Uptake of $^{22}$Na$^+$ and $^{45}$Ca$^{2+}$ into everted membrane vesicles from *Escherichia coli* was measured with imposed transmembrane pH gradients, acid interior, as driving force. Vesicles loaded with 0.5 M KCl were diluted into 0.5 M choline chloride to create a potassium gradient. Addition of nigericin to produce K$^+$/H$^+$ exchange resulted in formation of a pH gradient. This imposed gradient was capable of driving $^{45}$Ca$^{2+}$ accumulation. In another method vesicles loaded with 0.5 M NH$_4$Cl were diluted into 0.5 M choline chloride, creating an ammonium diffusion potential. A gradient of H$^+$ was produced by passive efflux of NH$_3$. With an ammonium gradient as driving force, everted vesicles accumulated both $^{45}$Ca$^{2+}$ and $^{22}$ Na$^+$. The data suggest that $^{22}$Na$^+$ uptake was via the sodium/proton antiporter and $^{45}$Ca$^{2+}$ via the calcium/proton antiporter. Uptake of both cations required alkaline pH$_{in}$. A minimum pH gradient of 0.9 unit was needed for transport of either ion, suggesting gating of the antiporters. Octyl glucoside extracts of inner membrane were reconstituted with *E. coli* phospholipids in 0.5 M NH$_4$Cl. NH$_4$-loaded proteoliposomes accumulated both $^{22}$Na$^+$ and $^{45}$Ca$^{2+}$, demonstrating that the sodium/proton and calcium/proton antiporters could be solubilized and reconstituted in a functional form.

Secondary cation/proton exchange systems catalyze the extrusion of ions from cytosol to the extracellular milieu or extracytosolic compartments. Examples in eukaryotic systems include the Na$^+$/H$^+$ exchangers of renal brush border (1), and mitochondria (2), and the Na$^+$/Ca$^{2+}$ exchanger of sarcodermal (3). In the *Escherichia coli* cytoplasmic membrane four antiporter systems have been identified. Evidence for a Na$^+$/H$^+$ antiporter was first obtained in intact cells by West and Mitchell (4) and subsequently demonstrated both in everted (5-7) and right-side-out membrane vesicles (8-10). This system clearly functions as a transformer, converting the electrochemical proton gradient into an electrochemical sodium gradient for driving Na$^+$-solute cotransport systems (11) and is coupled to the proton motive force, both in *vivo* and in *vitro* (11, 12). In alkalophilic bacteria a Na$^+$/H$^+$ exchanger is involved in regulation of cytosolic pH (11). The evidence for a similar role in *E. coli* is controversial (13, 14); a second monovalent cation/proton exchanger, the potassium/proton antiporter (15), also catalyzes sodium/proton exchange and has also been implicated in regulation of cytosolic pH in *E. coli* (16). Similarly Nakamura et al. (17) have suggested the involvement of a potassium/proton antiporter in regulation of cytosolic pH in *Vibrio alginolyticus*. Finally, two divalent cation exchangers have also been identified, a calcium/proton antiporter and a calcium-phosphate symporter/proton antiporter (18-20). Calcium extrusion is also driven by the proton motive force both in *vivo* (21) and in *vitro* (19, 20).

Elucidation of the molecular mechanism of transport requires purification of the porter proteins and reconstitution of those proteins in an active form in liposomes. Methods using the detergent octyl glucoside have been used successfully to purify several bacterial transport proteins, including the *E. coli* lactose carrier (22, 23) and the phosphate/glucose 6-phosphate antiporter of *Streptococcus lactis* (24). Toward that goal we report here solubilization of *E. coli* membranes with octyl glucoside and reconstitution of the sodium/proton and calcium/proton exchange activities by dilution in the presence of *E. coli* lipid. Activity in reconstituted liposomes was assayed by accumulation of $^{45}$Ca$^{2+}$ or $^{22}$Na$^+$ driven by an imposed ΔpH.

The efficacy of different methods for generating ΔpH was examined in everted membrane vesicles. When NH$_4$Cl-loaded vesicles were diluted into ammonium-free buffer, a ΔpH, acid interior, of up to 3.5 units was generated, and this ΔpH was capable of driving accumulation of both $^{22}$Na$^+$ and $^{45}$Ca$^{2+}$. Uptake of either ion showed a requirement for an alkaline pH$_{in}$, as we had previously found for the respiratory-driven exchange activities (7, 19). These results are in agreement with those of Bassilana et al. (9, 10), who showed that efflux of $^{22}$Na$^+$ from right-side-out membrane vesicles required an alkaline pH$_{in}$. The pH$_{in}$ in right-side-out vesicles is equivalent to the pH$_{in}$ in everted vesicles. They also reported an apparent ΔpH threshold of about 1 unit of $^{22}$Na$^+$ efflux, which they interpreted as an inhibitory effect of low internal pH. We find a similar threshold ΔpH of 0.9 unit for uptake of either $^{22}$Na$^+$ or $^{45}$Ca$^{2+}$. The lack of dependence of the threshold on external pH may reflect gating of the antiporters by the chemical gradient of protons, as suggested by Lamy and Silverman (25) for the sodium/proton antiporter of *Halobacterium halobium*.

**EXPERIMENTAL PROCEDURES**

**Materials**—$^{45}$CaCl$_2$ was obtained from ICN Radiochemicals. $^{22}$NaCl was obtained from New England Nuclear. Crude *E. coli* phospholipids were purchased from Avanti Polar Lipids, Birmingham, AL and were acetone/ether washed according to the method of Newman and Wilson (22). Acridine orange, nigericin, and gramicidin S were purchased from Sigma. Ionophores were used as ethanolic solutions. Other compounds were reagent grade and obtained from commercial sources.

**Bacterial Strains and Growth Conditions**— Cultures of *E. coli* W3133-2ts (26) were grown with shaking overnight at 37 °C in a triethanolamine-buffered minimal medium (27) with 0.5% glycerol as an additional carbon source.
Preparation of Everted Membrane Vesicles—Membrane vesicles were prepared by French press lysis of cells as described previously (28). The buffer consisted of 10 mM Tris-HCl, pH 7.0, 0.5 mM dithiothreitol, 5 mM MgCl₂, and either 0.5 mM KCl (Buffer A) or 0.5 M NH₄Cl (Buffer B), as indicated. During lysis and for storage 0.25 M sucrose was substituted for MgCl₂ in each buffer. The lysed suspension was treated with 7.5 μg/ml of DNase and 2.5 mM MgCl₂ for 10 min at 23 °C and centrifuged at 10,000 × g for 10 min to remove unbroken cells. The supernatant suspension was centrifuged at 100,000 × g for 1 h and washed once with the same buffer. The vesicles were suspended in the same buffer at approximately 20 mg/ml protein, quickly frozen, and stored at -70 °C. Prior to use vesicles were quickly thawed at 57 °C. Sucrose was removed by 10-fold dilution with the appropriate buffer, centrifugation, and suspension in the same buffer. Trypsin-treated vesicles were prepared by incubation of approximately 1 mg of membrane protein with 0.6 mg of trypsin for 30 min at 23 °C in 2 ml of Buffer B, followed by addition of 3 mg of soybean trypsin inhibitor. The vesicles were centrifuged and suspended in 40 μl of Buffer B.

Acridine Orange Fluorescence Assays—Formation of ΔpH was estimated from the quenching of acridine orange fluorescence (28). The reaction mixture consisted of 10 mM Tris-HCl, pH 8.0, containing 0.5 mM choline chloride and 5 mM MgCl₂ (Buffer C). Acridine orange was added to 2 μM. In assays where ΔpH was generated by a potassium gradient, 20 μl of potassium approximately 120 μg of protein) of K⁺-loaded vesicles was added to 2 ml of Buffer C containing acridine orange. Quenching was initiated by addition of 0.1 M of sucrose. In assays where ΔpH was generated by an ammonium gradient, quenching was initiated by addition of 1 ml (approximately 30 μg) of protein) of NH₄Cl-loaded vesicles to 3 ml of Buffer C containing acridine orange. Antiporter activity was estimated from the increase in fluorescence following addition of cation. Fluorescence was measured in stirred cuvettes with an Aminco-Bowman spectrophotometer with excitation at 430 nm and emission at 570 nm.

Transport Assay—Uptake assays were performed in Buffer C containing 1 mM [²²Na]Cl (approximately 3.2 × 10⁷ cpm/nmol) or 0.5 mM [⁴⁴CaCl₂] (approximately 4 × 10⁷ cpm/nmol). Where indicated concentrations of isotopes and pH of Buffer C were varied. When ΔpH was generated by a potassium gradient, 4-6 μl (approximately 150 μg of protein) of K⁺-loaded vesicles in Buffer A was added to 2 ml of Buffer C containing isotope. After 3 min at 23 °C, the reaction was initiated by addition of nigericin to 5 μg/ml. When ΔpH was generated with an ammonium gradient, the reaction was initiated by addition of 4-6 μl (approximately 150 μg of protein) of NH₄Cl-loaded vesicles to 3 ml of Buffer C containing isotope. Antiporter activity was estimated from the change in fluorescence following addition of cation. Fluorescence was measured in stirred cuvettes with an Aminco-Bowman spectrophotometer with excitation at 430 nm and emission at 570 nm.

Solubilization and Reconstitution of Antiporter Activities—Inner membranes were solubilized on a 30-55% linear sucrose gradient and stored at -70 °C in 5 mM sodium ethylenediamine tetracetate, pH 7.0, containing 0.25 M sucrose. Purified inner membranes (approximately 1 mg of protein) were solubilized in a 0.1 M MOPS-KOH buffer, pH 7.0, containing approximately 3 mg of acetone/ether-washed E. coli phospholipids, 1.2% octyl glucoside, 2 mM dithiothreitol, and 20% (v/v) glycerol in a final volume of 0.6 ml. The mixture was mixed by vortexing and incubated on ice for 20 min. The solution was centrifuged for 1 h at 150,000,000 × g at 4 °C, and the pellet discarded. Approximately 50% of the protein was solubilized by this procedure. Proteoliposomes were formed by the dilution method of Newlon and Wilson (22). The extract (0.2-1 mg of protein) was added to a solution containing approximately 8 mg of E. coli phospholipids and 1.2% octyl glucoside in 0.1 M MOPS-KOH, pH 7.0, in a final volume of 1.2 ml. The mixture was vortexed and incubated on ice for 20 min. It was then rapidly diluted into 50 ml of Buffer B lacking MgCl₂, incubated at 23 °C for 15 min, and centrifuged at 150,000 × g for 1 h at 4 °C. The proteoliposomes were washed once with Buffer B and suspended in 40 μl of the same buffer. Transport assays with proteoliposomes were performed as described above except that the pH of the assay buffer was 9.0 instead of 8.0. For assays of sodium uptake the specific activity of the [²²Na] was increased 10-fold and the concentration decreased to 0.1 mM. Also, 0.2-μm pore size filters were used for [²²Na] uptake. For [⁴⁴Ca] uptake 0.4- to 0.2-μm pore size filters gave equivalent results.

Protein Assays—Protein concentrations were determined by the method of Lowry et al. (29) using bovine serum albumin as standard or by Bio-Rad's modification of the method of Bradford (30) using bovine serum globulin as standard.

FIG. 1. Formation of imposed ΔpH in everted membrane vesicles. Everted membrane vesicles (left) or KCl (right) or 0.5 M NH₄Cl (right) were diluted into a fluorometer cuvette containing 0.5 M choline chloride and acridine orange, and fluorescence was measured as described under "Experimental Procedures." Left, K⁺/H⁺ exchange was catalyzed by addition of nigericin (arrows). Ca⁴⁺/H⁺ exchange activity was initiated by addition of 5 mM CaCl₂ (curve 2). Right, an ammonium diffusion potential was generated by a 3000-fold dilution of the vesicles. Na⁺/H⁺ exchange activity was initiated by addition of 5 mM NaCl (curve 4). The discontinuity in curve 3 represents 25 min.

Creation of pH Gradients—Respiring E. coli form a proton motive force acid and positive exterior. In everted membrane vesicles proton pumping produces a proton motive force of the opposite orientation, that is, acid and positive interior. In the experiments described below the high concentration of chloride salts collapse any membrane potential, and thus the entire proton motive force is in the form of the transmembrane pH gradient. Imposed ΔpH of similar orientation and magnitude were created by two strategies. In the first, KCl-loaded vesicles were diluted into buffer containing choline chloride to create a potassium gradient. Nigericin was added to catalyze K⁺/H⁺ exchange, resulting in formation of ΔpH. As illustrated in Fig. 1 (curve 1), formation of ΔpH can be visualized from the quenching of the weak base acridine orange (18, 26). Addition of CaCl₂ at the point of maximum ΔpH formation caused a reversal of fluorescence quenching (Fig. 1, curve 2), indicative of calcium/proton antiporter activity (6, 19).

While a nigericin-induced ΔpH could be utilized for measurement of Ca⁴⁺/H⁺ exchange, it was unsuitable for assaying sodium/proton antiporter activity because nigericin itself can catalyze either K⁺/H⁺ or Na⁺/H⁺ exchange. Alternatively, vesicles loaded with NH₄Cl formed a ΔpH upon dilution into buffer containing choline chloride (Fig. 1, curve 3). The ΔpH is produced by intravesicular dissociation of NH₃ into NH₄⁺ and H⁺ and passive diffusion of NH₃ out of the vesicles, leaving behind H⁺. The magnitude of the ΔpH can be calculated from the initial ratio of total internal and external amine and the pKa of NH₃ of 9.25 (31). At the start of each experiment [pH]ₜᵢᵣᵣ = 7.0 (the pH of the preparation buffer) and [NH₄]₀ = 0.5 M. Thus, the initial concentration of the permeant species, NH₃, could be calculated to be 3 mM, high enough to allow for rapid formation of ΔpH. The gradient

1 The abbreviation used is: MOPS, 4-morpholinopropansulfonic acid.

2 H. Tsujibo and B. P. Rosen, unpublished results.
FIG. 2. Effect of pHe on ΔpH-driven transport of \(^{45}\text{Ca}^{2+}\) and \(^{22}\text{Na}^{+}\). Everted membrane vesicles loaded with 0.5 M KCl (A) or 0.5 M NH\(_4\)Cl (B and C) were diluted into 0.5 M choline chloride and \(^{45}\text{Ca}^{2+}\) (A and B) or \(^{22}\text{Na}^{+}\) (C), and accumulation assayed as described under “Experimental Procedures.” ΔpH was generated by addition of nigericin (A) or imposition of an ammonium diffusion potential (B and C). The pH of the assay buffer in A was: □, pH 8.0; △, pH 7.0; +, pH 6.0; and in B and C: □, pH 9.0; △, pH 8.0; +, pH 7.0; and ○, pH 6.0.

FIG. 3. Release of accumulated \(^{22}\text{Na}^{+}\) or \(^{45}\text{Ca}^{2+}\) by gramicidin. ΔpH was imposed by dilution of ammonium-loaded vesicles into choline chloride containing \(^{22}\text{NaCl}\) (left) or \(^{45}\text{CaCl}_{2}\) (right). At the indicated times (arrows) gramicidin was added to a final concentration of 10 μM (left) or 40 μM (right): □, control; △, + gramicidin; and +, no ΔpH (dilution in 0.5 M NH\(_4\)Cl).

Gradually dissipated (Fig. 1, curve 3, inset). Here addition of Na\(^+\) reversed the fluorescence quenching, suggesting that an ammonium gradient was capable of driving sodium/proton exchange.

Uptake of \(^{45}\text{Ca}^{2+}\) and \(^{22}\text{Na}^{+}\) Driven by Imposed ΔpHs—To demonstrate more directly the coupling of the ammonium gradient to cation fluxes, uptake of \(^{45}\text{Ca}^{2+}\) and \(^{22}\text{Na}^{+}\) was measured (Fig. 2). \(^{45}\text{Ca}^{2+}\) transport was driven by ΔpH, whether produced by nigericin-catalyzed K\(^+\)/H\(^+\) exchange or by an ammonium gradient. Similarly \(^{22}\text{Na}^{+}\) uptake was coupled to an ammonium gradient, with concentration ratios as high as 300:1. Because the ΔpH created by an ammonium gradient could be used for driving uptake of either sodium or calcium, that method was used for subsequent experiments.

Uptake was strongly dependent on [pH]\(_{\text{out}}\), with little activity observed at pH 7 or below. Greatest uptake was found at pH 9.0 for both ions. The effect of [pH]\(_{\text{out}}\) being more alkaline than the pK of NH\(_4^+\) was not examined. The sodium gradient dissipated at about the same rate as the ΔpH (compare Fig. 2C and Fig. 1, curve 3). The calcium gradient dissipated more slowly. Addition of gramicidin caused efflux of either ion from the vesicles (Fig. 3). Again, efflux of "Na" was rapid while efflux of "Ca" was relatively slow. These results indicate that Na\(^+\) was free in solution, while Ca\(^{2+}\) was in equilibrium between free and bound states within the vesicles.

**Discrimination between Cation/Proton Antiporters**—There are four separate antiporters which catalyze cation/proton exchange in *E. coli* (6, 20). Both the sodium/proton antiporter and the potassium/proton antiporter have sodium as a substrate (6, 7, 15). To determine the contribution of each to the observed uptake of \(^{22}\text{Na}^{+}\), the effects of trypsin and K\(^+\) were examined. The potassium/proton antiporter is extremely trypsin sensitive, while the sodium/proton antiporter is not sensitive (15). Trypsin treatment resulted in approximately a 30% decrease in \(^{22}\text{Na}^{+}\) accumulation (Fig. 4). Addition of 10 mM KCl as a competitive inhibitor of the potassium/proton antiporter produced about a 15% decrease in the level of accumulation (Fig. 4) and about a 25% decrease in \(V_{\text{max}}\) (Table I). These results are consistent with 70–80% of the \(^{22}\text{Na}^{+}\) flux being catalyzed by the sodium/proton antiporter.

Two antiporters catalyze Ca\(^{2+}/\text{H}^+\) exchange activity, one of which is a calcium/proton antiporter, and the other exchanges both calcium and phosphate for protons (19, 20). The latter requires phosphate for \(^{45}\text{Ca}^{2+}\) transport. Since these assays are done in the absence of phosphate, that system is unlikely to contribute significantly to the observed \(^{45}\text{Ca}^{2+}\) uptake. The Ca\(^{2+}/\text{H}^+\) antiporter is sensitive to trypsin treatment, while the calcium-phosphate/proton antiporter is resistant. As seen in Fig. 4, trypsin treatment inhibited \(^{45}\text{Ca}^{2+}\) accumulation by 60%, comparable to the effect of trypsin on respiration-driven \(^{45}\text{Ca}^{2+}\) via the Ca\(^{2+}/\text{H}^+\) antiporter (20).

**Kinetics of \(^{22}\text{Na}^{+}\) and \(^{45}\text{Ca}^{2+}\)**—Uptake assays were performed over a 100-fold range of substrate concentration for time periods of 7 s–1 min. The reactions were found to be...
**FIG. 4. Effect of K⁺ and trypsin on cation uptake.** ΔpH was imposed by dilution of ammonium-loaded vesicles into choline chloride containing ²²NaCl (left) or ⁴⁵CaCl₂ (right). Trypsin treatment of vesicles was performed as described under "Experimental Procedures." ○, control; ●, +10 mM KCl; △, trypsin-treated vesicles; +, no ΔpH.

**TABLE I**

<table>
<thead>
<tr>
<th>Cation</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (nmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>²²Na⁺</td>
<td>1.2 ± 9.2</td>
<td>900</td>
</tr>
<tr>
<td>²²Na⁺ + 10 mM KCl</td>
<td>2.4 ± 0.5</td>
<td>670</td>
</tr>
<tr>
<td>⁴⁵Ca²⁺</td>
<td>0.9 ± 0.1</td>
<td>170</td>
</tr>
</tbody>
</table>

Nonlinear even over very short time periods. When the data were analyzed by linear regression of plots of $s/v$ versus $s$ for each time point, the observed $K_m$ values were constant with time, while the apparent $V_{max}$ values decreased with increasing time. To estimate the true $V_{max}$, a plot of apparent $V_{max}$ values as a function of time was extrapolated to $t = 0$. The results are summarized in Table I. Both antiporters have $K_m$ values in the millimolar range, similar to those determined for the respiration-driven exchange activities (7, 19). More accurate kinetic determinations will require use of sampling techniques in the millisecond range.

**Effect of Driving Force on ²²Na⁺ and ⁴⁵Ca²⁺ Uptake**—In the experiment shown in Fig. 2 the external pH was varied under constant ΔpH. In the experiment shown in Fig. 5 the magnitude of the pH gradient was varied by substitution of varying amounts of NH₄Cl for choline chloride in the assay buffer, but at constant ionic strength and osmolarity. No uptake of either cation was observed below a ΔpH of about 0.9 unit. ²²Na⁺ uptake was linear with ΔpH between 1 and 3 units at an external pH of 8. ⁴⁵Ca²⁺ uptake was linear over a similar range when measured in buffer at pH 9, but the driving force became saturating at about 2.3 units at pH 8. The data shown in Fig. 5 were obtained after 1 min for ²²Na⁺ or 30 s for ⁴⁵Ca²⁺ (but normalized to 1 min) to allow time for formation of maximal ΔpH (see Fig. 1).

**Solubilization and Reconstitution of ²²Na⁺ and ⁴⁵Ca²⁺ Uptake**—Inner membrane vesicles were solubilized with octyl glucoside and reconstituted by dilution with E. coli phospholipids. The reconstituted proteoliposomes accumulated both ²²Na⁺ and ⁴⁵Ca²⁺ to levels comparable to those of native vesicles (Fig. 6). Accumulation of either ²²Na⁺ (not shown) or ⁴⁵Ca²⁺ (Fig. 6, right) required a ΔpH. Both ²²Na⁺ and ⁴⁵Ca²⁺ accumulation were sensitive to gramicidin, indicating that the cations were in free solution inside of the proteoliposomes. With both cations the amount of isotope associated with liposomes alone was comparable to proteoliposomes with gramicidin (not shown) and probably represents nonspecific binding.

**DISCUSSION**

A preliminary step to purification of membrane-bound solute porter proteins is development of an assay. If the carrier can catalyze nonenergy-dependent transport, e.g. homologous or heterologous exchange, then uptake of isotope can be visualized by exchange with preloaded nonradioactive solute. This approach has proven successful with the lac carrier of E.
vesicles forms a \( \Delta p \H \), whose magnitude can be calculated when a proton motive force is applied. Proteoliposomes containing only purified antiporter proteins would lack the cellular primary proton pumps which establish the proton motive force in cells or vesicles. We have investigated methods for imposing a \( \Delta p \H \) acid interior, in everted membrane vesicles and liposomes. Two methods proved successful. Nigericin-mediated K\(^+/\H\) exchange using K\(^+\)-loaded vesicles created a \( \Delta p \H \), acid interior (Fig. 1), of magnitude sufficient to drive substantial uptake of \(^{45}\Ca^{2+}\) (Fig. 2). Because nigericin recognizes Na\(^+\) as well as K\(^+\), this method could not be used for \(^{22}\Na^{+}\) uptake. A more general method using ammonium gradients was used. NH\(_3\) can permeate passively across lipid bilayers while NH\(_3\) is relatively impermeant. Imposition of a gradient of NH\(_4\)Cl results in passive downhill ammonia efflux from vesicles. Dissociation of NH\(_4\) to NH\(_3\) + H\(^+\) within the vesicles forms a \( \Delta p \H \), whose magnitude can be calculated from the relationships (31)

\[ \Delta p \H = \text{pH}_{\text{out}} - \text{pH}_{\text{in}} \]

and, with a \( K_a = 9.25 \),

\[ \text{pH}_o = -\log([\text{NH}_3]/[\text{NH}_4]^+)(10^{-9.25} + 10^{-14+\text{pH}_{\text{in}}} - 10^{-9.25}) \]

This imposed proton gradient was capable of energizing accumulation of both \(^{22}\Na^{+}\) and \(^{45}\Ca^{2+}\) (Fig. 2) and provides a convenient assay for antiporter activity. Calcium/proton antiporter activity has been measured previously by respiration-driven uptake of \(^{46}\Ca^{2+}\) into everted membrane vesicles (19). Nakamura et al. (32) has used amine-loaded cells of Vibrio alginolyticus to drive uptake of Na\(^+\), probably by reversal of the sodium/proton antiporter of that organism. However, characterization of the sodium/proton antiporter in everted membrane vesicles has been performed mainly by examination of the effect of Na\(^+\) on the \( \Delta p \H \)-dependent quenching of acridine orange fluorescence, an indirect assay (5–7). Uptake of \(^{22}\Na^{+}\) into everted membrane vesicles has been measured by flow dialysis (7, 8), but the accumulation ratios were less than 10 compared to the 250-300-fold accumulation reported here. Moreover, only steady-state measurements can be made using flow dialysis. Bassilana et al. (9, 10) have made elegant kinetic measurements of \(^{22}\Na^{+}\) efflux from right-side-out membrane vesicles. For reconstitution studies, however, isotope efflux assays are less convenient than direct measurements of uptake.

From the time course of acridine orange fluorescence quenching (Fig. 1), \( \Delta p \H \) was formed in less than 20 s and dissipated spontaneously within 30 min (Fig. 1). Since acridine orange accumulates in response to \( \Delta p \H \), the proton gradient must form faster than 20 s; exactly how fast cannot be determined by that method. This presents a problem for determination of kinetic parameters, since the basic assumption in kinetics is that one substrate is varied with everything else held constant. If the driving force (and the internal concentration of the co-substrate, H\(^+\)) is changing at very early time points, then true initial rates cannot be obtained. However, the reactions were not linear over times greater than 10 s, so again, true initial rates could not be determined. Since the apparent \( K_a \) did not change during the first minute, the experimental values may be fair approximations of the affinity of the carriers for cation. Indeed the values for both Na\(^+\) and Ca\(^{2+}\) are similar to those determined from acridine dye measurements (7, 19). Bassilana et al. (10) reported a \( K_a \) for \(^{22}\Na^{+}\) efflux of 3.5 mM at pH 7.7, which is close to the our value of 2.4 mM obtained in the presence of K\(^+\) (Table I). The values for \( V_{\text{max}} \), on the other hand, probably represent an underestimate. Because the calculated \( V_{\text{max}} \) values decreased with time, the \( V_{\text{max}} \) was estimated by extrapolation to zero time. After correction for the contribution of the potassium/proton antiporter, the value of 670 nmol/mg/min for \(^{22}\Na^{+}\) is in reasonably good agreement with the \( V_{\text{max}} \) obtained from efflux of \(^{22}\Na^{+}\) from right-side-out vesicles (10).

Bassilana et al. (10) reported a nonlinear dependence of \(^{22}\Na^{+}\) efflux from right-side-out vesicles when measured as a function of \( \Delta p \H \). They considered two possibilities: (1) intrinsic gating of the sodium/proton antiporter, as has been suggested for the sodium/proton antiporter of H. halobium (25), or 2) an effect of low pH on the carrier. They concluded that the latter best explained their data. First, the apparent gating was observed only at pH\(_{\text{out}} < 7\), while gating should be dependent on \( \Delta p \H \) but independent of absolute pH. Second, the \( K_a \) decreased with increasing pH\(_{\text{in}}\). As shown in Fig. 5, uptake of either \(^{22}\Na^{+}\) or \(^{45}\Ca^{2+}\) into everted membrane vesicles exhibits an apparent gating, with a threshold of approximately ~55 mV. Similarly, uptake of both ions is pH dependent, with the rate of uptake increasing with increasing pH\(_{\text{in}}\). Note that pH\(_{\text{out}}\) in everted membrane vesicles corresponds to pH\(_{\text{in}}\) in right-side-out membrane vesicles. These results are in agreement with those of Bassilana et al. (10) and suggest that both Na\(^+/\H\) antiporter and Ca\(^+/\H\) antiporter have a dissociable group with a \( K_a \) below 7".
and 9 (Fig. 5). The pHmax as a function of ΔpH would necessarily be different in those two cases as well. Thus, the threshold ΔpH occurred at pHmax of 7 in one case and 8 in the other. A single dissociable group could not account for that result, leaving open the possibility of an actual energetic barrier to catalysis by antiporters.

This investigation of the catalytic mechanism requires purified functional antiporters. As a first step we have solubilized everted membrane vesicles with octyl glucoside and demonstrated that the activities could be reconstituted into proteoliposomes (Fig. 6). We previously used octyl glucoside to solubilize and reconstitute 45Ca2+ transport into asolectin liposomes (33), but the specific activity of the proteoliposomes was low compared to the original vesicles, perhaps because asolectin was used instead of E. coli phospholipids or perhaps because the driving force was inadequate. In those experiments ΔpH was created by rapid mixing of vesicles at low pH in 45Ca2+-containing buffer at high pH. This pH jump assay has several disadvantages: first, the gradient dissipates rapidly; second, transient variations in pHmax causes transient changes in binding of 45Ca2+. Tsujiya et al. (34) have reported the reconstitution of the sodium/proton antiporter using octyl glucoside extracts of E. coli membranes. In that study the driving force was a reconstituted respiratory chain, and only the effect of Na+ on the ΔpH-dependent quenching of acridine dye fluorescence was examined. The use of an ammonium gradient to drive isotope uptake should have several advantages: first, an artificial driving force it would not be possible to carry out purification past the separation of carrier from respiratory chain. Second, the signal-to-noise ratio in an isotope uptake experiment is much greater than in the indirect fluorescence assay. Present efforts are directed toward characterization of the reconstituted activities, and longer range toward fractionation and purification of the antiporters.

REFERENCES