Plant Physiol. (1984) 75, 269–272
0032-0889/84/75/0269/04/$01.00/0

Short Communication

Use of ^{23}Na-Nuclear Magnetic Resonance To Follow Sodium Uptake and Efflux in NaCl-Adapted and Nonadapted Millet (*Panicum miliaceum*) Suspensions[1]

Received for publication December 27, 1983 and in revised form March 14, 1984

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ABSTRACT

Cellular Na^+ transport was followed *in vivo* by ^{23}Na nuclear magnetic resonance (NMR) using anionic dysprosium-based shift reagents to resolve internal and external $^{23}Na^+$ resonances. Proso millet (*Panicum miliaceum*) cell suspensions adapted for rapid growth on 130 mM NaCl had biphasic ^{23}Na efflux kinetics when shifted to low Na^+ medium, while nonadapted cells had little measurable Na^+ efflux after preloading with $^{23}NaCl$. Uptake of ^{23}Na was also observed using ^{23}Na NMR. The resonance frequency of the external Na^+-dysprosium (III) triphosphate, relative to that of the ^{23}Na in the cells, was sensitive to pH, permitting the pH of the external medium to be followed during the course of *in vivo* experiments.

Sodium ions are directly toxic to many of the cytosolic enzymes of plants (2). The prevention of sodium-induced growth inhibition of plant cells involves the sequestering of Na^+ ions outside the cytoplasm, particularly by transporting Na^+ into the vacuoles and back out into the medium. An understanding of the transport of Na^+ ions by plant cells is needed to aid in unraveling the mechanisms by which certain plants are able to adapt to saline stress.

In earlier work, tobacco cell cultures (*Nicotiana tabacum* var Samsum) were shown to adjust to growth on 130 mM NaCl by the accumulation of Na^+, and Cl^-, presumably into the vacuole (6). The efflux of cellular Na^+ was resolved into two components, with the fast component (0.6–1.7 min $t_{1/2}$) attributed from the free space and cytoplasm, and the slow component (1.6–4.9 h $t_{1/2}$) attributed from Na^+ in the vacuole. In barley (*Hordeum vulgare*), the pattern of Na^+ efflux was found to correlate with the degree of NaCl tolerance (10, 13). There was a faster efflux of ^{22}Na from the more tolerant cultivar California Mariout than by the more NaCl-sensitive Arivat into solutions with equimolar concentratiaons of ^{23}Na.

NMR[2] provides a unique means for noninvasively monitoring cellular phenomena (1, 11, 12, 15–20). NMR techniques are well suited to the study of Na^+ transport; data can be obtained with excellent time resolution, *in vivo* and in real time without the need for extraction, grinding, or filtration of the tissue. Efflux or influx time courses can be obtained from a single sample. Good ^{23}Na NMR signals can be obtained from solutions containing 1 mM Na^+ in 1 to 2 min, so that Na^+ transport can be followed with good time resolution. Anionic shift regeants such as DyTP (3, 4) can be used to separate intra- and extracellular ^{23}Na resonances so that the ^{23}Na concentration in each compartment may be observed simultaneously.

In this report, we show that (a) $^{23}Na^+$ influx and efflux can be measured using ^{23}Na NMR, (b) different Na^+ efflux kinetics were observed for NaCl-adapted *versus* nonadapted millet suspension cells, and (c) the resonance frequency of the ^{23}Na DyTp-shifted peak was affected by the pH of the external medium.

MATERIALS AND METHODS

The method of Gupta and Gupta (3) which utilizes the paramagnetic, anionic, and impermeant nature of DyTp to shift external Na^+ resonances was used. The DyTp complex binds Na^+ with a sufficient strength to alter its NMR resonance frequency by several ppm, thus enabling the resolution of intra- and extracellular Na^+. The DyTp is not taken up by the plant cells. The changes in the internal and external Na^+ were then determined by the relative intensities of their respective resonance signals over a time course during upshift and downshift conditions.

The suspension cell line of proso millet, *Panicum miliaceum* cv Abarr, was derived from a single callus regenerated in a protoplast culture isolated from rapidly growing albino embryogenic suspensions (5). This protoplast-derived line was grown as callus for 3 months and in suspensions for 10 months before the start of these experiments. Suspension transfers were made weekly by adding Miracloth-filtered cell clumps (0.4–0.5 g fresh weight) to 15 to 20 ml of Linsmaeir and Skoog (5, 6, 8) medium plus 2.5 μg/ml 2,4-D and 4% (w/v) sucrose in a 125-ml cotton-stoppered flask. Suspension growth was monitored by the visual scale of Hooker and Nabors (9). After 5 to 7 d, the suspensions had a fresh weight of approximately 2 g.

Experiments were started by harvesting a flask of exponentially growing cells by Miracloth filtration and subsequent resuspension in fresh medium with or without NaCl. Cell clumps under these conditions were aerobic for at least 50 min and could be maintained aerobic indefinitely by slowly bubbling O_2 in the medium as judged from the ^{31}P NMR spectra of the cells (data not shown), and from the lack of change in the splitting of the sodium signals (see "Results and Discussion").

For NMR observation, a sample (~1 g) of millet cells adapted

[1] Supported by the United States Department of Energy.

[2] Abbreviations: NMR, nuclear magnetic resonance; DyTp, dysprosium (III) triphosphate.

for growth on 130 mM NaCl was removed from its culture flask and placed, along with 1 to 3 ml of the same medium, into a 10-mm NMR tube. After the addition of 100 μl of a 100 mM DyTp solution to give a final concentration of between 3 and 10 mM shift reagent, ^{23}Na NMR spectra were taken every 1 to 3 min with the aid of a Bruker WM 300 NMR spectrometer operating at 79.4 MHz in the Fourier-Transformation mode. The spectra were the result of 512 scans using a 90° (20 μs) pulse repeated every 0.35 s using a 6 kHz sweep and 4K data points. The sample was thermostated at 293 K. To follow Na^+ efflux, DyTP was added and several control spectra were taken to ensure equilibrium splittings were reached. The external medium was then diluted by a factor of two with Na-free medium and ^{23}Na NMR spectra were accumulated as above. To follow influx, nonadapted cells were shifted to medium containing 130 mM NaCl and 100 μl of a DyTP solution. To measure the pH dependence of the splitting of the Na signals, we titrated a sample containing 2 ml of NaCl (130 mM) and 100 μl DyTP with HCl with respect to a 130 mM NaCl standard in a coaxial NMR tube system.

RESULTS AND DISCUSSION

When nonadapted cells are upshifted to medium containing 130 mM NaCl, they show a lag in growth for 1 week and then adapt by resuming growth at a rate similar to cells on the control medium. Both adapted and nonadapted cells ceased growth after 1 week, when they were shifted to medium with 260 mM NaCl. The cell suspension clumps turned black, but these clumps resumed slow growth when plated on agar medium (1% w/v) containing 130 mM NaCl (7).

The resolution of internal and external Na^+ resonances with the aid of DyTP is shown in Figure 1. The NMR peak at 0.00 ppm arises from the interior Na^+, while that at −6.98 ppm comes from external Na^+. These signals are 0.74 to 1.08 ppm wide (full width at half maximum). To determine the sensitivity of ^{23}Na NMR detection in our instrument, we examined an analytical

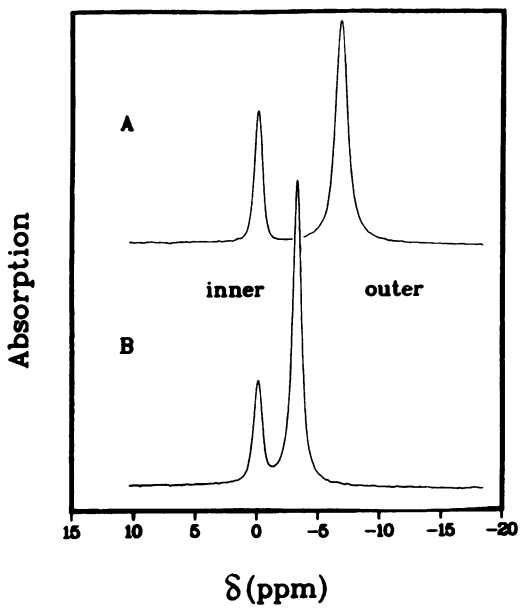


FIG. 1. ^{23}Na-NMR spectra of about 1 g of millet cells in the presence of DyTp at pH 6.9, obtained in 3 min. A, Initial spectrum taken just after DyTp addition showing resolution of the Na^+ resonances into two components: an upfield resonance from extracellular Na^+ ions and a downfield signal from intracellular Na^+. B, A spectrum of the same sample as in (A) taken 90 min after a 2-fold dilution of the extracellular medium with Na^+-free medium. The decrease in the chemical shift difference from (A) to (B) was a result of dilution of the DyTP.

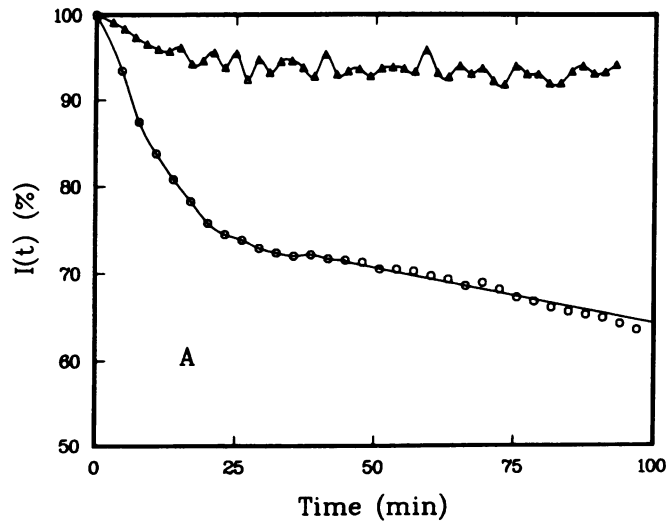
sample of 130 mM NaCl. With 3 min of signal averaging, the signal to noise ratio was in excess of 650:1, or 2.89/(mM min). The resolution and sensitivity of ^{23}Na NMR were more than adequate to follow Na^+ kinetics in plant cells, since it has been shown that in NaCl-adapted *N. tabacum* cells (6) the fastest efflux time constant was usually a few minutes. It should be noted that the exact value of the splitting (in ppm) between the two sodium signals is a function of the final DyTP concentration and the medium pH (see below).

After 90 min, the signal from interior Na^+ had decreased considerably (Fig. 1B) while the signal from exterior Na^+ had increased. The time course of the changes in peak heights (Fig. 2A) shows that the efflux of Na^+ from these NaCl-adapted cells can be described by a minimum of two time constants; (a) a fast component ($t_{1/2} = 9.15 \pm 0.37$ min) which may reflect the efflux of Na^+ from the cell wall and cytoplasm; and (b) a slow component ($t_{1/2} = 506 \pm 36$ min) which may represent the efflux of Na^+ from the vacuole. Nonadapted cells, *i.e.* those cells upshifted from basal medium to 130 mM NaCl and which had not yet resumed normal growth, had little measurable Na^+ efflux (Fig. 2A). DyTp was impermeant as shown by the continued separation of internal and external peaks (Fig. 1). This separation was maintained during the course of experiments. The cells were incubated with DyTP for about 10 min (see "Materials and Methods") prior to dilution of the extracellular medium with no change in the splitting of the Na signals. For this reason it is believed that equilibrium was established between the DyTP-accessible Na and the DyTP so that the efflux data presented in Figure 2 do not arise from diffusion of the DyTP into the cell wall space, but rather represent true movements of the Na.

The uptake of Na^+ by cells shifted from a medium containing no added NaCl to one containing 130 mM NaCl is shown in Figure 2B, with the steady initial influx of Na^+ being measured by ^{23}Na NMR. A linear least squares fit to this data from six experiments gave a slope of 0.757%/min, with a correlation coefficient of 0.91, and a standard deviation of ±14.7%. In these influx experiments, the initial intracellular sodium concentration in the nonadapted cells was measured to be 0.93 mM or 0.82 μmol/g tissue. The larger statistical scatter evident in Figure 2B derives from the smallness of this initial concentration and the resulting poorer signal to noise ratio for the intracellular signal.

By measuring the intra- and extracellular volumes (0.88 and 0.78 ml/g, respectively), the intra- and extracellular signal intensities, and the extracellular Na concentration, we can calculate from the NMR spectra the amount of Na contained in 1 g of millet cells. From this we derived the absolute rates of Na transport. For influx the cells took up Na^+ at a rate of 74.1 ± 8.2 nmol/g·min ($n = 6$). The efflux data from the NaCl-adapted cells (Fig. 1A) was biphasic as mentioned above. The initial and final slopes were found to be −1.39 and −0.122%/min, respectively. Extrapolation of the slow component to $t = 0$ indicated that 76.85% of the intracellular Na resided in this compartment. The data could be fitted to a two-compartment model where the time dependence of the intracellular sodium signal could be written as $I(t) = 23.15\ e^{-t/9.15} + 76.85\ e^{-t/506}$ which implies that 23.15% of the intracellular sodium was present in the fast compartment. We calculated the two fluxes as follows. The fast compartment had 23.15% of the total Na while the slow compartment had 76.85%. The total intracellular Na was 114 μmol/g tissue. The fast and slow compartments had 26.39 and 87.6 μmol Na/g, respectively. The slopes referred to above then give fluxes of 367 and 107 nmol Na/g·min, respectively.

As with the ^{31}P signal for Pi (14), the resonance frequency of the external Na^+ was found to be pH dependent (Fig. 3A). This data was obtained from a coaxial analytical sample (see "Materials and Methods"). Any acidification of the medium due to cellular metabolism could be readily monitored by the difference



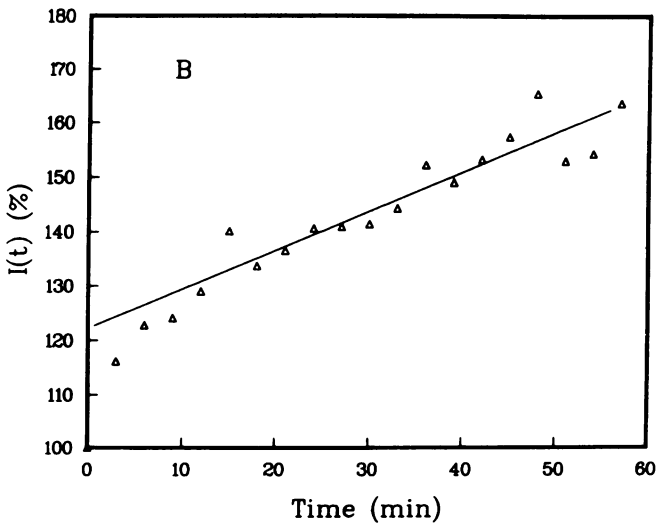
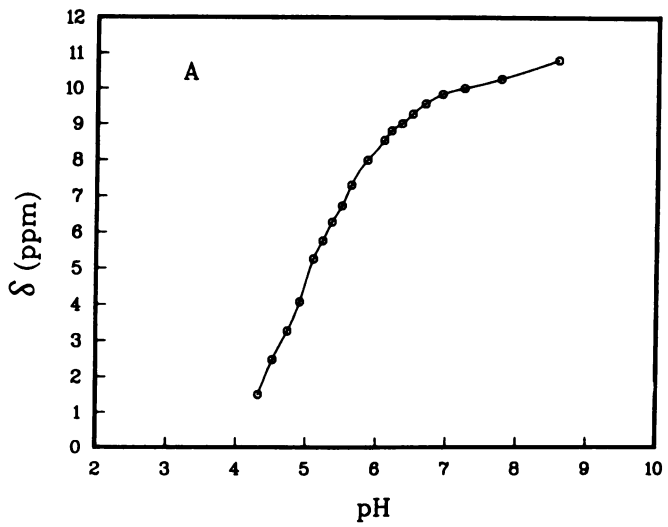


FIG. 2. Net efflux (A) and influx (B) of ^{23}Na in the presence of DyTp at pH 6.9 in the external medium. The per cent change in integrals [$I(t)$(%)] of internal ^{23}Na peak *versus* time is shown. For efflux (A), the NaCl-adapted cells (○) were grown for 3 weeks on 130 mM NaCl and had resumed normal growth, while nonadapted cells (△) were preloaded for 5 d. For influx, nonadapted millet cells were placed in medium containing 130 mM NaCl plus DyTp. The influx data (B) are the averages of six separate experiments.



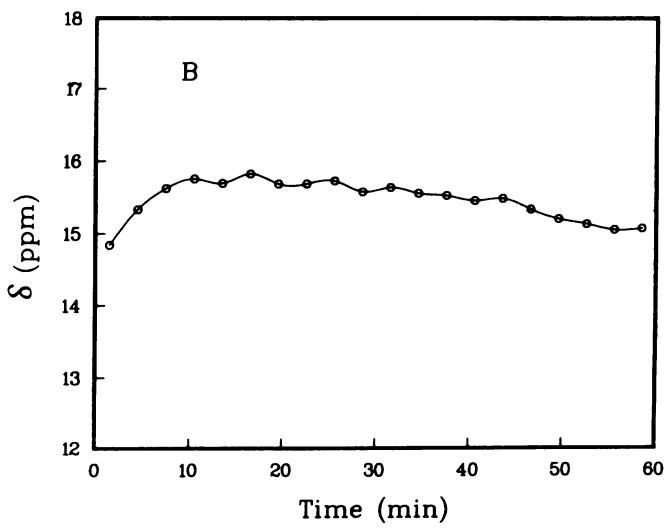


FIG. 3. A, Effect of medium pH on separation [δ(ppm)] of external and internal ^{23}Na NMR peaks caused by DyTP in a coaxial NMR sample tube containing 130 mM NaCl in the center and 130 mM NaCl plus DyTP in the outer tube. B, Time dependence of the chemical shift difference [δ(ppm)] between the intra- and extracellular ^{23}Na NMR signals in the presence of DyTP for millet cells grown without added NaCl. This latter data was measured during one of the influx experiments shown in Figure 2B.

between the internal and external Na^+ resonance frequencies (Fig. 3B). We found that the chemical shift difference was approximately constant over 50 min (Fig. 3B) in a sample of millet cells indicating that the extracellular pH was approximately constant at 6.71. This also implies that the DyTP was not taken up by the cells, and that the oxygenation was sufficient to prevent metabolic acidification of the medium.

In conclusion, we have demonstrated that ^{23}Na NMR can be used to monitor the kinetics of Na transport in real time, from single samples of millet cells. Similar techniques should be useful for monitoring the fluxes of other cations, notably potassium.

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