Use of $^{23}$Na Nuclear Magnetic Resonance Spectroscopy To Determine the True Intracellular Concentration of Free Sodium in a Halophilic Eubacterium

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We present new data obtained by $^{23}$Na nuclear magnetic resonance spectroscopy, which can distinguish free intracellular sodium from cell-bound sodium, showing that the intracellular concentration of Na$^+$ the halophilic eubacterium Vibrio costicola is only 5 to 20% of that in the extracellular medium. Previous methods could not distinguish free intracellular Na$^+$ from that bound to cell structures, and it was believed that in halophilic eubacteria the total monovalent cation concentration inside matched that of the NaCl outside. Information obtained by the newer technology raises fundamental questions about the ways in which these organisms and others which live in hypersaline environments function and cope with osmotic stress.

Halophilic bacteria belong to two major kingdoms. The archaeabacterial extreme halophiles generally require 2 to 3 M NaCl and can grow in saturated brines (16, 25). The composition of their cell envelopes differs from that of eubacterial cells, and they have high proportions of negatively charged proteins in their membranes, ribosomes, and other cell components. They are known to accumulate intracellular potassium ions to balance the extracellular sodium concentrations (16, 25).

The second group, the eubacterial halophiles, are far more widely distributed, both environmentally and among families and genera (19, 22, 32), and they are of great ecological and economic importance in agriculture, food industry, and other more specialized processes. The eubacterial halophiles also show more wide-ranging salt requirements and tolerances, i.e., they are more adaptable. The mechanisms and components which enable them to grow over such a wide range (from 3- to 10-fold, according to species) of salt concentrations are still largely unknown. One of the most important questions still unresolved involves the true intracellular ion concentrations relative to those in the medium and how the organisms deal with the osmotic and ionic gradients across their cell membranes. This problem has been highlighted by studies which showed that in vitro protein-synthesizing systems from such organisms (35) and most of their intracellular enzymes were inhibited by NaCl concentrations at which the cells had been grown (12, 17, 35). On the other hand, studies of intracellular salt concentrations in such organisms suggested that the total concentration of intracellular cations was more or less equal to the cation concentration in the extracellular milieu (3, 5, 15, 20, 23, 27, 28, 33). However, it was pointed out (15, 28) that the available methods could not differentiate between ionic concentrations in the cytosol and ions combined with, bound to, or occluded by cellular structures and components (2, 17, 18). These measurements of total, cell-associated ions failed, therefore, to identify the real intracellular concentrations of osmotically active free ions (18). The discovery of an Na$^+$/H$^+$ antiporter activity (Na$^+$ pump) coupled to respiratory-driven proton efflux (9, 10, 30) and direct measurements of sodium gradients by equilibrium dialysis (31) led to the expectation that the intracellular free Na$^+$ concentration would be less than that in the external medium. However, although it was clear that a sodium gradient across the bacterial cell membrane existed, the magnitude of the difference between intracellular and extracellular concentrations in intact bacteria was unknown. We now present the first direct measurement of the free intracellular concentration of sodium ions in a eubacterial halophile, using the technique of $^{23}$Na nuclear magnetic resonance (NMR) spectroscopy.

Use of $^{23}$Na NMR spectroscopy to measure intracellular sodium. The ability to measure intracellular concentrations of sodium ions by using $^{23}$Na NMR spectroscopy (1, 4, 8) provided the opportunity for this first determination of such Na$^+$ concentrations in the moderately halophilic eubacterium Vibrio costicola, grown and examined at various salt concentrations. The $^{23}$Na free-induction decay is potentially very complex. The use of a method that includes a membrane-impermeable shift reagent to displace the extracellular signal has alleviated this problem. However, our preliminary experiments showed that 2.0 M was the upper limit of NaCl at which useful resolution of the intracellular and extracellular peaks for the determination of intracellular Na$^+$ could be obtained. Therefore, we had to limit our examinations to cultures grown in media containing 0.6 to 2.0 M NaCl.

 Cultures of V. costicola NRC 37001 were grown as described previously (13) in 0.3% (wt/vol) Proteose Peptone plus 0.3% (wt/vol) Tryptone medium containing NaCl as indicated. Cultures in the mid- or late-exponential growth phase were harvested without delay by centrifugation at room temperature in order to avoid temperature shock with possible leakage of ions. The bacterial pellets were resuspended carefully in the same medium containing the appropriate concentration of NaCl and the shift reagent (25 or 50 mM dysprosium tripolyphosphate) to give a concentration equivalent to 40 to 75 mg of bacterial protein ml$^{-1}$ (final volume, 2.0 ml). The NMR spectra were then recorded without further delay.

A typical spectrum for a bacterial suspension in 1.0 M
FIG. 1. $^{23}$Na NMR spectrum of *V. costicola* grown and resuspended in 1 M NaCl, measured with a Bruker AM400WB instrument at a frequency of 105 MHz. The bacterial cell suspension (2.0 ml) was placed in a 10-mm-diameter NMR tube. Inside this tube, another 5-mm-diameter NMR tube containing the shift reagent in D$_2$O was introduced as a reference for intensity measurements. This reference tube containing a known concentration of sodium was calibrated against a 1 M NaCl solution; it gave a single peak that was shifted upfield by the presence of a high concentration of the shift reagent, which also showed that the dysprosium tripolyphosphate was fully effective in shifting the extracellular sodium signal. The chemical shift values (in parts per million) of the separated resonances due to extracellular and intracellular sodium are given relative to that of dysprosium tripolyphosphate, which is shown as zero.

NaCl is shown in Fig. 1; the resonances corresponding to intracellular and extracellular sodium have been separated by the shift reagent (which cannot penetrate the cell barrier and fully shifts the extracellular $^{23}$Na signal) so that the areas under the two peaks can be measured. Because the resonances of the intracellular Na$^+$ were so much smaller than the extracellular ones (because of the much smaller intracellular volumes as well as the lower intracellular concentrations), the vertical scale was increased by four- or eightfold to facilitate more accurate measurements. Even with such magnifications and the higher concentration of dysprosium tripolyphosphate (50 mM), the resolution of the two resonances at the higher salt concentrations (1.6 and 2.0 M) was often imperfect. Therefore, multiple measurements of several bacterial cultures grown and resuspended at the different salt concentrations were made. Very careful measurement of the areas under the relevant peaks gave the data presented in Table 1. The values are the means of determinations which fell within the range of twice the standard deviation. It is quite clear that these values for detectable intracellular Na$^+$ concentrations never approach the salt concentrations to which the bacterial cells had been exposed during growth and subsequent resuspension. The NMR method necessitated the use of thick bacterial suspensions in order to be able to record spectra with the least possible delay. It is appreciated that such suspensions would become anoxic during recording of the spectra, which would probably slow the activity of the Na$^+$/H$^+$ antipporter; therefore, the rate of Na$^+$ efflux would decline, and our values of free intracellular Na$^+$ are probably overestimates. Despite this reservation, the highest values found do not exceed about 25% of the external NaCl concentrations. This is in sharp contrast to the values of total cell-associated sodium concentrations reported in earlier studies of this and similar organisms (5, 20, 23, 28, 33). Our results here also confirm the hypothesis (15, 18, 28, 35) that these organisms are able to keep their free intracellular concentrations of Na$^+$ at levels (i.e., <0.15 M) which are tolerated by their intracellular enzymes (12), including those of protein biosynthesis (35). Such enzymes, therefore, may be largely similar to those of the nonhalophilic relatives of these organisms (12, 17).

**Status of intracellular sodium.** Our results also provide preliminary answers to two further questions. First, does the intracellular Na$^+$ concentration vary according to the external salt concentration? The present data give no evidence that it does, at least over the range of external salinity that we have been able to investigate. If anything, the intracellular sodium concentration seems to decrease at higher external salt concentrations, although the differences observed are of doubtful statistical significance ($P > 0.1$).

Second, is there any evidence for sodium bound or made invisible to the NMR probe? This is clearly implied by the large difference in values for cell-associated sodium in *V. costicola* (and other bacteria) determined by earlier methods (28) and our values reported here. It should be emphasized, perhaps, that even allowing for the possible effects of anaerobiosis, the concentrations of intracellular Na$^+$ determined as described above are minimal values: they may represent (or contain) only 40% of the sodium inside bacterial cells, including the weakly bound fraction able to exchange with ions in solution (21). Any sodium that is tightly bound will not be seen by the NMR probe (7). Although we have no way of estimating any weakly bound Na$^+$, we can calculate that if the intracellular sodium ion concentrations estimated by us represented only 40% sodium inside the cells, the total concentrations would range from 0.140 to 0.435 M (Table 1); that range is still well short of the extracellular NaCl concentrations and of the cell-associated Na$^+$ determined previously (28).

In the wider view, our results here serve to refocus the outstanding questions as to how these eubacterial halophiles can grow over such a wide range of salt concentrations...
(i.e., from 3- to 10-fold, according to species) by means of phenotypic adaptation, that is, adaptation of the whole culture (6). The exact nature of the molecular and metabolic changes that are involved in this adaptation is the wider subject of this study. Thus, changes in phospholipid composition of cell membranes in response to changes in external salt concentrations have been reported previously (13). The present results emphasize the role of the cell membrane and indicate that it may be exposed to different ion concentrations on its two faces. In addition, it would be involved in the uptake and/or synthesis of osmoregulatory substances (compatible solutes) recently demonstrated for eubacterial halophiles (11, 24, 34). Finally, there is the question of the mechanisms by which these organisms can sense the crucial ion concentrations—in the environment or within the membrane—and trigger the events that constitute the haloadaptive response (14, 26).

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