Measurement of Intracellular Sodium Concentration and Sodium Transport in Escherichia coli by $^{23}$Na Nuclear Magnetic Resonance

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Escherichia coli is known to actively extrude sodium ions, but little is known concerning the concentration gradient it can develop. We report here simultaneous measurements, by $^{23}$Na NMR, of intracellular and extracellular Na$^+$ concentrations of E. coli cells before and after energization. $^{23}$Na spectra in the presence of a paramagnetic shift reagent (dysprosium tripolyphosphate) consisted of two resonances, an unshifted one corresponding to intracellular Na$^+$ and a shifted one corresponding to Na$^+$ in the extracellular medium, including the periplasm. Extracellular Na$^+$ was found to be completely visible despite the presence of a broad component in its resonance; intracellular Na$^+$ was only 45% visible. Measurements of Na$^+$ were made under aerobic and glycolytic conditions. Na$^+$ extrusion and maintenance of a stable low intracellular Na$^+$ concentration were found to correlate with the development and maintenance of proton motive force, a result that is consistent with proton-driven Na$^+$/H$^+$ exchange as a means of Na$^+$ transport. In both respiring and glycolytic conditions, at an extracellular Na$^+$ concentration of 100 mM, the intracellular Na$^+$ concentration observed (4 mM) corresponded to an inwardly directed Na$^+$ gradient with a concentration ratio of about 25. The kinetics of Na$^+$ transport suggest that rapid extrusion of Na$^+$ against its electrochemical gradient may be regulated by proton motive force or intracellular pH.

Bacteria, like higher cells, extrude Na$^+$. They thus maintain an inwardly directed Na$^+$ gradient which can be utilized to drive various processes such as accumulation of nutrients (1, 2) or motility (3). The existence of a Na$^+$ extrusion system in Escherichia coli was initially inferred from the finding that the concentration of Na$^+$ in the cytoplasm was lower than in the external medium (4). Based on the observation that addition of Na$^+$ to an anaerobic cell suspension caused acidification of the external medium, West and Mitchell (5) suggested that Na$^+$ transport may be mediated by Na$^+$/H$^+$ exchange. Na$^+$ efflux was demonstrated both in intact E. coli cells and in right-side-out membrane vesicles derived from them and was shown to be dependent on the presence of proton motive force (6-9) and independent of the level of ATP (9). These observations are consistent with the operation of a proton-driven Na$^+$/H$^+$ antiport as a means of generating the Na$^+$ gradient. This hypothesized mechanism of Na$^+$ transport is generally accepted.

Little is known, however, about the intracellular Na$^+$ concentration of E. coli and therefore of the Na$^+$ chemical potential relative to that of the proton; such information is essential for an evaluation of the Na$^+$/H$^+$ antiport hypothesis. A major limitation has been the lack of a convenient method for estimating intracellular Na$^+$ in energized cells or vesicles; most studies have employed radioisotope tracer techniques and therefore have focused on transport kinetics (7-9). Tsu-chiya and Takeda (6) have assessed changes in intracellular Na$^+$ indirectly by monitoring extracellular concentration with a Na$^+$-selective electrode, but were able to do so only over a restricted concentration range. The only direct measurements of intracellular Na$^+$ were performed by flame photometry (4, 10). This approach, however, requires separation of the intracellular and extracellular compartments, with attendant errors resulting from incomplete removal of the extracellular fluid, binding of Na$^+$ to the outer surface of the membrane, and the possibility of inward leakage if the cells cannot be maintained in an energized state during the separation procedure. These problems are aggravated by the fact that the concentration of Na$^+$ in energized cells is, unlike that of K$^+$, quite low.

Recently, a new approach for measuring intracellular Na$^+$ has been described, which involves $^{23}$Na NMR spectroscopy in the presence of a paramagnetic shift reagent (11, 12). It permits the simultaneous observation of resonances deriving from intracellular and extracellular Na$^+$ and thus the estimation of both concentrations. The technique has been applied successfully to the measurement of intracellular Na$^+$ levels in several eukaryotic systems (11-18). In this paper, we describe its use for the measurement of steady state concentrations and transport of Na$^+$ in E. coli.

EXPERIMENTAL PROCEDURES

Bacterial Strains—E. coli strain MRE 600, wild type except for low RNase activity (19), was used throughout.

Chemicals—DyCl$_3$ was prepared by neutralizing Dy$_2$O$_3$ (Sigma) with hydrochloric acid. Potassium tripolyphosphate was prepared by passing the sodium salt (Sigma) over a column containing the potassium form of AG 50W-X8 resin (Bio-Rad). [U-$^{13}$C]Taurine, tetra-$^3$H phenylphosphonium, and $^3$H$_2$O were purchased from Amersham. All other chemicals were of reagent grade.

NMR Measurements—Bacteria were grown to $10^9$ cells ml$^{-1}$ (OD$_{600}$ = 0.7) at 37°C in M9 minimal medium (20) plus 0.4% glucose. They were cooled to 5-10°C with continuous oxygenation, centrifuged at 4000 x g for 10 min, washed once with ice-cold 20 mM Pipes, 170 mM KCl, 5 mM KH$_2$PO$_4$ (adjusted to pH 6.8 with KOH), and 1

The abbreviations used are: Pipes, 1,4-piperazinedinedithanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid; pH$_{intr}$, intracellular pH; pH$_{ext}$, extracellular pH; $\Delta$H, transmembrane pH difference; CCDF, carbonyl cyanide m-chlorophenylhydrazone; TPP$^+$, tetraphenylphosphonium; $\Delta$N/2, line width at half-height; $T_2$, transverse relaxation time.

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resuspended in the same buffer to a cell density of 10–15 mg dry weight ml⁻¹. These cell suspensions were kept on ice (maximum 3 h) until approximately 30 min before the experiment, at which time they were washed in the buffer to be used for the experiment and resuspended to 80 mg dry weight ml⁻¹; in all cases, this buffer lacked carbon and nitrogen sources. Immediately before the experiment, the suspensions (by combining two 5 ml portions of tripolyphosphate in a molar ratio 1:3, or as specified, and used at a final dysprosium concentration of 6 mM) was added together with additional buffer to bring the final cell density to 65–70 mg dry weight ml⁻¹. Thirteen ml of cell suspension were placed into a 30-mm sample tube and allowed to warm up to 22 °C in the spectrometer for 15 min, while continuously passing gas through the suspension (21). N₃O₃CO₃ (95±5%) and O₂/CO₂ (95±5%) were used for aerobic and aerobic conditions, respectively. C CCP, used to collapse proton motive force, was added as an anodic solution; ethanol by itself at the final concentration used (1%) had no effect on either proton or Na⁺ gradients.

A Bruker WH 360 wide-bore spectrometer was used throughout. For 23 Na measurements, it was operated at 95.26 MHz with 90° pulses applied every 0.07 s, an interval sufficient to allow complete nuclear relaxation. Chemical shifts were referenced to Na⁺ in buffer. For phosphorus measurements, a frequency of 145.78 MHz was used with 45° pulses every 0.1 s. Intracellular pH was obtained from the chemical shift of intracellular P₃O₁₀⁻. Extracellular pH was estimated from the chemical shift of extracellular P₃O₁₀⁻ and confirmed with a pH electrode immediately after the experiment.

**Calculation of Intracellular Na⁺ Concentration** —The following formula was used to calculate intracellular Na⁺ concentration:

\[
\frac{[\text{Na}⁺]_{i}}{[\text{Na}⁺]_{o}} = \frac{A_{i} \times V_{o}}{A_{o} \times V_{i}} \times \frac{\text{visibility}_{i}}{\text{visibility}_{o}}
\]

(Ao and Aᵢ are the areas of the intracellular and extracellular Na⁺ resonances and Vo and Vi are the intracellular and extracellular volumes. The term visibility refers to the calibration factor relating the NMR signal intensity for a given amount of Na⁺ in a solution to that expected for the same amount of Na⁺ in buffer alone, where the visibility was taken to be 100%.)

**Measurement of Membrane Potential** —Membrane potential was estimated essentially as described by Zaretzky et al. (25). E. coli were grown to late logarithmic phase and harvested as for an NMR experiment except that cooling with oxygenated water was omitted and all buffers contained 0.5 mM EDTA to facilitate uptake of TTP⁺. Typically, 2 ml of cell suspension (0.3 mg dry weight ml⁻¹) were incubated with stirring in the buffer used for the corresponding NMR experiments. [H³]TPP⁺ was added to a final concentration of 4 x 10⁻⁸ M (1 µCi ml⁻¹). After 30, 35, 40, and 45 min, a 50-µl aliquot of the suspension was added to 2 ml of buffer, filtered (Nucleopore polycarbonate filters, 0.4 µm pore, 25 mm diameter), and washed with a further 2 ml. Filters were dried and placed into scintillation fluid (Aquasol, New England Nuclear) and the radioactivity was counted. Non specific binding of TTP⁺ was estimated as the radioactivity associated with cells de-energized by the addition of 50 µM CCCP. Membrane potential was calculated from the Nernst equation as in Ref. 26 and is presented as an average of the determinations at the four time points.

**RESULTS**

**23 Na NMR of E. coli Suspensions** —The 23 Na NMR spectrum of an anaerobic suspension of E. coli MRE 600 cells consisted of a single resonance (Δν/Δ = 38 Hz) deriving from both intracellular and extracellular Na⁺ (Fig. 1A, solid line). Upon addition of the paramagnetic shift reagent, K₃Dy(P₃O₁₀)₉, the signal split into two resonances separated by 7 ppm (Fig. 1B). The chemical shift of the small resonance at 0 ppm (Δν/Δ = 140 Hz) was unaffected by the shift reagent, even over extended time periods. Since dysprosium tripolyphosphate is known to be impermeable to cell membranes, we have assigned this resonance to intracellular Na⁺.

Interaction of Na⁺ with the shift reagent is known (11) to cause its resonance to shift upfield and therefore the large resonances, corresponding to extracellular Na⁺, derived from the extra- and intracellular Na⁺, respectively, and were evaluated as described under "Results."

The areas of the intracellular and extracellular Na⁺ resonances were obtained by computer integration. For the intracellular resonance, the spectra were resolution-enhanced by Gaussian multiplication because otherwise it was difficult to discern the resonance when a large inwardly directed Na⁺ gradient ([Na⁺]ᵢ/[Na⁺]ₒ ≥ 30) existed. Gaussian multiplication altered the resonance areas in a line width-and line shape-dependent manner and therefore the intracellular and extracellular resonances could not be compared directly. The line width of the intracellular resonance was constant within each experiment and its line shape appeared to be Lorentzian under all conditions studied. Therefore, we were able to correct the integrated area following Gaussian multiplication by a factor relating to the intensity for the same amount of Na⁺ in buffer alone, where the visibility was taken to be 100%. Visibilityᵢ and visibilityₒ refer to extracellular and intracellular Na⁺, respectively, and were evaluated as described under "Results."

**Determination of Intracellular Volume** —Intracellular aqueous volume was measured essentially as described by Stock et al. (22) using [U-¹³C]taurine (0.02 pCi ml⁻¹) and [H₂]₃P₀₄ (0.25 pCi ml⁻¹) as markers for the extracellular and total aqueous volumes, respectively. The "extracellular" space includes the periplasmic space since taurine (Mᵩ = 125) can cross the outer membrane (23). Respiration and glycogenolyzing E. coli cells resuspended in the buffer used in the corresponding NMR experiments were found to have intracellular volumes of 1.25 µl (dry weight)⁻¹ and 1.5 µl (dry weight ml⁻¹), respectively.

**Respiration Rates** —Respiration rates were estimated by measuring the rate of oxygen consumption at 25 °C with cells at 8 mg dry weight ml⁻¹ in the buffer used in the corresponding NMR experiments. Oxygen consumption was monitored with an oxygen electrode (Yellow Springs, Model 53).

**ATP Assay** —Three ml of cell suspension (60 mg dry weight ml⁻¹), in the buffer used in the corresponding NMR experiments, was maintained at 25 °C and vigorously aerated. One-tenth ml aliquots were removed, rapidly mixed with 0.2 ml of 2 N perchloric acid, and kept at 0 °C for 15 min. To each aliquot, 0.3 ml of 0.2 M potassium phosphate, pH 7, was added to increase the buffering capacity and it was then neutralized with 3 M KOH, centrifuged at 15,000 × g for 10 min, and the ATP content of the supernatant was assayed by the luciferin-luciferase method of Kimmich et al. (24). ATP levels were also estimated from the intensity of the β-ATP resonance in 31P NMR spectra.
Because E. coli (like other Gram-negative bacteria) possesses a second aqueous compartment, the periplasmic space, it was necessary to determine whether periplasmic Na+ contributed to the "extracellular" or "intracellular" signal. Since Na+ ions can freely cross the outer membrane, they may be assumed to be in fast exchange between extracellular medium and periplasm. We therefore attribute the "extracellular" resonance to both extracellular and periplasmic Na+.

**Line Shape Analysis**—Visual inspection of the $^{23}$Na spectrum in Fig. 1A suggests that the resonance is not a simple Lorentzian and may contain more than one component. Indeed, we found that the line shape could be simulated by the sum of a narrow ($\Delta v_{1/2} = 33$ Hz) and a broad ($\Delta v_{1/2} = 458$ Hz) Lorentzian line centered at the same frequency (within 0.02 ppm) and with areas representing 40% and 60% of the total, respectively (Fig. 1C). The fit was excellent, as can be seen by superposition of sample points from the simulated spectrum (circle symbols in Fig. 1A), on the experimental spectrum. We conclude that the presence of two components in the spectrum of Fig. 1A is primarily due to the extracellular Na+ resonance which constitutes 97% of the total signal (cf. Fig. 1B). This conclusion is supported by the fact that the extracellular resonance (Fig. 1B), when resolved from the intracellular one, had a line shape resembling that in Fig. 1A and deviating substantially from a simple Lorentzian with the same $\Delta v_{1/2}$. The complex line shape of the extracellular resonance was only seen when cells were present at quite high density; the spectrum of a 4-fold diluted suspension (15 mg dry weight ml$^{-1}$) had a simple Lorentzian appearance (not shown).

The line shape of the intracellular resonance was more difficult to analyze because of the underlying broad shoulder of the extracellular resonance. In a spectrum where the two resonances were better resolved than those in Fig. 1B and where intracellular Na+ contributed 10% of the signal, the intracellular resonance resembled a single Lorentzian line (not shown).

**NMR Visibility of Na+**—The NMR visibility of Na+ in E. coli suspensions was estimated using an approach similar to that of Ogino et al. (14) and Gullans et al. (16), which involved a comparison of changes in the areas of the intracellular and extracellular resonances following Na+ extrusion. Since Na+ removed from the cytoplasm necessarily appeared in the extracellular medium, the ratio of the decrease in the intracellular resonance to the increase in the extracellular resonance yielded the relative visibility of the two Na+ pools.

When cells were harvested as described under "Experimental Procedures," the intracellular Na+ signal comprised only 4–6% of the total. To increase the intracellular Na+ and allow more accurate estimation of the changes in resonance areas, cells were first loaded with Na+ by incubating them for 2 h at 4 °C in a buffer containing 160 mM NaCl. They were then pelleted, resuspended in a Na+-free buffer, and used immediately to minimize leakage of Na+ from the cells. Intracellular Na+ contributed approximately 20% of the signal in these suspensions, the extracellular Na+ presumably resulting from incomplete removal of the high Na+ buffer in the last wash and from leakage of intracellular Na+. The cell suspensions were then placed in the NMR spectrometer and, following oxygenation to cause Na+ efflux, the changes in the areas of extracellular and intracellular resonances were compared. In this way, visibility$_{ex}$ was found to be 45% of visibility$_{in}$. Extracellular pH (6–7.5) and ionic strength (150–400 mM) had no effect on this ratio. In order to estimate visibility, relative to buffer, visibility$_{ex}$ had also to be evaluated. This was accomplished by adding a known amount of Na+ to the cell suspension and to buffer alone and comparing the relative increases in signal. In this manner, visibility$_{ex}$ was found to be essentially identical with that in buffer alone. The transportable intracellular Na+ was therefore 45% visible with respect to buffer. Table I summarizes these visibility measurements.
**TABLE I**

<table>
<thead>
<tr>
<th>ATP content (mM)</th>
<th>Shift reagent*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Membrane potential (mV)</td>
<td>135</td>
</tr>
<tr>
<td>6.9</td>
<td>7.0</td>
</tr>
<tr>
<td>Growth rate (doubling time in min)</td>
<td>50</td>
</tr>
</tbody>
</table>

**TABLE II**

<table>
<thead>
<tr>
<th>Cellular parameter</th>
<th>Shift reagent*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absent</td>
</tr>
<tr>
<td>Respiration rate</td>
<td>6.5</td>
</tr>
<tr>
<td>Growth rate (doubling time in min)</td>
<td>7.5</td>
</tr>
</tbody>
</table>

**Effect of Shift Reagent on the Physiology of E. coli**—We have employed several criteria to evaluate the suitability of the shift reagent for m v i v o studies of E. coli. The rate of cell growth was markedly reduced, with the normal doubling time at 37 °C in minimal medium (50 min) increasing to 150 min in the presence of 6 mM shift reagent (Table II). Similar growth inhibition was observed with triplyphosphate alone, suggesting it was responsible. Since triplyphosphate is a strong chelating agent for multivalent cations, we suspected it might be removing essential metal cations from the medium. This suspicion was confirmed when, after testing a variety of metal cations (Mg²⁺, Ca²⁺, Co²⁺, Mo²⁺, Zn²⁺, Fe²⁺, and Fe³⁺), we found that 10 mM MnCl₂ increased the growth rate to close to normal within 5 min (doubling time = 75 min).

Although the shift reagent impaired growth, other physiological parameters—respiration rate, ATP level, ΔpH, and membrane potential—were found to be unaffected by it (Table II), indicating that the metal deficiency did not affect the bioenergetic state of the cells, at least in the short term.

We could not, of course, measure Na⁺ gradients in the absence of shift reagent. However, since addition of MnCl₂ caused no enhancement of the gradient, we conclude that our measurements reflect the Na⁺ extrusion properties of well energized cells.

**Development and Maintenance of a Na⁺ Gradient under Aerobic Conditions**—It has been shown that the proton electrochemical gradient generated by E. coli under aerobic and glycolytic conditions drives Na⁺ extrusion (measured as unidirectional flux) (9). Furthermore, indirect assessment of changes in intracellular Na⁺ has indicated that net extrusion occurs upon energization (6). In neither case was intracellular Na⁺ concentration measured directly. We have now applied Na⁺ NMR spectroscopy to the measurement of intracellular Na⁺ concentration in E. coli under aerobic and glycolytic conditions.

Fig. 2, A and B depicts the changes in the intracellular Na⁺ and proton concentrations (estimated by 23Na and 31P NMR, respectively) when anaerobic suspensions of E. coli, at pH₃, approximately 6.5 and [Na⁺] = 95 mM, were supplied with oxygen in the absence of an exogenous carbon source. The proton concentration gradient (ΔpH) served as an indicator of the total proton motive force and is presented in Fig. 2C. Before oxygenation, there was a small inwardly directed ΔpH of 0.4 unit which decreased slowly, indicating that proton motive force was not being actively maintained. During this time, no significant change in intracellular Na⁺ occurred, despite the existence of a Na⁺ gradient ([Na⁺] = 96 mM, [Na⁺] = 42 mM, ΔpNa = 0.4) resulting from the cell preparation protocol.

Upon oxygenation, proton pumping lowered the intracellular proton concentration within 4 min to a constant value corresponding to pH₃ = 7.5. With a 1–2-min lag, intracellular Na⁺ concentration also began to drop. The sharp decrease in intracellular Na⁺ concentration was associated with a drop in intracellular Na⁺ concentration within 5 min and could be maintained for at least 40 min,
period prior to CCCP addition. Since $p$H$_{in}$ remained constant, basic metabolites and as a result took up protons from the external medium (at a rate of approximately 4 nmol (mg dry weight)$^{-1}$ min$^{-1}$ at $p$H$_{ext}$ = 6.5). The resulting change in $p$H$_{in}$ would not be noticeable at cell densities commonly employed for bioenergetic studies (0.1–10 mg dry weight ml$^{-1}$). At the cell densities needed for NMR studies (approximately 70 mg dry weight ml$^{-1}$), however, the rise in $p$H$_{in}$ can be significant if the external buffering capacity is low. This was the case in Fig. 2, where $p$H$_{in}$ rose from 6.45 to 6.65 during the 14-min period prior to CCCP addition. Since $p$H$_{in}$ remained constant at 7.5 (Fig. 2B), a value characteristic of well energized cells (21, 35), the increase in $p$H$_{in}$ was entirely responsible for the drop in $\Delta p$H (Fig. 2C). Close inspection of the intracellular Na$^+$ concentration (Fig. 2A) revealed that it was increasing gradually during this time ($\Delta p$Na decreasing from 1.45 to 1.25). This might reflect a direct effect of $p$H$_{in}$ on the activity of the Na$^+$/H$^+$ exchange (or other Na$^+$ transport systems) but is more likely to be a consequence of a reduced driving force ($\Delta p$H) for Na$^+$ transport. A detailed study of the relationship between $\Delta p$Na and other bioenergetic parameters will be published separately.\(^5\)

Collapse of the proton motive force by CCCP, indicated by the influx of protons and the decrease of $\Delta p$H to almost zero, led to an inward movement of Na$^+$ down its concentration gradient (initial rate of increase in [Na$^+_i$] = 3.5 mM min$^{-1}$) until the intracellular and extracellular Na$^+$ concentrations were almost equal, typically 40–50 min after CCCP addition.

Development and Maintenance of a Na$^+$ Gradient under Glycolytic Conditions—The initial features of Na$^+$ transport under glycolytic conditions (Fig. 4, A–C) were similar to those observed in respiring cells. Upon glucose addition to anaerobic cells at pH$_{ext}$ approximately 6.7 and [Na$^+_i$] = 100 mM, proton extrusion commenced immediately (Fig. 4B). Na$^+$ extrusion lagged by approximately 2 min but proceeded rapidly thereafter (Fig. 4A). By about 6 min, intracellular Na$^+$ had attained a minimum value comparable to that observed under aerobic conditions (3.6 mM, $\Delta p$Na = 1.4). However, it began to increase much more rapidly than in the aerobic case.

This could not be attributed to an increase in pH$_{ext}$, which actually decreased (from 6.7 to 6.1 in the 8 min following glucose addition) as a result of extrusion of protons deriving from acidic metabolites of glycolysis. Rather, the increase in intracellular Na$^+$ appeared to be due to progressive de-energization of the cells. The de-energization could be seen in the decrease in pH$_{ext}$, which, being more severe than that of $p$H$_{in}$, resulted in a decrease in $\Delta p$H. Also, the concentration of ATP—the direct energy source for development of proton motive force under glycolytic conditions—declined sharply 8 min after glucose addition (measurements, not shown, made by $^{31}$P NMR).

The principal factor contributing to the de-energization is likely to have been loss of the primary energy supply (glucose). Based on the cell density, the initial glucose concentration in this experiment and the rate of glucose consumption measured by Ugurbil et al. (28) in E. coli under similar conditions, we estimate that 95% of the glucose would have been depleted within 4–6 min. In addition, the large acid load produced by metabolism could have also contributed to de-energization. Thus, the rise in the intracellular Na$^+$ beginning about 6 min after addition of glucose is analogous to the rise following the addition of CCCP to respiring cells, since in both cases the cells were becoming de-energized.
DISCUSSION

In this study, we have established conditions for simultaneously monitoring extracellular and intracellular Na\(^+\) in *E. coli* by \(^{23}\text{Na}\) NMR and have shown that the technique is suitable for examining both steady state concentrations and transport. It has allowed, for the first time, direct measurement of the intracellular Na\(^+\) concentration of intact, well energized *E. coli* cells.

The technique requires high cell densities, which can make it difficult to maintain environmental parameters, such as pH or the concentration of an exogenous energy source, at constant values. It also requires the use of a nonphysiological compound—the shift reagent, dysprosium tripolyphosphate—which had no adverse effects for the purpose of short term bioenergetic studies, but cannot be considered completely benign in view of its ability to sequester multivalent cations that may be essential for cell viability. In spite of such limitations, \(^{23}\text{Na}\) NMR spectroscopy provides opportunities that have been lacking thus far for measurement of Na\(^+\) in bacterial cells.

Spectroscopic Aspects—The \(^{23}\text{Na}\) resonance deriving from extracellular Na\(^+\) in suspensions of *E. coli* (Fig. 1, A and B) is a superposition of a narrow and a broad line and therefore deviates from a simple Lorentzian line shape. Similar line shapes have been observed previously in \(^{23}\text{Na}\) spectra of suspensions of lipopolysaccharide (36) and red blood cells (13).

The \(^{23}\text{Na}\) nucleus carries a spin of \(3/2\) and, as reviewed in Ref. 29, is subject to quadrupolar interactions which may derive either from rapid exchange between free and bound Na\(^+\) or from diffusion of Na\(^+\) between ordered charged domains surrounding charged polyelectrolytes or assemblies such as membranes. Where these interactions are present, theory predicts that they will produce two different types of transitions with distinct transverse relaxation times (\(T_2\)) and line widths. The probabilities of the transitions with short and long \(T_2\) (and hence broad and narrow line widths) are 3/5 and 2/5, respectively. We have demonstrated that the line shape of the extracellular Na\(^+\) resonance observed in *E. coli* suspensions may be simulated with an excellent fit (Fig. 1, A and C) by two Lorentzian components with areas in the above proportion, suggesting that Na\(^+\) in the vicinity of the cell surface of *E. coli* is indeed experiencing quadrupolar interactions.

Both the outer bacterial surface and the periplasm contain a variety of negatively charged macromolecules such as lipopolysaccharides and other acidic polysaccharides (23) and membrane-derived oligosaccharides (30). These might provide suitable sites for Na\(^+\) binding and—since most of them are organized into membranes—might also give rise to extended ordered domains of Na\(^+\). It is uncertain precisely which of these mechanisms gives rise to the non-Lorentzian line shape we observed. However, it has been noted (36) that binding of Na\(^+\) to the negatively charged groups of purified bacterial lipopolysaccharide results in the appearance of narrow and broad components in the \(^{23}\text{Na}\) resonance.

The visibility of extracellular Na\(^+\) was found to be close to 100%, despite the presence of a broad component. In contrast, Magnuson and Magnuson (31) obtained reduced visibility of total Na\(^+\) (extracellular and intracellular Na\(^+\)) were not distinguished) in suspensions of Gram-negative bacteria. Visibility was 40% at high cell densities (comparable to those we used), increasing to 100% as the cell density was lowered. It is likely that the discrepancy stems from their use of continuous-wave NMR versus our use of Fourier-transform NMR. Continuous-wave NMR is relatively insensitive to detection of broad lines.

At high cell densities, probably only the narrow component was detectable, while at lower cell densities the broad component had narrowed sufficiently to be detectable also. This interpretation is in agreement with our finding that, at low cell densities, the entire signal could be characterized by a single Lorentzian.

Our observation that the intracellular Na\(^+\) resonance has a simple Lorentzian line shape could have two explanations. Intracellular Na\(^+\) might be characterized by a single \(T_2\) and hence a Lorentzian resonance. Alternatively, quadrupolar interactions might result in two different \(T_2\)s, with one so short that the resonance was broadened beyond detectability. These two alternatives make different predictions about the visibility of intracellular Na\(^+\). In the first, the intracellular Na\(^+\) should be 100% visible. In the second, the visibility should be 40% because transitions corresponding to the longer \(T_2\) represent 2/5 of the total signal (29). The experimentally determined value of intracellular visibility (45 ± 5%) is consistent with the latter prediction, indicating that the intracellular Na\(^+\) pool is experiencing quadrupolar interactions with either cytoplasmic macromolecules or the inner face of the cell membrane. Values close to 40% have been obtained with other systems, such as yeast cells (15) and tissue from muscle, kidney, brain, and liver (29), but several examples of 100% visibility have also been noted (in red blood cells, Refs. 13 and 14; in proximal kidney tubules, Ref. 16). The nature of the differences between the intracellular environments of the various cell types is unclear.

Na\(^+\) Extrusion by Energized *E. coli* Cells—Our measurements in respiring and glycolyzing *E. coli* cells demonstrate that the development and maintenance of proton motive force are accompanied by rapid Na\(^+\) extrusion and the maintenance of a substantial inwardly directed Na\(^+\) gradient. This would be expected if a major means of Na\(^+\) transport in *E. coli* was proton-driven Na\(^+/\text{H}^+\) exchange.

We obtained a value of 4.3 mM for the intracellular Na\(^+\) concentration in endogenously respiring *E. coli* when the extracellular Na\(^+\) was 95 mM. This value is much lower than those ([Na\(^+\)]) approximately 50 mM) obtained by other methods under similar conditions (4, 10). The latter values probably represent overestimates caused by artifacts intrinsic to the methods used (see introduction).

It should be noted incidentally that all estimates of intracellular Na\(^+\) (including ours) represent a formal concentration and thus an upper limit to the activity, the activity coefficient not having been taken into account.

When cells were switched from anaerobic to aerobic or glycolytic conditions, proton extrusion began immediately but there was a lag of 1–2 min before any Na\(^+\) extrusion occurred, by which time the proton motive force was appreciable. The delay in Na\(^+\) extrusion could mean either that a threshold proton motive force is needed or that pHm must be above a critical value. The first alternative has a precedent in the Na\(^+/\text{H}^+\) antiport observed in *Halobacterium halobium* where rapid Na\(^+\) extrusion occurs only when the proton motive force is above approximately ~150 mV (33). Evidence in favor of the second alternative derives from studies with *E. coli* membrane vesicles, which demonstrated that the unidirectional downhill efflux of Na\(^+\)—presumably mediated by Na\(^+/\text{H}^+\) exchange—is kinetically blocked at pH values below 6.5 (32)

The kinetics of net Na\(^+\) transport were found to be sensitive to the energetic state of the cell. Energization under both respiratory and glycolytic conditions elicited a rapid efflux of Na\(^+\) against its electrochemical gradient. The kinetics of downhill Na\(^+\) movement in de-energized cells (under anaerobic conditions or in the presence of CCCP) were much slower.
in agreement with the results of other studies (6, 9). In
addition, the rates of Na+ movement differed in the two de-
energized states even when the size of the inwardly directed Na+
gradient was the same. Comparison (Fig. 2A) of the 10-
minute interval immediately prior to oxygenation with the 10-
minute interval beginning at t = 28 min illustrates this point.
For both intervals, the initial value of ΔNa was 0.35, but in
the former case intracellular Na+ did not change appreciably
while in the latter case it increased by a factor of 1.6 during
the interval. Let us assume that the slow movement of Na+
in de-energized cells is still mediated by Na+/H+ exchange, i.e.
that the apparent inhibition is incomplete (as has been
demonstrated in H. halobium (33)). With this assumption,
the influx of Na+ would be limited by any inwardly directed pH
gradient that was present and possibly also by an inside-
negative membrane potential, depending on the stoichiometry
of the exchange. Under anaerobic conditions, there was an
inwardly directed ΔpH of 0.5-0.4, comparable within experi-
mental error to the size of the Na+ gradient; also, a residual
membrane potential might have been generated by the dissi-
pation of various ion gradients. In contrast, in the presence
of CCCP, both ΔpH and membrane potential were close to
zero, leaving Na+ free to flow down its concentration gradient
without an opposing force. Thus, the observed kinetics of
passive Na+ movement can be accounted for qualitatively by
the operation of Na+/H+ exchange. However, we cannot ex-
clude the possible contributions of additional pathways (e.g.
a Na+ leak or Na+/K+ exchange) for Na+ transport in de-
energized cells. The existence of leakage pathways has been
postulated to play a vital role in Na+ circulation in E. coli at
alkaline pH (34) and it would not be surprising if similar
pathways existed at more acidic pH values.

In summary, 23Na NMR employed in conjunction with a
paramagnetic shift reagent has enabled us to simultaneously
determine extracellular and intracellular Na+ concentrations in
E. coli. We have applied this technique further to a quan-
titative study of the magnitude of the Na+ gradient as a
function of the magnitude and composition of the electro-
chemical proton gradient. The results of that study will be
published in a forthcoming paper.

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