g*, 4 min, 4°C), suspended to 50% hematocrit with pHWS, and kept on ice ready for use.

Portions of this suspension were lysed for determinations of hemoglobin, hematocrit, specific activity (in ^{22}Na-loaded cells), and cellular Na^+ concentration. The actual pH_i was determined by taking tightly packed DIDS- and neptazane-treated cells, lysing with four volumes of 0.02% Acationox® detergent and measuring the pH of the lysate as previously reported (Escobales & Canessa, 1986). The measured cell pH was usually 0.2 pH units above the pH_o of the acid-loading solution. The cell Na^+ content was determined by atomic absorption spectroscopy with suitable standards. The cation content of the cells was expressed per liter original volume, as determined by relating the absorbance at 540 nm of the cell lysate to that of a known volume of red cells. To express this as mM concentration the value was divided by the fractional water content (0.65% vol/vol). The cell volume was estimated by measuring the hemoglobin per liter of fresh and loaded cells prior to measurements of initial transport rates. The cell volume of Na_i- and pH_i-loaded erythrocytes was normally 99 ± 4% (n = 6) of the initial cell volume. Cell lysis was determined at t = 0 and at the end of the flux determination of acid-loaded cells.

E. Composition of the Flux Media

The efflux media used in the Na^+/Na^+ EXC experiments contained (in mM): 150 (NaCl + KCl) to give external Na^+ 0 to 150 mM, 0.15 $MgCl_2$, 0.1 ouabain, 0.4 neptazane, 10 glucose, 10 Tris-MES/MOPS (to give desired pH_o at 37°C); for flux media with pH below 7.0, Tris-MES was used. The neptazane was added just prior to the start of the experiment. These media were osmotically adjusted so that cells with pH_i 6 to < 7, pH_i > 7 to 7.6, and pH_i > 7.6 were incubated in hypertonic (360 mOsm), isotonic (300 mOsm) and hypotonic (250 mOsm) media, respectively, at different pH_o (6 to 8).

In the Na^+/H^+ EXC experiments, the media was always hypertonic (360 mOsm) since the process was always measured in acid-loaded cells. When pH_i was changed (for titration experiments) then the osmolarity of the external media was adjusted accordingly as outlined above. The Na^+ influx media, for isotope studies, contained (in mM); 0 to 150 NaCl (substitution of NaCl by KCl), 20 KCl, 0.15 $MgCl_2$, 0.1 ouabain, 0.4 neptazane, 10 glucose, 40 sucrose, 10 Tris-MOPS (pH 7.4 at 37°C). For the H^+ efflux experiments unbuffered Na^+ and K^+ media were used. The K^+ media contained (in mM): 170 KCl, 0.15 $MgCl_2$, 0.1 ouabain, 0.4 neptazane, 10 glucose; in the Na^+ medium NaCl replaced KCl. The osmolarity of these solutions was adjusted between 360 and 320 mOsm by the addition of 40 mM sucrose when cells with pH_i < 7.0 were used.

F. Measurement of Na^+/Na^+ Exchange

Na^+/Na^+ EXC was measured as the difference in Na^+ efflux between Na^+ medium (150 mM) and Na^+-free K^+ medium (150 mM), i.e., as external Na^+-stimulated Na^+ efflux. Na^+ concentration of the media was measured by atomic absorption spectrophotometry. ^{22}Na-loaded cells, with the required internal Na^+ and pH_i, were suspended at 2% (Na^+ medium) and 4% hematocrit (K^+ medium) in 7 ml of ice-cold flux media as described in the section above. Following distribution of the cell suspensions into 4-ml tubes on ice, for no more than 10 min, Na^+ efflux was started by warming up the tubes to 37°C. Initial rates were determined by taking triplicate samples between 0 and 40 min every 5 min; these experiments indicated that Na^+ efflux was linear up to 20 min, but declined thereafter, especially in cells with low values of Na_i. To stop the transport reaction, the tubes were centrifuged for 2 min, 2000 × *g* at 4°C and the supernatant removed (0.8 ml) for radioactivity counting. The Na^+ efflux was calculated (mmol/liter cell × hr, flux unit = FU) from the specific activity of the loaded cells and the cpm of ^{22}Na appearing in the efflux media as a function of time (Canessa et al., 1986).

G. Measurement of Na^+/H^+ Exchange

1. *Na^+ Influx Driven by a H^+ Gradient*

Acid- and Na^+-loaded cells were suspended at 5% hematocrit in 4 ml of Na^+ influx medium containing ^{22}Na (1 μCi/ml). Na^+ influx was measured at pH_o 6.0 and 7.4. The red cells were added to the Na^+ influx media, preincubated at 37°C, and at 1, 6 and 11 min duplicate 200-μl aliquots were taken and placed in pre-cooled Eppendorf tubes containing 0.4 ml dibutyl phthalate oil and 0.7 ml influx wash solution. The samples were centrifuged at 14,000 × *g* for 15 sec and the supernatant carefully aspirated and the walls cleaned with cotton tips. The bottom of the tube, containing the cell pellet, was then removed by cutting and placed in 1 ml of 0.02% Acationox® detergent. The trapping of ^{22}Na was estimated by centrifuging cold suspension and it was in the order of 100 cpm. The lysates were centrifuged at 2000 × *g* for 5 min at 4°C and portions used for counting radioactivity (800 μl) and hemoglobin determinations (50 μl diluted 1/25 with 0.02% Acationox®). Fluxes were calculated (FU) from the specific activity of the influx media and the change in cpm/liter RBC as a function of time (Canessa et al., 1986).

2. *H^+ Efflux Driven by a Na^+ Gradient*

Proton extrusion rates were measured using a pH stat system (Metrohm/Brinkmann System, Westburg, NY). Na^+/H^+ EXC was estimated as the difference in H^+ efflux in Na^+ and K^+ efflux media prepared as described above. The pH of the flux media was adjusted, by addition of 0.02 N KOH, to equal the pH_i of the red cells. At the start of the experiment 200 μl of red cells (50% hematocrit) were added to 4 ml flux media in a water-jacketed chamber at 37°C under constant stirring. Immediately after the addition of the cells the pH was adjusted, by the addition of 0.02 N KOH by the titrator, to the set end point which in the majority of the experiments was 7.4. The amount of KOH added (volume in μl) per unit time to keep the pH_o at the set end point was recorded for at least 5 min. At the end of the experiment a portion of the suspension was taken for determination of hemoglobin to estimate the volume of RBC. Proton extrusion rates (FU) were calculated from the slope of the recordings (change in volume of KOH added per minute) taking into account the volume of red cells. A linear regression program was used to fit the best straight line through the experimental points. Problems with 'overshooting', i.e., pH_o exceeding the set end point, were avoided by adjustment of the dv/dt control (dosing rate) of the titrator.

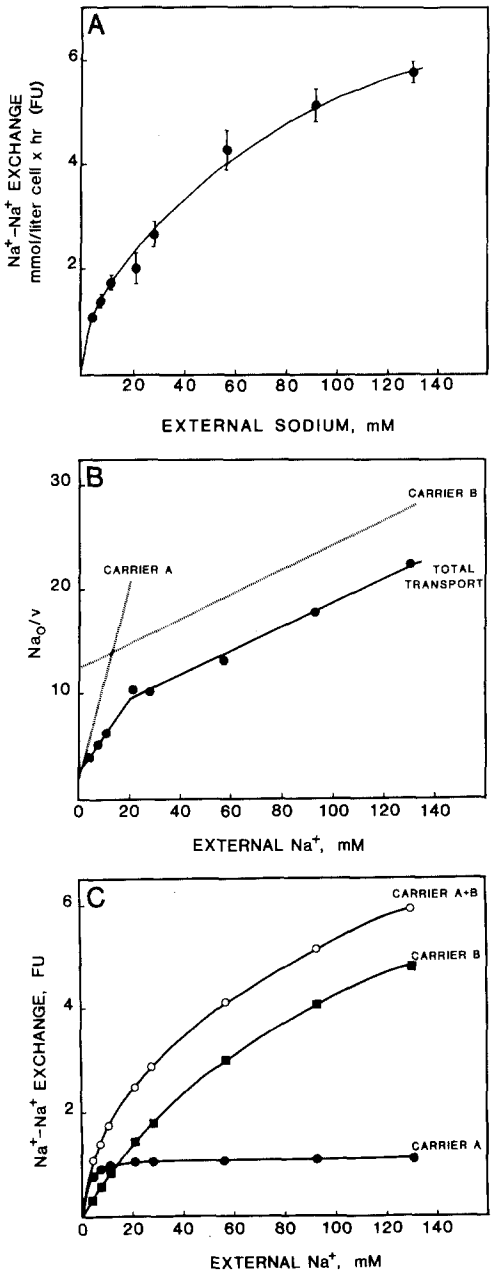

Fig. 1. Na^+/Na^+ exchange in rabbit red blood cells as a function of Na_o at pH_i = 7.4 = pH_o and Na_i 26 mM. (*A*) Measurements of Na^+ efflux (FU = mmol/liter cell · hr) were made into media containing (in mM): 150 (NaCl + KCl) to give external Na^+ 0 to 150 mM, 0.15 $MgCl_2$, 0.1 ouabain, 0.4 neptazane, 10 glucose and 10 Tris-MOPS (pH 7.4 at 37°C). Na^+/Na^+ exchange was calculated by subtracting Na^+ efflux into Na^+-free media from Na^+efflux into Na^+ media at each experimental point; values shown are mean ± SD of triplicate measurements, and the curve is representative of four other experiments performed in different rabbits. The shape of the curve is consistent with the presence of two saturable components of Na^+ efflux. (*B*) Hanes-Woolf plot of the dependence of Na^+/Na^+ exchange on Na_o (●—●) indicates that more than a single component is present since the line deviates from linearity. As explained in the text, fitting for two saturable components (dotted lines) for a model of two carriers/sites provided the best fit. Carrier/site *A* had V_{max} 1.14 ±0.58 FU and K_m 2.2 ± 3.7 mM and carrier *B* had V_{max} 8.8 ± 0.99 FU and K_m 108 ± 3.56 mM. Na^+/Na^+ exchange was measured at six Na_o below 20 mM but not all the points are drawn for the clarity of the figure. (*C*) Shows the calculated behavior of carrier/site *A* (●—●) and carrier *B* (■—■) as a function of Na_o; the curves were fitted using the kinetic constants given above which were obtained by successive approximations as described in section III A. The sum of these two components (○—○) gave calculated values that were tested for goodness of fit to the experimental values (*A*) and gave an X_2 = 0.118 value lower than a single carrier/site

III. Results

A. Activation of Na^+/Na^+ Exchange by External Na^+

The dependence of Na^+/Na^+ EXC in rabbit RBC on Na_o, under conditions of no pH gradient (i.e., pH_i = 7.4 = pH_o) and with Na_i fixed at 26 mM, is shown in Fig. 1*A*. Initial rates of ^{22}Na efflux were strongly dependent on the extracellular cation concentration. The efflux into Na^+-free medium at pH_o = 7.4 was around 1.0 mmol/liter cell · hr (FU = flux units); this value was subtracted from the flux at each of the Na_o concentrations in order to obtain the Na^+/Na^+ EXC component (i.e., Na_o-stimulated Na^+ efflux). To further confirm the presence of an exchange pathway, we also measured unidirectional Na^+ influx into Na_i-free cells and at Na_i 26 mM, Na_o 140 mM which gave values of 4.2 and 8.1 FU; furthermore, measurements of net Na^+ influx into both cells gave values of 4.5 FU. These data indicated that measurements of Na_o-stimulated Na^+efflux provide a better measurement of the Na^+/Na^+ exchange because diffusional Na^+ efflux into Na_o-free media is less than 10% of the total flux into Na^+media.

The shape of the velocity of Na^+/Na^+ exchange *versus* Na_o (Fig. 1*A*) appears to be biphasic; over Na_o 0 to 20 mM, there is a 'burst' of stimulation which thereafter decreases and gradually saturates at higher concentrations. If the velocity at low Na_o concentrations are omitted only a single low affinity component is detectable. This complex kinetic behavior would be observed if rabbit RBC Na^+/Na^+ EXC was being accomplished by any of the following models: (*i*) A single saturable system together with a diffusive component. (*ii*) A single saturable system with two sites of different affinity (site A and site B). (*iii*) Two different saturable systems, one a low capacity, high affinity system (carrier A), and the other a high capacity, low affinity system (carrier B).

Discrimination between rival mathematical models was fitted by nonlinear regression analysis to the experimental data (Mannervik, 1983). As indicated by Mannervik (1983), the following criteria was used to evaluate goodness of fit as well as discrimination for a good model: (*i*) convergence in the regression analysis; (*ii*) meaningful parameter values with low standard error; (*iii*) random distribution about zero levels of residuals with any dependent or independent variable; and (*iv*) low residual sum of squares compatible with the experimental variance.

A double-reciprocal plot (*not shown*) had significant bending demonstrating that more than one carrier or more than one site was present. The double-reciprocal plot (or Lineweaver-Burk plot) has a regression $y = 0.219\ (\pm 0.051) + 3.7\ (\pm 0.51)x$, $r = 0.9624$. The intercept (a) was significantly different from zero at the $P < 0.005$ level indicating that the presence of diffusion can be eliminated (Segal, 1975); this is in agreement with the definition of exchange as Na_o-stimulated Na^+ efflux which subtract Na^+ efflux into Na^+-free medium so as not to constitute another third component. The best fit for a Lineweaver-Burk plot gave a V_{max} of 7.56 ± 0.59 FU and a K_m of 43.6 ± 81 mM; furthermore, the residuals showed negative skewness. However, several texts of enzyme kinetics (Neame et al., 1972; Segal, 1975) consider that the double-reciprocal plot is the least reliable source of estimates of V_{max} and K_m because the plot tends to give large errors. We therefore analyzed the dependence of Na^+/Na^+ EXC on Na_o using a Hanes-Woolf plot (Fig. 1*B*). This type of analysis is preferred over the double-reciprocal plot because inversion of the data, without appropriate weighing, distorts the error span. A Hanes-Woolf plot (Na_o/v *versus* Na_o) of the data shown in Fig. 1 was obtained from the equation

$$\frac{Na_o}{v} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}} \cdot Na_o \qquad (1)$$

where Na_o was the external Na^+ concentration, v the measured flux of Na^+/Na^+ EXC, K_m represented the concentration of external Na^+ giving half-maximal flux and V_{max} was the maximum rate of flux. This plot yielded a line with a 'break' point (solid line, Fig. 1*B*) indicating that the efflux consists of more than a single component. With a single saturable component conforming to Michaelis-Menten kinetics this plot gives a straight line with a slope of $-1/K_m$ and a y intercept of K_m/V_{max}. The Hanes-Woolf plot of the total transport data (solid line, Fig. 1*B*) shows two distinct kinetic components. The regression for all points was $y = 5.29 + 0.137x$, $r = 0.9775$ yielding a $v_{max} = 38.5$ FU, K_m 7.29 mM, $r = 0.9775$ and a $X^2 = 163.4$, indicating that a model with a single site did not fit the experimental data ($P < 0.0001$). Then, we tested for the presence of two regressions ($y_1 + y_2 = y_t$). For Na_o concentrations between 4.5 and 21.4 mM, $y_1 = 2.29 \pm 0.209 + 0.3873 \pm 0.016388x$, $r = 0.9982$. For Na_o between 28.3 to 130 mM, $y_2 = 6.9 \pm 1.18 \pm 0.119 \pm 0.00156x$, $r = 0.9982$.The intercepts and slopes of the two regression lines were significantly different at the $P < 0.0001$ level. However, the Hanes-Woolf plot cannot be used to obtain kinetic constants for the individual carriers directly since the derived values from the linear estimates are incorrect; first, the data must be separated into its individual components.

Model 1, including a single saturable system and the presence of diffusion has already been eliminated because the double-reciprocal and Hanes-Woolf plots had intercepts at the ordinate significantly different than zero, indicating that the low affinity component is saturable (Segal, 1975). However, to determine the limits of the model fitting, we tested this model and obtained a diffusive component with a diffusion constant, $K_d = 0.018 \pm 0.09$; we then subtracted the 'diffusive' flux from the total flux and fit the remaining values to a single component in a double-reciprocal plot ($y = 0.3 + 3.3x$, $r = 0.982$) which gave V_{max} 3.99 ± 1.3 and $K_m = 18.9 \pm 10$ mM. The $X^2 = 0.229$ also provided a good fit of the model to the experimental data, but with substantially higher error than the model of two saturable sites ($X^2 = 0.119$ *versus* 0.229). The distribution of residuals exhibited larger error at low transport rates.

The presence of a single saturable transport system with two sites of different affinity appears to be favored by a Hill plot analysis (*see* Eq. (6), section IIID). Na^+/Na^+ EXC showed a curvilinear dependence on Na_o (*plot not shown*) with changes in slope and a n_{app} of 1.1 as expected for one system with two saturable sites with low cooperativity (Segal, 1975).

The possibility of two different saturable systems was examined using the kinetics of two carriers or two sites as described by Neame and Richards (1972) using the equation

$$v_{total}(v_A + v_B) = \underbrace{\frac{V_{max}A \cdot [S]}{K_mA + S}}_{\text{Carrier A}} + \underbrace{\frac{V_{max}B \cdot [S]}{K_mB + S}}_{\text{Carrier B}} \qquad (2)$$

where the v_{total} is the sum of the individual Michaelian components of Na^+ transported by carrier A plus carrier B. Using the procedure of successive approximations described by Spears, Sneyd and Loten (1971) reliable estimates of all four constants for

the two carriers/sites can be obtained provided that $K_mB > K_mA$ and $V_{max}B > V_{max}A$. First, a linear plot (Hanes-Woolf) was constructed from efflux measurements taken from Fig. 1*A* at nine Na_o concentrations above the approximate K_m of the low affinity component (carrier/site B, Na_o 50–130 mM). This plot gave a first estimate of $V_{max}B$ (8.9 ± 0.97 FU) and K_mB (68 ± 34.7 mM). The estimated constants were then used to calculate the efflux by carrier/site B at six different Na_o concentrations below the approximate K_m of the high affinity component (carrier A, Na_o 1–5 mM). The contribution of carrier B, in this region of Na_o, was then subtracted from the observed efflux and a Hanes-Woolf plot constructed from the corrected values. This plot gave a first estimate of $V_{max}A$ (0.73 ± 0.57 FU) and K_mA (1.3 ± 3.6 mM). These constants were in turn used to calculate the contribution of carrier A in the high Na_o region that was used to determine $V_{max}B$ and K_mB. The contribution of carrier A was subtracted from the observed efflux values, and the corrected values were plotted to obtain a second estimate of $V_{max}B$ (8.76 FU) and K_mB (93.5 mM). The better estimates of $V_{max}B$ and K_mB were then used to correct the observed efflux at low Na_o, and the corrected values were plotted to obtain a second (and closer) estimate of $V_{max}A$ (1.03 FU) and K_mA (1.9 mM). The entire procedure was then repeated for a third time and these values are reported. In Fig. 1*B*, the Hanes-Woolf plot of the measured total transport (solid line) is compared with calculated (dotted lines) parameter for the two carriers/sites (A and B). Figure 1*C* gives the calculated behavior of carrier/site A with a V_{max} of 1.14 ± 0.58 FU and K_m 2.2 ± 3.76 mM and carrier/site B having V_{max} 8.8 ± 0.00 FU and K_m 108 ± 3.56 mM (Fig. 1*C*). A best fit calculated using Enzyfitter computer program gave the following values: $V_{max}A$ = 2.62 ± 0.57, K_mA 6.18 ± 3.72, $V_{max}B$10.1 ± 0.97, K_mB 83.9 ± 3.47 with less standard error than Model 1. Thus, standard error of these parameters are between 4–8% while in Model 1, the adjusted parameters exhibit a 50% error. The distribution of residuals was analyzed weighing the larger experimental error of high fluxes and showed random distribution. Testing the two carriers/sites (open circles, Fig. 1*C*) of Model 1 against the experimental values shown in Fig. 1, it yielded a X^2 test for the goodness of fit of 0.118, indicating that it did not differ significantly from the Model and provided the minimum error in comparison with other models.

B. Effect of External H^+ on Na^+/Na^+ Exchange

The external Na^+-sites were titrated between pH_o 5.8 and 8.1 by measuring Na^+ efflux from cells with pH_i 7.35 and Na_i 23 mM into Na^+-rich (150 mM) and Na^+-free (0 mM) media, i.e., same intracellular conditions as for Fig. 1 but at different pH_o. As shown in Fig. 2*A*, net Na^+ efflux into the Na^+-free medium was independent of pH_o (approx. 1 FU) while the efflux into 150 mM Na^+-medium was markedly inhibited as pH_o decreased. The Na^+/Na^+ EXC component was obtained by subtracting these two values (Fig. 2*A*, squares) and thereby estimating the transport rate of a loaded carrier. As can be seen, Na^+ transport decreased monotonically with acid pH_o and at pH_o 5.8 there was still 1.8 FU of Na^+/Na^+ EXC, and it predicts that at pH_o 5.4 Na^+/Na^+ EXC will be negligible. From a Dixon plot (Fig. 2*B*) according to the equation

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \frac{(1 + H_o)}{K_H} \cdot \frac{1}{Na_o} \qquad (3)$$

where v = Na^+/Na^+ exchange rates; V_{max} = maximal rate; K_m = concentration for half-maximal activation; and K_H = inhibitor constant

the apparent pK of inhibition (half-maximal inhibitory concentration for H_o) for pH-sensitive Na^+/Na^+ EXC was found to be 6.55 (negative x intercept).

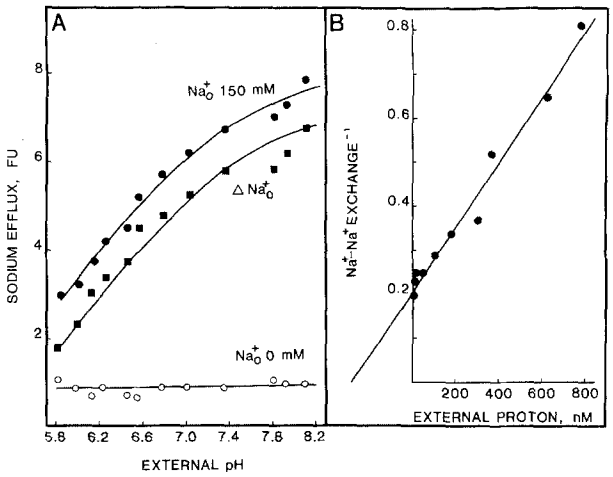

Fig. 2. The effect of pH_o on Na^+/Na^+ exchange in rabbit red cells at pH_i 7.35 and Na_i 23 mM. (*A*) shows the Na^+ efflux into Na^+-free media (○—○) and into 150 mM Na^+ media (●—●). The difference between the two curves (ΔNa_o) represents the Na^+/Na^+ exchange component (■—■). External H^+ inhibits Na^+ efflux into Na^+ but not Na^+-free media. Note there is a component of Na^+/Na^+ exchange still remaining at pH_o 5.8 which is named pH insensitive. Every point represents the mean of three experiments and the standard deviation has the size of the dots. (*B*) Shows a Dixon plot of the pH-sensitive Na^+/Na^+ exchange; these values were obtained by subtracting the pH-sensitive component from the exchange values shown in *A* (●—●). The plot gave a pK for external H^+ of 285 nM (pH 6.55) for inhibition of Na^+/Na^+ exchange

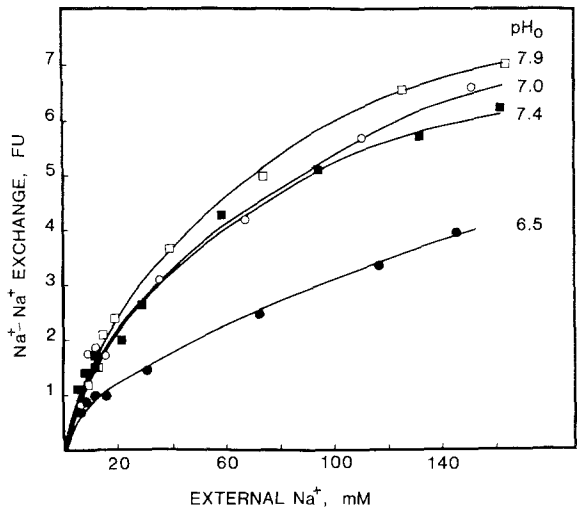

Fig. 3. Na^+/Na^+ exchange in rabbit red blood cells as a function of external Na^+ and H^+. The cells contained 26 mM Na^+ and had an pH_i of 7.4. The composition of the efflux media was identical to that used in Fig. 1; for $pH_o < 7.0$, 10 mM Tris-MES replaced 10 mM Tris-MOPS. Every curve represents a minimum of three experiments in one rabbit (*see* Table 4), being the coefficient of variation less than 5%. There was no significant differences between the curves at pH_o 7.4 and 7.0 below 100 mM Na_o

C. Kinetic analyses of the Interactions Between External H^+ and Na^+ on Na^+/Na^+ Exchange

The data presented in Fig. 1*A* and *B* shows that rabbit RBC Na^+/Na^+ EXC has two components with high (carrier A) and low affinities (carrier B) for Na_o, and Fig. 2 shows that the overall process can be inhibited by H_o. The next series of experiments were performed to determine the kinetics of interaction between external Na^+ and H^+ for each of these components. Na^+/Na^+ EXC was measured, with Na_i fixed at 26 mM and pH_i 7.4, as a function of both extracellular Na^+ and H^+ concentrations.

Figure 3 shows the effect of increasing Na_o on Na^+/Na^+ EXC measured in media with pH_o 6.5, 7.0, 7.4 and 7.9. The Na^+ fluxes at pH_o 7.0 and 7.4 were indistinguishable below 100 mM Na_o. The efflux was split into its two components (as outlined in section IIIA) and the kinetic interactions between Na^+ and H^+ were determined for carrier A (high affinity) and carrier B (low affinity). Figure 4*A* shows the interaction for the low affinity component analyzed by Hanes-Woolf plots; the negative intercept with the abscissa represents the K_m and the reciprocal of the slope represents V_{max} according to Eq. (1). As H^+ concentration was increased from 13 to 100 nM (pH 7.9 to 7.0) the K_m increased from 56 to 138 mM. The maximal exchange fluxes were similar between pH_o 7.9 and 7.0 as represented by the standard errors of the

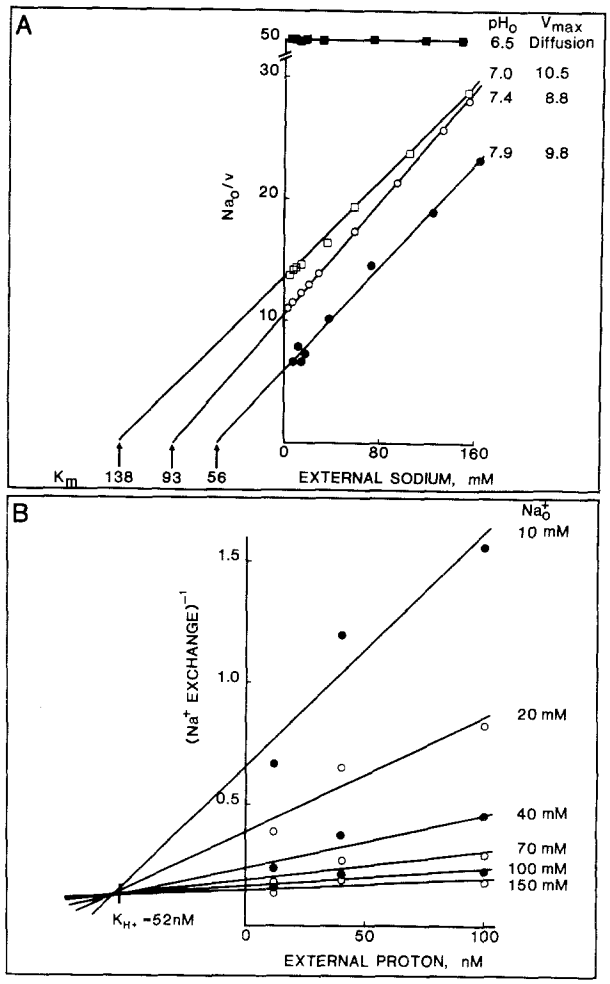

Fig. 4. (*A*) The effect of external H^+ on the K_m for external Na^+ of Na^+/Na^+ exchange performed by carrier/site *B*. Hanes-Woolf plot of the data is shown in Fig. 3. pH_i 7.4 and internal Na^+ 26 mM. Every point represents the mean ± SD of three experiments and the size of the dot gives the SD. The V_{max} of Na^+/Na^+ exchange remained fairly constant but the K_m for Na_o increased from 56 to 93 mM, indicating that H_o are competitive inhibitors. (*B*) Shows a Dixon plot of the data shown in Fig. 3. The point of intersection of the lines at different Na_o gives the K_H for inhibition of Na^+/Na^+ exchange (52 nM, pH 7.3) and the V_{max} real (7.7 FU) at the low affinity Na_o site. The curve were constructed using measured values of Na^+/Na^+ EXC or extrapolated values between two concentrations at a given pH_o

slopes of the three linear regression estimates. At pH_o 6.5, the low affinity component was not distinguishable from diffusion (shown as filled squares in Fig. 4*A*). The results of additional experiments are summarized in Table 1. The observation that H_o increases the K_m for Na_o but does not affect V_{max} indicates that H_o are competitive inhibitors of carrier/site B. The inhibitor constant for H^+ (K_H) for this component was determined from a Dixon plot, according to Eq. (3), as shown in Fig. 4*B*. The coordinates at the point of intersection

Table 1. Summary of the kinetic interactions of external Na^+ and H^+ at the external Na^- sites of the Na^+/Na^+ exchanger

pH_o	n	External Na^+ sites			
		Carrier/site A high affinity component		Carrier/site B low affinity component	
		V_{max} (FU)	K_m (mM)	V_{max} (FU)	K_m (mM)
7.9	1	Not detectable		9.8	56
7.4	3	1.0 ± 0.2	3.9 ± 3.4	9.7 ± 1.6	95 ± 12
7.0	1	1.1	1.8	10.5	138
6.5	4	1.6 ± 0.4	6.7 ± 2.2	Diffusion	

Cellular pH 7.4 and Na^+ 26 mM. Maximal velocity (V_{max}, mmol/liter cell · hr = FU) of Na^+/Na^+ exchange and K_m, the apparent half-saturation constant for external Na^+. Values shown are mean ± SD obtained in n number of rabbits in triplicate. Results depicted in Figs. 3–5 were obtained in red cells from a single rabbit.

represent $-K_H$ (52 nM, pH 7.3) and $V_{max}real^{-1}$ (7.7 FU), respectively.

The interactions of H^+ with the high affinity component of Na^+/Na^+ EXC (carrier/site A) are also summarized in Table 1. At pH_o 7.9 the high affinity component was not detectable. At all other pH_o values, the kinetic parameters for carrier A were not significantly different from each other, indicating that this process was insensitive to pH_o.

Thus, Na_o and H_o compete at the low affinity component of Na^+/Na^+ EXC (carrier/site B) but have no detectable effect on the high affinity component (carrier/site A) considering its large error (K_m = 3.9 ± 3.4 mM at pH 7.4). Because, we previously predicted that at pH_o 5.4, there is no Na^+/Na^+ EXC, most likely the high affinity Na^+ carrier/site may have a K_H substantially less than pH 6.5.

D. Activation of Na^+/Na^+ Exchange by Internal Na^+

In this set of experiments, pH_i and pH_o were fixed at 7.4 and Na^+ efflux into 0 and 150 mM external Na_o media measured varying internal Na^+ concentration; as previously stated, the differences in Na^+ efflux between the two media represented the countertransport component. Figure 5*A* shows the efflux of Na^+ into Na^+-free (0 mM, squares) and Na^+-rich (150 mM, circles) media. As Na_i increased, Na^+ efflux was stimulated into both media. Na^+ efflux into Na^+-free media did not follow simple diffusion kinetics (Fig. 5*A*, squares) but it was sigmoidally activated by Na_i; to account for this property, we henceforth designate Na^+ efflux into Na-free media as 'uncoupled' Na^+ efflux. The dependence of this flux component could be fitted by the equation:

$$v = \frac{V_{max}}{(1 + K_{Na}/Na_i)^n} \tag{4}$$

where V_{max} is the maximum Na^+ efflux, K_{Na} the apparent dissociation constant and n the number of internal Na^+ sites. Rearrangement of this equation as described by Garay and Garrahan (1973) gives:

$$\frac{Na_i}{v^{1/n}} = \frac{K_{Na}}{V_{max}^{1/n}} + \frac{Na_i}{V_{max}^{1/n}}. \tag{5}$$

A plot of $Na_i/v^{1/n}$ against Na_i yields a straight line with slope $V^{1/n}$ and negative intercept with the abscissa of $-K_{Na}$. A better fit was obtained with n = 3, as opposed to n = 2, indicating that occupancy of more than one Na_i site or more than a single conformational state is required for Na^+ translocation; the calculated K_{Na} was 42 mM.

We also analyzed the dependence of Na^+ efflux into Na^+-free media by the Hill equation:

$$\frac{v}{(V_{max} - v)} = \frac{(Na_i)^n}{K_m}. \tag{6}$$

A plot of $\log(v/V_{max} - v)$ *versus* $\log Na_i$ yields a straight line with slope equal to n_{app} (Hill coefficient) and intercept with the x axis of $\log K_m$ Na_i. The n_{app} was 2.2 (*plot not shown*), indicating strong positive cooperativity for Na_i. Uncoupled Na^+ efflux had a V_{max} of 12 FU and a K_m of 60 mM.

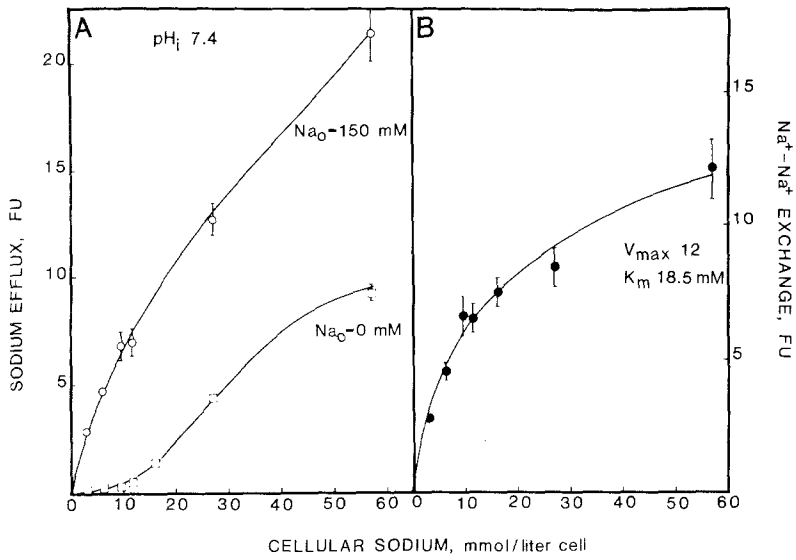

Fig. 5. The effect of internal Na^+ on Na^+/Na^+ exchange at $pH_i = pH_o = 7.4$. (*A*) Shows Na^+ efflux (FU, flux units) into 150 mM (O—O) and 0 mM (-□-□-) Na_o media. The efflux of Na^+ into Na^+-free media was sigmoidally dependent on Na_i. Hill plot analysis of the Na^+ efflux indicated a K_m for Na_o of 60 mM and a Hill coefficient (n_{app}) of 2.2. (*B*) Shows the dependence of Na^+/Na^+ exchange, estimated as Na_o-stimulated Na^+ efflux, on internal Na^+ (●—●). The SD of the flux measurements had the size of the dots. A Hanes-Woolf plot gave a V_{max} for Na^+/Na^+ exchange of 12 FU and a K_m for Na_i of 18.5 mM

Na_o-stimulated Na^+ efflux (Fig. 5*B*) showed saturation kinetics to give a V_{max} of 12 FU and a K_m of 18.5 mM for the internal Na^+ site. Mean values for K_m 19.7 ± 1.5 mM and V_{max} 11.8 ± 2.6 FU were obtained in three different rabbits. These findings also indicate that in the absence of Na_o, the unloaded carrier can outwardly transport Na^+ but with a high K_m (60 mM). If this flux component is part of the same system performing exchange diffusion, it appears, therefore, that Na_o decreases the K_m for Na_i from 60 to 18.5 mM.

E. Effect of Internal H^+ on Na^+/Na^+ Exchange

To determine the effect of H_i on Na^+/Na^+ EXC and the interaction of H_i with Na_i sites, Na^+ efflux was measured into Na^+ and K^+ media in cells with different Na_i and pH_i fixed at 6.6 (Fig. 6; these values were then compared with those obtained at pH_i 7.4 (Fig. 5*A* and *B*). Na^+ efflux into the Na^+-free media again did not follow diffusion kinetics but it had sigmoidal dependence on cell Na^+ (Fig. 6*A*). Kinetic analysis of these data according to Eq. (5) gave a V_{max} of 15 FU and K_{Na} 20 mM; from Eq. (6), a K_m for Na_i of 43 mM and n_{app} 2.0 were estimated.

As shown in Fig. 6*B* at pH_i 6.6 (filled circles) the kinetics of Na^+/Na^+ EXC clearly became sigmoidal and V_{max} increased to 21 FU, while at pH_i 7.4 Na^+/Na^+ EXC followed Michaelis-Menten kinetics (Fig. 5*B*) with a V_{max} of 12 FU. Thus, in contrast to the H_o inhibition of Na^+/Na^+ EXC (Figs. 2–4), H_i behaves as an allosteric activator of this ion transport pathway. Hill plots of the dependence of Na^+/Na^+ EXC from Na_i according to Eq. (6) (*plot not shown*) indicated that acid pH_i (6.6) increased the K_m for Na_i from 18 to 52 mM and n_{app} from 0.99 to 2.8. Furthermore, the modifier effect of H_i on Na^+/Na^+ EXC appears to be dependent on the concentration of Na_i; at low Na_i (<35 mmol/liter cell), H_i inhibits while at higher Na_i, H_i stimulates. The shape of the curves shown in Figs. 5*B* and 6*B* can be explained by competition of H_i for outwardly transporting Na^+ sites (Smith, Roberts & Kuchel, 1975). The sigmoidal activation of Na^+/Na^+ EXC at acid pH_i 6.6, as reflected by n_{app} 2.8, can be accounted for by the presence of more than one Na_i sites (Eq. (5)) or by multiple conformational states of the transporter to which internal Na^+ can bind at a single site (Segal, 1975). The apparent dissociation constant for Na_i was calculated to be 18 mM. Thus, an increase of H_i, decreases the affinity of Na^+/Na^+ EXC for Na_i and increases its V_{max}.

F. pH Titration of Internal Na^+ Sites of Na^+/Na^+ Exchange

The data presented in Fig. 6*B* demonstrate that the allosteric modifier effect of H_i on Na^+/Na^+ EXC depends on the Na_i status of the red cell. For this reason internal Na^+ site(s) were titrated at both 28 mM (Fig. 7) and 83 mM Na_i (Fig. 8); under these conditions, Na^+/Na^+ EXC is inhibited and stimulated by H_i, respectively. Throughout all these experiments, pH_o was set at 7.4. Figure 7 shows the titration (pH_i 5.6 to 8.0) of the H_i inhibitory effect in cells containing 28 mM Na_i. Na^+ efflux into Na^+-rich media was inhibited by increasing H_i whereas

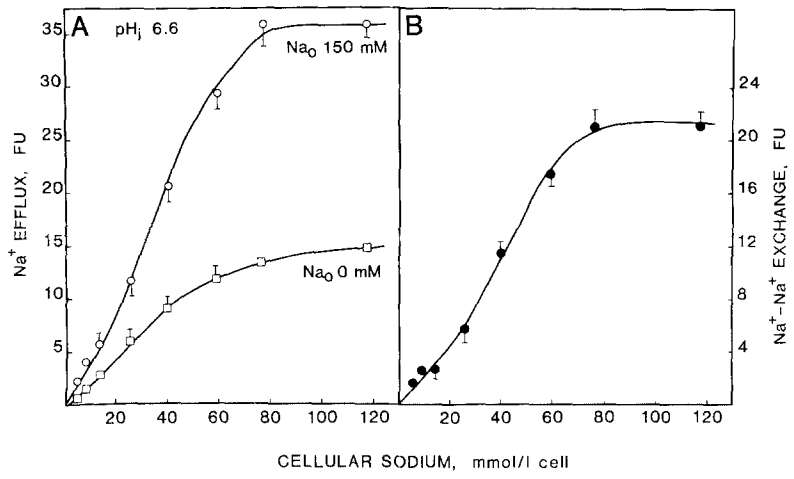

Fig. 6. The effect of internal Na^+ on Na^+/Na^+ exchange at pH_i = 6.6; pH_o = 7.4. (*A*) Shows the dependence on Na_i of Na^+ efflux into 150 mM (○—○—○) and 0 mM (□—□) Na_o media. As observed at pH_i 7.4 (Fig. 5) Na^+ efflux into Na-free media was sigmoidally activated (n_{app} = 2.2) by Na_i to give a V_{max} of 15 FU and a K_m for Na_i of 43 mM. Every point represents the mean of three experiments and the size of the dot gives the SD of the flux. (*B*) Shows the dependence on Na_i of Na^+/Na^+ exchange as Na_o – stimulated Na^+ efflux. At pH_i = 6.6, Na_i sigmoidally activated Na^+/Na^+ exchange (●—●) to give a V_{max} of 21 FU

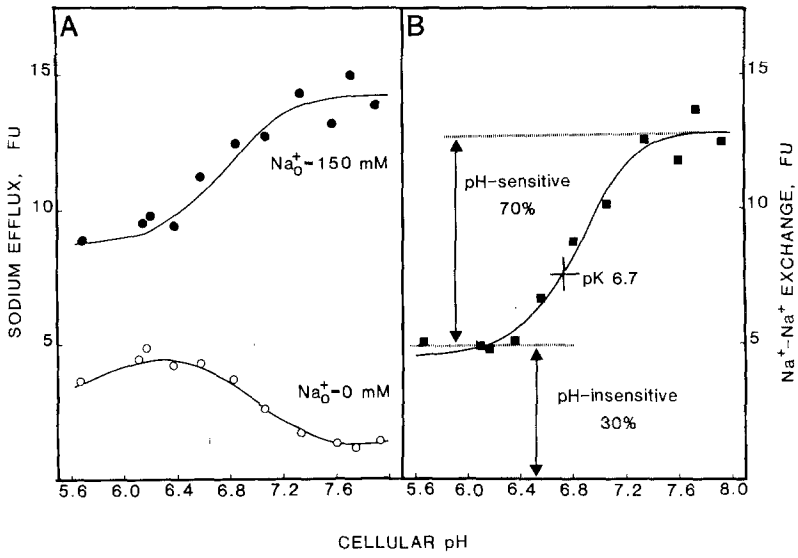

Fig. 7. The effect of internal H^+ of Na^+/Na^+ exchange at low Na_i: titration of the H^+ inhibitory site. (*A*) Shows Na^+ efflux as a function of pH_i into 150 mM (●—●) and 0 mM (○—○) Na^+ media. The pH_o was set at 7.4 and the red cells had an Na_i of 28 mM. Every point represents the mean of three experiments and the size of the dot gives the SD of the flux. (*B*) Shows Na^+/Na^+ exchange (■—■) over the pH_i range 5.6 to 8.0. The Na^+/Na^+ exchange was divided into pH-sensitive and pH-insensitive components (ratio 7:3). With Na_i 28 mM, the pH_i-sensitive component of Na^+/Na^+ exchange was sigmoidally inhibited by pH_i with pK 6.7

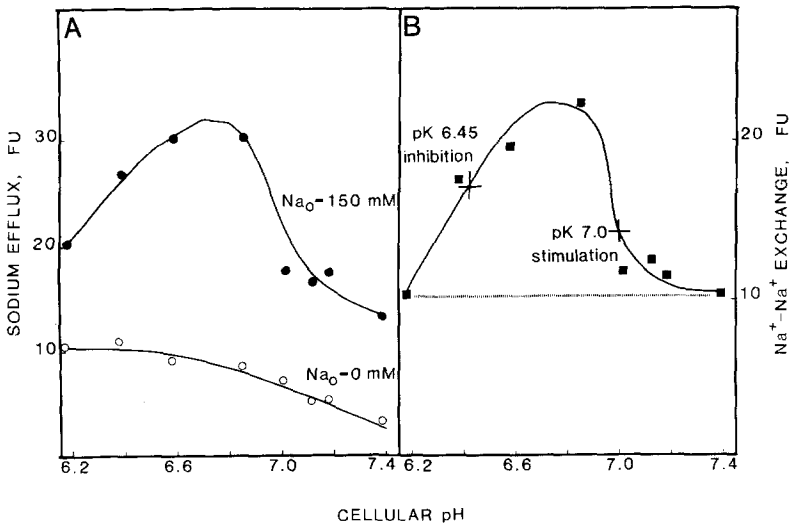

Fig. 8. Titration of the H_i stimulatory site of Na^+/Na^+ exchange. (*A*) Shows Na^+ efflux into Na^+-free media (○—○—) and 150 mM Na^+ (●—●) as a function of pH_i; the red cell Na^+ was 83 mM and the pH_o 7.4. Every point represents the mean of three experiments and the dot size gives the SD. (*B*) Shows that the dependence of Na^+/Na^+ exchange (□—□) on cell pH has a 'bell-shape' form with pK 7.0 for stimulation and pK 6.45 for inhibition

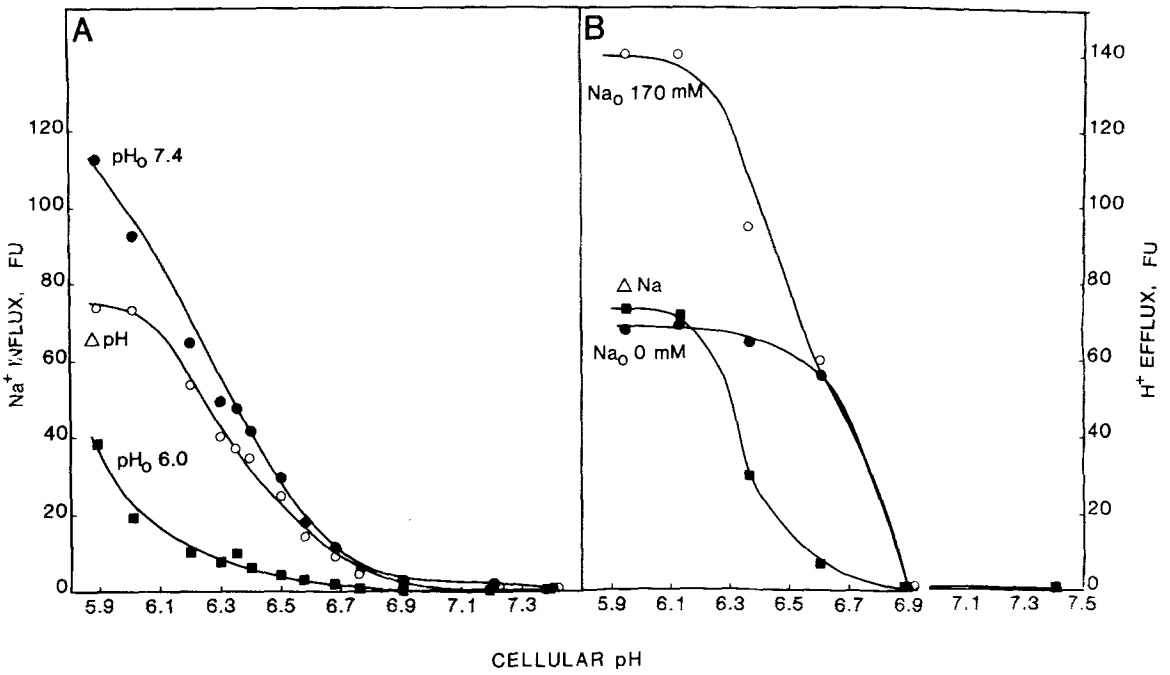

Fig. 9. Activation of Na^+/H^+ exchange by cell pH in Na^+-depleted cells; the measured Na_i was 1 mM. Every point is the mean of three experiments and the size of the dot gives the SD. (*A*) Depicts Na^+ influx measured at pH_o 7.4 (●—●) and pH_o 6.0 (■—■); the difference between the two curves (○—○) is a measure of H^+-driven Na^+ influx (Na^+/H^+ exchange). The V_{max} was 74 FU and the pK 6.4. (*B*) Shows H^+ efflux measured into 170 mM (—○—○) and 0 mM (■—■) Na^+ media. The difference (●—●) represents the Na^+-driven H^+ efflux. The V_{max} was 72 FU and the pK 6.3. A Hill plot analysis of the cell pH dependence of Na^+/H^+ exchange gave a n_{app} = 2.9 for H_o-driven Na^+ influx and 2.7 for Na_o-driven H^+efflux and pK's of 6.4 and 6.3, respectively

uncoupled Na^+ efflux was sigmoidally activated (Fig. 7*A*); when these two values were subtracted, the Na^+/Na^+ EXC component (Fig. 7*B*) was found to be sigmoidally inhibited from 12 to 5 FU, as pH_i decreased from 8 to 5.6, with a pK of 6.7. The Na^+/Na^+ EXC persisting at $pH_i < 6.0$ was found to be dependent on Na_i; at 28 mM Na_i there was 5 FU (Fig. 9*B*) and at 12 mM Na_i there was 1.5 FU (*data not shown*).

In contrast to the H_i inhibition of Na^+/Na^+ EXC at low Na_i, H_i has a stimulatory effect at higher Na_i values. The titration (pH_i 6.2 to 7.4) of the H_i stimulatory effect, in cells with Na_i 83 mM, is shown in Fig. 8*A* and *B*. This time, Na^+ efflux into Na media was 'bell shaped' and again the Na^+ efflux into Na-free media was stimulated as pH_i decreased (Fig. 8*A*). The Na^+/Na^+ EXC component (Fig. 8*B*) reached maximum values at pH_i 6.8 and it had a pK for activation of 7.0 and 6.45 for inhibition; at pH_i values less than this, Na^+/Na^+ EXC was inhibited and eventually returned to the flux observed at 7.4 when pH_i was 6.2. The interactions of H_i and Na_i with red cell Na^+/Na^+ EXC and uncoupled Na^+ efflux are summarized in Table 2.

G. The Effects of Inhibitors on Na^+/Na^+ Exchange

In rabbit RBC, at $pH_i = pH_o = 7.4$, no more than 10% of Na^+/Na^+ EXC was sensitive to 125 μM DIDS; because this inhibition occurred only at high DIDS concentration it might not reflect interaction with the anion exchanger. However, uncoupled Na^+ efflux was 50% inhibited by DIDS, suggesting that the unloaded carrier facing external sites might have amino-reactive sites. Phloretin, at 100 μM, was found to partially inhibit the Na^+/Na^+ EXC (25%). Under all the experimental conditions tested (i.e., different Na_i and pH_i) dimethyl-amiloride (DMA) was found to have no effect on the Na^+/Na^+ EXC component up to 100 μM. However, uncoupled Na^+ efflux stimulated by both Na_i and pH_i was inhibited 20 to 40% by 20 μM DMA (Table 3).

H. Effects of Internal Protons on Na^+/H^+ Exchange

Na^+/H^+ EXC was determined in acid-loaded rabbit red cells as Na^+ influx driven by an outward H^+

Table 2. Summary of the kinetic interactions of internal Na^+ and H^+ at the internal Na^+ sites for Na^+/Na^+ exchange and Na^+ efflux into Na^+-free media

A. Titration by Na_i	pH_i 7.4	pH_i 6.6
I. Na^+/Na^+ exchange (Na_o = 140 mM)		
V_{max} (FU)	11.8 ± 2.6	22.8 ± 3.7
K_m (mM) for Na_i	19.7 ± 1.5	55.3 ± 11.4
Hill coefficient (n_{app})	0.99 ± 0.01	2.8 ± 0.2
V_{max}/K_m ratio	0.60	0.41
II. Na^+ efflux at Na_o = 0 mM		
V_{max} (FU)	10.7 ± 1.5	14.0 ± 1.4
K_m (mM) for Na_i	56.0 ± 5.7	41.0 ± 3.2
n_{app}	2.2 ± 0.2	2.0 ± 0.1
V_{max}/K_m ratio	0.19	0.34
B. Titration by H_i	**Na_i < 57 mM**	**Na_i > 57 mM**
I. Na^+/Na^+ exchange (Na_o = 140 mM)		
Inhibition by H_i (pK)	6.7	6.5
Activation by H_i (pK)	—	7.0
II. Na^+ efflux at Na_o = 0 mM		
Activation by H_i (pK)	7.0	7.0

Values are mean ± SD from three separate experiments. pH_o 7.4. Results shown in Figs. 5 and 6 were obtained in one rabbit and are typical of that seen in the other two determinations.

Table 3. The effect of transport inhibitors on rabbit red cell Na^+/Na^+ exchange

	Cell Na^+ (mM)	Na^+ efflux (FU)			% Inhibition
		Na_o (0 mM)	Na_o (150 mM)	ΔNa_o Na^+/Na^+ EXC	
A. pH_i 7.4					
None	48	1.8 ± 0.1	10.0 ± 0.1	8.2 ± 0.2	—
+DIDS		0.8 ± 0.01	8.2 ± 0.5	7.3 ± 0.5	10
None	48	1.8 ± 0.1	10.0 ± 0.1	8.2 ± 0.2	—
+phloretin		1.2 ± 0.1	7.3 ± 0.6	6.1 ± 0.6	26
None	83	3.7 ± 0.1	13.7 ± 0.8	10.0 ± 0.8	—
+DMA		2.7 ± 0.2	13.0 ± 0.7	10.3 ± 0.7	0
B. pH_i 6.6					
None	28	4.9 ± 0.3	11.2 ± 0.6	6.3 ± 0.6	—
+DMA		3.6 ± 0.2	10.3 ± 0.6	6.6 ± 0.6	0
None	123	13.9 ± 1.0	33.3 ± 1.0	19.4 ± 1.4	—
+DMA		8.9 ± 0.1	30.9 ± 0.9	19.2 ± 0.9	0

Results shown are mean ± SD of three experiments. FU = mmol/liter cell · hr. The inhibitor concentrations were 125 μM DIDS, 100 μM phloretin, and 20 μM dimethyl-amiloride (DMA).

gradient (ΔpH_o) and as H^+ efflux driven by an inward Na^+ gradient, (ΔNa_o). Red cells depleted of internal Na^+ were acid loaded to pH_i between 7 and 6.0 and initial rates (Δt of 10 min) of net Na^+ influx determined at pH_o 6 and 7.4 (Fig. 9*A*). Na^+ influx increased steeply when pH_i decreased below 7.0 with the media at pH_o 7.4 and 6.0. The quiescent point of Na^+/H^+ EXC was selected at pH_o 6.0 because under such conditions, Na_o did not stimulate H^+ efflux (*data not shown*). The difference (ΔpH_o) between the two curves provided an estimation of H^+-driven Na^+ influx. As H_i increased from 25 to 1000 nM (pH 7.6 to 6.0), Na^+/H^+ EXC was sigmoidally activated. A V_{max} of 74 FU was observed at pH_i 6.0. From a Hill plot (*not shown*) of the dependence of H^+-driven Na^+ influx on cell pH (according to Eq. (6)) a K_m of 416 nM and a n_{app} of 2.9 were calculated.

Table 4. The effect of cell Na^+ and DMA on rabbit red cell Na^+/H^+ exchange

A. Cell Na^+ (mM)	Cell pH	Na_o (mM)	H^+ efflux (FU)	+DMA	δDMA
2	6.5	0	87.3 ± 6.6	74.5 ± 4.2	12.8 ± 5.5
		170	98.5 ± 5.7	76.4 ± 1.5	22.1 ± 4.2
		ΔNa	11.2 ± 5.5	None	9.3 ± 4.9
10	6.5	0	94.0 ± 6.1	89.0 ± 6.0	5.0 ± 6.1
		170	114.5 ± 12.5	83.5 ± 4.2	31.0 ± 9.3
		ΔNa	20.5 ± 5.0	None	26.0 ± 7.9
B. Cell Na^+ (mM)	Cell pH	pH_o	Na^+ influx (FU)	+DMA	δDMA
16.5	6.5	6.0	16.1 ± 0.8	11.9 ± 1.0	4.2 ± 1.2
		7.4	25.5 ± 2.5	9.9 ± 0.9	15.6 ± 2.7
		ΔpH_o	9.4 ± 2.6	None	11.4 ± 3.0
57	6.5	6.0	24.0 ± 1.1	23.5 ± 1.7	0.5 ± 2.0
		7.4	32.7 ± 2.0	33.1 ± 1.7	0
		ΔpH_o	8.7 ± 2.0	9.6 ± 1.5	—

Results shown are mean ± SD of three experiments of H^+ efflux and Na^+ influx. DMA, 20 μM; FU = mmol/liter cell · hr. In section A, pH_o = 7.4. In section B, Na_o = 150 mM.

H^+ efflux was also measured under pH-stat (7.4) in cells prepared as for Na^+ influx. Figure 9*B* shows the dependence of H^+ efflux on cell pH in the absence and presence of external Na^+; H^+ efflux increased sigmoidally with acid cell pH in K^+ media and Na^+ media. The difference between the two curves estimated the Na_o-driven H^+ efflux (Fig. 9*B*). V_{max} (72 FU) was also reached at pH_i 6.0. From a Hill plot (*not shown*) a K_m for H_i of 549 nM and n_{app} of 2.7 were obtained. These data provide experimental evidence for an electroneutral exchange process considering that the stoichiometric ratio between ΔpH_o-driven Na^+ influx and ΔNa_o-driven H^+ efflux is very close to 1 : 1.

As shown in Table 4, greater than 75% of Na^+/H^+ EXC was DMA sensitive (20 μM). In contrast, to the behavior of Na^+/Na^+ EXC, a substantial (5–10%) component of H^+ efflux into Na-free media was inhibited by DMA (Table 4,A).

I. The Effect of Internal Na^+ on Na^+/H^+ and Na^+/Na^+ Exchanges

Under conditions when Na^+/Na^+ EXC was inhibited by acid pH_i and low Na_i (Fig. 7*B*), Na^+/H^+ EXC was activated (Fig. 9*A* and *B*). These findings raised the possibility that Na^+/Na^+ EXC could be operating in a Na^+/H^+ EXC mode if the H_i inhibition of Na^+/Na^+ EXC resulted in H^+ transport rather than just simple inhibition. If this were correct, it would be predicted that at a 'crossover' point (between pH_i 6.4 to 6.6), alterations in Na_i should modify both processes such that when one was activated the other would be inhibited and vice versa.

To test this hypothesis, the effect of cell Na^+ on Na_o-driven H^+ efflux was studied at pH_i 6.5 (Table 4, A). An increase in cell Na^+ from 2 to 10 mM increased Na^+/H^+ EXC (Na_o-dependent H^+ efflux) from 11.2 to 20.5 FU. In this experiment, Na_o-dependent H^+ efflux was completely inhibited by 20 μM DMA, while in another was only 80% inhibited; however, the large coefficient of variation (10%) of Na_o-independent H^+ efflux makes difficult to detect a DMA-insensitive component of less than 3 FU. In subsequent experiments, Na^+/Na^+ and Na^+/H^+ exchanges were measured at pH_i 6.4 in cells with 2 and 10 mM Na_i. The results of these experiments are shown in Fig. 10*A*. At acid pH_i (6.6 and below) an increase in Na_i from 2 to 10 mM results in an increase of Na^+/Na^+ EXC from undetectable (<0.2 FU) to 2.5 FU. A similar increase of cell Na^+, at pH_i 7.4, increases Na^+/Na^+ EXC from <1 to 6 FU (Fig. 5*B*, filled circles). Thus, the pH_i-inhibited Na^+/Na^+ EXC was 3.5 FU (6–2.5). The increase in cell Na^+ also resulted in stimulation of Na^+/H^+ EXC from 11.2 to 20.5 FU (Fig. 10*A*). Therefore, the Na_i-stimulated Na^+/H^+ EXC (9.3 FU) is greater than the pH_i-inhibited Na^+/Na^+ EXC (3.5 FU); from these data, the presence of an internal Na^+ modifier site on the Na^+/H^+ exchanger can be inferred or alternatively that the two reactions (Na^+/Na^+ and Na^+/Na^+ EXCs) may occur on the same transporter but with different rate-limiting steps. It appears that the K_m

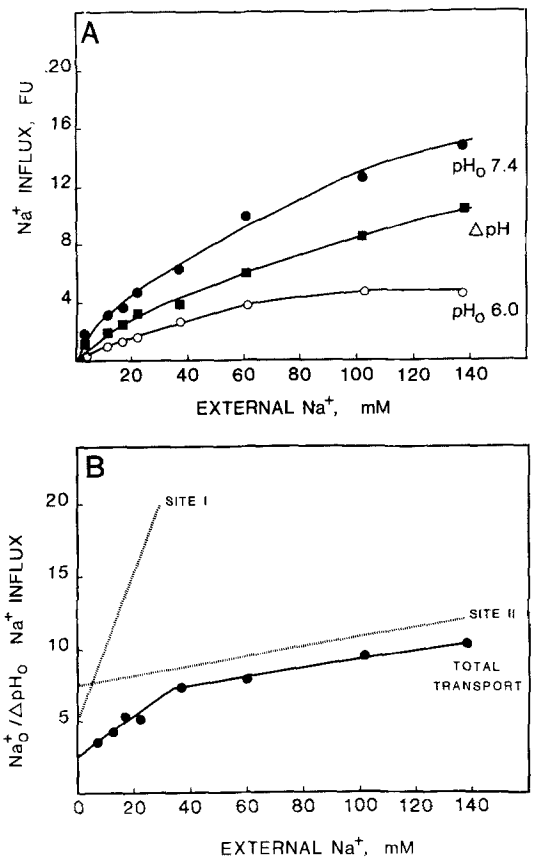

Fig. 10. H^+-driven Na^+ influx as a function of Na_o into acid-loaded and Na^+-depleted cells. (*A*) Shows the $^{22}Na^+$ influx into acid-loaded cells (pH_i 6.6) at pH_o 7.4 (●—●) and pH_o 6.0 (○—○—). The difference between these curves estimated the fraction driven by the outward H^+ gradient (ΔpH) (■—■—■). These measurements were performed in Na^+-depleted cells (Na_i 0.85 mM) to ensure that the contribution of Na^+/Na^+ exchange was negligible; under these conditions ΔpH_o Na^+ influx represents Na^+/H^+ exchange. Mean values of three experiments and SD = dot size. (*B*) Shows a Hanes-Woolf plot of Na^+/Na^+ exchange as determined by ΔpH_o Na^+ influx values from *A*. The solid symbols (●—●) represent the total influx measured which shows more than a single component. A model with two external sites ($X^2 = 0.239$) provided a better fit for the data than a model with a single site ($X^2 = 1.1$). The dotted lines represent the calculated kinetic parameters for the two external Na^+ sites; *site I* had a high affinity (K_m 11.1 mM) and *site II* a low affinity (K_m 248 mM). A model comprising a single site plus a diffusional component provided also gave a good fit ($X^2 = 0.20$) was excluded because the *a* intercept of the regression line ($y = 0.109 \pm 0.0199 + 4.16 \pm 0.35x$, $r = 099$) was significantly different from zero at the $P < 0.0025$ level

for the Na_i regulatory site of Na^+/Na^+ EXC is in the region of 20 mM (*data not shown*).

Previously, it was demonstrated that at acid pH_i (6.6) and high Na_i (>55 mM) Na^+/Na^+ EXC was activated (Figs. 6*B* and 8). For this reason Na^+/H^+ was measured as Na_o-driven H^+ efflux in cells containing very high Na^+, i.e., 80 mM. It can be seen in Fig. 10*B* that an increase in Na_i from 12 to 80 mM resulted in a decrease in Na^+/H^+ EXC from 22 to 8 FU yielding a Na_i-inhibited Na^+/H^+ EXC of 14 FU; a concomitant increase of Na^+/Na^+ EXC from 2 to 24 FU of Na^+/Na^+ EXC was also observed which gave a Na_i-stimulated Na^+/Na^+ EXC of 22 FU (Fig. 10*B*). Notably, at Na_i 80 mM, the pH_i-stimulated Na^+/Na^+ EXC and Na^+ influx driven by ΔpH_o were completely DMA insensitive up to 100 μM (Table 4,B). This is in contrast to the high DMA sensitivity of ΔpH_o-driven Na^+ influx at low internal Na^+ (Table 4, B). Thus, an increase in internal Na^+ (>50 mM) markedly inhibited DMA-sensitive Na^+/H^+ EXC and increased DMA-insensitive Na^+/Na^+ EXC.

In summary, these experiments indicate that inhibition by internal H^+ of Na^+/Na^+ EXC at low Na_i increases Na^+/H^+ EXC, while H_i stimulation of DMA-insensitive Na^+/Na^+ EXC at high Na_i inhibits Na^+/H^+ EXC and reduces its DMA sensitivity. These results are compatible with the hypothesis that a single transport system capable of operating in a DMA-sensitive Na^+/H^+ or DMA-insensitive Na^+/Na^+ EXC mode depends on the internal Na^+ and H^+ concentrations.

J. *Trans*-Effects of Internal Na^+ on Na^+/Na^+ Exchange

The data presented in Fig. 10*A* demonstrated that a small raise in Na_i, at acid pH_i, increased the rate of Na^+/H^+ EXC at the expense of Na^+/Na^+ EXC. However, the observation that the pH_i-induced inhibition of Na^+/Na^+ EXC was less than the Na_i stimulation of Na^+/H^+ EXC can be accounted for by the presence of an Na_i modifier site. This Na_i regulatory site could modify the K_m for Na_o of both Na^+/H^+ and Na^+/Na^+ EXC, and thereby control the amount of flux conducted by each of these modes of operation. In order to test this hypothesis, we studied the *trans*-effects of Na_i on both pathways.

Table 5 summarizes the *trans*-effects of Na_i and H_i on the K_m for Na_o of Na^+/Na^+ EXC at Na_i values where the transitions in modes of operation are observed. In these experiments Na^+/Na^+ EXC was measured at $pH_o = pH_i = 7.4$. At Na_i 26 mM Na^+/Na^+ EXC consists of high affinity (K_m 3.9 mM, carrier/site A) and low affinity (K_m 95 mM, carrier/site B) components (Table 5, I). Table 5, I also shows that as Na_i was raised to 58, 71 and 78 mM, in cells with pH_i 7.4, carrier/site A was no longer detectable but the K_m for carrier/site B decreased to 38, 33 and 22 mM, respectively. Furthermore, when Na_i increased from 26 to 78 mM, the V_{max}/K_m ratio increased from 0.11 to 0.61 (Table 5, I).

At pH_i 6.6 and Na_i23 mM, only Na^+/Na^+ EXC by carrier/site B was detected with a K_m for Na_o of

Table 5. *Trans*-modifier effect of cell Na^+ on the kinetic parameters of the external Na^+ sites of Na^+/Na^+ exchange in rabbit red cells

	Na_i (mM)	External Na^+ sites				
		Carrier/site A high affinity component		Carrier/site B low affinity component		
		K_m (mM)	V_{max} (FU)	K_m (mM)	V_{max} (FU)	V_{max}/K_m
I. pH_i 7.4						
	26	3.9	1.0	95	9.7	0.11
	58	not detectable		38	13.0	0.34
	71	not detectable		33	15.0	0.45
	78	not detectable		22	13.5	0.61
II. pH_i 6.6						
	23	not detectable		112	8.2	0.07
	86	not detectable		47	17.5	0.40

The external pH in all of the experiments was 7.4. FU = mmol/liter cell · hr.

112 mM, a value not significantly different from that observed at pH_i 7.4 (95 ± 12 mM). However, the K_m for Na_i increased from 18 mM at pH_i 7.4 to 52 mM at pH_i 6.5 (Figs. 6 and 7, Table 2). At pH_i 6.6, an increase of Na_i from 23 to 86 mM decreased the K_m for Na_o of Na^+/Na^+ EXC performed by carrier/site B from 112 to 47 mM (Table 5, II). Thus, the activity of Na^+/Na^+ EXC performed by carrier/site B at pH_i = 7.4 and 6.6 appears to be regulated by Na_i which increases the affinity for Na_o.

It was previously shown that when red cells with low Na_i (23 mM) were made acid (pH_i 6.6), Na^+/Na^+ EXC was inhibited because H^+ increased the K_m (from 18 to 52 mM) at the internal Na^+ sites (Figs. 6 and 7, Table 2), but it did not change the K_m at the external Na^+ sites for Na^+/Na^+ EXC. In contrast, when red cells with high Na_i were acidified to pH_i 6.6), Na^+/Na^+ EXC was stimulated because elevated Na_i again increased the affinity at the Na_o sites. Thus Na_i, but not H_i, modified the affinity of Na_o sites to perform Na^+/Na^+ EXC at neutral (7.4) or acid (6.6) pH_i. H_i alone has no effect on the Na_o sites but markedly decreases the K_m for Na_i to stimulate Na^+/Na^+ EXC.

In summary, our experiments demonstrate that Na_i and Na_o (section IIID, Fig. 5) have *trans*-effects in the affinity of Na sites for both exchange modes.

K. *Trans*-Effect of Internal Na^+ on Na^+/H^+ Exchange

In order to test the hypothesis that Na_i modulates the affinity of external Na sites of Na^+/H^+ EXC, Na^+ influx driven by an outward H^+gradient (ΔpH_o 7.4–6.0) was measured at pH_i 6.6 varying cell Na^+. Obviously, in Na^+-depleted cells, Na^+ influx through the Na^+/Na^+ EXC pathway is zero.

Na^+ influx was measured as a function of Na_o, in acid-loaded (pH_i 6.5) Na_i-depleted (Na_i 1 mM) cells at pH_o 6.0 and 7.4 (Fig. 10*A*). At pH_o 6.0, Na^+ influx saturates with increasing Na_o to give a V_{max} of 4.5 FU and a K_m of 24 mM and probably represents the residual influx which occurs even at low Na_i. The dependence of the H_o-driven Na^+ influx on Na_o (influx at pH_o 6.0 minus influx at pH_o 7.4 (Fig. 10*A*) was first analyzed using the reciprocal plot (*not shown*) with a regression $y = 0.109 \pm 0.0199) + 4.16$ ($\pm 0.35)x$, $r = 0.99$; the a intercept was significantly different from zero at the $P < 0.025$ level, indicating that a diffusional component could be excluded and the low affinity Na site was saturable. Subsequently, a Hanes-Woolf plot (Eq. (1)) indicated that a 'break' point was again observed (Fig.10*B*) and therefore more than one component/site of Na^+ influx was present. Fitting of the data to a single regression line with $y = 5.5 + 0.0619x$, $r = 0.96$ gave V_{max} 19.5 ± 2.4 FU and K_m = 127.6 ± 26.8 mM (*plot not shown*). A test for the goodness of fit gave a $X^2 = 1.1$, indicating that the differences between the model and the experimental values were not significant.

Further analysis of the Hanes-Woolf plot (section IIIA, Eq. (2)) by the procedure of successive approximations described by Spears et al. (1971) gave reliable estimates of all constants for two models: Model 1 with a diffusional plus a saturable and Model 2 for two saturable sites. The best fit for Model 1 gave V_{max} 2.81 ± 0.73, K_m 12.4 ± 6.6, K_d 0.058 ± 0.005; the distribution of residuals exhibited significant skewness to low flux values. For Model

Table 6. *Trans*-modifier effect of internal Na^+ on the kinetic parameters of Na^+ influx driven by a H^+ gradient in rabbit red cells

Na_i (mM)	External Na^+ sites				
	Site I high affinity component			Site II low affinity component	
	K_m (mM)	V_{max} (FU)		K_m (mM)	V_{max} (FU)
A.					
1	11.1	1.6		248	26.0
			Single site		
			K_m	V_{max}	
81			16	29.5	

B.	Influx due to Na^+/Na^+ exchange at 140 mM Na_o		Influx due to Na^+/H^+ exchange at 140 mM Na_o		
Na_i	%	FU	K_m	FU	Total flux
1	<2	0.3	248	26	26.3
81	50	13.2	14	12.3	25.5

These experiments were performed at cell pH 6.6 and external pH 7.4. The Na^+/H^+ exchange component was determined by subtracting the Na^+/Na^+ exchange from Na^+ influx at pH_o 7.4.

2 we obtained $V_{max}A = 1.64 \pm 0.69$ FU and $K_mA = 11.1 \pm 5.8$, $V_{max}B = 26 \pm 14$ FU and $K_mA = 248 \pm 248$ mM (Fig. 10*B*, dotted lines; Table 6). The test for the goodness of fit gave a $X^2 = 0.239$ which indicated that Model 2 did not differ significantly from the experimental data and exhibited random distribution of residuals. Thus, this analysis indicated that the two-site model provided a better fit (less error) than a one-site model ($X^2 = 1.1$); the latter was excluded.

When Na_i is increased, Na^+ influx will take place through two pathways: Na^+/Na^+ and Na^+/H^+ EXC. H^+-driven Na^+ influx will exclude influx due to the high affinity Na^+/Na^+ EXC (carrier/site A) component but will include the pH-sensitive (K_H 52 nM) component of Na^+/Na^+ EXC stimulated by Na_i (carrier/site B). Under such experimental conditions, the contribution of Na^+/H^+ EXC to Na^+ influx was calculated by subtracting the values of Na^+/Na^+ EXC from the ΔpH_o Na^+ influx measured in acid-loaded cells (pH_i 6.5–6.6) at pH_o 7.4. At Na_i 10 mM, Na^+ influx due to Na^+/Na^+ EXC (measured as Na_o-stimulated Na^+ efflux) represents ~10% of the total ΔpH_o Na^+ influx (Fig. 11*A* and *B*).

At Na_i 80 mM, the contribution of Na^+/Na^+ EXC increased to 50–70% of the ΔpH_o-Na^+ influx (Fig. 11*B*, Table 6). Measurement of the dependence of ΔpH_o-Na^+ influx from Na_o (Fig. 12) gave a single component with K_m 16 mM (Table 6) as evidenced by a linear Hanes-Woolf plot (*not shown*). Thus, under these conditions, the low affinity Na site of Na^+/H^+ EXC was no longer detectable. We also determined that at 19 mM Na_i, the K_m for Na_o for ΔpH_o was found to be 52 mM; these results demonstrate that the stimulation of Na^+/H^+ EXC by Na_i is caused by an increase in the affinity of the carrier for Na_o. At Na_i from 19 mM, pH_i 6.6, some stimulation of Na^+/Na^+ EXC also occurs but this is less than would be observed at neutral pH (Fig. 10).

The kinetic parameters for Na^+/H^+ EXC were calculated subtracting the values of Na^+/Na^+ EXC (measured as Na_o-stimulated Na^+ efflux in cells with pH_i 6.6 and 81 mM Na_i) from the values of ΔpH_o Na^+ influx under the same experimental conditions. As shown in Fig. 12, Na^+/H^+ EXC was sigmoidally activated by Na_o up to 40 mM with a n_{app} 3.2, K_m 14 mM (Table 6, B) and a V_{max} 17 FU (Hill plot, *not shown*). This observation is consistent with multiple Na_o transport sites on the Na^+/H^+ EXC pathway. Figure 12 also shows that as Na_o increased beyond 40 mM and the Na^+ gradient declined, Na^+/H^+ EXC was inhibited. Notice that $Na_i = Na_o = 80$ mM; there is still 14 FU of Na^+/Na^+ EXC.

In summary, at acid pH_i, low Na_i activated Na^+/H^+ EXC by increasing the affinity for Na_o to perform Na^+/H^+ EXC and inhibited Na^+/Na^+ by decreasing the affinity for Na_i to perform Na^+/Na^+. At higher Na_i (81 mM), the Na_i: H_i ratio regulates the system

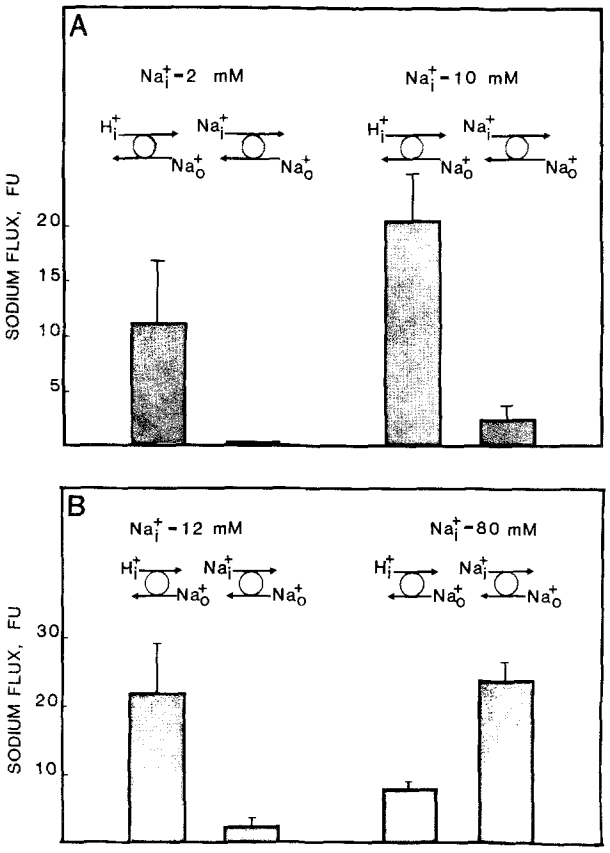

Fig. 11. The dependence of Na^+/Na^+ and Na^+/H^+ exchanges on Na_i at pH_o 7.4 and pH_i 6.4/6.5. Na_o was 0 or 150 mM. The Na_i concentration and the process being measured is indicated above each column; fluxes are shown as mean ± SD of three experiments. Na^+/Na^+ exchange was estimated as Na_o-stimulated Na^+ efflux and Na^+/H^+ exchange as Na_o-stimulated H^+ efflux. (*A*) Shows the effect of changes in cell Na^+ from 2 to 10 mM and (*B*) from 12 to 80 mM

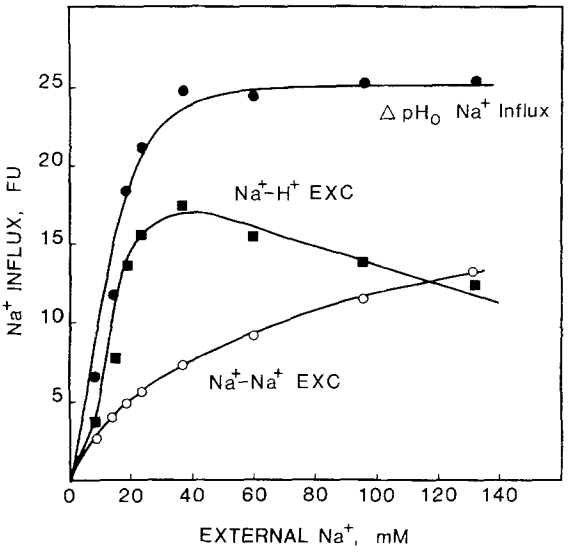

Fig. 12. The dependence of Na^+ influx on Na_o into acid-loaded cells (pH_i 6.6) containing 81 mM Na^+. Under these conditions the ΔpH_o Na^+ influx (●—●) comprises Na^+/H^+ and Na^+/Na^+ exchange yet still follows saturable Michaelian kinetics confirming that the two processes are mediated by the same transporter. The 'true' kinetic constants for Na^+/H^+ exchange (■—■) were determined by subtracting the values for Na^+/Na^+ exchange (○—○) from the ΔpH_o values. This analysis revealed that Na^+/H^+ exchange was sigmoidally activated (n_{app} 3.2) by Na_o up to 40 mM (V_{max} 17 FU) but thereafter decreased as increasing Na_o further stimulated Na^+/Na^+ exchange. At 115 mM Na_o the total Na^+ influx consisted of equivalent amounts of Na^+/H^+ and Na^+/Na^+ exchange. Mean values of three experiments

so that it performs more Na^+/Na^+ EXC than Na^+/H^+ EXC because Na_i increased the affinity of the low affinity Na_o sites (Table 5,II; Fig. 10*B*).

IV. Discussion

As an approach to investigate the hypothesis that the Na^+/Na^+ EXC pathway present in rabbit RBC is a mode of operation of the Na^+/H^+ exchanger, we studied the kinetic interactions of Na^+ and H^+ at both sides of the membrane with the Na^+/Na^+ and Na^+/H^+ exchange pathways.

A. An Ion Transport Model with Several Conformational Changes Can Account for Na^+/H^+ and Na^+/Na^+ Exchanges

The model shown in Fig. 13 adequately explains our observations in rabbit RBC. As in the case of the Na^+ pump, we propose that the transporter has two or more conformational states which may be mediated by phosphorylation. For simplicity, we distinguish conformations with H^+ regulatory site empty as E_1 and occupied as E_2. Conformation E_1 exists at $pH_1 = 7.4 = pH_o$ and can perform Na^+/Na^+ EXC (E_{1-1}) when Na_o is present (or Na^+/Li^+ EXC) at a slow rate (Fig. 1). At $Na_o = 0$, the carrier can return unloaded (Fig. 5*A*) and perform uncoupled Na^+ efflux in conformation E_{1-2}. Binding of H^+ to an H_i regulatory site induces a conformational change or a series of conformational changes (E_2) which allows the molecule to conduct different amounts of Na^+/H^+ and Na^+/Na^+ EXC depending on the $Na_i:H_i$ ratio. In the absence of Na_i (Fig. 9*A* and *B*), only Na^+/H^+ can be performed (E_{2-1}). At low Na_i, E_2 mainly performs Na^+/H^+ EXC (E_{2-2}) at the expense of Na^+/Na^+ EXC which is inhibited, while at high Na_i (E_{2-3}) the reverse is seen (Fig. 10*A* and 10*B*). The affinities of the internal and external sites of the Na^+/Na^+ and Na^+/H^+ antiporters are higher for $H^+ > Li^+ > Na^+$. In the E_2 conformation, a small fraction of the transporter also 'shuttles' back and forth across the membrane in an empty form, inhibitable by amiloride and its analog (Fig. 6*A*). The H^+-

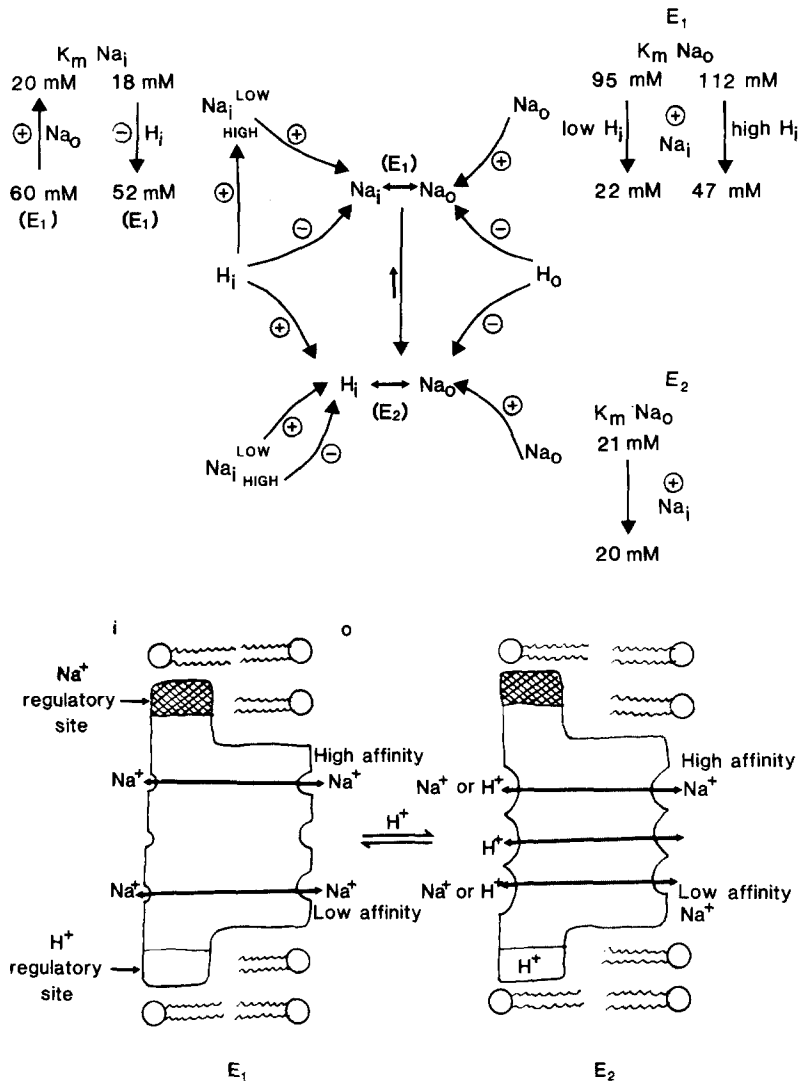

Fig. 13. Schematic representation of the *cis*- and *trans*- interactions of Na^+ and H^+ on Na^+/Na^+ and Na^+/H^+ exchange. Na_o stimulates (+) and H_o inhibits (—) both transport modes. The effects of H_i depend on the relative ratio of $Na^+ : H^+$. At pH_i 7.4 Na^+/Na^+ exchange is activated by Na_i. At higher H_i and low Na_i, Na^+/Na^+ exchange is inhibited due to a decrease in the K_m for Na_i; at higher values of Na_i, pH_i stimulates Na^+/Na^+ EXC. Under these same conditions, Na^+/H^+ exchange behaves the opposite, i.e., at low Na_i it is stimulated and at higher Na_i it is inhibited. Both exchange modes, in E_1 and E_2 conformational states, are *trans*-modified by Na_i which increases the affinity of Na_o sites; the transitions in the K_m values of the Na_o sites shown in the figure are those which occur as Na_i changes from 20 to 80 mM. (*B*) A proposed model of Na^+/H^+ exchange incorporates all the observed modes of operation of a Na^+ exchanger. The data is best explained by assuming that the carrier exists in two or more conformational states (E_1 and E_2) with internal H^+ and Na^+ regulatory sites which may reflect different states of phosphorylation. In the E_1 conformation the H_i regulatory site is not occupied ($pH_i = pH_o = 7.4$) and the system can perform Na^+/Na^+ exchange when Na_o is present. The binding of H^+ to an H_i regulatory site (pH_i 6.6) induces a conformational change (E_2) which can perform Na^+/H^+ and/or Na^+/Na^+ exchange depending on the $H_i : Na_i$; the flux through each pathway is controlled by the Na_i regulatory site

loaded carrier is sensitive to DMA only when in its E_2 conformation and conducting Na^+/H^+ EXC; E_1 and E_2 Na^+/Na^+ exchanges are DMA insensitive. However, in E_2 (acid pH_i) with the Na_o sites empty both the Na^+ and H^+ efflux are DMA-sensitive presumably because DMA can now gain easy access to an empty Na_o-binding site. These results imply that the binding of H^+ to the H_i regulatory site (at Na_i; H_i ratio 1.1×10^5) somehow confers most of the DMA sensitivity to Na^+transport.

B. Na^+/Na^+ Exchange Has Low and High Affinity Na^+ Sites

The dependence of Na^+ efflux, driven by Na^+/Na^+ EXC, on Na_o did not follow simple hyperbolic kinetics (Fig. 1) which indicated that more than one flux component was present. Jennings, Adams-Lackey and Cook (1985) studied the activation of Na^+/Na^+ EXC by Na_o at pH_i 7.2, Na_i 10 mM and found only a single Michaelis-Menten component of K_m 70 ± 20 mM; it is more than likely that the high affinity component was missed because only two Na_o concentrations below 20 mM were studied.

Our experimental data was analyzed by several kinetic models in order to obtain the best fit. After a diffusive component was eliminated, we examined two other models; a single carrier with two interacting sites and a two-carrier system (Fig. 1*B* and *C*). One component (carrier A/site) of Na^+/Na^+ EXC has high affinity (K_m 3.9 ± 3.4 mM) and low capacity (V_{max} 1.0 ± 0.2 FU); the other (carrier/site B) has low affinity (K_m 95 ± 12 mM) and high capacity (V_{max} 9.7 ± 1.6 FU). As pointed out by Segal (1975), the distinction between a single- or a two-carrier system is difficult and ultimately it may have to rely on other properties of the two components. The Hill analysis (n_{app} 1.1) favored one system with two sites with low cooperativity. Both components of Na^+/Na^+ EXC

appeared to be inhibited by H_o but with different pK. The low affinity carrier/site B was competitively inhibited by H_o with a very low pK (52 nM) while the high affinity, carrier/site A was inhibited by H_o with a pK 300 nM (Fig. 3*A* and *B*). Moreover, upon cell acidification to pH_i 6.6, Na^+/Na^+ EXC exhibited only a single component of low affinity (K_m 112 mM, Table 5, II). Thus, the high affinity carrier/site was sensitive to internal and external pH and the low affinity site was competitively inhibited by H_o. On the basis that H^+ interacted with the two components of Na^+/Na^+ EXC we propose, but not prove, that the data is more compatible with the model of one carrier with two sites of different affinities.

At pH_i 7.4, Na^+/Na^+ EXC showed hyperbolic dependence of Na_i (Fig. 5*B*) with K_m 19.7 ± 1.5 mM for Na_i and a V_{max} of 11.8 ± 2.6 FU (n = 3). Therefore, Na^+/Na^+ EXC is asymmetrically arranged in the membrane because the K_m for Na_o was 95 mM whereas the K_m for Na_i was 20 mM.

Na_i behaved as a *trans*-stimulator of Na^+/Na^+ EXC by regulating the affinity of the Na_o sites (Table 5) because the V_{max} increased from 5.8 (Fig. 1) to 10.6 FU (Fig. 8*B*) with a raise in Na_i from 26 to 83 mM (at fixed Na_o 140 mM). Similarly, the K_m for Na_i to stimulate Na^+ efflux was also modulated by Na_o (Fig. 5*A*) because it decreased from 60 to 18.5 mM when Na_o increased from 0 to 150 mM.

C. Na^+/Na^+ Exchange Has Alternative Modes of Operation

Several findings indicate that Na^+ efflux into Na^+-free media is not only diffusion driven but it may represent a different mode of operation of the Na^+/Na^+ or Na^+/H^+ exchangers. As recently discussed by Montrose and Murer (1988), even though the Na^+ exchange system is tightly coupled, under certain experimental conditions, it may operate in another mode. The kinetics of Na^+ efflux into Na-free media, at pH_i 7.4 and 6.6, demonstrated sigmoidal dependence on cell Na^+ (Figs. 5*A* and 6*A*) reflected by n_{app} 2.2 which also indicated strong cooperativity between Na_i sites. These observations clearly indicate that the uncoupled Na^+ efflux is not diffusive. Under these conditions 20 to 40% of Na^+ efflux was sensitive to DMA and stimulated by acid pH_i (Figs. 7*A* and 8*A*). H^+ efflux into Na^+-free media was also inhibited 10 to 20% by DMA as previously reported by Escobales and Rivera (1987). These components of Na^+ and H^+ efflux could reflect either nonspecific effects of DMA or partial reactions of the transport mechanism due to (*i*) a reversal of the carrier with Na_i being exchanged for H_o; (*ii*) electrodiffusive movement; (*iii*) uncoupled movement; or (*iv*) shuttling back and forth across the membrane with the Na_o sites empty, i.e., Na^+–H^+ cotransport. The data presented is not consistent with the presence of a significant component of cotransport since the H^+ efflux into Na-free media always exceeded the Na^+ efflux. Calculations of the membrane potential (E_m) in DIDS-treated cells from the Nernst equation, assuming that in RBC the $[Cl^-]_i/[Cl^-]_o$ ratio = $[H^+]_o/[H^+]_i$, with pH_o fixed at 7.4 gave E_m = −0.5 mV at pH_i 7.39 and E_m = −72.0 mV at pH_i 6.17. Electrodiffusive movement can be excluded as such E_m change would be expected to decrease H^+ and Na^+ efflux but in fact both are stimulated by acid pH_i.

D. Internal Na^+ Has *Trans*-Effect on Na^+/H^+ Exchange

Na^+/H^+ EXC has never been studied in Na^+-depleted cells but at a variety of internal Na^+ concentrations. Rothenberg et al. (1983) documented the effect of increasing Na_i in A431 cells but did not have the condition of zero Na_i. This study clearly shows that Na_i has important *trans*-interactions with the Na_o sites and *cis*-interactions with H_i sites.

Na^+/H^+ EXC was estimated as the H^+-driven Na^+ influx (Fig. 9*A*) and as the Na^+-driven H^+ efflux (Fig. 9*B*) in Na^+-depleted cells confirming a stoichiometry of 1 : 1. Internal H^+ sigmoidally activated the system and a Hill coefficient 2.8 indicating the presence of several interacting H_i sites; a V_{max} of 74 FU for Na^+ influx and 72 FU for H^+ efflux was achieved at pH_i 6.0 with similar K_m for H_i. Na^+/H^+ EXC, unlike Na^+/Na^+ EXC, was partially inhibited (between 80 to 90%) by 20 μM DMA. The Na_o dependence of Na^+ influx driven by an outward H^+ gradient proved to be markedly affected by cell Na^+ content (*see* section IIIK). In the absence of Na_i, pH_i 6.6, the dependence of H^+-driven Na^+ influx on Na_o (Fig. 10*A*) was best described by a model with two sites with low cooperativity (n_{app} 0.9). One Na_o site had high affinity (K_m 11.1 mM) and low capacity (V_{max} 1.6 FU) and another one, low affinity (K_m 248 mM) and high capacity (V_{max} 26 FU) site (Fig. 10*B*). However, the possibility that two separate carriers are responsible for H^+-driven Na^+ influx (i.e., DMA sensitive and insensitive) was not completely excluded. The absence of Na_i ensures that Na^+ entry does not take place via the Na^+/Na^+ EXC which is DMA insensitive. The substraction of Na^+ influx at pH_o 6.0 which inhibits Na_o-dependent H^+ efflux allows to eliminate diffusion pathways. In this particular case, the presence of two sites on a single carrier is more favored on the basis that an increase in Na_i changed the kinetics from two sites to single site (Table 6). When Na_i increased to 80 mM, only a single high affinity site for H^+-driven Na^+ influx was detectable (Fig. 12) with a calculated V_{max} equivalent to that seen for the low affinity site at depleted Na_i (Table 6). We also documented that an increase in

H_i changed the dependence of Na^+/Na^+ EXC on external Na^+ from a two-site kinetics to a single-site component (Table 5, II).

When red cells contained a high Na^+ content (81 mM), the Na^+/Na^+ EXC component was substracted from ΔpH_o Na^+ influx to derive transport by Na^+/H^+ EXC (Fig. 11*B*). Under such conditions, the dependency of Na^+/H^+ EXC on Na_o was sigmoidal and gave a n_{app} 3.2 (Fig. 12). This kinetic behavior is compatible with (*i*) multiple interacting sites or (*ii*) a single site which can exist in various conformational states. Recent work by Otsa et al. (1989) measuring the 'burst' kinetics of Na^+/H^+ EXC in rat kidney vesicles also demonstrated sigmoidal dependence on Na_o at low temperature which was interpreted as evidence for an oligomeric protein structure.

E. H^+ Have Asymmetric Effects on Na^+/Na^+ and Na^+/H^+ Exchanges

Our results clearly indicate that H^+ have asymmetric effects on Na^+/H^+ EXC; H_i allosterically modified Na^+/Na^+ EXC (Fig. 6*B*) and H_o inhibited (Fig. 2) with pK 6.55. The same pattern was exhibited by Na^+/H^+ EXC in rabbit RBC. Internal H^+ were sigmoidal activators of Na^+/H^+ EXC (Fig. 9) and increasing H_o inhibited Na^+ influx into acid-loaded cells (Fig. 10*A*). A summary of the *cis*- and *trans*-interactions of Na^+ and H^+ documented by this study are depicted in Fig. 13.

The effect of H_i on Na^+/Na^+ EXC was dependent on the level of Na_i (Figs. 5*B* and 6*B*). At low Na_i, an increase in H_i (Na_i : H_i ratio = 110,000) produced a competitive inhibition (pK 6.7, Fig. 7*B*) increasing the K_m for Na_i from 18 to 52 mM. At higher cell Na^+ (Na_i : H_i ratio = 330,000), a sigmoidal activation (pK 7.0) of Na^+/Na^+ EXC was observed (Fig. 8*B*). The n_{app} for the dependence of Na^+/Na^+ EXC on Na_i increased from 1.0 to 2.8 when pH_i decreased from 7.4 to 6.6, indicating that H_i induced strong cooperative interactions between the internal Na^+ sites. These findings provide compelling evidence that the occupancy of the internal Na^+ sites of the Na^+/Na^+ EXC is modulated by H_i and it is dependent on the Na_i : H_i ratio. The observation that in the absence of Na_o (at pH_i 6.6 and 7.4) the dependence of Na^+ efflux on Na_i (Fig. 5*A*) was also sigmoidal (n_{app} 2.2) indicates the presence of more than a single Na_i site or a single site in a transporter with multiple conformational states.

Several studies (Grinstein, Goetz & Rothstein, 1984; Aronson & Igarashi, 1986) have documented the allosteric activating effect of H_i on Na^+/H^+ EXC which has been interpreted as evidence for an H_i modifier site, independent of the substrate (H^+) transport sites. In this paper, we have provided compelling evidence that H_i modulates both Na^+/H^+ and Na^+/Na^+ EXC. Because amiloride analogs are certainly not specific inhibitors and not all Na^+ for H^+ exchange is blocked by these compounds, this is certainly a better demonstration. While our results can be interpreted in terms of separate regulatory binding sites for H_i and Na_i, competition for the same site is an alternative (Smith et al., 1975) that can account for the increased K_m for Na_i to perform Na^+/Na^+ EXC at pH 6.5. The asymmetric effect of H^+ on Na^+/Na^+ EXC explains why it is not possible to see stimulation of Na^+ efflux by H_o but stimulation by Na_i of H^+influx as reported by Grinstein in rat thymocytes (1984). In the absence of Na_o, at pH_i 7.4, the K_m for Na_i is very high (60 mM) and H_o inhibits without being transported. Thus, the failure of H_o to stimulate Na^+efflux cannot be used as an argument to support the contention that Na^+/Na^+ EXC cannot perform Na^+/H^+ EXC as proposed by Jennings (1985). Even though, several authors observed the H_o competitive inhibition of Na^+/Na^+ EXC in rabbit and beef red cells (Funder et al., 1984; Jennings et al., 1985) none of them studied the separate effects of H_i and H_o by blocking the $[Cl]^-/[HCO_3]^-$ exchanger with DIDS.

F. Are Na^+/Na^+ and Na^+/H^+ Exchanges Conducted by the Same Transporter?

Several experiments support the hypothesis that in rabbit RBC, Na^+/Na^+ EXC is a mode of operation of the Na^+/H^+ exchanger and exclude the presence of two independent transport systems. *First,* H_o are inhibitory and H_i are allosteric modifiers of both EXC processes. Over the same range of pH_i (*compare* Fig. 7*B* with Fig. 9), H_i caused sigmoidal inhibition of Na^+/Na^+ and sigmoidal activation of Na^+/H^+ EXC. Thus, the binding of H^+ to the H_i regulatory site promotes Na^+/H^+ EXC and inhibits Na^+/Na^+ EXC. However, these findings can also be accounted for by two independent transport systems only one of which was inhibited by pH_i; if this was the case, at $pH_i < 7.0$ the sum of the two countertransport components would not be kept constant as we documented in this study.

Second, at a 'crossover' point ($pH_i \sim 6.5$), Na_i was capable of stimulating both processes (Fig. 10) by regulating the affinity for Na_o of Na^+/Na^+ and Na^+/H^+ EXC (Tables 5 and 6) and, therefore, this *trans*-effect has a fundamental role in controlling Na^+ fluxes through both pathways. This regulatory effect of Na_i provides compelling evidence that the two types of countertransport are indeed mediated by the same carrier. At present, it is not yet clear if Na_i occupies the H_i regulatory site or a different site; however, the fact that H_i does not seem to modify

the affinity for Na_o, yet Na_i does, indicates that the Na_i site is distinct from the H^+ regulatory site. These findings can also explain the activation of Na^+/H^+ EXC by increasing internal Na^+ previously observed in fibroblast (Rothenberg et al., 1983; Pouyssegur et al., 1984; Zhuang et al., 1984).

Finally, evidence that a single transporter is mediating both Na^+/Na^+ and Na^+/H^+ EXC processes comes from the observation that under conditions that Na^+ influx proceeds through only Na^+/H^+ EXC in Na^+-depleted cells (pH_i between 6.0 and 7.0), two Na_o sites exist; similarly, under conditions that Na^+ transport proceeds through only Na^+/Na^+ EXC ($pH_i > 7.0$), two Na_o sites were observed. However, under experimental conditions that Na^+ influx occurred through both pathways (pH_i 6.5, Na_i 81 mM), being Na^+/H^+ EXC inhibited and Na^+/Na^+ EXC stimulated, the activation kinetics of Na^+/H^+ by Na_o became sigmoidal and therefore more than one Na_o site should be present (Fig. 12).

In our view, the strongest evidence in favor of two separate exchange systems is the lack of inhibition by amiloride and DMA of Na^+/Na^+ EXC under any of the conditions tested. Numerous studies have shown that amiloride (and its high affinity analogs), within a certain concentration range, are inhibitors of Na^+/H^+ EXC. In rabbit RBC Na^+/Na^+ EXC was completely DMA insensitive (up to 100 μM) whereas Na^+/H^+ EXC was only partially DMA sensitive (80 to 100%). However, we found that the amiloride sensitivity of Na^+ influx driven by Na^+/H^+ EXC is strongly dependent on cell Na^+. Even though a series of high affinity amiloride analogs have been developed to inhibit the Na^+/H^+ antiporter, in human RBC the most potent derivatives inhibit only 60% of ΔpH_o-driven Na^+ influx exchanging 1 : 1 with Na_o-stimulated H^+ efflux (Escobales & Canessa, 1986; Semplicini, Spalvins & Canessa, 1989).

It appears, therefore, that if the two exchangers are modes of operation of the same carrier then they are not both DMA sensitive; DMA inhibited the Na_i-stimulated Na^+/H^+ EXC, 'uncoupled' Na^+ and H^+ efflux but not basal ($pH_i > 7.0$) or acid-stimulated (pH_i 6.50 Na^+/Na^+ exchange (Table 4). This is unlike the effects of ouabain on the Na^+ 'pump' which also blocks its Na^+/Na^+ and K^+/K^+ EXC modes. However, the inhibitory effect of 2 mM amiloride of Na_i-stimulated Na^+ influx in renal microvillus membrane vesicles has been taken as proof of inhibition of Na^+/Na^+ EXC (Aronson, Nee & Suhm, 1982) despite the increased influx could be due to the Na_i stimulation of Na^+/H^+ EXC without concomitant outward Na^+ transport. The finding that the amiloride binding site can be genetically altered independently of the affinity of Na_o sites (Franchi, Cragoe & Pouyssegur, 1986) indicates that the antiporter has Na_o transport sites which do not interact with amiloride. We propose, that the affinity for amiloride may depend on the conformational (or phosphorylation) state of the antiporter elicited by internal and external Na^+ and H^+ levels.

In conclusion, we have demonstrated that the rabbit RBC Na^+/H^+ EXC can also mediate a pH_i-regulated Na^+/Na^+ EXC. This is accomplished by the binding of H^+ to the H_i regulatory site which induces a conformational change to allow Na^+/H^+ EXC. When this site is not occupied Na^+/Na^+ countertransport can occur. Na^+ influx through both pathways is controlled by the Na_i regulatory site and its *trans*-effects on the affinity for Na_o, irrespective of the transporter conformation state.

This research was supported by N.I.H. Grants NHLBI HL-35664 and 5P50-36568. We are very grateful to Dr. E.J. Cragoe from Merck, Sharp and Dohme Laboratories (Rahway, NJ) for the generous gift of dimethyl-amiloride. Preliminary reports of this work were reported at the Biophysical meeting and Society of General Physiology (Morgan & Canessa, 1987*a,b,c*).

References

Aronson, P.S. 1982. Red cell sodium-lithium countertransport and essential hypertension. *N. Engl. J. Med.* **307:**317

Aronson, P.S., Igarashi, P. 1986. Molecular properties and physiological roles of the Na^+–H^+ exchanger. *In:* Current Topics in Membranes and Transport. P.S. Aronson and W.F. Boron, editors. Vol. 26, pp. 57–75. Academic, London

Aronson, P.S., Nee, J., Suhm, M.A. 1982. Modifier role of internal H^+ in activating the Na^+/H exchanger in renal microvillus membrane vesicles. *Nature (London)* **299:**161–163

Berk, B.C., Aronow, M.S., Brock, T.A., Cragoe, E., Jr., Gimbrone, M.A., Jr., Alexander, R.W. 1986. Angiotensin II-stimulated Na^+/H^+ exchange in cultured vascular smooth muscle cells. *J. Biol. Chem.* **262:**5057–5064

Canessa, M. 1984. The polymorphism of red cell Na and K transport in essential hypertension: Findings, controversies, and perspectives. *In:* Erythrocyte Membranes 3: Recent Clinical and Experimental Advances. pp. 293–315. Alan R. Liss, New York

Canessa, M. 1989. Kinetic properties of Na^+/H^+ and Li^+/Na^+, Na^+/Na^+ and Na^+/Li^+ exchanges of human red cells. *Methods Enzymol.* **173:**176–191

Canessa, M., Adragna, N., Solomon, H.S., Connolly, T.M., Tosteson, D.C. 1980. Increased sodium-lithium countertransport in red cells of patients with essential hypertension. *N. Engl. J. Med.* **302:**772–776

Canessa, M., Brugnara, C., Cusi, D., Tosteson, D.C. 1986. Modes of operation and variable stoichiometry of the furosemide-sensitive Na and K fluxes in human red cells. *J. Gen. Physiol.* **87:**113–142

Canessa, M., Brugnara, C., Escobales, N. 1987. The Li^+–Na^+ exchange and Na^+–K^+–Cl^- cotransport systems in essential hypertension. *Hypertension* **10(Suppl. I):**4–10

Canessa, M., Morgan, K., Semplicini, A. 1988. Genetic differences in lithium-sodium exchange and regulation of the sodi-

um-hydrogen exchanger in essential hypertension. *J. Cardiovasc. Pharmacol.* **12(Suppl):**S92–98

Canessa, M., Spalvins, A. 1987. Kinetic effects of internal and external H^+ on Li/H and Li/Na exchange of human red cells (RBC). *Biophys. J.* **51:**567*a*

Duhm, J., Becker, B.F. 1979. Studies on lithium transport across the red cell membrane: V. On the nature of the Na^+-dependent Li^+ countertransport system of mammalian erythrocytes. *J. Membrane Biol.* **51:**263–286

Escobales, N., Canessa, M. 1986. Amiloride-sensitive Na^+ transport in human red cells: Evidence for Na/H exchange system. *J. Membrane Biol.* **90:**21–28

Escobales, N., Rivera, A. 1987. Na^+ for H^+ exchange in rabbit erythrocytes. *J. Cell Physiol.* **132:**73–80

Franchi, A., Cragoe, E., Jr., Pouyssegur, J. 1986. Isolation and properties of fibroblast mutants overexpressing an altered Na^+/H^+ antiporter. *J. Biol. Chem.* **261:**14614–14620

Funder, J., Wieth, J.O., Jensen, H.A., Ibsen, K.K. 1984. The sodium-lithium exchange mechanism in essential hypertension: Is it a sodium-proton exchanger? *In:* Topics in Pathophysiology of Hypertension. H. Villareal and M.P. Sambhi, editors. pp. 147–161. Nyjhoff, The Haghe

Garay, R.P., Garrahan, P.J. 1973. The interaction of sodium and potassium with the sodium pump in red cells. *J. Physiol. (London)* **231:**297–325

Glynn, I.M., Karlish, S.J.D. 1975. The sodium pump. *Annu. Rev. Physiol.* **37:**13–55

Grinstein, S., Goetz, J. D., Rothstein, A. 1984. $^{22}Na^+$ fluxes in thymic lymphocytes. II. Amiloride-sensitive Na^+/H^+ exchange pathway; reversibility of transport and asymmetry of the modifier site. *J. Gen. Physiol.* **84:**585–600

Haas, M., Schooler, J., Tosteson, D.C. 1975. Coupling of lithium to sodium transport in human red cells. *Nature (London)* **258:**425–427

Jennings, M.L., Adams-Lackey, M., Cook, K.W. 1985. Absence of significant sodium-hydrogen exchange by rabbit erythrocyte sodium-lithium countertransporter. *Am. J. Physiol.* **249:**C63–C68

Mannervik, B. 1983. Regression analysis, experimental error and statistical criteria in the design and analysis of experiments for discrimination between rival kinetic models. *In:* Contemporary Enzyme Kinetics and Mechanisms. D.L. Purich, editor. pp. 75–95. New York

Montrose, M.H., Murer, H. 1988. Kinetics of Na^+/H^+ exchange. *In:* Na^+/H^+ Exchange. S. Grinstein, editor. CRC Press, Boca Raton (FL)

Morgan, K., Canessa, M. 1987*a*. Interconversion of Na/Na to Na/H exchange depends on the ratio of internal Na^+ to H^+in rabbit red cells. *J. Gen. Physiol.* **90:**31*a*

Morgan, K., Canessa, M. 1987*b*. Kinetic effects of internal and external protons on Na/Na exchange of rabbit red cells. *Biophys. J.***51:**567*a*

Morgan, K., Canessa, M. 1987*c*. Modifier effects of internal H^+ and Na^+ on Na/Na and Na/H exchange in rabbit red cells (RBC). *J. Gen. Physiol.* **90:**32*a*

Murer, H., Hopfer, U., Kinne, R. 1976. Sodium/proton antiport in brush border membranes isolated from rat small intestine and kidney. *Biochem. J.* **154:**597–602

Neame, K.D., Richards, T.G. 1972. Elementary Kinetics of Membrane Carrier Transport. Halsted, New York

Otsa, K., Kinsella, J., Sacktor, B., Froehlich, J. 1989. Transient state kinetic evidence for an oligomer in the mechanism of Na^+/H^+ exchange. *Proc. Natl. Acad. Sci. USA* **86:**4818–4822

Pandey, G.N., Sarkadi, B., Haas, M., Gunn, R.B., Davies, J.M., Tosteson, D.C. 1978. Lithium transport pathways in human red cells. *J. Gen. Physiol.***72:**233–247

Pouyssegur, J., Sardet, C., Frenchi, A., L'Allemain, G., Paris, S. 1984. A specific mutation abolishing Na^+/H^+ antiport in hamster fibroblasts precludes growth at neutral and acidic pH. *Proc. Natl. Acad. Sci. USA* **81:**4833–4837

Rothenberg, P., Glaser, L., Schlesinger, P., Casel, D. 1983. Epidermal growth factor stimulates amiloride-sensitive $^{22}Na^+$ uptake in A431 cells. Evidence for Na^+/H^+ exchange. *J. Biol. Chem.* **258:**4883–4889

Sachs, J., Faler, L.D., Rabson, E. 1982. Proton hydroxyl transport in gastric and intestinal epithelia. *J. Membrane Biol.* **64:**123–135

Segal, I.H. 1975. Enzyme Kinetics. Wiley (Interscience), New York

Semplicini, A., Spalvins, A., Canessa, M. 1989. Kinetics and stoichiometry of the human red cell Na^+/H^+ exchanger. *J. Membrane Biol.* **1107:**219–228

Smith, G.D., Roberts, D.V., Kuchel, P.W. 1975. Active site directed effectors of allosteric enzymes. *Biochim. Biophys. Acta* **377:**197–202

Spears, G., Sneyd, J.G.T., Loten, E.G. 1971. A method for deriving kinetic constants for two enzymes acting on the same substrate. *Biochem. J.***125:**1149–1151

Villamil, M.F., Kleeman, C.R. 1969. The effect of ouabain and external potassium on the ion transport of rabbit red cells. *J. Gen. Physiol.* **54:**576–588

Zhuang, Y.X., Cragoe, E.J., Jr., Schaikewitz, T., Cassel, D. 1984. Characterization of potent Na^+/H^+ exchange inhibitors from the amiloride series in A431 cells. *Biochemistry* **23:**4481–4488

Received 8 March 1990; revised 13 June 1990