The relationship between the steady-state sodium gradient (ΔpNa) and the protonmotive force developed by endogenously respiring *Escherichia coli* cells has been studied quantitatively, using 23Na NMR for measurement of intracellular and extracellular sodium concentrations, 31P NMR for measurement of intracellular and extracellular pH, and tetrathyenylphosphoramidate for measurement of membrane potential. At constant protonmotive force, the sodium concentration gradient was independent of extracellular sodium concentrations over the measured range of 4–285 mm, indicating that intracellular sodium concentration is not regulated. The magnitude of ΔpNa was measured as a function of the composition and magnitude of the protonmotive force. At external pH values below 7.2, ΔpNa was parallel to ΔpH but showed no simple relationship to the membrane potential; above pH 7.2 the parallel relationship began to diverge, with ΔpH continuing to decrease but ΔpNa starting to level off or increase. Although plots of ΔpNa versus ΔpH had slopes of close to 1, the value of ΔpNa consistently exceeded that of ΔpH by ~0.4 units, indicating a partially electrogenic character to the putative H+/Na+ antiport. The apparent stoichiometry was 1.13 ± 0.01 at external pH below 7.2. The possible significance of this nonintegral stoichiometry is discussed according to a model in which two distinct integral stoichiometries (possibly 1H+/1Na+ and 2H+/1Na+) are available with some relative probability; the model predicts futile cycling of sodium ions and a dissipative proton current. In the course of this study, we discovered that the magnitude of the pH gradient developed by the cells was osmolarity-dependent, yielding steady-state intracellular pH values that varied from 7.1 at 100 mosm to 7.7 at 800 mosm.

In bacteria, in many other cells, the intracellular concentrations of the major cations, H+, K+, and Na+, are in general quite different from the extracellular concentrations. There is a high intracellular K+/Na+ ratio and a closely regulated intracellular pH (pHi), while in the extracellular medium the K+/Na+ ratio is often quite low and the pH can range from acidic to alkaline values. The framework for understanding how the differential composition of intracellular and extracellular compartments is developed and maintained is Mitchell's chemiosmotic hypothesis, which postulates that the energy of chemical bonds is transduced into the transmembrane electrochemical potential of a particular ionic species, and this potential then provides for the generation and maintenance of gradients of other ions and molecules (1). In most bacteria, protons extruded by coupling to oxidation-reduction reactions or hydrolysis of ATP are the primary source of transmembrane electrochemical energy.

At moderate extracellular K+ concentrations, the high intracellular K+ concentration is thought to result from uptake driven either by membrane potential (Δφ) alone via simple K+ uniport (2), or by H+/K+ symport (3, 4).

To account for the low intracellular Na+ concentration relative to the external medium (5), Mitchell (6) proposed that inward movement of H+ down its electrochemical gradient is coupled to Na+ extrusion via H+/Na+ antiport. Experimental evidence lending support to the existence of H+-driven H+/Na+ antiport exists for a variety of bacteria: halophiles and other marine bacteria (7–9), alkalophiles (10, 11) and neutrophiles (2, 12, 13). There are exceptions to this mechanism of Na+ extrusion: Streptococcus faecalis uses a Na+-ATPase (14, 15) and some marine organisms use a respiration-driven Na+ pump (16, 17).

In *Escherichia coli*, the first experimental evidence for H+/Na+ antiport came from a study by West and Mitchell (18), who observed proton extrusion following addition of Na+ to an anaerobic cell suspension. Since membrane-permeant ions had no effect on the Na+-dependent acidification of the medium, the authors concluded that the antiport is electroneutral. Later studies, primarily carried out in membrane vesicles from *E. coli*, have provided more direct evidence for the coupling between Na+ and H+ movements and have further characterized the properties of the antiport. It was demonstrated that addition of Na+ partially collapsed a pre-existing outwardly directed pH gradient in inverted vesicles (12, 19) and could drive the formation of a pH gradient in right-side-out vesicles (12). Because Na+ movements via H+/Na+ antiport could be driven by either Δφ or ΔpH (20), it was concluded, contrary to the original proposal, that the antiport could operate in an electrogenic manner. Schuldiner and Fishkes (12) have suggested that the stoichiometry of the antiport may be dependent on the external pH, behaving electroneutrally at acidic pH values and electrogenically at alkaline pH values. However, more recent studies (21) have demonstrated that, even at pHi values as low as 5.5, Δφ enhances Na+ efflux mediated by H+/Na+ antiport, a result that suggests an electrogenic process. Additional information

Anna M. Castle, Robert M. Macnab, and Robert G. Shulman

From the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511

*This work was supported by Public Health Service Grant AI 12202 (to R. M. M.) and National Science Foundation Grant PCM-8402670 (to R. G. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: pHe, intracellular pH; pH, extracellular pH; ΔpH, transmembrane pH difference; Δψ, membrane potential; ΔfNa, protonmotive force; ΔfNa+, sodiummotive force; NaL, intracellular Na+; NaE, extracellular Na+; pNaL, intracellular pNa; pNaE, extracellular pNa; ΔpNa, transmembrane pNa difference; Pipes, 1,4-piperazinediethanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid; Tes, N,N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Ricine, N,N-bis(2-hydroxyethyl)glycine.
on the properties of the H+/Na+ antiport process came from kinetic analyses of the effects of an imposed ΔPH or ΔΨ on the rate of Na+ efflux (22). An important conclusion was that the device responsible for the H+/Na+ antiport is regulated by intravesicular pH, low pH_{ra} (<6.6) being inhibitory.

Although the kinetics of the H+/Na+ antiport process have been extensively studied, information on the magnitude of the Na+ gradient (ΔpNa) developed by E. coli is scarce and inconclusive. Epstein and Schultz (23), using flame photometry to estimate [Na+]_{e}, reported that [Na+]_{e} was raised from 50 to 500 mM, ΔpNa increased from 0.2 to 0.6. In contrast, measurements of ΔNa accumulation in inverted membrane vesicles from E. coli have indicated that the gradient is constant (ΔpNa = 0.9) between [Na+]_{e} values of 0–50 mM and that it is independent of pH_{ra} (20). As we have discussed previously (24), one reason for the scarcity of information has been the lack of a suitable method for measurement of [Na+]_{e}, which, because of active extrusion, is quite low.

Recently it has become possible to monitor simultaneously both the Na+ and Na+ pools by means of ΔNa NMR which, carried out in the presence of a paramagnetic reagent that is membrane-impermeant, allows observation of a chemically shifted extracellular resonance and an unshifted intracellular resonance (25, 26). In a recent study, we demonstrated the use of this technique for measuring [Na+]_{e} and [Na+]_{i} in endogenously respiring and glycolyzing E. coli cells (24). The present study constitutes an extension of this work, in which we measure [Na+]_{e} in endogenously respiring E. coli cells under a variety of conditions, examine the question of regulation of [Na+]_{e}, and consider the magnitude of the resulting ΔpNa in relation to other energetic parameters of the cell. Specifically, the study focuses on the relationship between ΔpNa and the components of the proton motive force (Δμ_{p}), in the hope of providing a better understanding of Na+ transport in E. coli.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains—**E. coli strain MRE 600 is wild-type except for low RNase activity (27). E. coli strains CS 71 (gltC metB lacY1) (28) and RA 11 (29) were obtained from Drs. S. Schuldiner (Hebrew University, Jerusalem) and T. H. Wilson (Harvard Medical School), respectively.

**Chemicals—**Chemicals used and their sources are given in Ref. 24.

**Preparation of Cell Suspensions for NMR Measurements—**The procedures for growing, harvesting, and preparing strain MRE 600 for NMR measurements were as described (24), except that cells were maintained aerobically throughout the experiment, unless otherwise indicated in the text. Strain CS 71 was grown in medium M9 containing 0.4% glucose or 0.5% glycerol and 1 μg ml⁻¹ thiamine, and strain RA 11 was grown in medium 6I supplemented with 1% tryptone (Difco) and 1 μg ml⁻¹ thiamine. The procedures for harvesting these cells for NMR were the same as for strain MRE 600. The buffer used depended on the particular experiment, and is given in the text or the appropriate figure legend. For studies of [Na+]_{e} as a function of pH_{ra} at constant [Na+]_{e} and osmolarity, the composition of the resuspension buffer was chosen according to the desired value of pH_{ra}; for pH_{ra} values below 6.8; it contained 30 mM Pipes, 30 mM Mes, 5 mM KH2PO4, 80 mM NaCl, with KOH and NaOH added to adjust the pH to the desired value and KCl added to bring the osmolarity to 370 mosm. For higher pH_{ra} values, the resuspension buffer was modified by replacing 30 mM Mes with 60 mM Tes and adjusting the pH to approximately 0.5 units higher than the desired final value of pH_{ra}. To obtain pH_{ra} values as above 7.4, small aliquots of 310 mM Bisine, pH 12.5, were introduced into the cell suspension after it had been warmed up and oxygenated in the spectrometer. We found that the additional base and buffering capacity was necessary to maintain the higher pH_{ra} values, probably because under these conditions the cells generate and excrete acidic metabolites. Shift reagent was prepared by combining DyCl₃ and tripolyphosphate in a 1:3 molar ratio and added to the cell suspension at ~6 mM with respect to dysprosium, as described (24). In most experiments the shift reagent contained potassium tripolyphosphate; sodium tripolyphosphate was used as indicated in the text.

**RESULTS**

**Effect of External Na+ Concentration on ΔpNa—**In a typical experiment, the cell suspension was initially anaerobic and then oxygenated; [Na+]_{e} and [Na+]_{i} were monitored throughout. Oxygenation led to rapid extrusion of Na+ followed by stabilization of [Na+]_{e}, as we have described (24). The same final value of [Na+]_{e} was attained using either the above protocol, or one where the cells were pre-loaded with Na+ to an initial concentration 2-fold higher than usual, or one where the cells were maintained aerobically throughout the experiment. Since the final value of [Na+]_{e} was independent of the protocol, we consider it to be a true steady-state value for respiring E. coli cells.

Fig. 1 presents the steady-state value of [Na+]_{e} as a function of [Na+]_{i} in the range from 4 to 285 mM, at 25°C, pH_{ra} of
phosphate, sufficient to bring $[\text{Na}^+]$ to the desired value. The suspensions were temperature-equilibrated under anaerobic conditions and then oxygenated for the remainder of the experiment. Each point on the graph represents an average of 15-1 min spectra following the stabilization of $[\text{Na}_2^+]$ under aerobic conditions with pH$_{r}$ at 6.5-6.7. Inset, dependence of $\Delta p\text{Na}$, $\Delta p\text{H}$, $\Delta \psi$, and $\Delta \mu\text{H}$ on $[\text{Na}_2^+]$; for description of energy units, see Fig. 3.

6.6-6.7, and constant osmolarity (375 mosm). It is evident that the ratio $[\text{Na}_2^+)/[\text{Na}_+^+]$, and hence $\Delta p\text{Na}$, was independent of $[\text{Na}_2^+]$ over the whole range. The value of $\Delta p\text{Na}$, determined by linear regression, was 1.24 ± 0.02 (n = 12) or, in electrical units, −73 ± 1 mV.

Under the conditions of all these experiments, the value of $\Delta p\text{H}$ was constant at 0.8-0.9 (50 mV); also, $\Delta \psi$ had the same value (−125 mV) in the absence of added Na$^+$ as at quite high $[\text{Na}_2^+]$ (150 mM). Thus a constant $\Delta p\text{Na}$ was observed over a wide range of $[\text{Na}_2^+]$ values under conditions where both $\Delta p\text{H}$ and $\Delta \psi$, and hence the total $\Delta \mu\text{H}$, were constant. A schematic representation of this situation is given as an inset to Fig. 2.

We also examined the kinetics by which $\Delta p\text{Na}$ readjusted to a new steady state. Fig. 3 shows the changes in $\Delta p\text{Na}$ and the components of $\Delta \mu\text{H}$ upon a sudden increase in $[\text{Na}_2^+]$ from 1 to 100 mM. The initially large $\Delta p\text{Na}$ dropped within 2 min to slightly below the prestimulus value and subsequently (within 6 min) rose to a stable level comparable to that obtained in steady-state measurements. The magnitude and composition of $\Delta \mu\text{H}$ changed little: $\Delta \psi$ decreased slightly throughout the period, while $\Delta p\text{H}$ increased (as a result of a rise in pH$_{r}$) by 0.12 units immediately following the $[\text{Na}_2^+]$ jump and then remained constant.

Effect of pH$_{r}$ on $\Delta p\text{Na}$—Several studies with membrane vesicles and intact cells have indicated that gradients formed by secondary H$^+$-driven transport systems can be affected not only by the magnitude of $\Delta \mu\text{H}$ but also by the relative contributions of $\Delta p\text{H}$ and $\Delta \psi$ (33–35). We wished to examine how $\Delta p\text{Na}$ was affected in this regard. To do so, we chose several different ways of manipulating $\Delta \mu\text{H}$, the first being a simple variation of pH$_{r}$.

The rise in pH$_{r}$ can be attributed to the increase in osmolarity of the extracellular medium from ~270 to 450 mosm (see below).
Coupling of Sodium and Proton Gradients in E. coli

6.0  
6.5  
7.0  
7.5  
1.5  
1.0  
0.5  
0.0  
1.5  
1.0  
0.5  
I.0  
0.5  
I.0  
1.5  
I.0  
0.5  
0.0  

FIG. 4. Effect of pH on \( \Delta p_{\text{Na}}, \Delta p_{\text{H}} \), \( \Delta \phi \), and \( \Delta \bar{\mu} \). E. coli MRE 600 was suspended in a variety of buffers, as described under "Experimental Procedures." A, \( \Delta p_{\text{Na}} \) was determined at [Na\(^+\)] of 80 mM (○) and 180 mM (●) by \(^{23}\)Na NMR, each value deriving from a separate sample and representing an average of 6–8 4-min spectra. Within each experiment, pH was varied at most by 0.05 units at pH below 7.0 and 0.1 units at pH values above 7.0. \( \Delta p_{\text{H}} \) (○) was obtained by assuming \( p_{\text{H}} \) homeostasis using \( p_{\text{H}} \) of 7.5, a value confirmed at pH 6.4, 7.0, and 7.5. \( \Delta \phi \) values (×) represent an average of four determinations carried out during a 20-min interval. B, \( \Delta p_{\text{Na}} \) values replotted as a function of the corresponding \( \Delta p_{\text{H}} \) shown in A. Only points between 6.25 and 7.25 are included. In calculation of \( \Delta p_{\text{Na}} \) for strains RA 11 and CS 71 (data points indicated by arrows), intracellular volume was assumed to be the same as for strain MRE 600.

Effect of Benzoate on \( \Delta p_{\text{Na}} \)—A different type of manipulation of \( \Delta p_{\text{H}} \) was achieved by lowering pH at constant pH by addition of the membrane-permeant weak acid benzoate. With respiring cells at pH 6.1, [Na\(^+\)] = 140 mM, and constant osmolality (410 mosm), pH decreased from 7.5 in the absence of benzoate to 6.8 at 40 mM benzoate, whereas pH increased slightly (38), giving a net change in pH of 0.9 units (Fig. 6A; cf. similar results in Fig. 5 of Ref. 38). As has been observed previously in E. coli (39), \( \Delta \phi \) only partially compensated (by 50%) for the decrease in pH, so that there was a net decrease of \( \Delta \mu_{\text{H}} \) in the presence of benzoate. The parallel changes of \( \Delta p_{\text{Na}} \) and \( \Delta p_{\text{H}} \) (Fig. 6A) were strikingly similar to those shown above when \( \Delta p_{\text{H}} \) was manipulated by varying pH (Fig. 6B) between \( \Delta p_{\text{Na}} \) and \( \Delta p_{\text{H}} \) with a slope of 1.00 ± 0.09 (n = 12) and a displacement of \( \Delta p_{\text{Na}} \) from the corresponding values of \( \Delta p_{\text{H}} \), in this case by ~0.33 units.

As in the case when \( \Delta p_{\text{H}} \) was perturbed by altering pH, a sudden change in \( \Delta p_{\text{Na}} \) by shifting pH resulted in a somewhat slower readjustment of \( \Delta p_{\text{Na}} \) to a new value which corresponded well to that obtained from steady-state measurements (Fig. 7).

Effect of Osmolarity on pH.—In the course of this study, it became apparent that the osmotic strength of the external medium was affecting the pH achieved by endogenously respiring cells.

We first observed the effect in an experiment where 200 mM KCl was added (final concentration 210 mM) to respiring cells in a buffer of low osmolality (90 mosm). Follow the addition, pH rose from 7.15 to 7.6 within 2 min, then dropped to 7.5 and remained at that value for at least 15 min (Fig. 8). Because the \( K \) of the buffer (Pipes) is lower at high ionic strengths, there was also a decrease in \( p_{\text{H}} \), comparable to
Coupling of Sodium and Proton Gradients in E. coli

**Fig. 6.** Effect of benzoate on ΔpNa, ΔpH, Δψ, and ΔΔm. E. coli MRE 600 were resuspended in a buffer at pH 6.1 containing 30 mM PIPES, 50 mM Mes, 130 mM NaCl, 30 mM KCl, 5 mM KH₂PO₄, to which aliquots of 0.5 M benzoate, pH 6.0, were added; the final concentration shown was corrected for benzoate accumulated by the cells. A, ΔpNa (O) was determined by 23Na NMR. Each point represents an average of at least five 2-min spectra. ΔpH (●) was calculated from pHi, and pH,, estimated by 31P NMR. pHi values at 0 and 40 mM benzoate are indicated on the figure. Δψ (X) and ΔΔm were determined as described in Fig. 4. ΔpNa values were replotted as a function of the corresponding ΔpH shown in A. Also included are data from experiments performed at [NaCl] = 100 mM and pHi, = 6.0.

**Fig. 7.** Effect of a rapid pH,, shift on ΔpNa, ΔpH, Δψ, and ΔΔm. E. coli MRE 600 were resuspended in 100 mM Mes, 50 mM NaCl, 5 mM KH₂PO₄ adjusted to pH 6.1 with NaOH and KOH such that [NaCl] was 100 mM. At the time point indicated by the arrow, benzoate was added (final concentration 34 mM) to lower pHi, from 7.25 to 6.6. ΔpNa (O), ΔpH (●), and Δψ (X) were measured as described in Fig. 3.

that seen in a control experiment in the absence of cells.

We believe the change in pHi, reported by intracellular P₁ to be real. For example, it was not an artifact caused by a change in magnetic susceptibility, since there were no substantial changes in the chemical shifts of other major intracellular metabolites; the chemical shift of the α resonance of ATP was unaltered and only a small upfield shift (0.1 ppm) was observed for the β- and γ-ATP resonances. Nor was the effect a consequence of the suddenness of the addition of KC1, since cells washed with and resuspended in a buffer containing 210 mM KC1 also achieved a pHi, value of 7.5.

The effect was not ion-specific: KC1, NaCl, choline chloride, and sodium tripolyphosphate all caused pHi, to increase, as did a nonionic compound, sucrose. There was only one parameter common to the addition of the above compounds, an increase in the osmolarity of the medium. Indeed, when values of pHi, measured in buffers of different composition were plotted as a function of their osmolarity, the points fell on a single curve (Fig. 9). There was no correlation between pHi, and the ionic strength of the buffer, provided the osmolarity was the same.

The osmolarity of the extracellular medium is known to affect the size of the cytoplasmic compartment (Ref. 31 and Fig. 1) and the intracellular K⁺ concentration (23), and therefore might also affect the ionic strength of the cytoplasm, and hence the chemical shift of P₁, whose pK is ionic-strength dependent (40). However, an ionic-strength independent pH probe, methylphosphonate (30, 40), reported a similar dependence of pHi, upon osmolarity (Fig. 9). Because strain MRE 600 did not take up methylphosphonate, these experiments had to be carried out with a different strain of E. coli, CS 71; interestingly the steady-state value of pHi, in strain...
CS 71 was higher (by 0.2–0.3 units) than in strain MRE 600 under similar conditions.

**Effect of Osmolarity on the Composition of Δψ and on ΔpNa**—At constant pH_in, the changes of pH_in due to variation in external osmolarity were of course reflected in ΔpH (Fig. 10A). The steady-state value of Δψ declined gradually as extracellular osmolarity was increased from 120 to 750 mosm, producing a partial compensation (~50%) for the rise in ΔpH (Fig. 10A).

We next examined the effect of extracellular osmolarity on ΔpNa. Sucrose was added at various concentrations, at [Na+] = 50 mM and pH_in = 6.75 (Fig. 10A). As with the other two methods of varying Δψ, ΔpNa exhibited a linear correlation with ΔpH (Fig. 10B), with a slope of 0.89 ± 0.09 (n = 11). Once again, the line did not pass through the origin but gave an intercept corresponding to a ΔpNa of 0.49 units when ΔpH was zero.

**Generality of the Relationship between ΔpNa and ΔpH**—Fig. 11 summarizes the relationship between ΔpNa and ΔpH obtained from the three types of experiments in which we had manipulated the composition of Δψ (cf. Figs. 4B, 6B, and 10B). A single line (slope 0.95 ± 0.05; intercept 0.41 units, n = 34) represents all of the data well, indicating that changes in ΔpH, regardless of how they are accomplished, are reflected in ΔpNa.

**DISCUSSION**

In this study we have described the quantitative relationship between the Na⁺ gradient generated by respiring *E. coli* cells and the protonmotive force that is generally believed to be the driving force for Na⁺ extrusion. Further, we have examined the contributions of the proton electrical and chemical potentials to that gradient. Although a number of studies of Na⁺ fluxes have been reported (12, 21, 22, 41), we believe that the present study, together with our recently published description of Na⁺ levels during the process of energization (24), provides the first reliable measurements of cytoplasmic Na⁺ concentration in *E. coli*. The NMR approach we have used permits continuous measurement of extracellular and intracellular Na⁺ concentration in well-energized cells.

**E. coli Does Not Regulate Intracellular Na⁺ Concentration**—A major conclusion from this study is that *E. coli* does not exhibit Na⁺ homeostasis, i.e., as extracellular conditions are varied, [Na⁺] is not regulated to a constant value. Rather, its value is determined secondarily by [Na⁺] and by the magnitude of ΔpNa. The latter remains constant over a large range of values as long as the magnitude and composition of Δψ is also constant. Simply by varying [Na⁺] at constant Δψ, we were able to obtain [Na⁺] values as low as 0.4 mM and as high as 17 mM.

This finding is in accord with results obtained by Reenstra...
extrusion. A sudden change in $\Delta p\text{H}$ accomplished by changing either $p\text{H}_\text{in}$ (Fig. 5) or $p\text{H}_\text{ex}$ (Fig. 7) produced a more gradual but substantial change in $\Delta p\text{Na}$. In contrast, a sudden change in $\Delta p\text{Na}$ accomplished by changing $[\text{Na}^+]_\text{i}$ (Fig. 3) had little effect on $\Delta p\text{H}$ or $\Delta \psi$, and the change in $\Delta p\text{Na}$ itself cancelled within a few minutes. These results are what would be expected if a high-capacity proton pump (the respiratory electron transport chain) was responsible for developing a $\text{H}^+$ potential that in turn was responsible for developing $\text{Na}^+$ extrusion.

The fact that at moderately acidic $p\text{H}$ values ($p\text{H}_\text{ex} < 7.2$) $\Delta p\text{Na}$ closely followed $\Delta p\text{H}$ but not $\Delta \psi$ during various manipulations (Figs. 4, 6, and 10) suggested to us at first that the process might be electroneutral, i.e. occurring as a 1:1 antiport. This idea was supported by linear regressions of $\Delta p\text{Na}$ versus $\Delta p\text{H}$ which, for data obtained by three distinct types of manipulations, gave slopes of close to 1.

However, in each case we encountered a nonzero intercept of 0.3–0.5 units, so that $\Delta p\text{Na}$ consistently exceeded $\Delta p\text{H}$; this result is, of course, thermodynamically forbidden if $\Delta p\text{Na}$ is being developed by 1:1 antiport.

We have considered possible artifactual causes of the discrepancy. Of the parameters which enter the determination of $\Delta p\text{Na}$ (24), the most likely source of error was overestimation of intracellular volume. The error would have to be by a factor of at least 2.5 in order to account for a discrepancy of 0.4 units between $\Delta p\text{Na}$ and $\Delta p\text{H}$. This could be the case if the extracellular space marker (taurine) was excluded from the periplasm and the measured volume actually represented cytoplasm plus periplasm, but two observations argue against this possibility. First, measurements of cytoplasmic volume (31, 43) using a marker (sucrose) known to cross the outer membrane have yielded values similar to ours. Second, since the percentage of total volume representing the cytoplasm decreases from 70% to approximately 40% as the osmolarity of the medium is raised from 100 to 800 mosm (31), the discrepancy between $\Delta p\text{H}$ and $\Delta p\text{Na}$ would be expected to increase from 0.15 to 0.4 units in this osmolarity range. Our measurements show that this is not the case (Fig. 10).

Another possible source of error in estimating $\Delta p\text{Na}$ involves the NMR visibility of $\text{Na}^+$ in the cytoplasm versus buffer. We have reported previously our conclusions on this subject (24); briefly, they are that the cytoplasmic $\text{Na}^+$ is ~40% visible, a result that is expected where quadrupolar interactions yield transitions with different $T_2$ values, and where the shorter $T_2$ broadens the signal beyond detection. To account for the nonzero intercept, the visibility would have to be below 20%. This we consider to be outside the error limits of the measurement; it is also inconsistent with theoretical considerations (see, e.g. Ref. 44).

The NMR measurements indicate the amount of visible $\text{Na}^+$ but not its chemical activity. Measurements (not shown) of the activity of $\text{Na}^+$ in the external medium showed it to be unaffected by the presence of cells or of the shift reagent, indicating that this was not a likely source of systematic error. If the activity of cytoplasmic $\text{Na}^+$ were low, the thermodynamic gradient of $\text{Na}^+$ would have been underestimated, making the discrepancy between it and the $\text{H}^+$ gradient still greater.

A systematic error in $\Delta p\text{H}$ would most likely reside in the estimation of $p\text{H}_\text{in}$, which in this work was obtained from the chemical shift of intracellular $\text{P}$, in $^{31}\text{P}$ NMR spectra of $E$. coli suspensions. It has been claimed (45) that intracellular $\text{P}_i$ cannot give $p\text{H}_\text{in}$ values with greater certainty than 0.2–0.5 units, primarily because of effects of ionic strength and $\text{Mg}^{2+}$ on its $pK$. However, in $E$. coli the $p\text{H}_\text{in}$ values obtained from
P_i have been confirmed with an ionic-strength-independent probe, methylphosphonate (30, 40). The effect of Mg^{2+} was not controlled for but, since Mg^{2+} lowers the pH and thus the true pH_i corresponding to any given chemical shift, this would imply that, if anything, we were overestimating \( \Delta \phi \), and hence the discrepancy between \( \Delta \phi \) and \( \Delta \phi \) would be even greater than indicated on the figures.

The above considerations suggest that systematic errors cannot explain the observed relationship between \( \Delta \phi \) and \( \Delta \phi \). The finding that the relationship varies from strain to strain of E. coli supports this contention, since a systematic error would not be expected to be strain-dependent; in the case of strain RA 11, \( \Delta \phi \) was much higher (~1 unit) than \( \Delta \phi \).

We therefore conclude that the non-zero intercept is not artifactual in origin, but reflects the true relationship between \( \Delta \phi \) and \( \Delta \phi \). In other words, we conclude that devices with a H^{+}/Na^{+} stoichiometry of greater than unity participate in the generation and maintenance of \( \Delta \phi \).

**What is the H^{+}/Na^{+} Stoichiometry?**—Thermodynamic considerations dictate that if a H^{+}/Na^{+} device has a stoichiometry \( n \), and operates in the direction of H^{+}-driven Na^{+} extrusion, then \( \Delta \phi \) is equal to \( n \Delta \phi \) (potentials expressed in p units, and \( \Delta \phi \) positive). We may then define an apparent stoichiometry \( n_{app} \) by the equation \( \Delta \phi_{app} = n_{app} \Delta \phi_{H_0} \) (Equation 1). If we assume that the same stoichiometry applies throughout the range of our measured gradients (excepting those at pH_0 > 7.2), \( n_{app} \) may be estimated from a linear regression of \( \Delta \phi_{app} \) versus \( \Delta \phi_{H_0} \) to pass through the origin. For E. coli MRE 600 below pH_7.2, this yields \( n_{app} = 1.13 \pm 0.01 \), suggesting that the stoichiometry is slightly electrogenic. A similar calculation using the data above pH_7.2 yields \( n_{app} = 1.26 \pm 0.03 \), indicating that the stoichiometry is greater electrogenic character.

Equation 1 may be rewritten as \( \Delta \phi = \Delta \phi + (n_{app} - 1) \Delta \phi_{H_0} \), and so the observed slope of close to 1 in plots of \( \Delta \phi \) versus \( \Delta \phi \) (Figs. 4B, 6B, and 10B) is to be expected, provided either that \( \Delta \phi_{H_0} \) remains relatively constant as \( \Delta \phi \) is varied, or that \( n_{app} = 1.13 \) is small; both of these conditions are in fact met, since cells compensate to a considerable degree for changes in \( \Delta \phi \) by changes in \( \Delta \phi \). We therefore felt justified in using these equations as a first approximation. Fig. 12 gives the flux-force relations for the pairs of modes shown. At steady state the Na^{+} fluxes through Modes 1 and 2 are equal in magnitude and opposite in direction so that, setting \( \Delta \phi_{app} = \Delta \phi_{app} \) and rearranging, \( n_{app} \) as defined in Equation 1 is seen to be \( (m_1 + 2m_2)/(m_1 + m_2) \) for the conductances of Modes 1 and 2. It is evident that as long as both conductances are nonzero, \( n_{app} \) will in general be nonintegral. In case A the observed apparent stoichiometry of 1.1 would be obtained when the conductance of the 1:1 antiporter dominated over that of the 2:1 antiporter (i.e. \( m_1 = 9 m_2 \)), and in case B when the conductances for the antiport and the 2:1 antiporter were almost equal (i.e. \( m_1 = 0.8 m_2 \)).

At higher pH_i values, \( n_{app} \) increases, presumably because of an increase in the conductance of the electrogenic antiporter relative to either the electroneutral antiporter (case A) or the unipporter (case B).

The relationship between \( \Delta \phi \) and \( \Delta \phi \) observed in other strains of E. coli (cf. Fig. 4B) can be accommodated within the model by postulating different relative conductances of the two modes. In strain RA 11, in which \( \Delta \phi \) exceeded \( \Delta \phi \) by 1 unit, the relative conductance of the electrogenic antiport would be larger than in strain MRE 600 while in

---

*The futile cycling of Na^{+} that we propose here is a concept distinct from the Na^{+} recycling described in, for example, Ref. 51. Futile cycling is an ongoing process. Recycling is a process that, because of the finite reservoir of intracellular Na^{+}, is required to achieve substantial acidification of the cytoplasm at alkaline pH_i; it is a process that, at least in an idealized system, does not need to continue operating once the acidification has been achieved.*
strain CS 71, in which the difference between ΔpNa and ΔpH was only 0.1 units, its relative conductance would be smaller. This interpretation of the data for strain RA 11 is consistent with the results of Bassilana et al. (21) who have demonstrated that, in RA 11 vesicles, unidirectional flux of 22Na can be driven by either ΔpH alone or ΔpH alone at pH, values above 6.6, thus implying the presence of an electrogenic antiport.

In case A (Fig. 12A), the steady state is between an electrogenic and an electroneutral antiport, with the former operating (in the direction of H⁺-driven Na⁺ extrusion) far from equilibrium but with a low conductance and the latter operating in the reverse direction (Na⁺-driven H⁺ extrusion) rather close to equilibrium (since n is close to napp) but with a high conductance. Variable stoichiometry of H⁺/Na⁺ antiport in E. coli has been suggested by Schuldiner and Fishkes (12), who reported electroneutral behavior at pH, = 6.6 and electrogenic behavior at pH, = 7.5. Behavior at intermediate pH values, or the consequences of simultaneous use of different stoichiometries, was not discussed.

In case B (Fig. 12B), if the uniporter is a different device from the electroneutral antiporter, the situation is formally equivalent to a pump loaded down by leakage pathways for its substrate. If, on the other hand, it is the antiporter itself that has the capacity to operate as a uniporter, the situation is equivalent to what has been described by Eddy (49) as "carrier operation with slip." In either event, both modes are operating far from equilibrium since napp is far from both mechanistic stoichiometries.

Although both cases depicted in Fig. 12 are consistent with our data, we favor the interpretation of a 1:1 antiport and a 2:1 antiport (case A, Fig. 12A), since it (i) explains why values of napp of less than unity were never seen, (ii) makes the closeness of napp to unity understandable in terms of a lower bound rather than a coincidental value, and (iii) is in accord with the conclusions of other workers (12, 13, 21, 41, 42) that H⁺/Na⁺ antiport is responsible for the development of ΔpNa. There is also no evidence that Na⁺ can be accumulated by a uniport plus a 2:1 antiport under these conditions and cause such accumulation. However, we should emphasize that our data do not rule out the interpretation of a uniport plus a 2:1 antiport (case B, Fig. 12B). A rigorous choice must await further measurements, especially ones that could indicate the extent of the cycling.

It has been postulated that Na⁺ transport is involved in pH homeostasis and that the mechanism involves a shift from electroneutral to electrogenic H⁺/Na⁺ antiport at alkaline pH (30). The change in the relationship between ΔpNa and ΔpH that we observed above pH, = 7.2 is preliminary evidence in support of this postulate, but further investigation is needed. Measurements of the apparent stoichiometry over a wider pH range could yield valuable clues as to which types of devices are responsible for developing and maintaining the Na⁺ gradient and might help clarify the mechanism of pH homeostasis in bacteria.

pH,E in E. coli Is Affected by Osmolarity of Extracellular Medium—Two observations made in the course of these studies have indicated that, in spite of the well-established phenomenon of pH homeostasis in E. coli (30, 36, 51), there appears to be no unique value of pH,E that is achieved under all conditions, at least by endogenously respiring cells. First, we have noted significant strain-to-strain variation: pH,E values in E. coli CS 71 were always higher by 0.2–0.3 units than those in strain MRE 600. Second, pH,E was dependent on the
osmolarity of the extracellular medium, probably reflecting in some way the cellular response to osmotic stress.

The mechanisms of sensing osmotic stress are not understood in detail but are thought to involve K⁺ transport (52), regulation of porin biosynthesis (53), and uptake of osmoprotectants such as glycine, betaine, or proline (54, 55). The latter two mechanisms are unlikely to be responsible for the changes in pHₑ that we observed, since both require protein synthesis, which would not be occurring to any appreciable extent in buffer. Furthermore the timescale required for such synthesis would be at least 30 min, while pHₑ was found to change within 2 min following a change in osmolarity of the extracellular medium. K⁺ transport, on the other hand, can respond rapidly to osmotic changes, K⁺ being taken up following an osmotic increase and released following an osmotic decrease (23).

In K⁺-depleted cells of E. coli, it has been found that K⁺ uptake correlates with an increase of pHₑ (and thus ΔpH) with a concomitant decrease in ΔΔpH (56). Therefore the rise in pHₑ and drop in ΔDpH observed by us as the osmolarity of the extracellular medium was increased might be attributable to osmolarity-stimulated K⁺ uptake.

It should be remembered that our observations were made in endogenously respiring cells. It might be that growing cells can, in the face of osmolarity changes, achieve a higher degree of pH homeostasis.

Concluding Remarks—The present study of the bioenergetics of Na⁺ in E. coli has confirmed that, as has been strongly predicted by other studies, the Na⁺ gradient is closely linked to that of the proton. Based on the quantitative relationship, we suggest that ΔpHₑ in E. coli represents a steady state between Na⁺ extrusion and Na⁺ influx using different H⁺/Na⁺ stoichiometries. The futile cycling that would occur as a result of the presence of two devices with differing driving forces would of course represent an ongoing expenditure of energy. However, we are inclined to view such an expenditure as functionally important and not as an unfortunate lack of efficiency. If it were to contribute to the regulation of an important cellular parameter such as cytoplasmic pHₑ it would presumably convey net advantage to the cell.

Acknowledgments—We are grateful to M. Kihara for technical assistance, to Drs. S. Schuldiner and T. H. Wilson for provision of strains, and to Dr. W. Boron for use of his osmometer.

REFERENCES
1. Mitchell, P. (1963) Biochem. Soc. Symp. 22, 142–169
2. Hellingwerf, K. J., Friedberg, I., Lokkena, J. M., Michels, P. A. M., and Konings, W. N. (1982) J. Bacteriol. 150, 1183–1191
3. Bakker, E. E., and Harold, F. M. (1980) J. Biol. Chem. 255, 433–440
4. Stewart, L. M. D., Bakker, E. P., and Booth, I. R. (1985) J. Gen. Microbiol. 131, 71–85
5. Schultz, S. G., and Solomon, A. K. (1961) J. Gen. Physiol. 45, 365–369
6. Condliff, P. (1986) Biol. Rev. 11, 445–502
7. Lamy, J. K., and MacDonald, R. E. (1976) Biochemistry 15, 4608–4614
8. Eisenbach, M., Cooper, S., Garty, H., Johnstone, R. M., Rottenberg, H., and Caplan, S. R. (1977) Biochim. Biophys. Acta 465, 599–613
9. Niven, D. F., and MacLeod, R. A. (1978) J. Bacteriol. 134, 737–749
10. Mandel, K. G., Guffanti, A. A., and Kruwich, T. A. (1980) J. Biol. Chem. 255, 7391–7396
11. Koyama, N., and Nosoh, Y. (1985) Biochim. Biophys. Acta 812, 206–212
12. Schuldiner, S., and Fishkes, H. (1978) Biochemistry 17, 706–711
13. Tokuda, H., and Kaback, H. R. (1977) Biochemistry 16, 230–2316