**II. Amiloride-sensitive Na⁺/H⁺ Exchange Pathway; Reversibility of Transport and Asymmetry of the Modifier Site**

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**ABSTRACT** ²²Na⁺ flux and cytoplasmic pH (pHi) determinations were used to study the reversibility, symmetry, and mechanism of activation of the Na⁺/H⁺ exchange system in rat thymic lymphocytes. In acid-loaded cells, the antiport can be detected as an Na⁺-induced, amiloride-sensitive alkalinization. At pHi >7.0, amiloride-sensitive net H⁺ fluxes are not detectable. To investigate whether at this pH the transporter is operative in a different mode, e.g., Na⁺/Na⁺ exchange, ²²Na⁺ uptake was measured as a function of pHi. The results indicate that the antiport is relatively inactive at pH ≥7.0. Comparison of the rates of H⁺ efflux (or equivalent OH⁻ uptake) and Na⁺ uptake indicate that Na⁺/Na⁺ countertransport through this system is negligible at all values of pHi and that the Na⁺:H⁺ stoichiometry is 1:1. Measurements of pHi in Na⁺-loaded cells suspended in Na⁺-free medium revealed an amiloride-sensitive cytoplasmic acidification, which is indicative of exchange of internal Na⁺ for external H⁺. The symmetry of the system was analyzed by measuring the effect of extracellular pH (pHe) on Na⁺ efflux. Unlike cytoplasmic acidification, lowering pHe failed to activate the antiport. The results indicate that the amiloride-sensitive Na⁺/H⁺ exchanger is reversible but asymmetric. The system is virtually inactive at pHi ≥7.0 but can be activated by protonation of a modifier site on the cytoplasmic surface. Activation can also occur by depletion of cellular Na⁺. It is proposed that Na⁺ may also interact with the modifier site, stabilizing the unprotonated (inactive) form.

**INTRODUCTION**

It was recently reported (Grinstein et al., 1984a) that Na⁺ induces a rapid cytoplasmic alkalinization in acid-loaded rat thymocytes. The underlying mechanism was inferred to be an Na⁺⁺/H⁺ exchange, because of the strict Na⁺ dependence and the sensitivity to amiloride (Kᵢ = 2.5 μM), a diuretic known to...
inhibit other Na+/H+ antiports (Benos, 1982). Involvement of Na+ in the transport process was confirmed by measurements of cellular ionic contents, which revealed a substantial amiloride-sensitive Na+ gain during recovery from acid loading. Maximal Na+ influx rates, calculated from the H+ flux assuming 1:1 stoichiometry, approached 0.9 nmol/10⁶ cells·min.

A substantial Na+ flux (~0.1 nmol/10⁶ cells·min) has also been detected in normal (pHᵢ 7.0) cells, but, in contrast to acid-loaded cells, only a small fraction (~15%) is sensitive to amiloride (Grinstein et al., 1984b). The predominant component in this case appears to be a different system that promotes a rapid Na⁺/Na⁺ exchange under normal conditions. It is different not only in its amiloride sensitivity but also in its lack of sensitivity to Li⁺ and its response to changes in temperature.

These observations suggest that the amiloride-sensitive Na⁺/H⁺ exchanger active in acid-loaded cells is not an alternative operating mode of the Na⁺/Na⁺ exchange system of normal cells. They also indicate that the amiloride-sensitive Na⁺/H⁺ exchanger is relatively active when the cytoplasmic pH is normal and that it is activated when pHᵢ is reduced.

In this paper, the factors that determine the activity of the amiloride-sensitive Na⁺/H⁺ exchanger were examined by comparing the kinetic and pharmacologic properties of ²²Na⁺ uptake in cells at varying pHᵢ. A method was developed for rapid clamping of pHᵢ based on the use of ionophores and K⁺-rich solutions. Recordings of pHᵢ were used to verify the efficiency of the clamping procedure.

Activation of the Na⁺-induced alkalinization has also been reported when intracellular Na⁺ is depleted (Grinstein et al., 1984a). The mechanism responsible for this effect has not, however, been clear. In this communication, the role of Na⁺ was approached by comparing the fluxes of ²²Na⁺ in pHᵢ-clamped normal and Na⁺-depleted cells. Finally, ²²Na⁺ efflux and pHᵢ measurements were used to determine the reversibility and symmetry of the amiloride-sensitive exchanger. The results indicate that the rate of Na⁺/H⁺ countertransport is controlled kinetically by at least one modifier site that is activated by H⁺ and inhibited by Na⁺. Moreover, whereas the transport site(s) is accessible to Na⁺ and H⁺ on both sides of the membrane, the modifier is restricted exclusively to the cytoplasmic surface.

MATERIALS AND METHODS

Nigericin was purchased from Calbiochem-Behring Corp., San Diego, CA; bovine serum albumin was from Sigma Chemical Co., St. Louis, MO; all other reagents and solutions were the same as in the preceding communication (Grinstein et al., 1984b). The methods for cell isolation and characterization, cation content determination, pHᵢ measurement, ²²Na⁺ uptake and efflux determination, and acid production measurement were the same as in the accompanying paper. Because the ²²Na⁺ influx rates were considerably higher in acid-loaded cells, uptake was linear only for 1–2 min. A 1-min incubation was used for all the experiments described in this manuscript.

In some experiments, pHᵢ was driven to a desired value prior to the measurement of
Validation of cytoplasmic pH (pHi) clamping procedure. Cells were loaded with DCF as described and suspended in a solution containing (mM): 140 KCl, 20 NaCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, and 20 Tris-MES at the pH indicated at the right end of the trace. A composite of typical fluorescence recordings is illustrated. Where indicated, 0.4 μg/ml nigericin was added and recording continued. The samples were magnetically stirred throughout the experiment. Temperature, 20–22°C. Discontinuities in the trace indicate the intervals when the sample compartment was opened for additions.

Na⁺ fluxes. This was accomplished using nigericin, an alkali cation/H⁺ exchange ionophore. The ionophore, which has a much higher affinity for K⁺ than for Na⁺, sets \([K^+] / [K^+] = [H^+] / [H^+]\), so that if cells are suspended in media with approximately the same K⁺ concentration as the cytoplasm, [H⁺] will follow [H⁺]. In this way, pH can be manipulated simply by changing pH. Practically, this was accomplished by suspending the cells (2 × 10⁶/ml) in a medium containing (mM): 140 KCl, 20 NaCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, and 20 Tris-MES at the indicated pH. Na⁺ was present in the medium to prevent cellular Na⁺ depletion through the ionophore. When pH was to be clamped in Na⁺-depleted cells, NaCl was omitted from the solution. Nigericin (0.4 μg/ml, final) was then added and the cells were incubated for 3 min at room temperature. The ionophore was then removed by addition of concentrated albumin (10 mg/ml, final) and incubation for an additional minute, followed by sedimentation at 1,700 g for 35 s, and resuspension in the indicated (nigericin-free) solution.

The effectiveness of this procedure for pHi clamping was assessed by fluorimetric measurements with 5,6-dicarboxyfluorescein (DCF). Typical results are illustrated in Fig. 1, in which five traces are superimposed. The figure shows that in the absence of nigericin, the pH (≈7.0) is maintained in the high-K⁺ solutions of pH 6.6–7.2 for several minutes, but that more acidic media gradually acidify the cytoplasm. Upon addition of the ionophore, pH, rapidly changes and equilibrates with that of the external medium within 3 min. After equilibration has been attained, addition of Triton produces only a small fluorescence change¹ (not illustrated) at all values of pH.

¹ The magnitude and direction of the change vary with the degree of DCF loading and with the cell concentration used. The detergent not only relieves the red shift of DCF but also reduces light scattering, which can be significant when high cell densities are used.
A low Na⁺ concentration (17.5 mM) was used for influx measurements in pH⁻-clamped cells, in order to minimize changes in pH during the course of the flux measurement. Experiments under comparable conditions using DCF-loaded cells showed that pH changed by a maximum of 0.15 unit (starting pHᵢ, 6.2) during the 1-min period used for ²²Na⁺ uptake determinations. In some ²²Na⁺ efflux experiments, pHᵢ was manipulated as described above prior to the isotope-loading step. In these cases, in order to maintain pHᵢ at the desired level, loading with ²²Na⁺ was performed in the high-K⁺ solution used for pH⁻ clamping at the appropriate pHᵢ. Nigericin and albumin were omitted from the radioactive solution.

The pH⁻ clamping procedure affected cell volume marginally, if at all. When measured using the Coulter/Channelizer system (Coulter Electronics, Hialeah, FL), cell volume decreased by ≤6% at pHᵢ, 6.2 and was not significantly altered at pHᵢ, 7.2.

RESULTS

²²Na⁺ Uptake in Acid-loaded Cells

The rate of ²²Na⁺ uptake was determined in cells at varying pHᵢ but at a constant pHₑ of 7.2 (Fig. 2). These experiments were performed using cells pretreated briefly with nigericin in high-K⁺ media of varying pHᵢ, in order to modify pHᵢ (see Materials and Methods for details and validation). A low Na⁺ concentration (17.5 mM) was used to restrict Na⁺/H⁺ exchange and therefore to minimize pHᵢ.

![Figure 2](image-url)

**Figure 2.** Effect of pHᵢ on ²²Na⁺ influx in thymocytes in the presence (solid symbols) or absence (open symbols) of 100 µM amiloride. Triangles: untreated cells; pHᵢ values are taken from Grinstein et al. (1984a). Circles: pHᵢ-clamped cells. The cells were pretreated with nigericin/K⁺ at the indicated pH in order to modify pHᵢ (see Materials and Methods) prior to the uptake assay. Uptake was measured for 1 min in a medium containing 1 vol Na⁺ medium and 7 vol choline⁺ medium plus 10 µCi ²²Na⁺/ml at pHₑ, 7.2. Data are means ± SE of three experiments, each with duplicate determinations. Room temperature.
changes during the course of the measurement. Two important features of the data can be noted. First, the total rate of \( ^{22}\text{Na}^+ \) uptake is exquisitely \( \text{pH}_i \) dependent, increasing as \( \text{pH}_i \) decreases. The rate of \( ^{22}\text{Na}^+ \) uptake increases over sixfold between \( \text{pH}_i \) 7.2 and 6.0. This behavior is almost identical to the \( \text{Na}^+ \)-induced alkalinization reported earlier in cells acid-loaded in choline+ media (see Fig. 4 in Grinstein et al., 1984a), which suggests that \( ^{22}\text{Na}^+ \) uptake is increased by activation of \( \text{Na}_3^+/\text{H}_3^+ \) exchange. Second, the stimulation of \( ^{22}\text{Na}^+ \) uptake by internal acidification is largely abolished by 100 \( \mu \text{M} \) amiloride. Importantly, the amiloride-insensitive fraction of the flux is only slightly responsive to \( \text{pH}_i \). In fact, the moderate increase observed upon acid loading in the presence of amiloride may simply reflect residual (uninhibited) \( \text{Na}_3^+/\text{H}_3^+ \) exchange. Alternatively, the small stimulation may reflect the operation of the amiloride-resistant \( \text{Na}^+/\text{Na}^+ \) exchanger (Grinstein et al., 1984b) in the \( \text{Na}^+/\text{H}^+ \) mode.

Also shown in Fig. 2 (triangles) is the uptake rate of cells not subjected to the \( \text{pH}_i \) clamping procedure (i.e., with \( \text{pH}_i \) 7.0). As already shown in the preceding paper, only a small fraction of the influx was amiloride sensitive in these cells. Whereas the amiloride-insensitive fraction of the flux falls on the line obtained for \( \text{pH}_i \)-clamped cells, the total flux is somewhat lower than that in nigericin-treated cells at a similar \( \text{pH}_i \). This probably implies that a slight stimulation of the amiloride-sensitive component is produced by the clamping procedure itself. The precise mechanism of this activation is unclear, but it does not represent \( ^{22}\text{Na}^+ \) flux through residual nigericin, inasmuch as the latter is amiloride insensitive (Grinstein et al., 1984a).

**Na**⁺ Uptake as a Function of [**Na**⁺] in Acid-loaded Cells

As shown above and in the preceding paper, the fraction of the total flux that is amiloride sensitive at \( \text{pH}_i \) 7.0 is small, particularly at high [**Na**⁺]. Therefore, determination of the Na⁺ kinetics of the \( \text{Na}^+/\text{H}^+ \) exchanger under these conditions is unreliable. On the other hand, the amiloride-sensitive flux is the major component in acid-loaded cells, allowing accurate determination of the [**Na**⁺] dependence. The results are summarized in Fig. 3. For these experiments, \( \text{pH}_i \) was set at 6.3 with nigericin/K⁺ prior to the flux assay, which was performed in the presence or absence of amiloride. Total Na⁺ uptake increased as a function of [**Na**⁺] but did not fully saturate within the range studied (Fig. 3A). As expected, most of the flux under these conditions was blocked by the diuretic. A comparatively small fraction was amiloride insensitive. Unlike the amiloride-resistant fraction at \( \text{pH}_i \) 7.0 (Grinstein et al., 1984b), this fraction increased almost linearly with [**Na**⁺]. It is possible that a small amount of residual nigericin increases the flux, particularly at high [**Na**⁺], which tends to linearize the flux vs. [**Na**⁺] relationship. The contribution of this linear component causes the total flux to appear nonsaturable in the range studied (open circles, Fig. 3). However, the amiloride-sensitive component of the uptake, calculated from the difference of the two curves, is saturable, following Michaelis-Menten-type kinetics (Fig. 3B) that can therefore be linearized by the Lineweaver procedure (Fig. 3C). At \( \text{pH}_i \) 6.3, the amiloride-sensitive component has a \( K_m \) for **Na**⁺ of 51 mM. This value is similar to the reported \( K_m \) of 59 mM for the **Na**⁺-induced alkalinization.
**Na⁺ Uptake in Na⁺-depleted Cells**

The pH dependence of Na⁺ uptake and the close correspondence between $^{22}$Na⁺ uptake (Fig. 2) and Na⁺-induced cytoplasmic alkalization (Grinstein et al., 1984a) indicate that much of the amiloride-sensitive flux is Na₂H⁺ exchange. It is, however, possible that a diuretic-sensitive Na⁺/Na⁺ exchange is occurring concomitantly. To estimate the relative contribution of the two modes, $^{22}$Na⁺ uptake was assayed in Na⁺-depleted cells. This procedure would be expected to reduce Na⁺/Na⁺ exchange while possibly increasing Na₂H⁺ exchange. Thus, an
Figure 4. Effect of pH$_i$ on $^{22}$Na$^+$ uptake into Na$^+$-depleted cells. Thymocytes were Na$^+$-depleted for 60 min at 37°C as described in Materials and Methods. Their pH$_i$ was then clamped in an Na$^+$-free, high-K$^+$ medium with nigericin, the ionophore was removed with albumin, and the cells were resuspended for $^{22}$Na$^+$ flux measurements. Uptake was determined at 1 min in a medium containing 17.5 mM Na$^+$ (1 vol Na$^+$ solution plus 7 vol choline$^+$ solution) in the presence (solid symbols) or absence (open symbols) of 100 μM amiloride. The points are means ± SE of three experiments, each with duplicate determinations. pH$_i$ 7.2. Room temperature. The dotted line, included for comparison, represents total Na$^+$ uptake as a function of pH$_i$ in normal (not depleted) cells and is taken from Fig. 2.

The overall decrease in the uptake rate would be suggestive of a predominant Na$^+/Na^+$ exchange and vice versa.

The results of $^{22}$Na$^+$ influx measurements at varying pH$_i$ in Na$^+$-depleted cells (average [Na$^+$] was 3.7 ± 2.2 mM, n = 4) are shown in Fig. 4. The measurements were performed either in the presence or absence of 100 μM amiloride. For comparison, the pattern obtained with cells with normal [Na$^+$] (average 16 mM, from Fig. 2) is included as a dashed line. Two aspects of the data are remarkable: (a) the rate of influx is increased substantially in the depleted cells throughout the pH$_i$ range, which is inconsistent with an Na$^+/Na^+$ exchange mode; (b) at pH$_i$ ≥ 7.0, where the amiloride-sensitive component is virtually undetectable in normal cells, Na$^+$-depleted cells display a large amiloride-sensitive flux. These results closely resemble the effects of Na$^+$ depletion on the Na$^+$-induced alkalinization (Grinstein et al., 1984a) and strongly support the predominance of Na$^+/H^+$ exchange through the diuretic-sensitive pathway. In addition, the data suggest that depletion of Na$^+$ has effects on the exchanger that cannot be accounted for simply by competitive effects at the substrate site (see Discussion).

The Temperature Sensitivity of the Na$^+/H^+$ Antiport

The rate of $^{22}$Na$^+$ uptake was compared in acid-loaded cells at 22 and 37°C. pH$_i$ was fixed at 6.3 and influx was measured in a medium containing 17.5 mM Na$^+$ and osmotically balanced with choline$^+$. In four determinations, the amiloride-sensitive uptake at 22°C was 0.261 ± 0.025 nmol/10$^6$ cells·min. At 37°C, the
rate increased to 0.527 ± 0.019 nmol/10⁶ cells-min. This 102% increase is significantly larger than that reported for the amiloride-insensitive nonconductive Na⁺ flux at pH 7.0 (Grinstein et al., 1984b), which suggests that different transporters are involved.

**Effects of Li⁺**

Li⁺ was found to have virtually no effect on the amiloride-resistant flux of normal (pH 7.0) thymocytes (Grinstein et al., 1984b). A demonstration that Li⁺ interacts with the amiloride-sensitive Na⁺/H⁺ antiport would provide further evidence for the independence of the two systems. Indeed, Li⁺ has been shown to be the only alkali cation, other than Na⁺, capable of eliciting an acidification in acid-loaded thymocytes (Grinstein et al., 1984a) and has been shown to be transported by other amiloride-sensitive antiporters (Kinsella and Aronson, 1981; Rindler and Saier, 1981; Paris and Pouyssegur, 1983). Thus, it was expected that the uptake of $^{22}$Na⁺ through this system would be competitively inhibited by Li⁺. The results of six experiments measuring the effect of Li⁺ are summarized in Table I. Cells were acid-loaded to pH 6.3 and $^{22}$Na⁺ uptake was measured in the presence of 126 mM of either choline⁺ or Li⁺. Consistent with the results reported above, >80% of the flux measured in choline⁺-based medium is inhibited by amiloride. However, markedly different results were obtained in Li⁺ medium. The total uptake was inhibited by 81% and the inhibition was entirely due to a change in the amiloride-sensitive component, while the resistant fraction was not significantly altered. These data further indicate that the pH₁-stimulated $^{22}$Na⁺ fluxes traverse a pathway different from that operative at normal pH₁.

**Reversibility of Na⁺/H⁺ Exchange**

The Na⁺-dependent pH regulatory system of barnacle muscle has been shown to operate backwards when the ionic gradients are reversed (Russell et al., 1983). Similarly, the amiloride-sensitive antiport has been shown to catalyze Na⁺/H⁺ exchange in Na⁺-free media in the case of fibroblasts (Moolenaar et al., 1983) and in low-Na⁺ media in cultured heart muscle fibers (Piwnica-Worms and Lieberman, 1983). On the other hand, only a slow amiloride-insensitive cyto-

### Table I

**Effect of Li on $^{22}$Na Uptake at pH 6.3**

| Medium | Amiloride | $^{22}$Na uptake \( \text{nmol/10}^6 \text{ cells-min} \) |
|--------|-----------|---------------------------------------------------------|
| Choline | -         | 0.189±0.012                                             |
|        | +         | 0.023±0.003                                             |
| Li      | -         | 0.057±0.004                                             |
|        | +         | 0.025±0.002                                             |

Cells were acid-loaded with K⁺ plus nigericin to pH 6.3 as described in Materials and Methods. Uptake was measured for 1 min in media that contained 126 mM of the major cation indicated plus 14 mM Na⁺. They were prepared by combining 1 vol Na⁺ solution with 9 vol of choline⁺ or Li⁺ solution. Data are the means ± SE of \( n \) determinations.
plasmic acidification is recorded in normal thymocytes suspended in Na⁺-free medium (Grinstein et al., 1984b), with no evidence that it represents reversal of the Na⁺/H⁺ antiport. Because several parallel pathways for Na⁺ efflux exist in these cells, it is conceivable that proportionally little substrate is available for the backward operation of the exchanger, compared with that which flows through other routes. To test this possibility, thymocytes were loaded with Na⁺ and pH was recorded following resuspension in Na⁺-free solution. Na⁺ loading was accomplished by incubation in K⁺-free medium with ouabain. Typical pH recordings are illustrated in Fig. 5. In the presence of amiloride, the cells acidified at a rate of ~0.03 pH unit·min⁻¹. This result is consistent with those in the preceding manuscript and confirms the presence of Na⁺/H⁺ exchange. In the absence of the diuretic, on the other hand, a very large acidification was recorded. In extensively loaded cells (2 h loading at 37°C; final [Na⁺] = 102 mM), a high rate of acidification was apparent almost immediately after resuspension in choline⁺ medium. In cells loaded for 1 h at 20°C (final [Na⁺] = 38 mM), the acidification was clearly sigmoidal, with a slow initial component followed by a more rapid phase. The rate of the initial component was comparable to the amiloride-insensitive component.

The intracellular acidification recorded in experiments like those in Fig. 5 is due to transmembrane displacement of H⁺ (or OH⁻) and not to metabolically produced acid. This was confirmed by measurements of pH in weakly buffered media. An extracellular alkalinization was recorded that mirrored the internal acidification. Thus, the acidification of the cytoplasm can be presumed to represent Na⁺/H⁺ exchange, which indicates that the antiport is reversible.

![Figure 5](image_url)

**Figure 5.** Effect of Na⁺-free medium on pH in Na⁺-loaded thymocytes. The cells were loaded with Na⁺ by incubation in K⁺-free medium containing ouabain for either 1 h at room temperature or 2 h at 37°C. DCF acetoxymethyl ester was added to the cells during the last 30 min of the incubation. The cells were then washed twice and used for pH determinations as described. The traces (left to right) start upon resuspension of the cells in choline⁺ solution with or without 100 μM amiloride. pH calibration was performed after lysing the cells with Triton X-100 and titration, applying a correction factor to compensate for the red shift of the intracellular dye. The traces are representative of five similar experiments. Room temperature (20–22°C).
DISCUSSION

The experiments in this report support the existence of a substantial amiloride-sensitive Na⁺/H⁺ antiport in thymocytes and, together with earlier data, allow the calculation of the transport stoichiometry. Moreover, the data indicate that the transport function is reversible but that the system as a whole is asymmetric. These conclusions will be analyzed individually in the following sections.

Stoichiometry of the Amiloride-sensitive Na⁺/H⁺ Exchange

We had earlier detected the operation of a Na⁺/H⁺ antiport in thymocytes as an Na⁺-induced alkalinization in acid-loaded cells (Grinstein et al., 1984a). That Na⁺ was being taken up was demonstrated by the change in content measured photometrically in ouabain-poisoned cells. The present isotopic flux data with ²²Na⁺ support the idea that H⁺ is being extruded in exchange for Na⁺ and allow a more precise quantitation of the stoichiometry. H⁺ efflux at pH 6.3, pH 7.2, and \([Na⁺]\) = 17.5 mM was 2.8-3.3 mmol·liter⁻¹·min⁻¹, calculated from the rate of change of pH and the buffering capacity (Fig. 5 in Grinstein et al., 1984a). This is equivalent to 0.30-0.36 nmol H⁺/10⁶ cells·min. This can be compared to the amiloride-sensitive uptake of ²²Na⁺ under comparable conditions: 0.26 nmol/10⁶ cells·min (from Fig. 2, above). The ratio of the fluxes is reasonably close to unity, which supports the hypothesis of a 1:1 stoichiometry. This is also in agreement with the electroneutral nature of the exchange.

Comparison with Other Na⁺ Transport Pathways

A sizable Na⁺ permeability exists in normal (pH 7.0) thymocytes (Lichtman et al., 1972; Averdunk, 1976; Grinstein et al., 1984b). As in the case of the amiloride-sensitive antiport, a major fraction of the resting permeability is saturable, electroneutral, and selective for Na⁺ over K⁺. Moreover, the \(K_m\) for Na⁺ is similar in both systems (63 mM in resting cells vs. 51 mM in acid-loaded cells). However, the resting and acid-activated transport pathways differ in several important respects. (a) Only the latter is sensitive to amiloride. (b) The acid-activated antiport is more temperature sensitive than the resting flux in the 22–37°C range. (c) The amiloride-resistant fraction of the resting ²²Na⁺ flux, which constitutes ~85% of the total, is not affected by Li⁺, whereas the amiloride-sensitive flux in acid-loaded cells is completely eliminated by this cation, most likely by a competitive action. Competition is likely because Li⁺ was shown to support alkalization in acid-loaded cells (Grinstein et al., 1984a) and because it is an effective competitor in other cell types (Kinsella and Aronson, 1981; Vigne et al., 1981). (d) The amiloride-inhibitable system is considerably more sensitive to pH (Fig. 2). Taken together, these results suggest that the system underlying the major part of the Na⁺ fluxes in resting cells with normal pH is distinct from the amiloride-sensitive exchanger that is activated by lowering pH.

Na⁺ uptake through the apical face of tight epithelia is largely inhibited by amiloride (see Benos, 1982, for review). However, this system also differs in many respects from the amiloride-sensitive uptake of Na⁺ in thymocytes. The former is conductive (Koefoed-Johnsen and Ussing, 1958), whereas the latter is not (Grinstein et al., 1984a). The uptake in thymocytes, but not in epithelia, is
coupled to H⁺ efflux. Moreover, the concentrations of amiloride required for inhibition are significantly lower for epithelia ($K_i < 10^{-6}$ M; see Benos, 1982, for review) than for thymocytes ($K_i = 2.5 \times 10^{-6}$ M). Finally, the inhibitory potency of amiloride is reduced significantly in epithelia by removing divalent cations from the medium (Cuthbert and Wong, 1972). In contrast, no significant change was observed in the case of thymocytes (unpublished observations).

**Reversibility of Na⁺/H⁺ Exchange**

Resuspension of Na⁺-loaded cells in Na⁺-free solution resulted in a marked fall in $pH_i$ (Fig. 5). The magnitude and time course of the acidification were dependent on the extent of Na⁺ loading. If the medium was weakly buffered, an external alkalinization could be measured concomitantly, which would indicate transmembrane displacement of H⁺ (OH⁻). In addition, a large fraction of the acidification was amiloride sensitive, which suggests that backward operation of the Na⁺/H⁺ exchanger is involved.

In experiments like those of Fig. 5, maximal acidification rates of $\sim 0.15 \text{ pH unit} \cdot \text{min}^{-1}$ could be obtained. Assuming that the 1:1 stoichiometry applies also to the reverse flux and considering a buffering power of 25 mmol·pH⁻¹·liter⁻¹ (Grinstein et al., 1984a), the maximum rate of Na⁺ efflux was 3.75 mmol·liter⁻¹·min⁻¹ (0.43 nmol/10⁶ cells·min). This rate is comparable to the maximal amiloride-sensitive $^{22}$Na⁺ uptake rates measured in the forward direction (0.55 nmol/10⁶ cells·min; Fig. 3).

These data indicate that the transport site(s) of the Na⁺/H⁺ exchanger can operate reversibly. The similarity in the maximal rates of the fully activated antiport suggest that the transport site(s) may be symmetric. The reversible operation of the Na⁺/H⁺ exchanger has also been reported in renal brush border vesicles (Aronson, 1983), in fibroblasts (Moolenaar et al., 1983), in cultured heart cells (Piwitca-Worms and Lieberman, 1983), and in A431 cells (Rothenberg et al., 1983).

**Is There a Modifier Site?**

In thymocytes, no significant amiloride-sensitive Na⁺⁺/H⁺⁺ exchange activity is detectable at $pH_i \geq 7.0$ (Grinstein et al., 1984a). Thus, addition of Na⁺ to cells with $pH_i \geq 7.0$ suspended in Na⁺-free medium failed to produce a measurable alkalinization. In addition, incubation of normal cells with amiloride has no immediate effect on $pH_i$. Because the antiporter can operate reversibly (see above), it is conceivable that failure to effect net Na⁺⁺/H⁺⁺ exchange at $pH_i \geq 7.0$ is due to competition of Na⁺⁺ and H⁺⁺ for the transport site on the inner surface. If [H⁺⁺] became sufficiently low, the antiport might operate in an Na⁺⁺/Na⁺ exchange mode. However, the results in this report suggest that competitive inhibition by Na⁺⁺ is unlikely because the amiloride-sensitive fluxes at $pH_i \geq 7.0$ are much smaller (>10-fold) than the maximal rates of Na⁺⁺/H⁺⁺ exchange attained at lower $pH_i$ (see Figs. 2 and 4). It is similarly unlikely that the complex of the carrier with Na⁺ is transported much more slowly than the H⁺-carrier complex, inasmuch as high exchange rates can be obtained in the reverse (Na⁺⁺/H⁺⁺)
direction, i.e., when Na\(^+\) occupies the inside-facing transport site. Moreover, increasing [Na\(^+\)] increases the rate of reverse exchange.

The pH\(_i\) dependence of the transport activity in the pH 6–7 range is much more pronounced than expected. In view of the 1:1 Na\(^+\)/H\(^+\) stoichiometry and the Michaelis-Menten type of kinetics with respect to [Na\(^+\)], similar Michaelis-Menten kinetics would be predicted for [H\(^+\)]. However, a fivefold increase in [H\(^+\)] (between pH 7.0 and 6.3) produced a >10-fold increase in the rate of Na\(^+\)/H\(^+\) exchange. Thus, both when Na\(^+\)-induced alkalinization (Grinstein et al., 1984a) and \(^{22}\)Na\(^+\) uptake (Fig. 2) are measured, the system appears to be “turned on” by lowering pH\(_i\) below 7.0. The data are consistent with the existence of an allosteric modifier site on the cytoplasmic side, which activates amiloride-sensitive transport upon H\(^+\) binding (Aronson et al., 1982). This hypothesis would explain: (a) the sigmoidal dependence of transport on [H\(^+\)]; (b) the inactivation of transport at pH\(_i\) ≥7.0, even though the system is thermodynamically not at equilibrium at this pH\(_i\);\(^2\) (c) the sigmoidal time course of acidification in Na\(^+\)-loaded cells suspended in choline\(^+\) medium (middle curve in Fig. 5). According to the modifier hypothesis, the exchanger would be virtually inactive immediately after the cells are suspended in Na\(^+\)-free medium. An amiloride-resistant acidification process (discussed in detail in Grinstein et al., 1984b), would, however, slowly bring pH\(_i\) to the range where the amiloride-sensitive antiport is activated. This activation would lead to further acidification and positive feedback.\(^3\) Na\(^+\)/H\(^+\) exchange would finally slow down and eventually stop as Na\(^+\) is depleted and Na\(^+\)/Na\(^+\) approaches H\(^+\)/H\(^+\).

Experiments were performed to determine whether the rate of amiloride-sensitive unidirectional \(^{22}\)Na\(^+\) efflux would be increased by decreasing pH\(_i\), as reported by Aronson et al. (1982) for renal brush borders. The results, summarized in part A of Table II, indicate that acidification did not significantly increase the amiloride-sensitive flux. However, this may be the result of opposite neutralizing effects of activation of the modifier site on the one hand and competition of H\(^+\) and Na\(^+\) for the transport site on the other.

**Asymmetry of the Modifier Site**

Is the modifier site situated exclusively at the cytoplasmic surface of the membrane or are there equivalent sites on both sides? To probe for the existence of an externally facing modifier, the efflux of \(^{22}\)Na\(^+\) was measured at varying pH\(_o\) in the presence and absence of amiloride. The results of six determinations are

\[ p\text{H}_i = \ln \frac{[\text{Na}^+]_{\text{cyt}}}{[\text{Na}^+]_{\text{extr}}} + p\text{H}_o. \]

Under normal conditions (i.e., [Na\(^+\)] = 140 mM, [Na\(^+\)] = 14 mM, and pH\(_o\), 7.2), equilibrium would be reached at pH 8.2. This is a minimum estimate since it assumes that all the cellular Na\(^+\) (determined by flame photometry) is free in the cytoplasm.

\(^3\) We have demonstrated that the buffering power is relatively constant in the pH 6.2–7.0 range (Grinstein et al., 1984a). Therefore, pH\(_i\) changes are directly proportional to fluxes in these experiments.
**TABLE II**

**Effects of Intracellular and Extracellular pH on \(^{22}\)Na\(^{+}\) Efflux**

| Medium          | pH\(_i\) | pH\(_{eq}\) | Amiloride | Efflux rate constant | n  |
|-----------------|----------|-------------|-----------|---------------------|----|
|                 | mM       |             |           | 100 \(\mu\)M | min\(^{-1}\) |
| **Series A**    |          |             |           |                     |    |
| 140 choline     | 7.2      | 7.2         | -         | 0.069±0.004         | 6  |
|                 |          |             | +         | 0.042±0.005         | 6  |
|                 | 6.3      | 7.2         | -         | 0.055±0.008         | 6  |
|                 |          |             | +         | 0.056±0.004         | 6  |
| **Series B**    |          |             |           |                     |    |
| 122.5 choline plus 7.0 Na\(^{+}\) | 7.0      | 7.2         | -         | 0.064±0.005         | 6  |
| 17.5 Na\(^{+}\) |          |             | +         | 0.042±0.004         | 6  |
|                 | 6.6      | 7.2         | -         | 0.068±0.005         | 6  |
|                 |          |             | +         | 0.044±0.003         | 6  |
|                 | 6.0      | 7.2         | -         | 0.052±0.006         | 6  |
|                 |          |             | +         | 0.038±0.003         | 6  |

Thymocytes were loaded with \(^{22}\)Na\(^{+}\) as described in Materials and Methods. Efflux was measured in either choline medium (series A) or in 7/8 choline medium plus 1/8 Na\(^{+}\) medium (series B). In both cases, 1 mM ouabain was present. Where indicated, 100 \(\mu\)M amiloride was also added. In series A, pH\(_i\) was clamped with nigericin/K\(^{+}\) prior to \(^{22}\)Na\(^{+}\) loading, which was carried out in medium with 140 mM K\(^{+}\) and 20 mM \(^{22}\)Na\(^{+}\) at the appropriate pH\(_{eq}\). Means ± SE of six determinations.

shown in Table II. Briefly, there is no significant activation of efflux by acidifying the medium. This is in sharp contrast to the effects of pH\(_i\) on Na\(^{+}\) influx. This comparison is illustrated in Fig. 6, where the normalized Na\(^{+}\) flux was related to

![Figure 6](image-url)

**Figure 6.** Evidence for the asymmetry of the amiloride-sensitive Na\(^{+}\)/H\(^{+}\) antiport. The effect of pH on the trans side of the membrane on the rate of Na\(^{+}\) flux (cis to trans) is illustrated. Uptake data (circles) at varying pH\(_i\) are redrawn from Fig. 2. Efflux data at varying pH\(_{eq}\) are plotted from Table II. Open symbols: control. Solid symbols: with 100 \(\mu\)M amiloride. To facilitate comparison, error bars have been omitted and the flux rates have been normalized: unity is the control flux at pH\(_{eq}\) = 7.2. Under the conditions used, [Na\(^{+}\)] and [Na\(^{+}\)] are similar for both uptake and efflux determinations.
pH on the trans side of the membrane. Notice that the intra- and extracellular concentrations of Na⁺ were approximately the same and equal for influx and efflux determinations, which are therefore comparable. The results show a profound asymmetry in the behavior of the amiloride-sensitive carrier, and suggest that the H⁺-dependent modifier site is located exclusively on the internal surface. This asymmetry is consistent with a pH₇-regulatory role of the antiport.

**Effect of Na⁺ Depletion: Is It a Substrate Effect?**

Na⁺ depletion was found to activate Na⁺⁺-induced H⁺ efflux from thymocytes (Grinstein et al., 1984a) and other cells (Paris and Pouyssegur, 1983; Frelin et al., 1983). In acid-loaded cells, this can be rationalized as a decreased competition with H⁺ at the transport site. However, competition alone cannot account for the increased Na⁺ uptake in acid-loaded cells (Fig. 4) and particularly for the activation of the otherwise inactive exchanger at pHᵢ ≥ 7.0 (Grinstein et al., 1984a; Fig. 4). Thus, another mechanism must be responsible that involves a site different from the transport site.

For simplicity, it can be hypothesized that Na⁺ acts at the same modifier site that is activated by H⁺. It is conceivable that, when occupied by Na⁺, the site maintains the exchanger in its inactive conformation and that this is the preferred state at normal Na⁺ and pHᵢ ≥ 7.0. Activation of transport will occur when Na⁺ at the modifier site is replaced by H⁺. This is expected to happen both when [H⁺] is increased at constant [Na⁺], or when [Na⁺] is lowered at constant pHᵢ. The latter would explain the activation recorded in Fig. 4. Alternatively, a separate Na⁺ sensitive cytoplasmic modifier could also exist.

As discussed earlier, this modifier role of Na⁺ confers to the Na⁺⁺/H⁺ exchanger [Na⁺⁺]-regulatory properties. A reduction in [Na⁺⁺] would activate the system and increase Na⁺ uptake, at the expense of pHᵢ. The latter, however, is buffered and could also be compensated by Cl⁻/HCO₃⁻ exchange or by other regulatory mechanisms.

The rapid acidification observed when extensively Na⁺⁺-loaded cells are suspended in Na⁺⁺-free medium (Fig. 5) is apparently inconsistent with an inhibitory role of Na⁺ on the modifier. However, it is possible that the increased amiloride-insensitive Na⁺⁺/H⁺ exchange rapidly acidifies the cell to the level where inhibition by Na⁺ is overcome. On the other hand, prolonged treatment of the cells with ouabain might have resulted in activation of the exchanger. Although the mechanism of this putative activation is not understood, a similar phenomenon has been reported by Rothenberg et al. (1983) in A431 cells. Finally, it is conceivable that the activation observed upon Na⁺⁺ depletion is not a direct effect of the change in [Na⁺⁺], but is an indirect result of the depletion procedure. In this case, Na⁺⁺ loading would not produce the opposite effect (i.e., inhibition of exchange).

**Na⁺⁺/H⁺ Exchange and Volume Regulation**

The antiport can also be activated in lymphocytes by osmotic shrinking (Grinstein et al., 1983). In this case, however, activation is not associated with either cytoplasmic acidification or with a measurable decrease in [Na⁺⁺]. Importantly,
upon activation of the exchanger, pH in shrunken cells can reach levels that are more alkaline than the resting pH of isotonic cells. As discussed elsewhere in detail (Grinstein et al., manuscript submitted for publication), activation by shrinking appears to reflect a change in the pH sensitivity of the exchanger that undergoes an alkaline shift. Thus, a number of parameters determine the activity of the lymphocyte Na+/H+ antiport, which can therefore be involved in the regulation of cellular volume, cytoplasmic pH, and perhaps [Na+].

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