22Na+ Fluxes in Thymic Lymphocytes

I. Na+/Na+ and Na+/H+ Exchange Through an Amiloride-insensitive Pathway

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ABSTRACT The Na+ transport pathways of normal rat thymocytes were investigated. Na+ conductance was found to be lower than K+ conductance, which is consistent with reported values of membrane potential. In contrast, the isotopically measured Na+ permeability was >10-fold higher than that of K+, which indicates that most of the flux is electroneutral. Cotransport with Cl− (or K+ and Cl−) and countertransport with Ca2+ were ruled out by ion substitution experiments and use of inhibitors. Countertransport for Na+ or H+ through the amiloride-sensitive antiport accounts for only 15–20% of the resting influx. In the presence of amiloride, 22Na+ uptake was increased in Na+-loaded cells, which suggests the existence of Na+/Na+ countertransport. Cytoplasmic pH determinations using fluorescent probes indicated that under certain conditions this amiloride-resistant system will also exchange Na+ for H+, as evidenced by an internal Na+-dependent acidification of cells suspended in Na+-free media. The rate of cytoplasmic acidification is proportional to internal [Na+] but inversely related to extracellular [Na+]. Moreover, 22Na+ uptake is inhibited by increasing external [H+]. The results support the existence of a substantial amiloride-insensitive, electroneutral cation exchange system capable of transporting Na+ and H+.

INTRODUCTION

An Na+/H+ exchange system has recently been demonstrated in human peripheral blood lymphocytes (Grinstein et al., 1983) and in rat thymocytes (Grinstein et al., 1984a), detectable as an Na+-induced alkalinization of the cytoplasm or by the volume changes associated with Na+ gain in cells acidified with propionic acid (Grinstein et al., 1984b). The antiporter in thymocytes is electroneutral, amiloride sensitive, and alkali cation specific: it will transport Na+ and Li+ but not Rb+, Cs+, or K+. This exchanger appears to be similar to transport systems in a variety of other cell types reported to exchange extracellular Na+ (Na+) for intracellular H+ (H+). Functionally, the exchanger is presumed to be involved in transcellular salt transport in epithelia (Aronson, 1981, 1983; Boron and Boulaep, 1983; Weinman and Reuss, 1984), but is also present in nonepithelial tissues, where it...
participates in the regulation of cellular volume (Cala, 1980; Kregenow, 1981; Grinstein et al., 1983) and cytoplasmic pH (pHi) (Aickin and Thomas, 1977; Piwnica-Worms and Lieberman, 1983; Grinstein et al., 1984a). It may also play a role in mitogenic activation by serum (Villereal, 1981) and by specific growth factors (Moolenaar et al., 1983; Rothenberg et al., 1983), and may be a mediator in hormonal action (Moore, 1983).

In blood and thymic lymphocytes, the Na⁺/H⁺ exchange is not detectable at pH_i > 7.0, but is greatly stimulated by lowering pH_i. This observation could be explained by competition of internal Na⁺ (Na⁺) and H⁺ for the transport site on the inner surface of the membrane. Given the appropriate affinities for Na⁺ and H⁺, the internal site would be predominantly occupied by Na⁺ at normal [Na⁺] and pH_i (7.01 ± 0.06; Grinstein et al., 1984a), reducing the rate of Na⁺/H⁺ exchange. On the other hand, an increase in [H⁺] (acidification of the cytoplasm) should result in increased exchange. This hypothesis would also explain the reported activation of Na⁺/H⁺ exchange by depletion of Na⁺ (Grinstein et al., 1984a), even at pH_i values where it is otherwise quiescent (i.e., pH_i ≥ 7.0).

A corollary of the competition hypothesis is that at pH_i ≥ 7.0, when Na⁺/H⁺ exchange is no longer detectable, an electroneutral exchange of Na⁺ for Na₀ should be present. Moreover, Na⁺/Na⁺ exchange would be expected to decrease as Na⁺/H⁺ countertransport becomes significant. In principle, this Na⁺/Na⁺ exchange should be sensitive to the same inhibitors that block Na⁺/H⁺ countertransport.

Because the transport capacity reported for Na⁺/H⁺ exchange in thymocytes is very large (>10 mmol-liter⁻¹.min⁻¹ under optimal conditions), the hypothetical Na⁺/Na⁺ exchange would also be expected to be of considerable magnitude. Indeed, large Na⁺ fluxes have been reported in resting (normal pH_i) thymocytes (Lichtman et al., 1972; Averdunk, 1976) and in other cell types. In skeletal muscle, for instance, a substantial fraction of the permeability has been identified as an electroneutral Na⁺/Na⁺ exchange (Erlij and Leblanc, 1971). Na⁺/Na⁺ (Na⁺/Li⁺) exchange has also been described in human red blood cells (Pandey et al., 1978). In thymocytes, however, the Na⁺ fluxes have not been functionally defined.

The purpose of the present experiments was to study the fluxes of Na⁺ in resting (pH_i 7.0) thymocytes, with particular interest in their sensitivity to amiloride and in their electrical properties. The fraction of the flux transported by the antiport was defined and the remainder was separated into several components, based on conductance measurements, ion substitution experiments, and the use of inhibitors. The results indicate the presence of a large, electrically neutral Na⁺/Na⁺ exchange system. Furthermore, in the absence of extracellular Na⁺, the system can mediate the exchange of Na⁺ for H⁺. This exchange was not, however, sensitive to amiloride and differed in other significant respects from the amiloride-sensitive antiport (see also the accompanying paper), which suggests that two different pathways are involved.

MATERIALS AND METHODS

Reagents

2-[N-morpholino]ethanesulfonic acid (MES) and ouabain were purchased from Sigma
Chemical Co., St. Louis, MO; valinomycin was obtained from Calbiochem-Behring Corp., San Diego, CA; bis-(1,3-dithyliothiobarbituric)trimethine oxonol (bis-oxonol) was from Molecular Probes, Junction City, OR; phloretin was from Roth, Karlsruhe, Federal Republic of Germany (FRG); ethacrynic acid was from Serva, Heidelberg, FRG; amiloride was the gift of Merck, Sharp & Dohme, Montreal, Canada; 3,3’dipropylthiadicarbocyanine [diS-C₃-(5)] was the kind gift of Dr. A. Waggoner, Amherst College, MA; 5,6-dicarboxyfluorescein (DCF) acetoxymethylester was the gift of Dr. T. J. Rink, University of Cambridge, England; furosemide was obtained from Hoechst, Toronto, Canada; bumetanide was from Lovens Kemiske Fabrik, Denmark. Bu(carboxyethyl) carboxyfluorescein (BCECF) acetoxymethylester was synthesized by Dr. M. Ramjeesing of The Hospital for Sick Children.

**Isotopes**

Na⁺ and Rb⁺ were from Amersham Corp., Arlington Heights, IL.

**Solutions**

Stock solutions of diS-C₃-(5), DCF acetoxymethylester, BCECF acetoxymethylester, bumetanide, furosemide, and phloretin were made in dimethylsulfoxide. Valinomycin stocks were in ethanol. Amiloride, ethacrynic acid, and ouabain were made up directly in aqueous solutions. Na⁺ solution contained (mM): 140 NaCl, 1 KCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, and 20 Tris-MES, pH 7.2. Choline⁺ solution, Li⁺ solution, and K⁺ solution were prepared by isosmotic replacement of Na⁺ by choline⁺, Li⁺, or K⁺, respectively, but were otherwise identical. Cl⁻-free Na⁺ solution contained 140 Na gluconate, 1 K gluconate, 1 Ca (gluconate), 1 MgSO₄, 10 glucose, and 20 Tris-MES, pH 7.2. K⁺-free Na⁺ solution contained 141 NaCl and no KCl, and was otherwise like normal Na⁺ solution. Ca²⁺-rich Na⁺ solution was similar to the Na⁺ solution but contained 11 mM CaCl₂.

**Cell Isolation**

Thymocytes were isolated from male Wistar rats (200–250 g) as previously described (Grinstein et al., 1984a). The cells were maintained in HEPES-buffered RPMI 1640 solution at room temperature for up to 8 h. Cell viability, determined by trypan blue exclusion, was >95% throughout this period. With the exception of ethacrynic acid (see below), none of the procedures or reagents used affected viability.

Cell counting and sizing were performed with a Coulter Counter/Channelyzer combination (Coulter Electronics, Hialeah, FL). The median cell volume was 114 μm³.

**Cellular Cation Content Manipulation and Determination**

Cellular Na⁺ depletion was accomplished by incubation in Na⁺-free media as described (Grinstein et al., 1984a). Na⁺ loading was achieved by incubation of cells (2 × 10⁷/ml) in K⁺-free medium containing 2 mM ouabain for the indicated times and temperatures. Intracellular Na⁺ and K⁺ contents were measured by flame photometry and cellular concentrations were determined essentially as described (Grinstein et al., 1984a).

**pH, Manipulation and Determination**

pH, was measured fluorometrically using DCF or BCECF exactly as described (Grinstein et al., 1983, 1984a) using a Perkin-Elmer (Norwalk, CT) 650-40 fluorescence spectrophotometer attached to a Perkin-Elmer R-100 recorder. Calibration was carried out at the end of each experiment by disrupting the cells with 0.05% Triton X-100, followed by titration of the medium with concentrated Tris or MES. A correction factor, determined independently, was applied to all the measurements to offset the red shift undergone by the dye inside the cells (Thomas et al., 1979; Rink et al., 1982).
Membrane Potential ($E_m$) Measurements

Membrane potential was estimated fluorometrically using two different probes: bis-oxonol and diS-C$_3$(5). The positively charged cyanine dye diS-C$_3$(5) was used at 0.5 μM with 3 x 10$^6$ cells/ml as previously described (Grinstein et al., 1982). Calibration of fluorescence vs. potential was made by setting $E_m$ equal to the K$^+$ equilibrium potential ($E_{K^+}$) using 1 μM valinomycin. An intracellular K$^+$ concentration of 145 mM was used to calculate $E_{K^+}$ by the Nernst equation (Grinstein et al., 1984a).

The fluorescence of bis-oxonol (0.3 μM) was measured with excitation at 540 nm (2-nm slit) and emission at 580 nm (10-nm slit). Aliquots of the cell suspension (10$^6$ cells/ml, final) were added to medium containing the dye, and the membrane potential was recorded as described by Rink et al. (1980). Calibration was made by adding gramicidin (0.1 μM final) to cells suspended in isotonic media containing various ratios of Na$^+$ and N-methylglucamine (which does not permeate through gramicidin). $E_m$ was calculated assuming that Na$^+$ and K$^+$ permeation through gramicidin is comparable (Gomez-Puyou and Gomez-Lojero, 1977) and/or that intracellular K$^+$ exchanged rapidly with external Na$^+$ through the ionophore (Grinstein et al., 1982).

Isotopic Flux Measurements

For $^{22}$Na$^+$ uptake measurements, duplicate aliquots of 2 x 10$^7$ cells were suspended in 120 μl of the indicated media containing 10–25 μCi $^{22}$Na$^+$/ml for 1–3 min. The reaction was terminated by dilution of the samples with 1 ml of ice-cold choline$^+$ solution containing 100 μM amiloride, followed immediately by centrifugation through an oil-phthalate mixture as described (Grinstein et al., 1982). Extracellular trapping was estimated by addition of the isotope after dilution with the choline$^+$ plus amiloride solution and immediate centrifugation. The overlaying aqueous phase and most of the oil were aspirated and the tip of the tube was cut off and counted. Preliminary experiments showed that the rate of uptake was linear for at least 3 min.

For $^{22}$Na$^+$ efflux determinations, the cells were loaded in HEPES-buffered RPMI 1640 containing 50 μCi $^{22}$Na$^+/ml for 8 min at room temperature. The cells were then sedimented, the loading solution was aspirated, and the sides of the tube and the top of the pellet were quickly rinsed with choline$^+$ solution. The pellet was then resuspended in the indicated medium to a final concentration of 2 x 10$^7$ cells/ml and duplicate 1-ml aliquots were sedimented through oil as above at 0, 4, and 8 min after resuspension.

For $^{86}$Rb$^+$ efflux, 5 x 10$^7$ cells/ml were loaded in HEPES-buffered RPMI 1640 containing 5 μCi/ml $^{86}$Rb$^+$ for 3 h at room temperature. The cells were then sedimented and washed once in Na$^+$ medium before final resuspension in Na$^+$ solution containing 1 mM ouabain at 2 x 10$^7$ cells/ml. Samples were taken at the appropriate times as described for $^{22}$Na$^+$ efflux and the pellets were counted by scintillation in Aquasol after disruption in 2% sodium dodecyl sulfate.

Other Methods

Acid extrusion was measured as changes in external pH with an Orion (Cambridge, MA) 601A digital ionalyzer connected to an X vs. time flatbed recorder (model 7044; Hewlett-Packard Co., Palo Alto, CA) exactly as described (Grinstein et al., 1984a). The buffering power of the medium was determined by titration with KOH and HCl.

Unless otherwise specified, all the experiments were carried out at room temperature (20–22°C). The results are presented either as representative traces (e.g., Fig. 5) or as the means ± SE of the number of determinations specified.
RESULTS

Comparison of Na\(^+\) and K\(^+\) Conductances

The experiments in this and the following section were designed to estimate the fraction of the Na\(^+\) flux that is conductive. Because the small size of thymocytes precludes the use of microelectrodes, absolute conductance measurements could not be obtained. Instead, relative conductances (transference numbers) for Na\(^+\) and K\(^+\) were determined using fluorescence techniques, based on the ionic concentration dependence of the membrane potential. Fig. 1, A and C, summarizes the results of these experiments, using the cationic cyanine dye diS-C\(_3\)(5). A typical calibration curve for this dye is shown in Fig. 1A. The dotted line in Fig. 1C indicates the Nernst potential for K\(^+\) (\(E_{K^+}\)), calculated assuming an intracellular K\(^+\) concentration of 145 mM (Grinstein et al., 1984a). The circles in Fig. 1C indicate the membrane potential determined with cyanine at various extracellular cation concentrations. Reducing the concentration of Na\(^+\) by replacement with choline\(^+\) produced small changes in membrane potential. In contrast, increasing extracellular K\(^+\) substantially depolarized the cells. Transference numbers for Na\(^+\) and K\(^+\) were calculated as

\[
T_i = \frac{\Delta E_m}{RT \ln \frac{C_1}{C_2}}
\]

where \(\Delta E_m\) is the potential change recorded upon varying the concentration of the relevant ion from its initial \((C_1)\) to its final \((C_2)\) value. In the 5–140-mM interval illustrated in Fig. 1, the transference numbers for Na\(^+\) and K\(^+\) were \(T_{Na^+} = 0.21\) and \(T_{K^+} = 0.76\), respectively.

It has been reported that, under certain conditions, cyanine dyes such as diS-C\(_3\)(5) can block a K\(^+\) conductance in red cells (Simons, 1979) and lymphocytes (Rink et al., 1980). Moreover, because of their lipid-soluble cationic nature, the cyanines can accumulate in mitochondria, inhibiting respiration and depleting cellular ATP (Montecucco et al., 1979). It was therefore essential to validate the diS-C\(_3\)(5) results using an alternative approach. For this reason, the transference numbers for Na\(^+\) and K\(^+\) were also determined using bis-oxonol, a negatively charged membrane potential probe that is not accumulated in mitochondria and has not been reported to affect cation permeability.\(^1\) A calibration curve for bis-oxonol is shown in Fig. 1B, and the cation concentration dependence of \(E_m\), determined with this dye, is shown in Fig. 1C (triangles). The absolute value of \(E_m\) was slightly lower when determined with bis-oxonol as compared with the cyanine data. However, the external cation dependence yielded virtually identical results. The transference numbers in the 7.5–140-mM interval were \(T_{Na^+} = 0.15\) and \(T_{K^+} = 0.72\). Under similar conditions, \(T_{Cl^-}\), determined using gluconate substitution in Na\(^+\) medium, was \(\leq 0.10\) (not illustrated).

\(^{1}\) Experiments detecting Na\(^+\)/H\(^+\) exchange as osmotic swelling in Na propionate (Grinstein et al., 1984b) demonstrated that, at the concentrations used, neither diS-C\(_3\)(5) nor bis-oxonol affected the amiloride-sensitive antiport.
FIGURE 1. The membrane potential of thymocytes as a function of external Na\(^+\) and K\(^+\) concentrations. (A) Typical calibration curve, for diS-C\(_2\) (5), obtained by treating the cells (3 \times 10^6/ml) with 1 \(\mu\)M valinomycin at varying external K\(^+\) and measuring fluorescence intensity at equilibrium (6–8 min). The membrane potential under these conditions was assumed to equal \(E_{K^+}\), which was calculated from the Nernst formulation using an intracellular K\(^+\) concentration of 145 mM. (B) Typical calibration curve for bis-oxonol (0.3 \(\mu\)M), obtained by treating the cells (10^6/ml) with 0.1 \(\mu\)M gramicidin in isotonic media with varying ratios of Na\(^+\) and N-methylglucamine*. The membrane potential (abscissa) under these conditions was calculated as 59 \(\ln([\text{Na}^+]/[\text{Na}^+ + \text{K}^+])\), assuming that the permeabilities of Na\(^+\) and K\(^+\) through the ionophore are similar (Gomez-Puyou and Gomez-Lojero, 1977) and/or that K\(^+\) rapidly exchanges for Na\(^+\). Calibration by valinomycin is not possible, because of interaction with bis-oxonol. (C) Effect of varying Na\(^+\) and [K\(^+\)] on membrane potential. K\(^+\) and Na\(^+\) were isoosmotically replaced by choline* or N-methylglucamine*. Circles: data obtained with diS-C\(_2\) (5). Triangles: data obtained with bis-oxonol. The Nernst potential for K\(^+\) (\(E_{K^+}\)), calculated using [K\(^+\)] = 145 mM, is indicated by the dashed line. Note log scale in abscissa. The points are means \(\pm\) 1 SE of three experiments.

These results are very similar to those obtained for human blood lymphocytes (Grinstein et al., 1982), and are consistent with the membrane potentials reported for thymic (Grinstein et al., 1984a) and other types of lymphocytes (Deutsch et al., 1979; Rink et al., 1980) using various techniques.
Comparison of Na⁺ and K⁺ Isotopic Fluxes

The relative permeabilities of Na⁺ and K⁺ were estimated isotopically, measuring the efflux from ouabain-treated cells. The isotope was added to eliminate fluxes through the Na⁺-K⁺ pump. ⁸⁶Rb⁺, which is technically preferable to ⁴²K⁺, was used as a K⁺ analogue. That ⁸⁶Rb⁺ is a suitable analogue for K⁺ has been demonstrated for several tissues, including lymphocytes (Hamilton and Kaplan, 1977). Moreover, conductance measurements like those of Fig. 1 yield almost identical transference numbers for Rb⁺ and K⁺ (unpublished observations). As shown in Fig. 2, ⁸⁶Rb⁺ (K⁺) efflux from rat thymocytes is remarkably slow. The fractional loss is only 0.003 min⁻¹ (mean of four determinations). This very low rate does not reflect saturation of the transport system(s) for K⁺, as evidenced by experiments using partially K⁺-depleted cells. If saturation were occurring, reducing [K⁺] ought to increase the fractional efflux rate, which is proportional to the efflux rate constant. However, as shown in Fig. 2, the fractional efflux rate was not affected by reducing [K⁺] from the normal range of 16–17 nmol/10⁶ cells to 10.2 nmol/10⁶ cells (mean of four determinations) by a 2-h preincubation at 37°C in K⁺-free medium containing 1 mM ouabain. Thus, the efflux rate is linearly related to concentration in the K⁺ concentration range studied.

In agreement with earlier reports (Lichtman et al., 1972; Averdunk, 1976),

* A slow rate of ⁴²K⁺ equilibration has also been reported (Lichtman et al., 1972; Averdunk, 1976), which supports the suitability of ⁸⁶Rb as an analogue. Direct comparison with our results is not possible because of the different conditions used, but the ratio of ²²Na⁺ to ⁴²K⁺ permeability in these reports resembles the ²²Na⁺/⁸⁶Rb⁺ ratio reported here.
the isotopically measured Na⁺ permeability of thymocytes is high (Fig. 2). In
four experiments, the fractional loss averaged 0.056 min⁻¹ (see Table IV for
additional similar data). Therefore, the isotope permeability for Na⁺ is nearly
20-fold greater than that of K⁺ (⁸⁶Rb). This is in sharp contrast to the 3.5-4.5-
fold-higher K⁺ conductance reported in the previous section.

The large discrepancy between the isotopic and conductive permeability
determinations implies that a large fraction of the Na⁺ flux must be nonconduc-
tive, resulting from electroneutral cotransport with an anion, or from counter-
transport for another cation. These possibilities are examined in detail below.

Is Na⁺ Cotransported with Anions?

Electroneutral pathways that cotransport Na⁺ and Cl⁻ or Na⁺, K⁺, and Cl⁻ have
been described in a variety of cell types (Geck et al., 1980; Chipperfield, 1980;
Burg and Good, 1983). As a rule, these systems are tightly coupled so that
removal of one of the ions abolishes transport of the other(s), and they are fully
inhibitable by loop diuretics such as furosemide and bumetanide.

The possible existence of Na⁺/Cl⁻ or Na⁺/K⁺/Cl⁻ cotransport systems in
normal thymocytes was analyzed pharmacologically and by ion substitution
experiments. The results are summarized in Tables I and II. In Na⁺ medium,
the initial rate of ²²Na⁺ uptake at 20-22 °C was found to be ~0.1 nmol/10⁶ cells·
min. Removal of K⁺ or substitution of Cl⁻ by gluconate produced only small,
statistically insignificant increases in the rate of ²²Na⁺ uptake.

The inhibitorsensitivity of the uptake process is reportedin Table II. Bume-
etanide, a potent inhibitor of other cation-anion cotransport systems, had no
significant effect on the rate of ²²Na⁺ influx. Furosemide, at concentrations
that fully block cotransport in Ehrlich ascites cells (Geck et al., 1980), epithelia (Burg
and Good, 1983), and human and avian red cells (Chipperfield, 1980; Kregenow,
1981), inhibited ²²Na⁺ uptake in thymocytes by 22.5%, a marginally significant
effect (P < 0.05).

It is concluded that an external Cl⁻-dependent cotransport of Na⁺ (with or
without K⁺) cannot account for a significant fraction of the nonconductive Na⁺
uptake. The very low rates of K⁺ flux measured under comparable conditions
(Fig. 2) further rule out the participation of an Na⁺/K⁺/Cl⁻ cotransport system.

Na⁺/Cation Countertransport

A coupled Na⁺/Ca²⁺ exchange system is known to exist in the plasma membrane
of many cell types (Lee et al., 1980; DiPolo and Beauge, 1983). This antiport is
generally electrogenic, catalyzing the exchange of at least three Na⁺ per Ca²⁺,
and is therefore unlikely to underlie the observed nonconductive Na⁺ flux.
Moreover, cytoplasmic Ca²⁺ determinations with quin 2 have failed to provide
evidence for the existence of an Na⁺/Ca²⁺ countertransport in splenic (Tsien et
al., 1982) and thymic (Grinstein et al., 1984a) lymphocytes. Nevertheless, the
possibility of Na⁺/Ca²⁺ exchange was measured by means of ²²Na⁺ fluxes. As
shown in Table I, removal of extracellular Ca²⁺, which is expected to increase
²²Na⁺ uptake by diminishing competitive inhibition, had no effect on the influx
rate. Similarly, increasing external Ca²⁺ failed to produce the expected compet-
TABLE I

Effect of Extracellular Ions on $^{22}$Na$^+$ Uptake in Thymocytes

<table>
<thead>
<tr>
<th>Uptake rate (nmol/10$^6$ cells·min)</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.103±0.006</td>
</tr>
<tr>
<td>K$^+$-free</td>
<td>0.150±0.026</td>
</tr>
<tr>
<td>Cl$^-$-free</td>
<td>0.115±0.011</td>
</tr>
<tr>
<td>Ca$^{2+}$-free</td>
<td>0.098±0.010</td>
</tr>
<tr>
<td>Ca$^{2+}$-rich</td>
<td>0.152±0.011</td>
</tr>
</tbody>
</table>

Uptake was measured for 3 min in NaCl medium with the indicated substitutions at 20–22°C. Ca$^{2+}$-rich solution contained 11mM CaCl$_2$. Data are the means ± SE of the number of determinations indicated.

Grinstein et al. Amiloride-insensitive Na$^+$/Na$^+$ and Na$^+$/H$^+$ Exchange Pathway

Because of the low rate of K$^+$ (Rb$^+$) efflux (e.g., Fig. 2), a countertransport of Na$^+$ for K$^+$ cannot account for a substantial fraction of the electroneutral $^{22}$Na$^+$ uptake. Moreover, $^{22}$Na$^+$ efflux is unaffected by complete removal of extracellular K$^+$. Therefore, intracellular cations other than K$^+$ must be exchanging for Na$^+$. Two likely candidates are Na$^+$ and H$^+$. Na$^+$/Na$^+$ (or Na$^+$/Li$^+$) exchange systems have been described in a variety of mammalian cells. In skeletal muscle, the exchanger can be completely blocked by ethacrynic acid and other sulfhydryl group reagents (Erlij and Leblanc, 1971). In red cells, Na$^+$ exchange can be inhibited competitively by Li$^+$ and noncompetitively by phloretin (Pandey et al., 1978; Sarkadi et al., 1978). Finally, under certain circumstances, the Na$^+$-K$^+$ pump can catalyze an exchange of Na$^+$/Na$^+$ that is ouabain sensitive (Blostein, 1983). These inhibitors were tested on the $^{22}$Na$^+$ flux of thymocytes and the results are summarized in Table II. Phloretin, at concentrations equal to or higher than those required for full inhibition in red cells, had no significant effect on $^{22}$Na$^+$ uptake in thymic lymphocytes. Similarly, addition of 1 mM ethacrynic acid to the assay medium had no detectable effect. Because ethacrynic acid probably inhibits in muscle by reacting with sulfhydryl groups (Erlij and Leblanc, 1971), it is conceivable that a preincubation is required for inhibition to develop. To test this possibility, cells were incubated with 1 and 5 mM ethacrynic acid for 30 min at 37°C prior to the transport assay. Preincubation with 1 mM ethacrynic acid reduced the rate of $^{22}$Na$^+$ uptake by 33%. A significant decrease in cell viability was observed at 5 mM, so results at that concentration are not reported. A small inhibition (~15%) that was not statistically significant was obtained with 2 mM ouabain, a concentration that should completely inhibit the pump. The effects of Li$^+$ on Na$^+$ uptake are discussed in detail below. In summary, therefore, the predominant Na$^+$ uptake pathway of thymocytes is distinct from the Na$^+$-K$^+$ pump and from the Na$^+$/Na$^+$ (Na$^+$/Li$^+$) exchanger of human erythrocytes and may be related to the antiporter of skeletal muscle, though with a reduced reactivity toward sulfhydryl reagents.
Uptake was measured for 9 min in NaCl medium with the indicated concentrations of the inhibitors. Where indicated, the cells were preincubated with 1 mM ethacrynic acid for 90 min prior to the transport assay. Data are the means ± SE of n experiments. P was calculated using Student's t test.

**Effects of Amiloride**

As discussed in the Introduction, an active Na\(^+\)/H\(^+\) exchanger has been detected in acid-loaded thymocytes, and it is conceivable that the same system operates in cells with normal pH\(_i\) either in an Na\(^+\)/H\(^+\) exchanging mode, extruding metabolically produced H\(^+\), or as an Na\(^+\)/Na\(^+\) exchanger. These possibilities were considered experimentally using amiloride, an inhibitor of Na\(^+\)/H\(^+\) exchange (Benos, 1982).

As shown in Table II, only 15% of the \(^{22}\)Na\(^+\) uptake by normal cells (pH\(_i\) 7.01; Grinstein et al., 1984a) in Na\(^+\) medium is inhabitable by 100 μM amiloride, a concentration that is >20-fold higher than the \(K_i\) for the diuretic in these cells.\(^3\) In the experiments of Table II, the effect is not statistically significant, but the experiments in the next section indicate that in larger samples a small but significant fraction of the Na\(^+\) influx is indeed amiloride sensitive. These experiments suggest that the major part of Na\(^+\) uptake in resting cells utilizes a pathway that is distinct from the amiloride-sensitive Na\(^+\)/H\(^+\) antiport. Differences between the two systems are also evident by comparison of their Na\(^+\) concentration dependence, temperature sensitivity, and effects of Li\(^+\) (Grinstein et al., 1984a, c).

\(^3\) Because the inhibition by amiloride is competitive with [Na\(_2\)] (Grinstein et al., 1984a), the inhibition under these conditions can be estimated from

\[
K_{i/2} = K_i \left(1 + \frac{[\text{Na}_2]}{K_m}\right)
\]

where \(K_{i/2}\) is the concentration of amiloride needed for half-inhibition at the indicated [Na\(_2\)]. \(K_i\) for amiloride is 2.5 μM and \(K_m\) for Na\(_2\) is 59 mM (Grinstein et al., 1984a). Therefore, under the conditions of Table II, \(K_{i/2} = 8.4\) μM, and virtually complete inhibition of the amiloride-sensitive antiport is expected.


\textbf{Na}^+ \textit{Concentration Dependence of Uptake}

Fig. 3 summarizes experiments to determine the \textit{Na}^+ concentration dependence of the uptake, using choline\textsuperscript{+} to maintain isoosmolarity. Total $^{22}$\textit{Na}^+ influx increases hyperbolically as a function of [\textit{Na}$_{\text{a}}$]. Amiloride partially inhibited the flux at all [\textit{Na}$_{\text{a}}$]. The average inhibition was 23.4 \pm 3.9\%, a statistically significant effect ($P<0.01$). The data for the amiloride-insensitive fraction of the flux (solid symbols in Fig. 3) could be linearized by the method of Lineweaver-Burk (correlation coefficient = 0.984). From this line, the apparent $K_m$ for \textit{Na} was found to be 63 mM. The maximal velocity was 0.077 nmol \textit{Na}^+/10\textsuperscript{6} cells·min.

The magnitude of the amiloride-inhibitable component was not large enough relative to the experimental error (see Fig. 3) to allow determination of its kinetic parameters under these conditions. However, data are available for cells in which the amiloride-sensitive exchange is substantially stimulated by acidification of the cytoplasm (see Grinstein et al., 1984c). The $K_m$ was 59 mM, which is not significantly different from the value above for the amiloride-insensitive flux.

\textbf{Effects of Temperature and Li}^+

To further define the analogies or differences between the two systems, we explored the temperature dependence and effects of Li\textsuperscript{+}. Table III shows uptake data of cells suspended in media containing 14 mM \textit{Na} plus 126 mM of either choline\textsuperscript{+} or Li\textsuperscript{+}. Consistent with the results in Fig. 3, uptake in choline\textsuperscript{+} medium (with 14 mM \textit{Na}$_{\text{a}}$) was 0.020 nmol/10\textsuperscript{6} cells·min and 0.014 nmol/10\textsuperscript{6} cells·min, or 30\% less, in the presence of amiloride. In contrast, uptake in Li\textsuperscript{+} was only 0.013 nmol/10\textsuperscript{6} cells·min, and similar rates were obtained in the presence or

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure3.png}
\caption{External \textit{Na}^+ (\textit{Na}$_{\text{a}}$) concentration dependence of uptake into thymocytes. Normal thymocytes were incubated for 3 min in media containing the indicated [\textit{Na}$_{\text{a}}$] and osmotically balanced with choline\textsuperscript{+}, in the presence (solid symbols) or absence (open symbols) of 100 \textit{m}M amiloride. The data are means \pm SE of three experiments, each with duplicate determinations. pH\textsubscript{e} 7.2. Room temperature.}
\end{figure}
absence of the diuretic. Given that the rates of uptake were essentially the same in the presence of amiloride or Li\(^+\) (~30% lower than the control), the results imply that only the amiloride-sensitive fraction of the flux is inhibited by Li\(^+\). Direct measurements of inhibition of Na\(^+\)/H\(^+\) exchange by Li\(^+\) in acid-loaded cells (Grinstein et al., 1984c) are consistent with this conclusion.

The effect of temperature on fluxes was also analyzed. Uptake was measured in a medium with 17.5 mM Na\(^+\) and osmotically balanced with choline\(^+\). At 22°C in the absence of amiloride, uptake averaged 0.035 ± 0.003 nmol/10^6 cells·min (n = 6). Amiloride (100 μM) reduced the rate to 0.019 ± 0.002 nmol/10^6 cells·min. At 37°C, the total rate increased by 37% (to 0.048 ± 0.005 nmol/10^6 cells·min) and by only 21% (to 0.023 ± 0.003 nmol/10^6 cells·min) in the presence of amiloride, which suggests that the diuretic-sensitive component is more temperature dependent. This was confirmed in the accompanying paper (Grinstein et al., 1984c), using acid-loaded cells.

### Effect of [Na\(^+\)] on Na\(^+\) Uptake

As noted above, the amiloride-resistant flux of Na\(^+\) is largely nonconductive and anion independent. Because the flux was partly sensitive to ethacrynic acid, an inhibitor of Na\(^+\)/Na\(^+\) countertransport in muscle fibers, the existence of an analogous exchanger in thymocytes was considered further. For this purpose, 22Na\(^+\) uptake was measured in Na\(^+\)-loaded cells, in which exchange might be activated by the increased intracellular substrate concentration. Cells were Na\(^+\)-loaded by incubation for 2 h in K\(^+\)-free solution with 1 mM ouabain at 37°C. This treatment increased Na\(^+\) from the original 1.4 to 11.7 nmol/10^6 cells, while decreasing K\(^+\) from 16.6 to 7.1 nmol/10^6 cells. Uptake was measured in 17.5 mM Na\(^+\) medium in the presence of amiloride (100 μM), to circumvent possible effects of Na\(^+\) loading on the diuretic-sensitive component. Under these conditions, influx was 0.0148 ± 0.001 nmol/10^6 cells·min (n = 6) in control cells and increased by a factor of >2.5 to 0.0398 ± 0.007 nmol/10^6 cells·min in Na\(^+\)-loaded cells. These results support the existence of an amiloride-resistant Na\(^+\)/Na\(^+\) countertransport in thymocytes.

### 22Na\(^+\) Efflux Measurements

To further examine the possibility of Na\(^+\)/Na\(^+\) exchange, the efflux of 22Na\(^+\) was measured in the presence and absence of Na\(^+\). The various components of

---

**Table III**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Amiloride</th>
<th>22Na(^+) uptake</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline</td>
<td>-</td>
<td>0.020±0.001</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.014±0.002</td>
<td>8</td>
</tr>
<tr>
<td>Li</td>
<td>-</td>
<td>0.013±0.001</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.013±0.001</td>
<td>8</td>
</tr>
</tbody>
</table>

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.
the efflux were determined by means of the inhibitors ouabain and amiloride. The results are summarized in Table IV. Under the conditions studied, the glycoside inhibited only 35% of the efflux in Na⁺ medium. Amiloride also blocked ~55% of this flux.

Unexpectedly, the efflux was only marginally reduced by complete removal of Na⁺. In choline⁺ medium, ouabain blocked 23% of the flux and amiloride blocked 33%. The effects of ouabain and amiloride were approximately additive, which indicates that they act on separate pathways. This is important in light of a recent report (Soltow and Mandel, 1983) that at millimolar concentrations amiloride effectively inhibits the Na⁺-K⁺ pump.

### Coupling of Na⁺ Efflux with H⁺ Influx

The Na⁺ efflux that persists in ouabain-treated cells suspended in choline⁺ solution is unlikely to be accompanied by intracellular anions, inasmuch as cellular volume measurements of these cells showed only marginal shrinkage (<5%). If accompanied by a monovalent anion, the complete loss of Na⁺ should have resulted in ~10% shrinkage. Therefore, a cation presumably enters the cells in exchange for Na⁺, to preserve electroneutrality. One possible candidate is H⁺, which is accepted as an Na⁺ substitute in other systems.

Fluorescence measurements of pH were undertaken to investigate whether Na⁺ can exchange for H⁺ in Na⁺-free (choline⁺) medium. The results of typical recordings are presented in Fig. 4. In contrast to cells suspended in Na⁺ medium that maintain a constant pH, for extended periods (dotted line, Fig. 4), suspension in choline⁺ medium leads to a gradual cytoplasmic acidification. Typically, freshly isolated cells acidified at a rate of 0.01–0.02 pH unit-min⁻¹. As also shown in Fig. 4, this acidification is not brought about by reversal of the amiloride-sensitive Na⁺/H⁺ antiport, since it is observed in the presence of the diuretic. When cells were suspended in lightly buffered (0.5 mM Tris) choline⁺ solution, the medium became alkaline in parallel with cellular acidification, which indicates that translocation of H⁺(OH⁻) across the membrane occurred.
**Figure 4.** Effect of Na⁺-free medium on pHᵢ. Thymocytes were loaded with DCF and used for pHᵢ determination as described in Materials and Methods. The traces (left to right) start upon resuspension of the cells in choline⁺ solution with or without 100 μM amiloride. The experiment was terminated by disruption of cells with Triton X-100 and calibration of fluorescence vs. pHᵢ (not illustrated). A correction factor, determined using nigericin, was applied to compensate for the red shift undergone by intracellular dye (Thomas et al., 1979). The traces are representative of five similar experiments. Room temperature. The dotted line indicates the pHᵢ of cells maintained in Na⁺ medium.

**Figure 5.** Na⁺-driven H⁺ influx into thymocytes. (A) The pHᵢ of freshly isolated cells (upper trace) or cells loaded with Na⁺ (lower trace) was measured upon resuspension in choline⁺ solution containing 100 μM amiloride. The fluorescence of BCECF was recorded to monitor pHᵢ. Na⁺ loading was for 2 h in K⁺-free medium with 1 mM ouabain at 37°C. This treatment increased [Na⁺] from the control value of 1.36 to 11.33 nmol/10⁶ cells. Calibration of pHᵢ vs. fluorescence was as in Fig. 4. The traces are representative of four determinations. (B) Effect of [Na⁺] on Na⁺-driven acidification. Cells were stained with BCECF and Na⁺-loaded as in A. They were then resuspended in media containing 100 μM amiloride (the concentration of Na⁺ is indicated on the curve in millimolar) and osmotically balanced with choline. Similar results were obtained with N-methylglucamine as a substitute. The traces are representative of three experiments. Temperature, 22°C. pHᵢ 7.2.
Further evidence that the acidification is brought about by an exchange of Na\(^+\) for H\(^+\) was obtained with Na\(^+\)-loaded cells. As shown in Fig. 5A, the acidification rate is considerably higher in these cells than in thymocytes with a normal Na\(^+\) content. Rates of up to 0.05 pH unit-min\(^{-1}\) were obtained. Amiloride was present in all the traces of Fig. 5.

Fig. 5B provides evidence that external Na\(^+\) and H\(^+\) compete for the same site in the purported amiloride-resistant exchanger. pH\(_{0}\) was measured in Na\(^+\)-loaded cells suspended in amiloride-containing solutions with increasing Na\(^+\) concentration. Clearly, the magnitude of the acidification is inversely related to [Na\(^+\)], which supports a competitive interaction. The Na\(^+\) concentration dependence of the inhibitory effect is roughly the same as the concentration dependence for Na\(^+\) influx (Fig. 3), which is consistent with the suggestion that the amiloride-insensitive Na\(^+\)/Na\(^+\) exchanger can also operate in an Na\(^+\)/H\(^+\) exchange mode in the absence of Na\(^+\). Additional evidence that Na\(^+\) and H\(^+\) interact at the external surface of the amiloride-resistant pathway was obtained measuring the pH\(_{0}\) dependence of Na\(^+\) uptake. A reduction of pH\(_{0}\) from 8.0 to 6.0 resulted in a 60% reduction in the flux (Table V). Both the amiloride-resistant and amiloride-sensitive components were pH\(_{0}\) dependent, but the latter was considerably more sensitive. At pH 8.0, the amiloride-sensitive component was a substantial fraction of the total flux (40%), but it was almost completely blocked at pH\(_{0}\) 6.0. The suppression of the amiloride-sensitive system at pH\(_{0}\) 6.0 is consistent with earlier results (Grinstein et al., 1984a).

### Table V

<table>
<thead>
<tr>
<th>pH(_{0})</th>
<th>Amiloride</th>
<th>Na(^+) Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 (\mu M)</td>
<td>nmol/10(^6) cells·min&lt;sup&gt;−1&lt;/sup&gt;</td>
</tr>
<tr>
<td>6.0</td>
<td>-</td>
<td>0.0118±0.0007</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.0121±0.0019</td>
</tr>
<tr>
<td>7.0</td>
<td>-</td>
<td>0.0188±0.0008</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.0151±0.0022</td>
</tr>
<tr>
<td>8.0</td>
<td>-</td>
<td>0.0267±0.0015</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.0187±0.0022</td>
</tr>
</tbody>
</table>

Uptake was measured in media of the indicated pH containing 14 mM Na\(^+\) and osmotically balanced with choline\(^+\). Room temperature (20-22°C). The data are means ± SE of four determinations.

**Discussion**

As illustrated in Fig. 6, the Na\(^+\) fluxes in rat thymocytes can be subdivided into a number of components. One of these is ouabain sensitive and presumably represents flux through the Na\(^+\)-K\(^+\) pump. It constitutes ~30% of the efflux (Table IV) and a smaller fraction of the uptake. A second component is conductive and mediates the modest changes in membrane potential recorded upon changing extracellular [Na\(^+\)]. The conductive component may represent Na\(^+\)
channels, but would also include cotransport of Na\(^+\) with some organic substrates such as neutral amino acids. The absolute magnitude of the conductive component cannot be determined accurately with the available methods, but it can only be a small fraction (<5%) of the total flux. This was concluded by comparison with K\(^+\) (Rb\(^+\)), which has a 3.5–4.5-fold-higher conductance than Na\(^+\) (Fig. 1) but a nearly 20-fold-lower rate of isotopic exchange (Fig. 2). These results lead to the conclusion that most of the isotopic Na\(^+\) flux must represent nonconductive pathways.

The predominance of nonconductive Na\(^+\) fluxes was further suggested by the application of Ussing’s (1949) equation for independent fluxes, as modified by Hodgkin and Keynes (1955):

\[
\frac{M_{\text{out}}^{\text{Na}}}{M_{\text{in}}^{\text{Na}}} = \exp[(E_m - E_{\text{Na}}) n'F/RT],
\]

where \(M_{\text{out}}^{\text{Na}}\) and \(M_{\text{in}}^{\text{Na}}\) are the passive Na\(^+\) efflux and uptake, respectively, \(E_m\) is the membrane potential, \(E_{\text{Na}}\) is the Na\(^+\) equilibrium potential, and \(n'\) is an empirical number whose deviation from unity is a measure of nonindependent behavior (e.g., exchange diffusion, single filing). Using \(E_m = -65\) mV (Fig. 1) and \(E_{\text{Na}} = +59\) mV (calculated from \([\text{Na}^+] = 14\) mM), \(n'\) could be calculated using the efflux and uptake measurements reported in Tables I and IV. Alternatively, because the efflux measurements from the rate constant may be slightly overestimated,\(^4\) efflux could also be estimated assuming that in steady state total

\(^4\) The calculations of flux from the efflux rate constant and cellular \([\text{Na}^+]\) are probably overestimates, since they assume that the entire Na\(^+\) pool is exchangeable within 8 min, the time used for loading. Longer loading periods were impractical, since cell viability was compromised at the high cell concentrations required. In the steady state, the calculated efflux exceeded the measured uptake by 40–80%, which indicates that overestimation indeed occurred.
efflux is equal to the uptake, and then subtracting the fraction (35%; see Table IV) that is ouabain sensitive. By either method, n' values of <0.1 are obtained, which confirms that most of the Na⁺ flux is neither conductive nor independent.

The ion-coupling mode of the nonconductive component was studied by ion substitution and by using inhibitors. The nonconductive flux is unlikely to be an Na⁺/Cl⁻ or Na⁺/K⁺/2Cl⁻ cotransport, inasmuch as ²²Na⁺ uptake was unaffected by the elimination of external Cl⁻ or K⁺ and was not inhibited by the typical cotransport blocker bumetanide and was inhibited only partially by furosemide (Tables I and II). An Na⁺/Ca²⁺ countertransport is similarly unlikely, based on Ca⁺ substitution experiments.

About 20% of the flux is sensitive to amiloride and to Li⁺ (Tables II and III and Fig. 3). In these characteristics, it is similar to the Na⁺/H⁺ exchange system activated in thymocytes by acidification of the cytoplasm (Grinstein et al., 1984a, b). A fraction of the efflux of Na⁺ is also inhibited by the diuretic (Table IV), which suggests that at normal pHt the Na⁺/K⁺ exchanger may be operating as a slow Na⁺/Na⁺ exchanger. This conclusion is consistent with the observation that under the same conditions amiloride-sensitive acid secretion is minimal.

The largest fraction of the nonconductive flux, accounting for at least half of the total flux, appears to be a different Na⁺/Na⁺ exchange pathway. It differs from the Na⁺/H⁺ antiport not only in its insensitivity to amiloride (Table II) and Li⁺ (Table III) but also in its pH (Table V) and temperature dependence, although the apparent affinity for Na⁺ of the two systems is not substantially different. The amiloride-resistant exchanger is also insensitive to ouabain and bumetanide but is partially inhibited by ethacrynic acid (Table II). This exchanger is different from that of human red blood cells (Pandey et al., 1978; Sarkadi et al., 1978), since it is insensitive to both Li⁺ and phloretin. Instead, it may be akin to the exchanger of muscle (Erlij and Leblanc, 1971), inasmuch as both are inhibited by ethacrynic acid but not by Li⁺.

In the absence of external Na⁺, the amiloride-insensitive system can also act as an Na⁺/H⁺ exchanger. This conclusion is supported by the following findings. (a) In ouabain- and amiloride-treated cells, Na⁺ efflux was only marginally reduced by removal of Na⁺. (b) Under these conditions, a cytoplasmic acidification and an alkalinization of the medium are recorded concomitantly with Na⁺ efflux (Figs. 4 and 5). The magnitude of the H⁺ fluxes involved can be calculated from ΔpH, using a buffering capacity of 25 mmol·liter⁻¹·pH⁻¹ (Grinstein et al., 1984a). In normal cells, a flux of 0.027–0.041 nmol H⁺/10⁶ cells·min is required to produce the observed pHt drop. This can be compared to the amiloride- and ouabain-insensitive fraction of the ²²Na⁺ efflux (0.066 nmol/10⁶ cells·min) calculated from the fractional efflux rate and the intracellular Na⁺ concentration (14 mM) measured by flame photometry. Thus, a substantial fraction of the ouabain- and amiloride-resistant efflux of Na⁺ is balanced by H⁺ uptake. (c) The addition of increasing concentrations of extracellular Na⁺ is inhibitory to the cytoplasmic acidification (Fig. 5), with a concentration dependence similar to that of Na⁺ uptake (Fig. 3).

Both the amiloride-sensitive and -insensitive exchangers may contribute to the regulation of cytoplasmic pH. When pHt is drastically lowered, the amiloride-
sensitive system becomes highly activated, becoming the primary route for Na⁺ uptake and the predominant pH-regulatory mechanism. Under certain conditions, this system is also involved in the regulation of cellular volume in anisotonic media (Grinstein et al., 1983). At normal pH, however, this system is relatively quiescent, constituting only ~15% of the Na⁺ flux. As noted above, at least part of this flux is Na⁺/Na⁺ exchange. Under these conditions, the predominant flux involves the amiloride-insensitive system. In Na⁺-free media, this exchanger will countertransport Na⁺/H⁺. This finding raises the possibility that even at normal [Na⁺], where the sites are predominantly occupied by Na⁺, a low level of Na⁺/H⁺ exchange may be taking place. If the exchanger is symmetric, transport should proceed in the direction of net Na⁺ gain and H⁺ extrusion. In this capacity, the exchanger could be participating in the maintenance of pH by extruding H ions that leak into the cell driven by Eₘ or are produced metabolically. This system may be operating continuously to regulate pH, whereas the higher-capacity amiloride-sensitive Na⁺/H⁺ antiport may be activated significantly only when pH falls drastically.

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